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Corresponding Author:	Mingxiao Feng Johns Hopkins University School of Medicine Baltimore, MD UNITED STATES
Corresponding Author's Institution:	Johns Hopkins University School of Medicine
Corresponding Author E-Mail:	mfeng8@jhmi.edu
Order of Authors:	Mingxiao Feng
	Sheng Wu
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TITLE:

An Improved Time- and Labor- Efficient Protocol for Mouse Primary Hepatocyte Isolation

AUTHORS AND AFFILIATIONS:

Mingxiao Feng[1]*, Sara Divall[3], Sheng Wu[1,2]*

[1]Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

[2]Department of Physiology, Temple University School of Medicine, Philadelphia, Pennsylvania, USA

[3]Department of Pediatrics, Seattle Children's Hospital, Seattle, Washington, USA

Email addresses of co-authors:

Mingxiao Feng (mfeng8@jhmi.edu)

Sara Divall (sara.divall@seattlechildrens.org)

Sheng Wu (sheng.wu@temple.edu)

*Corresponding author:

Mingxiao Feng (mfeng8@jhmi.edu)

Sheng Wu (sheng.wu@temple.edu)

KEYWORDS:

primary hepatocyte; liver; metabolism; mouse; isolation; hepatic; glucose

SUMMARY:

Primary hepatocytes are a valuable tool to study liver response and metabolism *in vitro*. Utilizing commercially available reagents, an improved time- and labor-efficient protocol for mouse primary hepatocyte isolation was developed.

ABSTRACT:

Primary hepatocytes are used extensively in liver *in vitro* research, especially in glucose metabolism studies. A base technique has been adapted based on different needs, like time, labor, cost, and primary hepatocyte usage, resulting in various primary hepatocyte isolation protocols. However, the numerous steps and time-consuming reagent preparations in primary hepatocyte isolation are major drawbacks for efficiency. After comparing different protocols for their pros and cons, the advantages of each were combined, and a rapid and efficient primary hepatocyte isolation protocol was formulated. Within only ~35 min, this protocol could yield as much, if not more, healthy primary hepatocytes as other protocols. Further, glucose metabolism experiments performed using the isolated primary hepatocytes validated the usefulness of this protocol in *in vitro* liver metabolism studies. We also extensively reviewed and analyzed the significance and purpose of each step in this study so that future researchers can further optimize this protocol based on needs.

INTRODUCTION:

The liver serves as one of the most important organs in the vertebrate body due to the vital role it plays in numerous life-supporting functions like food digestion, blood circulation, and detoxification. Usage of mouse primary hepatocyte *in vitro* culture is increasingly popular in studies of carbohydrate metabolism and hepatic carcinoma. Therefore, it is important to develop a convenient method for mouse primary hepatocyte isolation while maintaining its innate physiological function. Due to its function as a hub of glucose metabolism, the liver is also central to glucose production and storage[1]. Experiments with primary hepatocytes *in vitro* are a must to most glucose metabolism studies. Therefore, for years, various research groups have developed protocols for mouse primary hepatocyte isolation.

The general procedure of mouse hepatocyte isolation is to first flush out blood in the liver with an isosmotic liquid such as phosphate-buffered saline (PBS) or Hanks' Balanced Salt Solution (HBSS) and then use collagenase-containing solution to dissociate hepatocytes. These protocols share a general procedure but differ in reagents and steps based on different needs. However, preparing required reagents and performing isolation steps take time. In developing the present protocol, efficiency was set as a priority, with all reagents ready-to-use and available from the market, and as few steps as possible. The overall goal of this protocol is to provide a fast and labor-efficient method to isolate primary hepatocytes from mouse, without jeopardizing the isolated primary hepatocyte purity and viability.

PROTOCOL:

All procedures were approved by the Johns Hopkins Animal Care and Use Committee. C57BL/6 female mice (8-week old) were used in this study.

1. Preparation:

1.1. Mix William's E Medium (GlutaMAX Supplement) with 10% FBS and 1% antibiotic-antimycotic solution to make the culture medium.

1.2. Filter 25 mL of collagenase-dispase medium (e.g., Liver Digest Medium) through a 0.45 µm syringe filter to remove particle debris.

1.3. Warm 50 mL of double-distilled H_2O (ddH_2O), 35 mL of perfusion Medium (e.g., Liver Perfusion Medium) (or 50 mL at the first time using this protocol), and 25 mL of filtered collagenase-dispase medium in a 45 °C water bath for 30 min.

1.4. Within a sterile tissue culture hood, mix 2 mL of 10x HBSS and 18 mL of Percoll in a 50 mL tube to make 20 mL 1x Percoll-HBSS and keep on ice or 4 °C.

