Purpose: Primary hepatocytes provide scientists with a valuable tool for evaluating metabolic, biochemical, and molecular functions in a physiologically relevant, readily controlled *in vitro* experimental system. However, as is the case for all primary cells, there are unique considerations that must be taken into account to minimize batch-to-batch variability and ensure the quality, reliability, and reproducibility of data. The following protocol outlines the materials and methods for the (relatively) time- and cost-effective isolation and 2D-culture of high-quality, responsive, and functionally-consistent primary mouse hepatocytes. These techniques were developed using the C57BL/6 strain of mice (6-20 weeks of age; 20-40g total body weight), have been scaled successfully in rats, and may, with minimal adjustments, be applicable to related species.

Background: Historically, primary hepatocytes have been utilized for the study of drug metabolism, xenobiotic transformation, metabolite processing, hormonal function, and numerous other purposes. When properly isolated, hepatocytes strongly adhere to a variety of surfaces, including standard vacuum-plasma-treated polypropylene cell culture dishes, gelatin, collagen, and various ECM-based coatings, and remain functionally viable for at least 48 hours post-plating. The majority of hepatocyte-based studies are completed within this time frame, and while many authors have published data using > 3 day-old cells, longer-term (> 2 days) preservation of hepatocyte morphology and function appears to have more to do with the culture and/or substrate conditions, and less on the actual isolation procedure.

Our experience is limited to the isolation and short-term culture of hepatocytes, and towards this end, our goal is to simply provide the information needed for the isolation of healthy, fully functional cells. Instructions for the performance of select functional assays will be added in the near future, as these will serve as benchmarks to assess the quality and consistency of isolated hepatocytes.

We have successfully and consistently performed a number of assays in isolated hepatocytes, including:

Readout	Tested agonists*	Tested antagonists*	Tested metabolic substrates*
ATP (intracellular)	Rotenone		Glucose
Beta-oxidation	Carnitine	Etomoxir	Palmitate (3-H)
EROD	PCBs, BNF	Multiple	
Glucose output	Glucagon, forskolin	Insulin, AICAR, Metformin	Lactate, pyruvate, glycerol
Glycogen synthesis	Insulin	Glucagon, forskolin	Glucose, lactate, pyruvate, dihydroxyacetone, glycerol
Glycogenolysis	Glucagon, forskolin	Insulin	
Immunoblotting	Insulin, Adenoviruses, Lipofectamine	Kinase inhibitors	Free fatty acids, glucose
Lactate ouptut			Glucose
LDH release	Rotenone, hypotonic lysis		
Lipogenesis	Insulin		Glucose (14-C)
Oil Red O staining			BSA-coupled palmitate, oleate, stearate, & linoleate
qRT-PCR	Multiple	Multiple	Multiple

*All tested agonists/antagonists/substrates listed are those which we have observed to perform in a manner consistent with the known mechanism of action as well as the published literature.

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*Materials for isolation**

1) Peristaltic pump capable of 1-10mL/minute.
2) Reservoir capable of holding between 50-100mL of solution
3) Bubble trap
4) Waterbath @37C
5) Sterilized 100mL wide-mouth glass bottles
6) Sterile 50mL conical tubes
7) 70 micron filter; disposable mesh or reusable stainless steel [1]
8) 10cm petri/cell culture dishes
9) Sterilized dissection tools; at minimum, one pair of scissors and two pairs of fine-tip forceps
10) 70-75% EtOH and detergent, both in spray bottles (iodine optional)
11) Anesthetic, either injectable (PentoBarb) or inhalable (Isofluorane)
12) Vacutainer brand butterfly cannula; recommended gauge for mice = 23-3/4 [2]
13) Face mask
14) Absorbent bench pads (2 per mouse)
15) Elevated working surface/platform/board
16) Lab/masking/duct tape

Reagents for isolation

1) 1x Hank's buffered salt solution (HBSS), without magnesium or calcium, with 0.5mM EGTA [3]; 60-70mL
2) DMEM-low glucose with 1x Penn-Strep and 15mM HEPES (Digestion medium) [4]; 90mL
Make sure the DMEM you use contains calcium
3) Isolation medium [5]; 120mL
4) Collagenase, Type IV [6]; sufficient quantity for 100 units Collagenase Digestive Units (CDU)/mL [7]
5) Autoclaved water

Materials & Reagents for plating

1) Type I Collagen, from rat tail; BD #354236 [8]
2) 0.02N AcAc, sterile filtered, for diluting Type I Collagen
3) Culture dishes/plates, collagen coated and rinsed with sterile 1x PBS before use [9]
4) Trypan Blue, 0.4%
5) Hemacytometer

*A comprehensive materials and reagents list may be found at the end of this document.

