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TITLE:

Isolation of Tissue Extracellular Vesicles from the Liver

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KEYWORDS:
Liver, extracellular vesicles, collagenase, ultra-centrifugation, diagnostics, perfusion

SUMMARY:
This is a protocol to isolate tissue extracellular vesicles (EVs) from the liver. The protocol describes a two-step process involving collagenase perfusion followed by differential ultracentrifugation to isolate liver tissue EVs.

ABSTRACT:
Extracellular vesicles (EVs) can be released from many different cell types and detected in most, if not all, body fluids. EVs can participate in cell-to-cell communication by shuttling bioactive molecules such as RNA or protein from one cell to another. Most studies of EVs have been performed in cell culture models or in EVs isolated from body fluids. There is emerging interest in the isolation of EVs from tissues to study their contribution to physiological processes and how they are altered in disease. The isolation of EVs with sufficient yield from tissues is technically challenging because of the need for tissue dissociation without cellular damage. This method describes a procedure for the isolation of EVs from mouse liver tissue. The method involves a two-step process starting with *in situ* collagenase digestion followed by differential ultra-centrifugation. Tissue perfusion using collagenase provides an advantage over mechanical cutting or homogenization of liver tissue due to its increased yield of obtained EVs. The use of this two-step process to isolate EVs from the liver will be useful for the study of tissue EVs.

INTRODUCTION:
Extracellular vesicles (EVs) are membrane-bound vesicles that are released from many different types of cells in the body. EVs contain a cargo of molecules that include RNA, DNA, and protein. Transfer of this cargo by EVs from one cell to another is postulated as one mechanism by which

cells within tissues communicate with each other[1]. The majority of information regarding the cargo or roles of EVs in normal health and diseases has been derived from studies on EVs obtained from cells in culture or collected from the circulation or other body fluids[2]. In order to understand their physiological roles *in vivo*, a robust method is necessary for the isolation of tissue EVs that captures all populations of EVs and avoids cellular damage or contamination[3]. The overall goal of the method described herein is to isolate tissue EVs from mouse livers.

Most cell types in the liver have been shown to produce EVs, and the study of EV-based signaling is advancing basic knowledge and understanding of hepatic diseases. However, the combined impact of EVs from different cell types within tissues is only partially understood. Isolation of EVs from liver tissues is necessary in order to understand the *in situ* contributions of EVs within the tissue milieu. The approach described herein is based on a two-step perfusion to enhance tissue dissociation and minimize cell damage. Subsequently, EVs are isolated from the dissociated liver tissue. Approaches using two-step perfusion for isolation of hepatocytes have been used since the early 1950s[4]. These methods for hepatocyte isolation have been modified and continuously improved and are now standard approaches for isolation of hepatocytes in cultures, in cell suspensions, and from tissues[5-7]. In the first step, the liver is subjected to a non-recirculating perfusion with calcium-free buffer, Hank's balanced salt solution (HBSS). In the second step, the liver is perfused with collagenase to dissolve the extracellular matrix for further separation of desmosomal cell-to-cell junctions. An optimal treatment time for collagenase dissolution is 7 to 10 minutes. A shorter duration of treatment will cause incomplete dissolution and retain cell contacts in the liver, whereas a longer duration may cause liver damage or portal vein disruption. EVs are then isolated using differential centrifugation to remove cells and cellular debris. This results in EV collection in high yields that can be used for further downstream analyses or studies.

PROTOCOL:

All studies involving animals were performed in accordance with a protocol that was approved by the Mayo Clinic Institutional Animal Care and Use Committee.

1. Bench Preparation

1.1. Prepare a water bath and set it to 37 °C. The other necessary equipment and set-up are shown in **Figure 1**.

1.2. Measure 100 mg of collagenase type IV and add it to a 125 mL flask containing 100 mL of HBSS in a water bath (40 °C). Ensure that the collagenase has been dissolved by swirling the liquid in the flask, and also make sure that the flask is submerged completely in the water bath. For greater efficiency, allow the collagenase to dissolve for at least 30 min even though it may appear to dissolve instantly.

1.3. Submerge a 125 mL flask containing 50 mL of HBSS in a water bath at 40 °C. This will be used for initial flushing.

1.4. Spray down the surface of all instruments such as the scissors and forceps with 70% ethanol. Prepare a few clean cotton swabs.

1.5. Rinse out the tubing of the pump by running 70% ethanol through the pump and remove any residual ethanol by rinsing it twice with running water.

1.6. Lay an absorbent bench pad on the lab bench and place a hard box container on top. This will be needed to contain any excess liquids during the mouse perfusion. Wrap a polystyrene foam pad with aluminum foil and place it inside the container.

1.7. Place a sterile 10 cm culture dish close to the bench. This will be used to hold the digested liver after the perfusion is completed.

1.8. Pour 10 mL of collagenase medium (step 1.2) into a sterile culture dish in advance.

1.9. Using a winged blood collection set, connect the 23-gauge to the free end of the pump tubing (cutting the wings off the butterfly cannula can lead to better handling). Pass until the tip of the cannula of blood collection is filled.

2. Animal Preparation

2.1. Before starting anesthesia using isoflurane, make sure that an adequate amount of supply gas is available for the duration of the procedure. Turn on the oxygen (O_2) supply to the induction chamber at 1-2 L, then turn on isoflurane between 2-4% using a flowmeter.

2.2. Put the mouse into the induction chamber and close the top door. Monitor the mouse until it is recumbent.

Note: The gases in the chamber will keep the mice anesthetized for several minutes.

2.3. Place the mouse on a polystyrene foam pad wrapped with aluminum foil. Switch the flow from the induction chamber to a nosecone. Ensure that anesthesia is adequate. If the mouse has started responding, gently restrain it in a nose cone until the it is fully anesthetized.