NOTE: 1x Percoll-HBSS can be kept at 4 °C for at least 6 months.

1.5. Within a sterile tissue culture hood, pour 30 mL of wash medium (e.g., Hepatocyte Wash Medium) into a clean Petri dish, and keep on ice.

1.6. Submerge the pumping tube in the water of a 45 °C water bath. Results are most reliable if the room temperature is at 25 °C.

1.7. Prepare 2 mL of 1x anesthetics by mixing 225 µL of Ketamine HCL, 93.75 µL of Xylazine, and 1681 µL of 1x PBS.

1.8. Anesthetize one mouse using an approved method. Here the mouse was intraperitoneally injected with 150 µL of 1x anesthetics. Perform the tests for loss of reflexes such as reaction to toe pinching to ensure full anesthesia.

1.9. Secure the mouse on its back onto the dissection pad by four limbs, by either pining or using water-proof tape or other methods approved by the institution's Animal Care and Use Committee (or equivalents).

1.10. Prepare sterilized forceps and scissors for dissection. To avoid possible contamination, conduct all steps within a sterile hood.

2. Procedure:

2.1. Using a peristaltic pump, start pumping warmed-up ddH_2O at a speed of 4 mL/min for 5 min. Change the pumping tube from water to a warmed-up perfusion medium.

2.2. Disinfect the anesthetized mouse's abdomen with 70% EtOH and cut open with a scissor to expose the liver, portal vein, and inferior vena cava (IVC).

2.3. Stop the peristaltic pump. Insert a 24 G catheter (e.g., Closed IV Catheter, 24 G, 0.75 IN) into IVC. Start pumping and cut the portal vein open.

2.4. Continue pumping until the flushed-out liquid is clear (around 3–5 min). Press the portal vein every minute to let liquid reach every corner of the liver. Be careful not to let air bubbles enter the IVC.

NOTE: This step is to flush out as much blood as possible from the liver.

2.5. Change the pumping tube from the perfusion medium to the collagenase-dispase medium. Continue pumping until all 25 mL of the collagenase-dispase medium is depleted.

2.6. Press the portal vein every minute to let liquid reach every corner of the liver.

NOTE: At this stage, the complete loss of blood is fatal to the mouse. The death of the mouse can be confirmed by a lack of heartbeat after the experiment. Dispose of the carcass as per facility policies.

2.7. Isolate the whole liver out without gallbladder to the 30 mL wash medium in the Petri dish on ice.

2.8. Tear it up into pieces with forceps to release primary hepatocytes into solution. This step would turn the wash medium into a cloudy solution full of released primary hepatocytes and small liver pieces.

2.9. Filter the cloudy solution in step 2.8 through a 70 µm cell strainer into the 20 mL 1x Percoll-HBSS in a 50 mL tube on ice. Mix by inverting the tube 20 times.

2.10. Centrifuge at 300 x *g* for 10 min at 4 °C.

2.11. Within the tissue culture hood, aspirate the supernatant. Wash the pellet with cold 30 mL of wash medium.

2.12. Centrifuge at 50 x *g* for 5 min at 4 °C.

2.13. Remove the supernatant and resuspend the pellet in 25 mL of the culture medium (or appropriate other volumes) within the tissue culture hood.

2.14. Count the cell number and plate the cells on desired culture plates according to the experimental design.

NOTE: Primary hepatocytes properly isolated from one 8-week-old mouse are usually sufficient to be plated on four 6-well plates or four 12-well plates.

REPRESENTATIVE RESULTS:

In order to test the efficiency, the present primary hepatocyte isolation protocol was performed on 8-week old female C57BL/6 mice. The attachment and purity of isolated primary hepatocytes were tested. Primary hepatocyte isolation is used for a wide variety of experiments on liver physiology, such as hepatic drug effects and glucose metabolism, pharmaceutical biomarker activity[2], insulin sensitivity, and glucose production. Therefore, the activities of the primary hepatocytes isolated with this protocol were tested in the following experiments.

Coating was not required for primary hepatocyte attachment in the present protocol

Primary hepatocytes isolated were plated at 5 x 10^5 cells/well on a 6-well plate. After 1 h, a firm attachment was observed, and primary hepatocytes fully expanded 12 h after plating (**Figure 3**). This indicated that 12 h after plating, cells were ready to be utilized for experiments. Based on the nucleus morphology of mouse hepatocytes within the liver, mononuclear hepatocytes were enriched at borders, while binuclear/polynuclear hepatocytes, a signature of terminal differentiation, were in the middle[3,4]. A significant amount of cells imaged displayed typical dual-nucleus (diploid) morphology, indicating the success of isolating and purifying live primary hepatocytes. This also indicates that collagen coating is not a requirement for primary hepatocyte attachment with this protocol.