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A. Pre-isolation preparation

1. Prepare all necessary reagents. For each mouse, you will need (see details for reagents in *materials*):

a. 60-70mL HBSS for initial flushing/washing; 37C
b. 90mL DMEM-low glucose (digestion medium); 37C
c. ~120mL isolation medium; 4C
d. Sufficient collagenase to make 90mL of digestion solution at 100 CDU/mL; store desiccated at 4C until ready to use

Again, note that these quantities are based upon the C57 strain of mice weighing 20-40g.

2. (Preferably under the hood) Dispense pre-measured collagenase aliquot into digestion medium, swirl gently, and allow to dissolve for 30-60 minutes at 37C. We have observed that the efficiency of the collagenase is more consistent (based on total cell yield) when it is allowed to dissolve for at least 30 minutes, even though it appears to go into solution instantly. Put HBSS into waterbath to warm at this time as well.

3. Prepare work surface. A sterile environment is preferable, but not necessary for the initial isolation. Spray down all non-porous surfaces with 70-75% ethanol.

4. Rinse out the pump/tubing by running 70-75% ethanol through and allowing it to sit for >20 minutes. Remove residual ethanol by rinsing twice with autoclaved water. Make sure the bubble trap is thoroughly cleaned and rinsed as well.

5. Take two absorbent bench pads and lay one pad down on the bench; use the other pad to cover a hard board (e.g. a stiff piece of cardboard or Styrofoam). As this is a non-recirculating perfusion, all liquids will run to waste; it is advisable to fold the bench pad in such a manner that excess liquids will be trapped within, to avoid making a mess.

5a. If desired, you may set the board inside a shallow-wall container. This is optional for the quantity of liquid used during a mouse perfusion (the pads should provide sufficient holding capacity), but may be necessary when working with larger volumes of liquid (i.e. rat perfusions).

5b. You may find it more comfortable to elevate the hard board slightly, as this will aid draining of liquids, as well as bring the work surface closer to your eyes.

6. Bring a 10cm culture dish (sterile) to the bench. This will be used to hold the digested liver once the perfusion is complete.

6a. If using a reusable Dual Mfg 75 micron filter, make sure that it is cleaned/sterilized beforehand [10]

7. Put the following on ice: Bottle containing isolation medium (~120mL)
50mL sterile conical tube

8. Ensure the following are warm: HBSS
Digestion medium w/collagenase

9. Attach your cannula to the free end of the tubing. You may find it preferable to cut the "wings" off of the butterfly cannula, as it reduces the effort needed to hold the needle tip, as well as offering better handling.

10. Immediately before knocking out your mouse, pour warmed HBSS into the reservoir and prime the pump, making sure that the bubble trap contains sufficient HBSS (at least 1/4, but no more than 1/3 full) and that there are no air bubbles in the system, particularly past the bubble trap. If bubbles are present in the tubing past the bubble trap, turn on the pump and elevate the affected section of tubing to drive the bubbles out. Do not be concerned about losing a few mL of HBSS.

> 10a. After priming the pump and ensuring that there are no visible air bubbles, turn the flow rate to a slow drip (1-2mL/min) and shut the pump off.

B. Cannulation, perfusion, and digestion

1. Anesthetize the mouse. Isofluorane is recommended, as it has minimal impact on liver metabolism.

2. Secure the mouse, ventral side up, to the working platform; tape or pin down all four limbs. Tape is preferred to minimize distress, should the animal wake from anesthesia.

3. Thoroughly clean the abdomen/chest region, using EtOH + detergent, and/or iodine. Work quickly but thoroughly at this step, as flora from the fur is the first potential source of contamination. If the animal defecates, be sure to clean it up and disinfect the area.

4. Pick up a pair of scissors and your straight forceps. Make an incision in the lower abdomen, cutting through the fur and muscle layer; try to puncture through the muscle layer as soon as you make your incision (this is why sharp-tipped scissors are recommended). Do NOT nick any internal organs, particularly the intestines. Pull up on the fur while cutting to minimize this risk.

5. Continue to cut vertically until the liver, portal vein (PV), and inferior vena cava (IVC) are sufficiently exposed. It is easiest to make alternating cuts on either side of the skin while pulling up. Make a final incision on the left (your left) side of the abdomen, near the navel region, so that blood can readily drain once you cannulate and perfuse.

6. Start the pump (which should be set at a low flow rate already) and wait until HBSS begins to flow from the tip of the cannula; with HBSS flowing, swiftly, and in one motion, insert the cannula into the PV.