2.4. Ensure anesthesia by monitoring respiration and response to stimulation during the procedure. Adjust the rate flowmeter as needed to ensure adequate anesthesia. The paws must be nonresponsive to the pinch test under anesthesia. Additional details may be obtained from the laboratory guide for the care of animals.

2.5. Tape or pin down all four limbs of the mouse.

2.6. Clean the skin over the abdomen by spraying with 70% ethanol and wiping it off with gauze and an alcohol pad. This step is critical to avoid contamination from the mouse fur.

2.7. Make a wide-open cut through the skin from the anterior to the pelvis bone using a set of sterile scissors. Be careful not to cut any internal organs**.** Displace the intestines to the right side by using a cotton tipped applicator to gently to expose the portal vein (PV) and inferior vena cava (IVC).

3. Cannulation and Perfusion (0.5 h)

3.1. Using curved forceps, place a thread underneath the portal vein and tie a knot loosely to prepare cinching tightly after cannulation.

3.2. Insert the cannula (23G from the blood collection set) into the portal vein 5-10 mm below the ligature. Do not insert the cannula past the first portal branch, otherwise the right anterior lobe can be inadequately perfused. The cannula can be fixed or fastened with thread using a stopper knot.

3.3. Start the pump to infuse in HBSS at a low flow rate (1-2 mL/min). Once the cannulation is confirmed to be successful, the liver will begin to blanch.

3.4. Cut the IVC to relieve pressure and allow excessive fluid within the liver to drain. This is best performed using the operators other hand so the cannula is not moved.

3.5. Slowly increase the flow rate to 8 mL/min and complete the perfusion using the entire 50 mL volume of HBSS through the liver, over the next 5 min.

3.6. Pour the collagenase containing medium (step 1.2) into the beaker just before the HBSS is starting to run out. Ensure that air bubbles are not present and do not flow into the liver when changing the medium.

3.7. Apply transient pressure to the IVC at 5 s intervals by clamping with forceps. This will cause the liver to swell and help with tissue digestion and dissociation (7-8 min). As digestion progresses, the liver will swell and become white. The liver can swell uniformly to approximately twice its original size.

3.8. Turn off the pump and remove the cannula once digestion is completed. The completion of liver digestion will depend on the size of the mouse and condition of the liver. A dent in the liver can be observed if a cotton-tipped applicator is used to gently probe the liver.

3.9. Remove the gallbladder from the liver, being careful not to tear it. Using a washed pair of scissors and forceps, extract the liver from the mouse into a sterile 10 cm culture dish containing phosphate-buffered saline (PBS) for surface washing. Transfer the liver carefully into the sterile 10 cm culture dish containing collagenase medium (from step 1.8). This is a critical step for avoiding contamination from the mouse blood and bile.

3.10. Grab and tear apart the liver with two clean forceps while gently shaking the cells from the liver. As this happens, the medium will become clouded. All hepatocytes can be shaken away, leaving behind the connective tissue and vascular tissue.

3.11. Triturate the cell solution many times using a 25 mL serological pipette until the undigested parts of the liver are shaken off. Pour it off into a 50 mL conical tube with 70 µm nylon cell strainers to filter any undigested connective tissue. Wash the dish with HBSS to collect the remaining cells and fill up the 50 mL conical tube.

3.12. Centrifuge softly the 50 mL conical tube in a swinging bucket rotor at 50 x g for 10 -20 min at 4 °C.

3.13. Transfer the supernatant to a new 50 mL conical tube.

4. Isolation of EVs (5 h)

4.1. Centrifuge the supernatant at 300 x *g* for 10 min at 4 °C. Transfer the supernatant into a new 50 mL conical tube.

4.2. Centrifuge the supernatant at 2000 x *g* for 20 min at 4 °C to remove cell debris and aggregates.

4.3. Transfer the supernatant to a round-bottom tube and centrifuge the supernatant at 10,000 x g for 70 min at 4°C.

4.4. Collect the supernatant and place into a polycarbonate ultracentrifuge tube and centrifuge at 100,000 x g for 70 min at 4 °C.

4.5. Collect the pellet in an ultracentrifuge tube which is then washed by re-suspending in PBS. Centrifuge the supernatant further at 100,000 x g for 70 min at 4 °C.

4.6. The final pellet comprising of cellular nanovesicles can be directly used for experiments or re-suspended with 1000 µL of PBS and stored at -80 °C.

5. Assessment of Quality and Yield of Isolations

5.1. Assess the size distribution and concentration using nanoparticle tracking analysis or tunable resistive pulse sensing following the instrument manufacturer's protocols.

5.2. Perform further isolation and purification of specific vesicle populations by various approaches such as the addition of a sucrose gradient or cushion, immunoaffinity techniques, or size exclusion chromatography, based on specific experimental needs.

REPRESENTATIVE RESULTS:

The apparatus needed for these isolations comprises of standard laboratory equipment, making this a relatively simple and cost-effective approach. Isolations have been performed from twelve- to thirty-week-old male and female Balb/c or FVB mice. The tray holding the mouse is lined with aluminum foil inside a hard-walled container that collects excess fluids during the perfusion. The flasks containing HBSS or collagenase-containing medium are submerged in a water bath (40 °C) ready to be used. Two sterile 10 cm culture dishes are used. One is needed for surface washing with PBS, and the other for hepatocyte separation from the connective tissue components.