Purity was enriched in the isolated primary hepatocyte population

Various cell type-specific gene markers have been used in previous protocols to check isolated primary hepatocyte purity (**Table 1**). TTR (Transthyretin), CD95 (Cluster of differentiation 95, also known as Fas), ASGR1 (Asialoglycoprotein receptor 1), and ASGR2 are markers for hepatocytes. After isolation, the mRNA levels of these hepatocyte markers were significantly increased by about 2 folds, compared to the whole liver (**Figure 4A–D**,**H**).

This protocol also greatly reduced the interference from other hepatic cells. The presence of immune cells, stellate cells, and endothelial cells were lower, shown by the sharp decrease of mRNA levels of CD45 (immune cell marker), COL1A1 (Collagen, type I, alpha 1, stellate cell marker), and TIE2 (Endothelial cell kinase 2, endothelial cell marker), compared to the whole liver (**Figure 4E–H**). These suggest that this protocol could purify the primary hepatocyte population from hepatic cells and thus reduce the possible interference from other cell types in experiments.

Activity of pharmaceutical biomarkers was preserved

Biomarkers on hepatocytes have been extensively used for drug targeting and delivery. The activity preservation of pharmaceutical biomarkers thus is a key point in primary hepatocyte isolation and is a standard to test the usefulness of primary hepatocyte isolation[2]. Hepatocyte markers ASGR1 and ASGR2 are used in this manner[2]. We first tested the time-course expression level of these two markers before and after primary hepatocyte plating. After plating, their mRNA level decreased considerably with time, but levels remained considerable compared to the whole liver until the 12 h time point after plating, especially for ASGR1 (**Figure 5A**,**B**). The expression trend was consistent with a previous report[2] and indicated comparable primary hepatocyte healthiness. Various pathogens target CD81, a hepatocyte membrane-bound protein, to facilitate their entrance into cells and infection, like hepatitis virus[5], *Plasmodium falciparum,* and *Plasmodium yoelii*[6]. Other hepatocyte membrane-located proteins, like TLR4 (Toll-like receptor 4), are also targeted by pathogens and important for hepatocyte immune response[7]. After plating, the expression level of CD81 was consistent until 48 h (**Figure 5C**). TLR4 expression level generally increased, but not until after 48 h, when it reached a level higher than *in vivo* (**Figure 5D**). These suggest that primary hepatocytes isolated by this protocol can also be used for CD81 and TLR4 studies within at least 48 h after plating. Together, these results indicate that primary hepatocytes isolated are valid for use in studies related to pharmaceutical biomarkers. It is worth noting that RNA and protein levels may be inconsistent because of influences from post-transcriptional activities like signal peptide-induced RNA migration, posttranslational modification and/or protein degradation. Therefore, protein level and bioactivity verification of pharmaceutical biomarkers identified by mRNA may be necessary if required by the experimental paradigm.

Isolated primary hepatocytes were insulin-sensitive

Primary hepatocyte performance in experiments of glucose metabolism was also analyzed. Insulin, a hormone playing a central role in glucose metabolism, decreases glucose level, promoting hepatic glucose uptake and storage through phosphorylating AKT and FOXO1 (Forkhead box O1). Therefore, an insulin sensitivity assay was carried out with isolated primary

hepatocytes. After 16 h, cells were starved for 3 h, with a serum-free medium. At the last 0.5 h of starvation, 100 nM insulin was administrated to the culture medium. As shown in **Figure 6A–C**, insulin significantly promoted the phosphorylation of both AKT at Ser473 and FOXO1 at Ser256, indicating the sensitivity of primary hepatocytes to insulin. This suggests that the isolated primary hepatocytes from the present protocol are useful in insulin/glucose metabolism studies.

Isolated primary hepatocytes were capable of glucose production

Not only are they a center for glucose storage, but hepatocytes are also responsible for glucose production. To test whether the primary hepatocytes we isolated are useful in studies of glucose production, the cells were starved for 10 h in the presence of glucagon to stimulate glucose production. The starvation medium was then collected for glucose assay, while cells were harvested for western blot. Phosphoenolpyruvate carboxykinase (PEPCK) is an essential component in liver glucose production, controlling its rate[8]. The protein level of PEPCK was significantly increased after glucagon treatment, suggesting that the glucose production pathway was activated (**Figure 7A**,**B**). This activation was further confirmed by an increased level of glucose production (**Figure 7C**). This phenomenon was also confirmed with other glucose production stimulators like forskolin plus IBMX (**Figure 7A–C**). However, due to the limitation of this experiment, we could not verify whether glucose production was exclusive via gluconeogenesis or whether there is a component of glycogenolysis as well.