> 6a. If performed properly, the liver should ***instantly*** begin to blanch. Once you have confirmed that the cannulation is successful, quickly cut the IVC to relieve pressure in the system and allow perfusate to drain to waste (having an assistant perform this task is highly recommended). Once the IVC has been cut, the liver should finish blanching, and become pale in color. Blanching is easiest to monitor under bright, directed lighting.
>
> 6b. If the liver does NOT blanch instantly, particularly after the IVC has been cut, there are two likely sources of error: occult air bubbles blocking microcapillaries, or user error (i.e. missing the portal vein and cannulating a pancreatic duct instead). The former is more common, which is why it is essential that HBSS

is allowed to drip from the cannula tip during perfusion (the continued presence of liquid displaces any unseen air in the bevel/tip region of the cannula.

6c. Alternatively, you may perform a retrograde perfusion by cannulating the IVC and cutting the PV for drainage. Klaunig et al. (1980) found that PV cannulation resulted in higher yields than IVC cannulation, although the difference was not dramatic, nor were viability estimates significantly different. Due to the significantly larger size of the IVC, you may find it easier to work with; our experience, however, is all based on PV cannulation with IVC drainage.

7. Once the IVC has been cut, increase the flow rate to 7-9mL/minute. Allow the entire volume of HBSS to perfuse through the liver. The flow may be increased in increments of 1-2mL/second (have your assistant monitor and adjust the flow).

7a. A quick test for successful cannulation can be performed by applying light pressure on the IVC; at $\geq$8mL/minute, all lobes of the liver should instantly begin to swell.

8. When the reservoir (NOT the bubble trap) is just about to run out of HBSS, pour in 70mL of the digestion medium (fill the reservoir to the top). It is not necessary, nor is it recommended to stop the pump. There will obviously be some dilution (by HBSS) of the initial few milliliters of digestion medium, but this is inconsequential. Pour the remaining digestion solution (~20mL) into the 10cm plate. Close the plate quickly (again, these are tasks that are greatly facilitated by a second pair of hands).

8a. One technique which may increase yield and reduce total digestion time is to periodically (5-10 times during digestion) apply pressure to the IVC for 5-second intervals (as mentioned in step 7a). This will cause the liver to swell, and the increased pressure during the clamping aids in dissociation, and therefore, final yield. We strongly recommend this be performed as standard operating procedure.

9. As digestion progresses, you should see the liver begin to swell. This presumably occurs as a result of collagenase breaking down the elastic structure of the liver. When this swelling starts, you should be very close to the end of digestion.

9a. If you performed step 8a, you cannot use this as an indicator, as the liver will swell and contract as you apply and release pressure. Therefore, to monitor completeness of digestion, note that as digestion continues, the liver will contract less with each IVC clamping cycle, due to progressive loss of elasticity.

10. There is no definite rule as to when digestion is complete. Generally, once the liver has begun to swell, 1-3 minutes of additional digestion should suffice. You may or may not see small clear/transparent sections on the lower lobes; furthermore, the liver will take on the texture of a wet piece of cloth, and appear almost soggy.

11. The extent to which you allow the liver to digest is almost entirely dependent upon how skillful you are in excising the organ; one or two minutes of prolonged digestion will not affect viability if a sufficiently gentle (i.e. low tryptic activity) collagenase is used.

12. When you are satisfied with the digestion, turn off the pump and carefully remove the cannula.

C. Extraction and purification

1. Using your straight-tipped forceps, gently expose the central region the liver by carefully working through the lobes. Locate the bundle of fibers connecting the lobes. At this point, switch to your curved, fine-tipped forceps and securely grip the center of this bundle. Do not rush this step, as it is essential that you have a solid hold on the liver for a complete excision.

2. Gripping the liver, pull the organ away and towards you (try to get behind the liver, and free the lobes from the chest cavity), exposing the chest cavity and the various connective tissues holding the liver in place. Carefully cut these connections, slowly pulling the liver forward and out as you cut. You may wish to switch to a fresh pair of scissors for this step, as your previous pair may be covered in blood and fur. Take care to avoid nicking any internal organs.

3. Once a sufficient number of connections have been severed, you should, with moderate effort, be able to pull the liver free. Do NOT tear the gall bladder; if you wish to do so, remove the gall bladder at this step.

4. Immediately place the liver into the 10cm dish containing digestion medium, and cover the dish (assistant).

5. [Preferably in the hood] Using two pairs of forceps (either another sterile set, or the same set, cleaned with EtOH and/or flamed), tear apart the lobes of the liver; if still present, avoid disrupting the gall bladder.