In this method, the liver is perfused in a non-continuous manner *via* the portal vein in preference to cannulation from the inferior vena cava. An alternative and commonly used perfusion approach is to perform retrograde perfusion by cannulating the inferior vena cava and cutting the portal vein for drainage. However, portal vein cannulation is easy to access and involves a short distance to the liver, as the portal vein feeds directly into the liver[8]. The selection of an insertion point for cannulation is crucial for optimal success **(Figure 2).** The cannula is placed past the branches of the stomach and pancreatic veins but not beyond the first portal branch (right and left hepatic portal veins). Once the optimal insertion location in the portal vein is identified, curved forceps are used to place a thread underneath portal vein and tie a loose knot. The needle of cannula is fixed with thread using a stopper knot to stop the needle from falling out.

Figure 3 outlines the overall processing scheme for the differential centrifugation for liver tissue EVs isolation. Ultracentrifugation removes cells, debris and other impurities. The first four centrifugation steps (50 x g, 300 x g, 2,000 x g, 10,000 x g) are designed to remove hepatocytes, intact other cells, dead cells, or cell debris respectively. **(Figures 4A** and **4B)**. After these steps, ultracentrifugation is again performed at 100,000 x g to collect the pellet **(Figure 4C)**. The pellet is washed by re-suspending in PBS and subjected to a final ultracentrifugation at 100,000 x g. The pellet after ultracentrifugation is absolutely visible and viscid in this procedure compared to EVs from conditioned culture media. Pipetting many times is required until any brown aggregates are out of sight and completely dissolved. The final pellet is re-suspended with 1000 µL of PBS **(Figure 4D).** Ultracentrifugation removes impurities and other soluble contaminants from the plasma, which can affect functional experimental outcomes. The centrifugation is be carried out at 4 ˚C.

From mouse liver, this method yields a tissue EV concentration that ranges from 1.74 to 4.00 x 10^{12} with a mean of 3.46 x 10^{12} particles per mL as determined by nanoparticle tracking analysis (NTA) **(Figure 5).** The mean size of the isolated liver tissue EVs was 157.7 nm, with a mode size of 144.5 nm and EV sizes ranging from 100-600 nm by NTA. The yield of EV will depend on factors such as the liver weight and losses within the perfusate or ultracentrifugation steps.

FIGURE LEGENDS:

Figure 1: Bench preparation. The materials and their locations are: (A) pump, (B) warmed 125 mL flask containing HBSS and water suction port, (C) nose cone connected to an Isoflurane vaporiser, (D) water exhaust port of the pump connecting with needle, (E) tray lined with aluminum foil inside a hard walled container, and (F) 10 cm culture dish in which collagenase medium is poured in advance.

Figure 2: Cannulation site. The anatomy of the mouse abdomen is shown. Using curved forceps, a thread is placed underneath the portal vein (PV) and a loose knot is tied. The insertion location is near the liver, 5-10 mm below the ligature, but not beyond the first portal branch (left and right hepatic portal veins). The cannula is fixed or fastened using thread with a stopper knot. This knot serves as a marker of the PV location if the cannula is dislodged.

Figure 3: Schematic of centrifugation steps. The goal is to remove unwanted cells and other components and isolate EVs. The first four centrifugation steps are designed to remove hepatocytes and other cells, dead cells, or cell debris using differential centrifugation. After these steps, ultracentrifugation is performed at 100,000 x g to collect the pellet of EVs. The pellet is washed by re-suspending in PBS and subjected to a final ultracentrifugation at 100,000 x g. All the centrifugation steps are carried out at 4 ˚C.

Figure 4: Differential centrifugation. (A) After centrifugation at 50 x g for 10 min, a pellet containing hepatocytes is observed. (B) A round-bottom tube is used for centrifugation at 10,000 x g for 70 min to remove cell debris. (C) A polycarbonate ultracentrifuge tube is used for centrifugation at 100,000 x g for 70 min. The pellet is collected in one tube and washed by re-suspending with PBS. (D) The final pellet is re-suspended in 1000 µL of PBS.

Figure 5: Representative result. The size and concentration of liver tissue EVs can be determined by nanoparticle tracking analysis (NTA).

DISCUSSION:

This protocol describes an optimal and reproducible method for the isolation of hepatic tissue EV using a two-step perfusion process *via* the portal vein followed by differential ultracentrifugation. Important steps of the procedure include cannula placement, collagenase concentration and digestion time, flow speed of the medium, handling of the tissue after digestion, and classical differential ultracentrifugation.

Cell separation is achieved by separation from connective tissue components after digestion using collagenase type IV. The concentration of collagenase used for perfusion can range from 0.1 to 5 mg/mL. There can be considerable batch-to-batch variation in the efficacy of collagenase for tissue digestion. Concentrations of collagenase from 0.5 to 5 mg/mL were tested, but the concentration used did not have a major impact on the yield of EVs obtained. Using a higher concentration of collagenase will result in a more rapid swelling and whitening of the liver. The goal is to obtain satisfactory cell dissociation without excessive contamination or damage. An optimal concentration of collagenase used in these isolations is 1-2 mg/mL perfused for 7-8 min at a flow rate of 8 mL/min. A perfusion procedure that is too long will increase the risk of destroying the thin connective tissue within the liver as well as increase technical risks such as needle dislodgment from the portal vein or air trapping with the vein.

The most challenging aspect of this protocol is the cannulation of the portal vein. This can be challenging to perform, particularly in mice in the 18- to 25-g size range. The techniques for collagenase perfusion were originally developed for use in rats and subsequently adopted for use

in mice after numerous modifications and adjustments. Cannulation using a 23G blood collection set is easier than placement of a catheter in blood vessels of small luminal diameter. Fixing the cannula using thread with a stopper knot is recommended to avoid dislodgement and the knot also serves as a marker of portal vein location in case the cannula comes off the vessel.