FIGURE AND TABLE LEGENDS:

Figure 1: Bench setup. (**A**) The bench setup for primary hepatocyte isolation. (**B**) The cartooned bench setup for primary hepatocyte isolation.

Figure 2: Mouse dissection, perfusion, and primary hepatocyte purification. (**A**) The dissected mouse, with arrows pointing to the liver, IVC, and portal vein. (**B**) Catheter insertion into IVC. (**C**) Pressuring portal vein causing enlargement and stiffness of liver lopes, indicating successful perfusion. (**D**) Softened liver lopes after perfusion, indicating the success of collagenase digestion. (**E**) The position of the gallbladder in the isolated liver (arrow pointing to the gallbladder). (**F**) Gallbladder removal. (**G**) Teared-up liver in hepatocyte wash medium. (**H**) Mixing 1x Percoll-HBSS with filtered primary hepatocyte before centrifuge. (**I**, **G**) primary hepatocyte pellet after centrifuge. (**K**) Primary hepatocyte resuspension within hepatocyte wash medium. (**L**, **M**) Primary hepatocyte pellet formed within hepatocyte wash medium after centrifuge.

Figure 3: Primary hepatocytes after plating. Images were taken after (**A**)1 h, (**B**, **C**) 12 h, (**D**) 24 h, (**E**) 36 h, (**F**) 48 h, (**G**) 72 h, and (**H**) 96 h primary hepatocyte plating. Scale bar = 100 µm.

Figure 4: Enhanced primary hepatocyte purity after isolation. RNA was isolated before (from the whole liver) and after (from isolated primary hepatocytes) primary hepatocyte isolation, followed by reverse transcription PCR, according to a previous protocol[9]. The primary hepatocyte purity was assessed by real-time PCR with primers for hepatocyte markers (A) TTR , (B) CD95, (**C**) ASGR1 and (**D**) ASGR2, while also with immune cell marker (**E**) CD45, (**F**) Stellate cell marker COL1A1, and (**G**) endothelial cell marker TIE2. (**H**) Heatmap was generated for the expression changes of cell type markers before and after primary hepatocyte isolation. GAPDH

(Glyceraldehyde 3-phosphate dehydrogenase) was used as an internal control. Primers used here were for genes (Primer sequences are in **Table 2**. Sequence references are cited here): *TTR*[10]; *CD95*[11]; *ASGR1*[12]; *ASGR2*[13]; *CD45*[14]; *COL1A1*[15]; *TIE2*[16]; *GAPDH*[17]. Graphs and heatmap were generated with GraphPad Prism 8. Error bar indicates Standard Deviation, and two-tailed unpaired (since primary hepatocyte and whole liver samples were unpaired because this protocol requires intact liver to begin with) t-test significance is indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). N=7.

Figure 5: Expression of pharmaceutical biomarkers. RNA was isolated from the whole liver and primary hepatocyte after plating, followed by reverse transcription PCR. The expression of pharmaceutical biomarkers was assessed by real-time PCR with primers for (**A**) ASGR1, (**B**) ASGR2, (**C**) CD81, and (**D**) TLR4. GAPDH was used as an internal control. Primers used here were for genes (Primer sequences are in **Table 2**. Sequence references are cited here): *CD81*[18]; *TLR4*[19]. Graphs were generated with GraphPad Prism 8. N = 5.

Figure 6: Insulin sensitivity preserved after primary hepatocyte isolation. (**A**) Western blot of p-AKT (S473), AKT, p-FOXO1 (S256), FOXO1, and GAPDH after insulin treatment. (**B**) p-AKT (S473)/AKT in densitometry of western blot. (**C**) p-FOXO1 (S256)/FOXO1 in densitometry of western blot. Insulin sensitivity assay was carried out 16 h after primary hepatocyte plating. Primary hepatocytes were starved for 3 h in William's E Medium (GlutaMAX Supplement) without FBS but with 1% antibiotic-antimycotic solution and then treated with 100 nM insulin for 30 min, before being harvested for protein lysis with 1x RIPA buffer. Graphs were generated with GraphPad Prism 8. Western blot imaging was carried out with LI-COR Odyssey CLx. Error bar indicates Standard Deviation, and two-tailed paired t-test significance is indicated by asterisks (* $p < 0.05$; ** $p < 0.01$). N = 4.