> 5a. If the liver has been properly digested, you should see the medium turn cloudy as you tear, and the liver should mostly dissolve into the medium. Ideally, all that will be left is a stem of connective tissue, but often, you will have some residual lobe matter as well. If, upon tearing, you end up with chunks of liver and little to no clouding of the media, something went wrong during the digestion. See troubleshooting section.

6. Once torn apart, grab the remaining section of the liver and shake gently to free residual cells. Discard any solid particles that remain, including the gall bladder (if you chose not to remove it in step 3, the gall bladder should remain intact during this process; if it burst, you either used too much force, or accidentally tore it open with your forceps).

> 6a. A torn gall bladder should not have any adverse effect on cell viability or function, but this is not something we have tested. In the interest of batch-to-batch consistency, try to minimize gall bladder bursting.
>
> 6b. Reminder: all steps involving handling of hepatocytes must be performed with care, using sterile equipment. The cells are very fragile at this point and are susceptible to shearing damage.

7. In the same 10cm dish, triturate the suspension three times using a 25mL serological pipette.

> 7a. If you are using a Dual Mfg stainless steel filter, make sure you pre-wet the bottom of the filter at this time, or even before you start tearing the liver. Simply pipette 2-3 mL of isolation medium onto the bottom of the filter, swirl until the entire surface area is wet, and discard the remaining medium.

8. Filter the suspension through a 70-75-micron membrane. If you are using the Dual Mfg filter this step should be very fast; with BD disposable falcon tube nylon filters, this will take some time, and possibly a few filters (a sterile eyedropper pipette might be easier for falcon tube strainers).

> 8a. If using the Dual Mfg filter, your cells will be in a 95mm dish; transfer them into a sterile, clean 50mL conical tube. For the falcon tube strainers, your cells will already be inside a 50mL conical tube and ready to use.

9. Spin at 4C for two minutes at 50 xg in a swinging-arm centrifuge. The centrifuge does not have to be refrigerated, although this is preferred.

10. (in the hood) Aspirate the supernatant (should be cloudy/opaque) using a sterile glass Pasteur pipette and add 25mL of cold isolation medium. Triturate gently a few times to break up the cell mass at the bottom and resuspend.

11. Repeat steps 9 and 10 two more times, for a total of three washes. The medium should be almost completely clear by the second wash, and clear by the third.

12. After the final spin, aspirate the medium and add 25-45mL of cold isolation medium. You want your cells to be concentrated enough so that you do not have to re-spin them prior to plating, but not so concentrated that they will be difficult to count. Resuspend cells gently with a 25mL serological pipette. Remove an 80uL aliquot and transfer it into a microfuge tube for counting/trypan blue staining.

D. Staining, quantitation, and plating

1. Add 20uL of 0.4% trypan blue to your 80uL cell suspension aliquot. Pipette up and down several times to mix.

2. Allow to stain for ~1 minute at room temperature.

3. Pipette up and down again, and take a 10uL aliquot and dispense onto a hemacytometer.

4. Count all non-blue, non-blebby cells. Note that trypan blue OVERESTIMATES viability. Cells which take up the dye are certainly non-viable; however, cells which do not stain may nonetheless be damaged and useless. In our experience, viable, healthy hepatocytes have a bright, clear, smooth appearance and are relatively small and rounded up. Dead/damaged cells generally are swollen and appear rough and granular. Viability MUST be over 85% *at minimum* to ensure batch-to-batch consistency.

> 4a. From a typical 8-12 week-old C57 mouse liver, you can expect total yields on the order of 30-50 million cells. The average yield should be ~40 million, and yields below 30 million indicate that something was wrong with the procedure (most likely attributable to digestion/cannulation).

5. After counting cells (make sure you correct for the 25% dilution factor due to trypan blue), make a working dilution for plating. A general estimate for dilution is to add three million (viable) cells per plate, at 10mL total volume per plate, or 300,000 cells/mL.

6. As a general guideline, we recommend:

a. 8mL/plate for 10cm plates (7-9mL OK)
b. 1.7mL/well for 6-well plates (1.5-2.0mL OK)
c. 800uL/well for 12-well plates (700-820uL OK)
d. 360uL/well for 24-well plates (340-450uL OK)

These volumes have been found to give the most consistent plating results, as they minimize the tendency for cells to cluster in the middle or on the edges. See Troubleshooting #13 for a special note about 24-well or smaller plates.