For downstream analysis, it is extremely important to have minimal contamination from cells. There are several important considerations in the handling of tissue after digestion. First, the forceps and scissors are changed when the liver is extracted to avoid blood contamination. Second, it is critical that the gallbladder be carefully removed from the liver to avoid tearing and unwanted contamination from bile. Third, once the liver has been removed from the mouse, the liver is washed very gently using PBS to remove any blood. Minimizing contamination with cells should be given a higher priority than reduction in the yield of EVs obtained.

Ultracentrifugation is the most commonly used method for the isolation and purification of EVs[8-11]. This approach will remove most parenchymal cells such as hepatocytes or cholangiocytes and non-parenchymal cells such as Kupffer cells, sinusoidal endothelial cells, and stellate cells, In addition, cell debris, cell aggregations, and dead cells will also be removed by differential centrifugation. Further purification and isolation of specific populations can be performed by size exclusion chromatography to remove any non-vesicular protein aggregates or lipoproteins.

A limitation of this protocol is that it may not capture all tissue vesicles, given the possibility that some vesicles may be removed in the perfusate. If a global assessment is needed, collection of perfusate and isolation of vesicles within perfusate should be considered. A further limitation is the potential for cell damage. To monitor the potential impact of excessive cell death, cell viability can be monitored and incorporated within quality parameters for tissue EV isolations. In conclusion, this procedure describes an optimized workflow using a two-step perfusion technique *via* the portal vein followed by differential ultracentrifugation for obtaining liver tissue EVs from mouse livers at a high yield. These tissue EVs are suitable for downstream analyses such as characterization of biomolecular composition and other studies that aim to characterize their physiological or pathophysiological roles or potential applications as disease markers.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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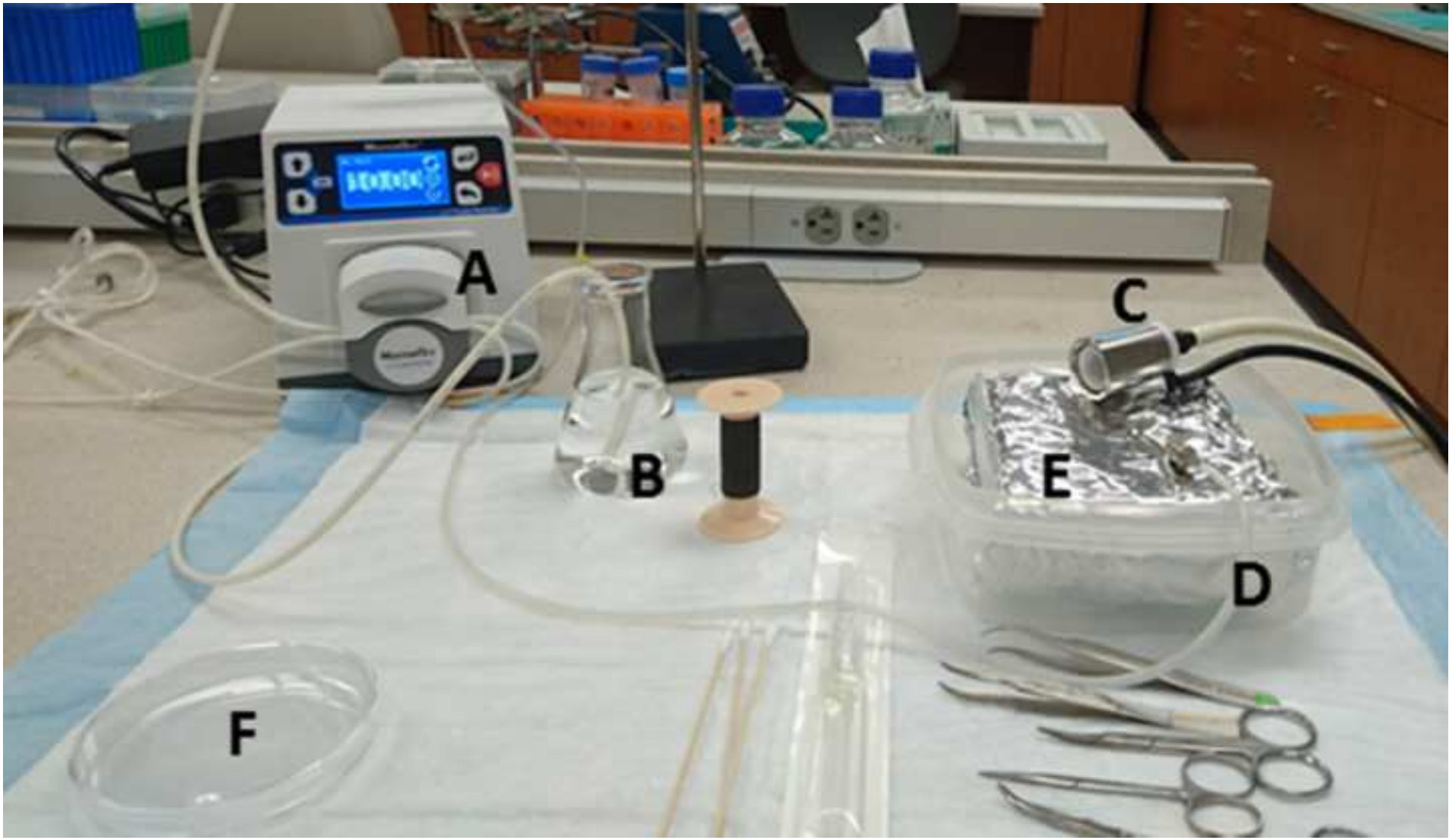
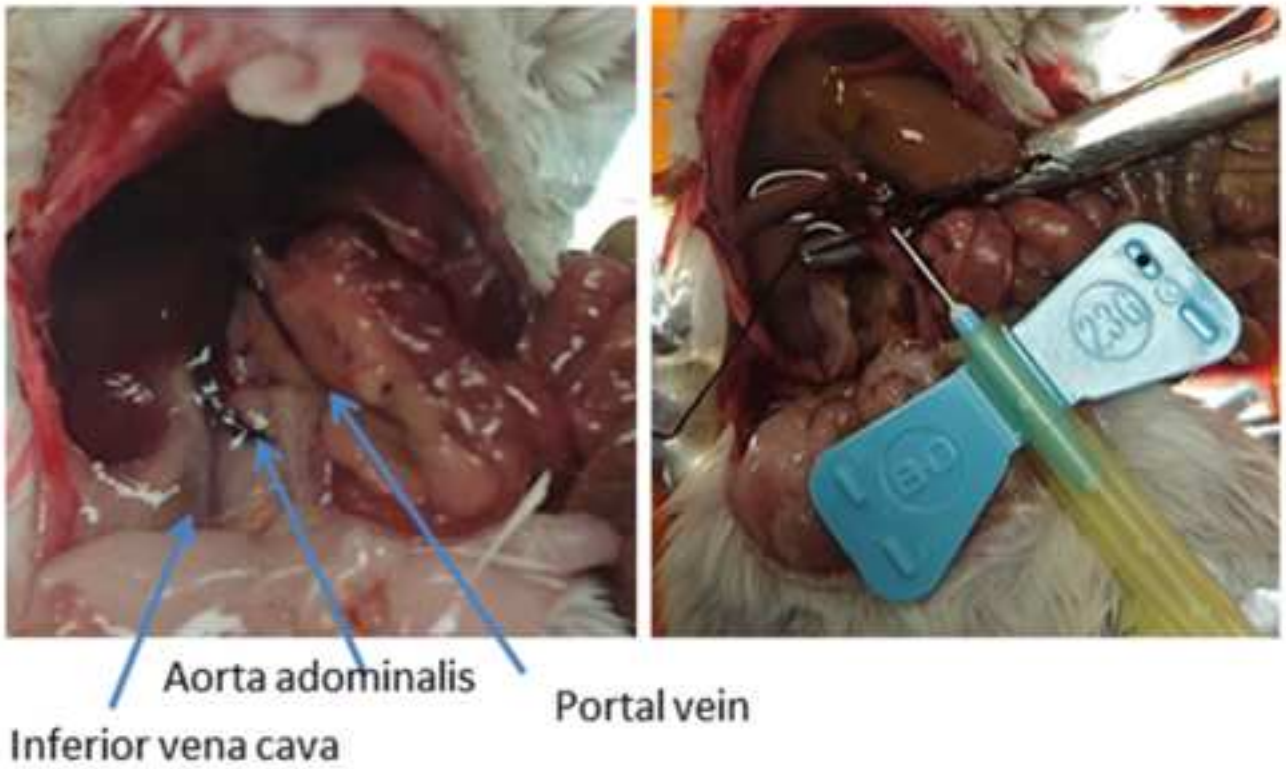
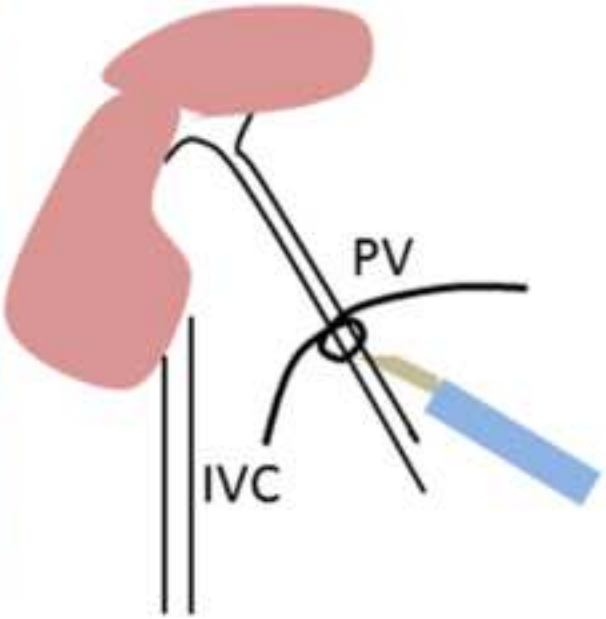