Figure 7: Glucose production assay. (**A**) Western blot of PEPCK and GAPDH after glucagon or forskolin+IBMX treatment for 10 h. (**B**) Densitometry of PEPCK protein level comparing to GAPDH after either glucagon or forskolin+IBMX treatment for 10 h. (**C**) Glucose level comparing to protein level after either glucagon or forskolin+IBMX treatment for 10 h. Glucose production assay was carried out 16 h after primary hepatocyte plating. Primary hepatocytes were starved for 10 h in glucose- and phenol red-free DMEM (added with 2 mM L-Glutamine, 2 mM Sodium Pyruvate, 20 mM Sodium L-Lactate, 1% Pen Strep), with either 50nM glucagon or 10 μM forskolin and 200 μM IBMX. The medium was harvested for glucose concentration measurement with Glucose Assay Kit, while protein was harvested for western blot. Western blot imaging was carried out with LI-COR Odyssey CLx. Error bar indicates Standard Deviation, and two-tailed paired t-test significance is indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). N = 4.

Table 1: Comparison of primary hepatocyte protocols.

Table 2: List of primers: Primers used for genes *TTR*, *CD95*, *ASGR1*, *ASGR2*, *CD45*, *COL1A1*, *TIE2*, *GAPDH, CD81*, and *TLR4*

DISCUSSION:

Various primary hepatocyte isolation protocols have been developed. They also have been kept optimized and adapted based on different needs (**Table 1**). Isolation protocols are generally composed of two parts: perfusion (including enzyme digestion) and purification.

The perfusion can be performed with the entire liver *in vivo*[2,20–23] or with dissected liver lobes[24]. The perfusion of dissected lobes was generally restricted to the left and medium lobes for technical ease. Theoretically, *in vivo* perfusion should yield more primary hepatocytes due to the usage of the entire liver with all lobes. Keeping the liver in place during perfusion may also reduce cell death since hepatocytes *in vivo* can be bathed in fluid, either blood or perfusion buffers, at all times during this step.

The maintenance of sterile conditions also plays an important role in this step. If the condition permits, it is better to conduct perfusion in a clean hood. All the tools with direct touch to tissues should be sterile, which can be achieved by autoclave. In order to prevent any contamination from bacteria and fungi, an antibiotic-antimycotic solution was added into the culture medium.

The IVC and portal vein are two main vessels connecting the liver with other organs. Most protocols utilize either of these veins to insert needles for perfusion: IVC[2,20,21,23], portal vein[22]. The position of the portal vein is at a skewed angle, which leads to difficulty in positioning and stabilizing the inserted needle. A suture is thus usually needed to tie the needle in place with a surgical knot, which must be done very carefully as the vein is prone to damage. Generally, the diameter of the portal vein is also smaller than IVC, complicating needle insertion. Considering this, the use of IVC can be more convenient, although in some protocols, the needle is also surgically knotted to the IVC[20]. It has been reported that perfusion with IVC insertion resulted in less viable primary hepatocytes than portal vein[25]. However, in the present protocol, we successfully used IVC insertion, and the isolated primary hepatocyte viability (>96% in optimized conditions, measured with Trypan Blue staining according to manufacturer's protocol) was as high, if not more, as other protocols (varies between 80% and 96% based on protocols and conditions[2,22–24]).

There are two aims for the perfusion step: to flush blood out of liver lobes and digest the liver to release primary hepatocytes. In order to achieve these objectives, at least two buffers should be used, one for blood flux (Flux Buffer), and one for digestion (Digestion Buffer). Most of the protocols prepare HBSS-based Flux Buffer and add collagenase within to make it capable for digestion, as Digestion Buffer. The preparation of these buffers is time-consuming, and the buffers could vary slightly in some delicate properties, like pH, from batch to batch, therefore introducing uninvited variables. Considering this, utilizing commercially available buffers saves valuable labor and time while minimizing these variables. Gibco developed a protocol for primary hepatocyte isolation from adult rats[26]), based on the use of commercially available Liver Perfusion Medium (as Flux Buffer), and Liver Digestion Medium (as Digestion Buffer). Mouse and rat, as rodents, share high similarities in body properties; one may integrate these two rat-optimized buffers into mouse primary hepatocyte isolation. Indeed, Gonçalves et al.[24], successfully carried out the perfusion of dissected liver lobes with these buffers, raising the promise of their use in liver perfusion *in vivo*. Here we successfully tested Liver Perfusion Medium

and Liver Digestion Medium in the present mouse primary hepatocyte protocol, which yielded high-quality viable primary hepatocytes (**Figure 3** and **Figure 4**).

The temperature of the perfusion buffer determines how well primary hepatocytes survive the isolation. If the temperature is too high, the collagenase within Digestion Buffer may have reduced activity; if too low, the primary hepatocytes may suffer cold shock, causing possible compromised isolation yield. The time that buffer takes to flow through perfusion tubing also determines the temperature of buffer when it reaches the liver. In previous protocols, 40 °C[2] or 42 °C[21] water bath was used. This temperature may be subject to change based on lab conditions, like the environment temperature, length of the perfusion tube, and the type of Peristaltic Pump. In the present protocol, after multiple times of testing, we optimized the water bath temperature to be 45 °C to warm up the buffers.