7. Before plating all cells, you should plate a single well and double-check under the microscope that the actual density is close to what you assume it to be, based on your counting. Make sure to shake the plate thoroughly (in a back-and-forth, and NEVER in a circular fashion) and allow the cells to settle for ~30 seconds before looking at them. In a typical prep of >90% viable cells, you should aim for approximately 60-70% confluence at this time. This level of confluence allows for cell-cell contact, while maintaining sufficient space for the hepatocytes to grow to their full size, and will yield a final confluence of 90-95%.

8. After confirming that the cell density is OK and making any necessary adjustments, plate cells down. Make sure you resuspend cells after every minute or two, as hepatocytes are quite dense and settle rapidly. Generally, you should resuspend cells after every two 12-well plates, or every (one) 24-well plate.

9. Thoroughly shake your plates in a linear fashion before placing them in the incubator; this step is critical, as the cells tend to sink straight down and attach, and failure to spread them out in a homogenous monolayer at this time will result in a patchy, inconsistent lawn.

10. Allow cells to attach for 45-60 minutes @37C after plating.

E. Post-plating and overnight culture

1. After cells have attached, wash once with DMEM-low and add back culture media (with 10% FBS) for 3-4 hours.

1a. At this time, if your cells are healthy, nuclei should start to become visible under low power. Cells will still be rounded, but somewhat larger; note that clusters of cells tend to recover more rapidly than single cells.

1b. Use caution when changing the media at this time, as the hepatocytes are still relatively fragile and can be easily damaged or disrupted by direct contact; pipette only down the side of the well, and never directly on top of the cells.

2. After the first 4-5 hours of plating, we suggest that cells be kept in serum-free medium. This helps to maintain their morphology, and there are no adverse effects to serum-free medium. The most critical time during which serum must be present is the first hour of plating, where is has been suggested that the various proteins found in serum aid in attachment.

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Notes

[1]	Disposable, sterilized cell strainers:	BD	#352350	70um
	Reusable cell strainers:	Dual Mfg	#US3-200S	74um
	(Brass frame/stainless steel mesh; #200 mesh/74um; 3" diameter x 1" depth)			

Disposable BD strainers fit over standard 50mL conical tubes

Reusable Dual Mfg strainers work best with 95mm diameter culture plates; the lip on the strainer frame sits on top of the 95mm plate perfectly, while 100mm plates are a bit too wide, causing the strainer to sit entirely on the bottom of the plate.

Dual Mfg strainers are autoclavable, although we have not found it necessary to autoclave them every time. Washing well with detergent and hot water, followed by thoroughly spraying with 70-75% ethanol is sufficient. Make sure you allow the ethanol to dry (either air-dry in the hood, or use a heat gun) before use. Furthermore, make sure you wet the bottom of the strainer immediately before use with sterile media, to facilitate flow-through.

Do NOT expose cell strainers to direct fire- the solder used to seal the mesh to the frame will melt. Likewise, if using a heat gun, do not use too high a temperature setting (<300F).

[2] These cannulas have a large beveled tip, which aids flow rate, but also requires some experience to properly insert into the portal vein. A viable alternative is to use fine-bevel Teflon-sheathed cannulas.

[3] See formulation sheet for ingredients. The HBSS is formulated based on Invitrogen #14175, with the addition of 0.5mM EGTA and 25mM HEPES, sufficient NaOH to bring the pH to 7.4@37C. NO oxygenation of any buffers or media is necessary.

[4] We have had success with Mediatech/Cellgro DMEM-Low #10-014-CM, which contains 200mg/L CaCl2 (~1.8mM).

[5] See formulation sheet for ingredients. This contains 10% FBS, which helps to quench collagenase activity; the fibrinogens in serum may also aid in cell attachment, even in the presence of a collagen coating. If you wish to conserve media, perform the initial wash and final resuspension in isolation medium, and the intermediary two washes with serum-free medium (i.e. 50/50 DMEM-High/F-12).

DMEM-High:	Mediatech/Cellgro #10-013-CV
F-12:	Hyclone #SH30026.01

[6] The type and batch of collagenase you choose for isolation is absolutely critical. We and others have found that many batches of Type I collagenase, which is the most common form used in the literature for primary hepatocyte isolation, is far too harsh, and tends to damage cells and/or cleave receptors, compromising viability and function. We highly recommend a Type IV collagenase, often used for beta cell isolation, as this type contains ~10x less tryptic activity.

Worthington is strongly recommended as a source for collagenase; their customer service and quality control are exceptional, and their collagenase sampling program allows you to test batches of collagenase before committing to a specific lot.