Figure 2





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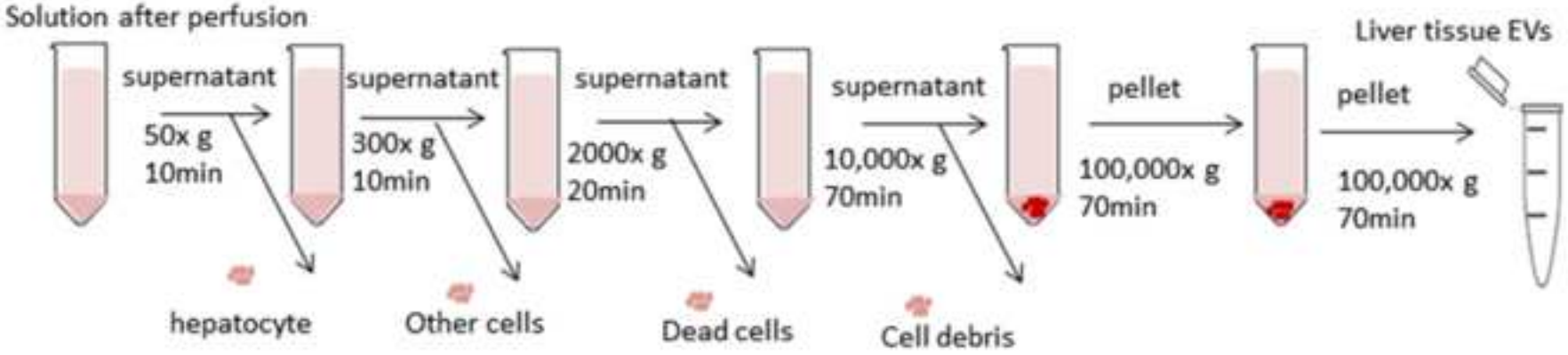