The purity of isolated primary hepatocytes plays an important role in subsequent experiments, as the higher the purity ratio, the less possible interference. Although the liver is mainly composed of hepatocytes, accounting for 60%–80% by mass[27], various types of other cells are also present, like immune cells, stellate cells, and endothelial cells, which are important for hepatic immunological activities. Each of these cells could post a potential interference in later-on experiments[28]. For instance, stellate cells in the liver also respond to pathogen invasions and liver injuries, involving in scar formation[29]. In numbers, 5%–8% of the total hepatic cell population is contributed by stellate cells[30]. Normally, stellate cells are in quiescence, but this status can be broken when stresses are present. Therefore, stellate cell activities may be triggered by stresses introduced during isolation or subsequent treatment if some of these cells remain in the final isolated primary hepatocyte pool. Other types of cells also contribute to a considerable portion of the hepatic cell population, like liver sinusoidal endothelial cells (LSECs), accounting for 20% of the total hepatic cell population[31]. How to eliminate the presence of these cells has been a puzzling part in primary hepatocyte isolation process. Therefore, purification is a key part in primary hepatocyte isolation, which usually takes advantages of the weight and size differences between different types of cells. By optimizing the centrifuge speed and/or purification buffer, primary hepatocytes can be pelleted down to the bottom of the tube.

Separating live cells from dead cells is also critical in obtaining healthy primary hepatocytes and accurate cell counting. Usually, counting cell number is a must after purification since the results of numerous experiments vary as cell confluence changes. Gradient centrifuge reagents can be used in this step to fulfill this mission, like Percoll. 36%–40% of Percoll in centrifuge pellets down live cells and keeps dead cells in supernatant. We successfully optimized centrifugation speed and time to reduce the non-primary hepatocyte cell population in the present protocol. Cell type-specific markers were used to test the purity of isolated primary hepatocytes here, like other protocols. The hepatocyte markers used here were TTR[2], CD95[24,32], ASGR1[33], and ASGR2[34]. Markers for other types of cells include CD45 (immune cell marker), COL1A1 (Stellate cell marker), TIE2 (Endothelial cell marker)[2,35]. With these markers, the primary hepatocytes isolated with the present protocol showed a high level of purity (**Figure 4**), comparable to the previous protocols[2,24].

During perfusion, collagenase in Digestion Buffer loosens attachment between cells by breaking down collagen within the extracellular matrix. This leads to difficulty in cell attainment during plating. Most of the previous protocols require/recommend pre-coating of plates with collagen[2,22] or gelatine[24] for better attachment of primary hepatocytes. To our knowledge, while Salem et al.[21] and Li et al.[20] did not discuss this step, other protocols assessed in this study clearly stated/recommended the usage of pre-coated plates[2,22-24]. In the present protocol, we found that the plate coating was not required for plates of certain types. While we are not sure whether this was because different types of plates, especially if they are from different manufacturers, have different surface smoothness, and whether this varied smoothness, if ever exists, plays an important role in primary hepatocyte attachment, it is beneficial to note so that another step (plate-coating) could be skipped for efficacy. It is also important to note that this protocol generates a significant proportion of terminally differentiated, e.g., diploid hepatocytes, along with mononuclear hepatocytes, similar to *in vivo* hepatic sinusoid.

In this study, the advantages of previous primary hepatocyte isolation protocols were combined, and the process was simplified as much as possible, using commercially available reagents and eliminating unnecessary steps. The centrifugation steps were successfully reduced to two, which is, to our knowledge, the fewest in published protocols. To confirm the purity and bioactivity of isolated primary hepatocytes, the level of various hepatic cell markers was assessed, confirming that the present protocol could greatly enhance primary hepatocyte purity and reduce other hepatic cell populations, such as immune cells, stellate cells, and endothelial cells. The activity of pharmaceutical biomarkers like ASGR1, ASGR2, CD81, and TLR4 was well-preserved in primary hepatocytes isolated with present protocol, which also had confirmed insulin sensitivity and glucose production activity. The main limitation of this protocol is the expense since all reagents were purchased commercially for efficiency. We did not specifically verify that glycogenosis was intact in primary hepatocytes isolated using the present protocol, and this may need further research for related studies. This protocol has similar perfusion steps to previous ones, like Salem et al.[21], Severgnini et al.[2] and Li et al.[20], and Korelova et al.[23], using IVC insertion. Their perfusion and digestion buffers, which may require extra labor to prepare, may also work with the present protocol with little modification of enzyme digestion time. Therefore, combining reagents of prior protocols with steps of the present protocol may also be beneficial, both time- and economically friendly.