[7] There are various standards used for assessing collagenase activity; most collagenase powders (including those from Worthington) contain other enzymatic activity which have been found to be important to digestion. As a result, collagenase selection is not an exact science, and batches must be tested. To ensure consistency, it is recommended that collagenase be quantified solely on the basis of its actual action of collagen digesting activity. In other words, 100 CDU/mL refers to the collagenase activity, exclusive of other enzymatic (e.g. tryptic) activity- hence the importance of selecting the right type and batch of collagenase.

We have found 100 CDU/mL to be an optimal concentration of collagenase; to adjust for liver size/animal age, simply vary the total volume and/or flow rate. For example, you may use 100 CDU/mL even for a rat, so long as you scale the flow rate and total volume as needed.

[8] Type I Collagen from rat tail: BD #354236. This comes in 100mg lots of varying concentration (generally ~3.5mg/mL) dissolved in 0.02N acetic acid. The concentration of collagen you use to coat your wells can have dramatic effects on the attachment, growth, and performance of your cells. According to BD, the concentration of collagen they use for coating is between 5-8ug/cm^2; cm^2 refers to the surface area of the bottom of the well- check the manufacturer's specifications, as surface area may not scale linearly with well size. We recommend 5ug/cm^2 as a general rule. Plating by this method is not only more consistent than the standard "pipette 1mg/mL collagen in, remove excess, repeat for next well" method, but also much more economical.

[9] For actual coating, add the desired volume of collagen/0.02N acetic acid, shake/tap the plate to distribute the collagen evenly, and let dry under UV for 1.5-2 hours, or overnight. Rinse with PBS before use. You may use a repeater pipette to speed the process up- just make sure that you allow the plates to have sufficient UV exposure if you are using non-sterile repeater tips. For coating, simply add the required quantity of collagen in enough volume of 0.02N AcAc to easily coat the bottom of the dish, but not so much as to significantly coat the sidewalls (to minimize waste and evaporation time). For example, ~100-150uL of total volume/well for a 12-well plate, and 50-75uL/well for a 24-well plate.

Collagen coated plates may be sealed with parafilm and stored at 4C for months; it is generally recommended, though, that you use plates within two weeks.

[10] See notes in [1] for cleaning/sanitizing reusable cell strainers.

Troubleshooting

1. Liver does not completely blanch/only one lobe blanches/odd blanching (vascular appearance)/other blanching anomalies.

This is almost always caused by air bubbles entering the portal vein. Check the entire length of tubing after the bubble trap for occult air bubbles, particularly the section of the tubing leading to the cannula, as well as the actual cannula itself. Ensure that a slow drip is present during actual insertion of the needle, as this will push out occult air pockets in the bevel of the needle.

Other causes include improper cannulation (i.e., partial cannulation of portal vein), cannulation of a neighboring duct (evidenced by inflation of the pancreas), clotted blood (caused by allowing the animal to die too soon before cannulation- you generally have less than 30s after death, unless you injected heparin beforehand), or improper buffer osmolality (is your HBSS really 1x?).

2. Liver immediately swells (excessively) upon increasing flow rate after initial cannulation, even after IVC has been cut.

Check the osmolality of your HBSS, and make sure you are actually perfusing HBSS, and not H20.
Make sure the blood has not clotted already- is there any liquid coming out of the IVC?
Make sure you did not introduce air into the system after cannulation- did you compromise the portal vein at any location aside from the insertion point? Was there an air bubble in an earlier section of the tubing you missed?

3. Liquid drains from the insertion point in the portal vein, in addition to coming from the IVC.

This is likely to be caused by a nick in the portal vein, due to movement of the needle tip within the vein. If it is a minor leak, and you still see the majority of the liquid draining from the IVC, you should be OK. If, however, you do not see the liver swelling at all when you clamp the IVC (i.e. nothing within 5 seconds), the leak may be too severe for perfusion to continue effectively.

4. An organ besides the liver swells, and upon perfusion of the digestion medium, appears to be digested as well.

This organ is almost certainly the spleen, as the portal vein is formed from the junction of the superior mesenteric vein and the splenic vein. This is common when the portal vein is cannulated at too low (distal from the liver) a location, or when excessive systemic pressure forces perfusate into the splenic vein (blood clotting/congealing). Cannulate at a location more proximal to the liver, as this will bypass the splenic vein, which in turn directs the majority of the flow to the liver.

It has been our experience that so long as there is sufficient flow going to the liver, digestion of the pancreas/spleen, while obviously not desirable, should not have any negative impact on the actual isolation procedure. In such instances, digestion time may have to be increased to accommodate the lower flow.