Figure 4

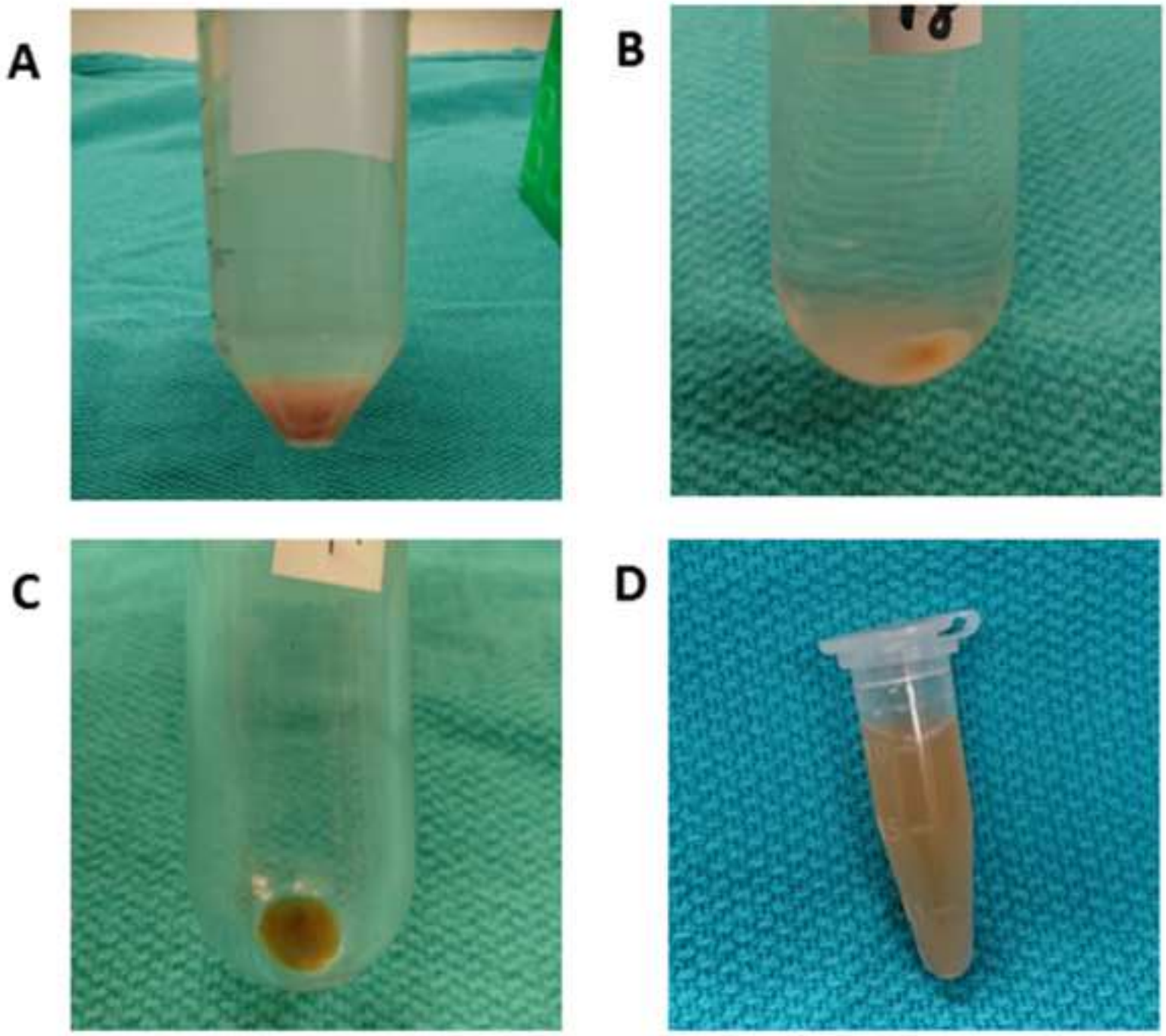

Figure 5

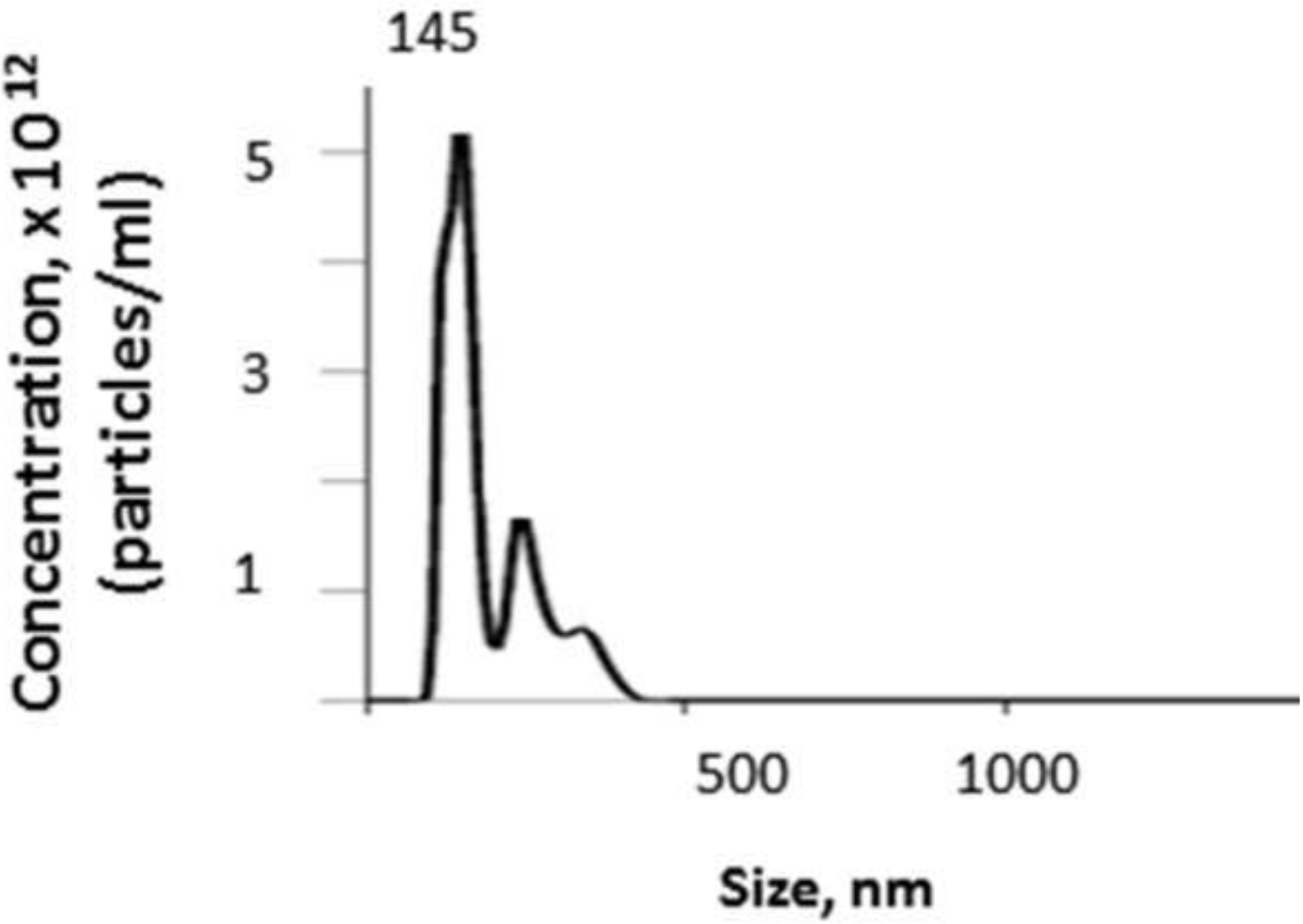

Table of Materials

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Equipment	Company	Catalog Number
125 mL Erlenmeyer flask	Fisher scientific	FB500125
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Curved non-serrated scissors	Fine Science Tools	14069-12
Curved forceps	Fine Science Tools	13009-12
Masking tape	Home supply store	
Aluminum foil	Home supply store	
Styrofoam pad	Home supply store	
Absorbent Bench Underpad	Scientific inc.	B1623
Masterflex L/S Digital Miniflex Pump	Cole-parmer	ZX-07525-20
Water bath	Thermo Electron Precision	2837
Blood collection sets	Becton Dickinson	367292
Petri dish, clear lid 100x15	Fisher Scientific	FB0875712
Falcon 70µm Nylon Cell Strainers	Fisher scientific	352350
50 mL conical tubes	Fisher Scientific	12-565-270
Cotton Tipped Applicators	Moore medical	69622
25mL Serological Pipet	Falcon	357525
Ohmeda Isotec 4 Isoflurane Vaporiser	BioSurplus	203-2751
O2 gas		
Isoflurane		
Levo Plus Motorized Pipette Filler	Scilogex	74020002
Centrifuge 5804R	Sigma-aldrich	22628048
Beckman Coulter Optima L-100 XP	Beckman	969347
Beckman Coulter Avanti JXN-26	Beckman	B34182
70 Ti Fixed-Angle Rotor	Beckman	337922
JA-25.50Fixed-Angle Rotor	Beckman	363055
Nalgene Round-bottom tube	Thermo Scientific	3118-0028
Polycarbonate ultracentrifuge tubes with cap assembly	Beckman	355618

Reagents	Company	Catalog Number

HyClone Hank's Balanced Salt Solution (HBSS), Ca/Mg free	Fisher scientific	SH30588.01
Collagenase, Type IV, powder	Fisher scientific	17104019
Phosphate Buffered Saline (PBS)	Fisher scientific	SH30256.01

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JoVE58649R1

We thank the Editors and reviewers for their thoughtful review and comments. A detailed annotated response to the comments follows:

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: Noted

2. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .svg, .eps, .psd, or .ai file.

Response: We have done this

3. Figure 3: Please include a space between numbers and their corresponding units (i.e., 50 x g, 10 min, etc.).

Response: These changes have been made

4. Figure 5: Please change 10e12 to 1012, and change ml to mL.

Response: We have revised Figure 5 accordingly

5. Please provide an email address for each author.

Response: Email addresses have been included

6. Short Abstract: Please revise it to be in complete sentences.

Response: We have made changes as appropriate

7. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

Response: We have rephrased the introduction

8. Please spell out each abbreviation the first time it is used.

Response: We have made changes as appropriate

9. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Response: We have made changes as appropriate

10. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Response: We have made these changes

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Response: We have made changes as appropriate

12. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: We have made changes as appropriate

13. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

Response: We have made changes as appropriate

14. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Response: We have made changes as appropriate

15. 1.5: What type of water is used here, running water or deionized water?

Response: Running water was used here.

16. 2.1: Please specify the age, gender and type of mouse used.

Response: The range of age, gender and type of mouse from which isolations have been performed is now listed in the representative results section.

17. 2.5: Please specify all surgical instruments used in the protocol.

Response: We have specified as appropriate

18. 3.3: Please specify the low flow rate used here.