5. Liver swells on its own after a few minutes of digestion

This is normal provided you have perfused roughly 50% or more of your target volume. If, however, you notice that total yield is high, but viability low, this may be an indication that your collagenase concentration is too high and/or you are using too harsh a collagenase. Remember that 100 CDU/mL is a concentration we and others have found to be optimal with batches of collagenase we have tested; your own results may vary. We have not, however, ever experienced an instance where known working collagenase (which had been stored and handled properly) suddenly went bad. If you know your collagenase was working last week, or even last month, look to other sources of error.

6. The perfusion started out fine, but the needle slipped (happens for any number of reasons). Can I re-cannulate?

Assuming that the initial cannulation and blanching were ideal, our experience is that re-cannulation can be perfectly fine, assuming you have the skill and luck needed to cannulate an already compromised, collapsed vein. The recommended course of action is to lower the flow rate back to 1-2mL/min and cannulate again as quickly as possible. Each successive attempt lowers your chances of success, as the portal vein is quite fragile to begin with. Do not be surprised if your success rate for re-cannulation is several-fold lower compared to cannulation of a fresh portal vein.

7. The liver does not appear digested, even after perfusing all of the collagenase solution.

First, confirm that the liver indeed is not digested well, particularly if you do not have extensive experience in isolation of primary hepatocytes. Excise it and tear it apart in your plate of digestion medium- if you do not see the medium become cloudy after tearing and shaking, and if the liver comes apart in solid chunks, there is almost certainly a problem with digestion.

Digestion problems are generally related to improper perfusion, assuming total collagenase concentrations are not drastically lower than 100 CDU/mL. Blanching of the liver must occur almost instantly and completely upon perfusion with HBSS- you should see the liver start to turn pale when you first cannulate; once the IVC is cut and the flow rate turned up, the liver should suddenly and completely turn pale. If it does not, you will almost certainly fail to digest it, no matter how much you attempt to force the solution in (i.e. increasing the flow rate, clamping the IVC).

Also, make sure that the DMEM you chose has sufficient $CaCl_2$ (the one we recommend has ~1.8mM).

Temperature is almost never a culprit here- a few degrees below 37C should have negligible impact on digestion efficacy.

In order of importance, as concerns digestion:

A. Blanching/initial cannulation
B. Sufficient washing with HBSS/presence of 0.5mM EGTA in HBSS
C. Presence of sufficient CaCl2 in digestion medium
D. Concentration of collagenase
E. Length of perfusion
F. Flow rate
G. Temperature

8. Yield and/or viability problems

It is nearly impossible to efficiently troubleshoot both yield and viability issues concurrently. Yield issues should be dealt with first, and then viability.

Low yield is almost always related to blanching and/or collagenase concentration. Blanching is covered in the previous section, so we will focus here on collagenase. Regardless of viability, your primary goal should be high total yield. This generally means >20-25 million cells for a 20-25g mouse. If your yield is consistently ~25% less than that, consider raising your collagenase concentration by 30%. If your yield is consistently <10 million, double the concentration of collagenase. If you still have issues, try a few other batches of Worthington Type IV collagenase. As a troubleshooting step, try Type I collagenase as a positive control for digestion-- you should have very high yield, and very likely will have low viability. If even 100 CDU/mL of Worthington Type I collagenase does not solve your yield problems, there is something fundamentally wrong with your system (i.e. EGTA concentration of HBSS is too low or too high, CaCl2 concentration of digestion medium is too low, cannulation technique is poor, etc).

If increasing collagenase does not bring your yield up to $\geq$20-25 million, the problem likely resides in the actual perfusion. Your flow rate may be compromised by air trapped in the tubing, or the flow rate may simply be too low. Always refer to the appearance of the liver when digestion is complete- is it soggy and enlarged, with the consistency of a wet paper towel? Is it difficult to excise (intact) due to its fragility? Does it readily tear apart and cloud the medium? Do you have a readily visible pellet after spinning down?

Once yield issues have resolved, viability, if problematic, can be dealt with by tweaking collagenase type and/or concentration. Flow rate and digestion times may need to be tweaked for much larger (>35g) or much smaller (<20g) mice, but their contribution to total yield and viability is small compared to the quality of the cannulation and collagenase type.

For working with significantly smaller or larger animals, make initial adjustments to the flow rate first, and only change the digestion time if absolutely necessary. Collagenase concentration should remain static, assuming you have a known working stock and concentration. We have scaled this protocol to rats by increasing the flow rate from 10mL/min to 22mL/min, with only a slight increase in digestion time (~10-15%) and no change to collagenase concentration (held at 100 CDU/mL). Remember, when isolating primary cells, time = quality; it should take no longer than 15-20 minutes from the instant you cannulate until you have excised the liver.