Response: We have mentioned the flow rate used.

19. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Response: Revised

20. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: Done

21. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

Response: We have made changes as appropriate.

22. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response: Noted

23. Representative Results: Please move details about the protocol to the Protocol section, and move the discussion about the protocol to the Discussion. As Figure 5 shows the size and concentration of liver tissue EVs, please briefly mention the methods to measure the size and concentration of liver tissue EVs. For figures showing the experimental set-up, please reference them in the Protocol.

Response: We have made these changes as appropriate. Figure 1 is referred to in the Protocol session.

24. Discussion: Please also discuss any limitations of the technique.

Response: We have mentioned limitations within the discussion.

25. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

Response: We have made changes as appropriate

26. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Response: We have made changes as appropriate

27. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.
Response: We have made changes as appropriate

28. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

Response: We have made changes as appropriate

Reviewers' comments:

Reviewer #1:

1. The characterization of vesicles is inadequate to determine whether these are liver derived vesicles alone or whether there are other contributing particles such as lipoprotein particles and/or protein aggregates. To convincingly demonstrate that these are only liver derived vesicles, the authors would have to do either density gradient fractionation or column based separation and quantify vesicles versus protein aggregates versus lipoproteins. In addition, the characterization of vesicles in terms of nanoparticle tracking analysis alone is insufficient; they need to show via additional complementary methods including immunoblotting and electron microscopy with immune electron microscopy that they are isolating extracellular vesicles.

Response: We have included reference to this in the discussion section

2. Page 1, line 32. The method is really isolating vesicles ex vivo and not in vivo.

Response: We agree and have removed reference to in vivo.

3. Line 164. It should be clarified that this is the 10mL collagenase medium that was set aside in step 1.8.

Response: We have added this notation.

4. Line 139. The low flow rate for HBSS infusion should be included.

Response: We have included the flow rate.

5. Line 236. A range is provided 1.74 x 10e15 to 4 x 10e15 with a mean of 3.46 x 10e12 per mL. The mean does not fit within the range. Besides this calculation, number of vesicles obtain per liver or per gram of liver tissue should be also provided.

Response: Thank you for bringing this to our attention. The mean was 3.46 x 10e15 per mL. We have now corrected this.

6. While vesicles released during dissociation of the liver may be one way to collect liver derived vesicles, the authors do not demonstrate or compare how this method may compare with vesicles collected during perfusion of the liver by collecting perfusate. While this may not be important, as there is no established "gold standard," this should be included in the discussion as a possible alternative.

Response: We concur and have now included this within the discussion.

7. Page 1: Line 35: The first line of 'Introduction' is not grammatically correct. Please check.

Response: Thank you for bringing this to our attention. We have now corrected this.

8. Figure 5: Y axis is showing concentration 10e12 (particles/ml), but the average yield is 3.46X1015. In write up, there is also discrepancy between the ranges of EV concentration (1.74X1015 to 4.00X1015) and mean of 3.46X1012 particles per ml. Please recheck the values.
Response:

Response: Thank you for bringing this to our attention. We have now corrected the typos and revised Figure 5.

Reviewer #2:

For a balanced view, the potential drawbacks of the protocol shall be discussed. One common issue with the two-step perfusion of the liver is cell damage. The author shall add a step after the perfusion to check the cell viability. This can be easily done using Trypan blue. High viability is essential, as dying or dead cells will release intracellular components to the buffer affecting the purity of EVs.

Response: We thank the reviewer for the comments, and have included these considerations within the discussion.

In fact, though the author claims the method will produce EVs of high purity, they did not mention how the purity was checked.

Response: We have removed mention of purity of EVs.

During the process of perfusion, EVs can possibly be flushed out. How do the authors address this concern?

Response: We concur and have included these considerations within the discussion.

Another issue is the normalization of the yield of EVs. It appears that the yield of EVs with this method can only be normalized to the number of mouse used. Under pathological conditions, when the ratio between liver and body weight is significantly changed, this normalization approach can be problematic. I believe adding some discussions on these issues will make the manuscript more objective.

Response: We concur with the comments. While the yield can be normalized per mouse, we do not expect that the method will isolate 100% of the tissue EV. A loss of EV during ultracentrifugation can also be expected. Thus the comparison of yield is not likely to be useful. These issues are discussed in the representative results and discussion sections.

Reviewer #3:

Exosomes are secreted nanovesicles. Will liver perfusion and collagenase digestion cause the release of some kind of immature "exosomes"? Or will it contain other microvesicles - e.g. apoptotic bodies that are released from dead/dying cells resulted from liver perfusion?

Response: This protocol will isolate extracellular vesicles. These include exosomes, as well as microvesicles. Within the discussion, we have mentioned the use of cell viability as a quality measure to monitor for excessive cell death.

The authors didn't provide any western blots of EV markers. Thus, the quality of the liver tissue-derived EVs is not sure.

Response: We are not aware of any EV protein markers that provide information about quality of EVs. There are no universal markers of EVs.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done

2. Please provide more references to support your manuscript.

Done

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s). Please remove the parentheses before and after the reference numbers.

Done

4. Please use standard SI unit symbols and prefixes such as µL, mL, L, g, m, etc., and h, min, s for time units.

Done

5. Please do not highlight any steps describing euthanasia or anesthesia.

Steps are now not highlighted

6. Step 2.7: What's the size and depth of the incision? What is used to make a cut?

Additional detail provided

7. Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Done

8. Step 3.7-3.8: Please ensure that all text is written in imperative tense.

Text revised

9. 4.6: Please ensure that all text is written in imperative tense.

Text revised

10. 5.1-5.2: Please ensure that all text is written in imperative tense.

Text revised

11. Please ensure that the references appear as the following:
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