9. After spinning down cells, the pellet is substantial, but total yield is low

Having a large pellet does not guarantee that you will have high yield and/or viability. On the other hand, if you have almost no pellet, it DOES guarantee that you have few cells.

If the digestion was complete, and the previously listed criteria met, this should never be an issue. However, it is possible that the cells were sufficiently damaged (during digestion, filtering, and/or washing) such that the majority of the pellet is composed of cell particulate. If your hemacytometer contains almost entirely cell particles/debris, this is likely the case. If so, treat the cells more gently, and/or cut back on your collagenase concentration by 30%.

10. Hepatocytes do not attach to plate---or, hepatocytes attach, but do not appear healthy

It is entirely possible to have a batch of cells that have been damaged during the isolation process, yet do not take up trypan blue. These are most often larger, vacuolated cells that do not reflect light well. Refer to previous sections pertaining to yield and viability.

As it is nearly impossible to perform all steps aseptically, there is always the remote possibility of contamination. If you find that your cells are not recovering as they should, keep a close watch for bacteria.

The type of plate matters for cell health, due to the surface area:volume relationship, which magnifies the "edge effect" as microwells become smaller. In our experience, it is much easier to have healthy, confluent, homogeneous monolayers in 12-well and larger plates, but much more difficult in 24-well and smaller plates. This is almost entirely due to the fact that hepatocytes tend to crowd in the middle of smaller wells, creating a ring of cells around the edge of the well, and a massive confluence of cells in the middle of the plate, which tends to be less than healthy due to excessive crowding. We therefore recommend that you learn to culture in 12-well or larger plates, and scale down only when you are confident in your technique.

We have had limited success with standard tissue culture-treated collagen-coated plates, whether we coat them ourselves or purchase ready-to-use plates. However, initial tests suggest that UNTREATED (non tissue culture-treated) polystyrene plates may be a viable option. While these plates are generally used when cell attachment is not desired, you will be coating the bottoms with collagen, so this is a non-issue; however, the lack of tissue culture treatment means that the plastic is hydrophobic. This reduces the magnitude of the meniscus, and cells therefore behave essentially as they would in 12-well (or larger) plates. The charge of the plates could also be a factor- in any event, we suggest Corning # 3738. We are in the process of confirming this hypothesis and testing even smaller-well culture dishes.

Finally, keep in mind that no matter how proficient your technique, there will be animal-to-animal variability, such that some batches of cells will look better than others.

11. Hepatocytes look normal, but there are bare patches , particularly in the center and/or edges

Most commonly, this is due to insufficient shaking immediately after plating, and/or too much or too little volume used, and/or too many cells plated.

Make sure you thoroughly shake the plate in a linear fashion after plating.

Double-check the cell density by plating a single well, shaking it, allowing it to settle for 30 seconds, and looking at it under low (100x) power. You should strive for 60-70% confluence at this time, assuming >90% viability.

When coating plates with collagen, ensure that the entire bottom surface is exposed to collagen, and that any coated regions are not scraped by your pipette tip.

Finally, refer to #9 for a discussion on cell size and plating issues.

12. There are many dead cells stuck to live hepatocytes

A. Improve your technique so that viability >90%

B. Wash cells within 45 minutes after initial plating, to prevent any dead cells from having enough time to stick.

C. This is unavoidable to some extent, and generally not problematic.

13. Hepatocytes do not evenly attach in smaller (i.e. <12-well) plates/cells tend to congregate in the middle of the plate and around the edges, forming a "bullseye" pattern.

This problem potentially encompasses two issues. First, we have observed that scaling hepatocytes into smaller wells requires higher-quality cells. Second, the narrower diameter of smaller wells increases the capillary effect, creating a larger meniscus that tends to pull cells towards the center before they have a chance to settle evenly.

The issue of cell quality is a complex one, and is addressed on other parts of this protocol. A solution for the latter issue, uneven cell distribution/bullseye effect, is to use NON tissue-culture treated plates (i.e. Corning #3738, Costar(R) 24 well not treated multiple well plates). Standard "Tissue culture treated" plates undergo a process that makes the plastic more hydrophilic, while NON tissue culture treated plates retain the intrinsic hydrophobic properties of the plastic. Since the bottom of the plate will be coated with collagen, the lack of tissue culture treatment has no bearing on cell attachment; it it the side walls we are concerned with. The increased hydrophobicity appears to greatly reduce the "bullseye" effect, and in our experience, allows for an even monolayer of hepatocytes. Note that the non-treated plates also have different charge properties, and we cannot say for sure whether this plays any role; in any event, if you find that standard tissue culture treated 24 (or 96)-well plates make even plating difficult, try the non tissue culture treated versions.