In summary, an improved time- and labor-efficient protocol for primary hepatocyte isolation from mouse liver was developed. This protocol utilizes commercially available reagents entirely and can be completed in ~35 min, from dissecting mouse to plating primary hepatocytes, thus providing a useful technique to primary hepatocyte-related studies.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

A

B

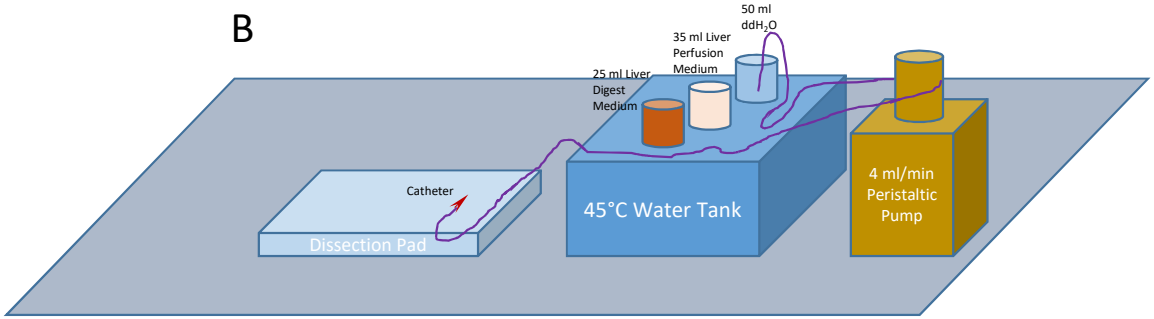

Figure 2

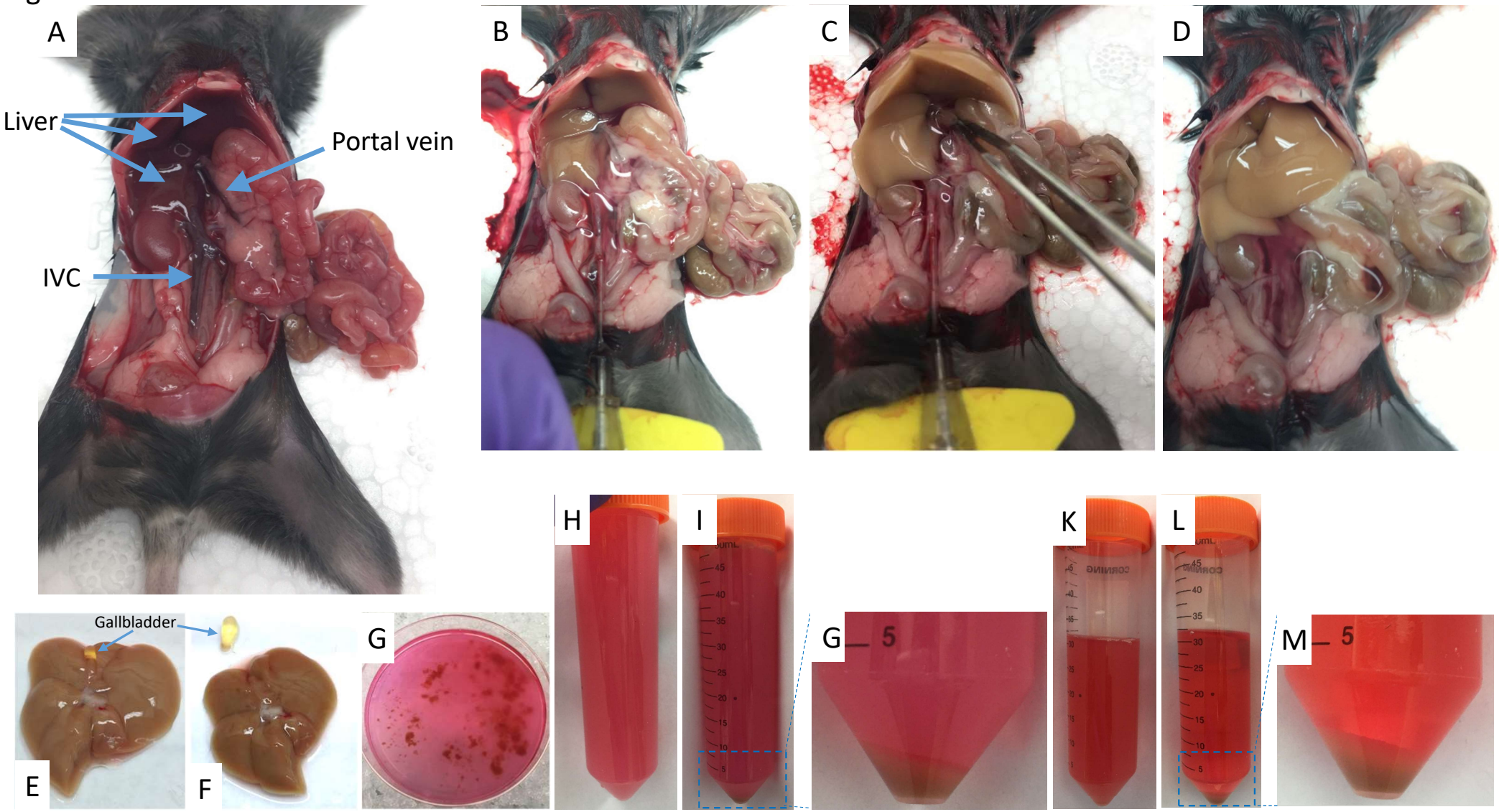

Figure 3

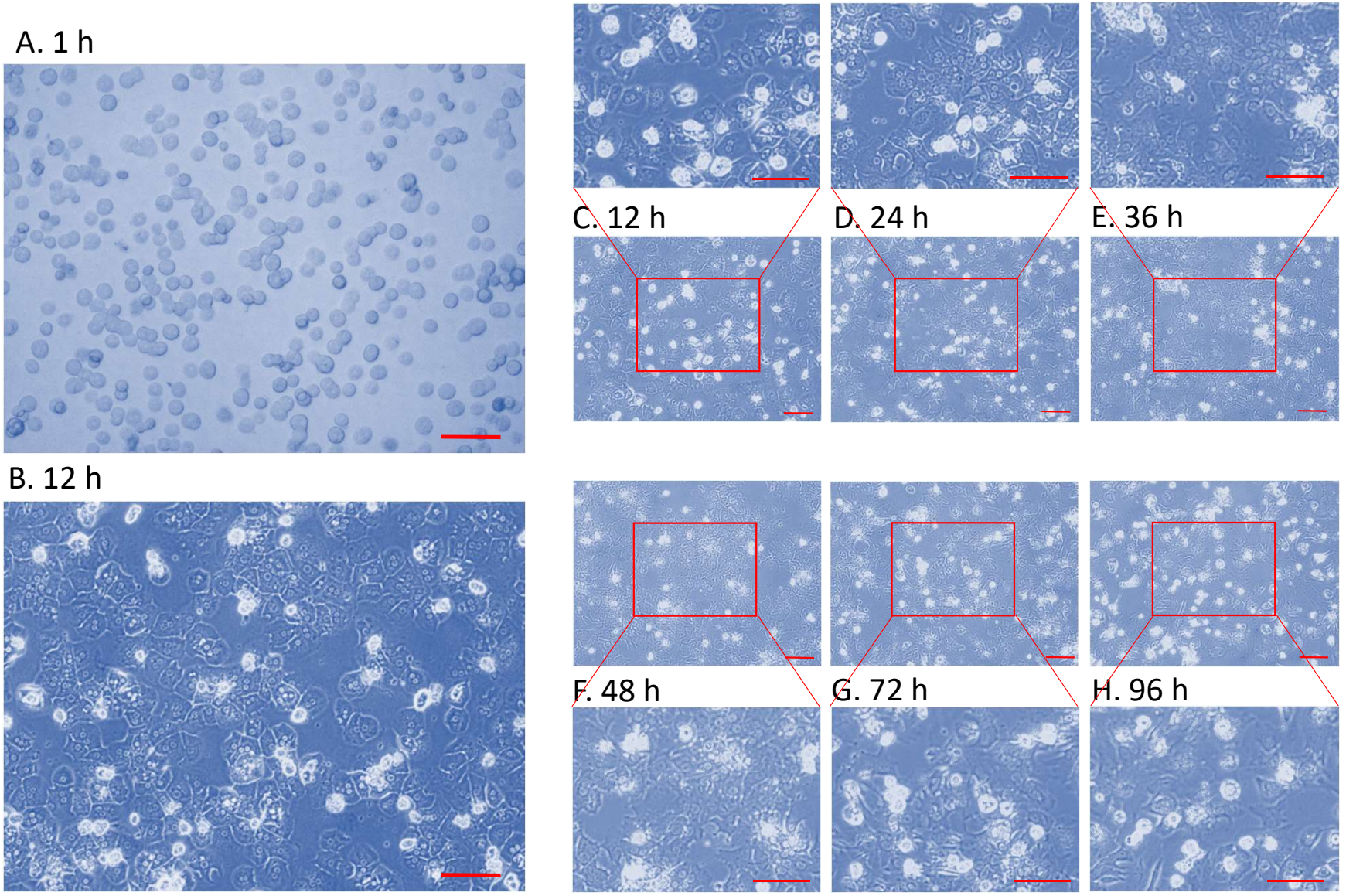

Figure 4

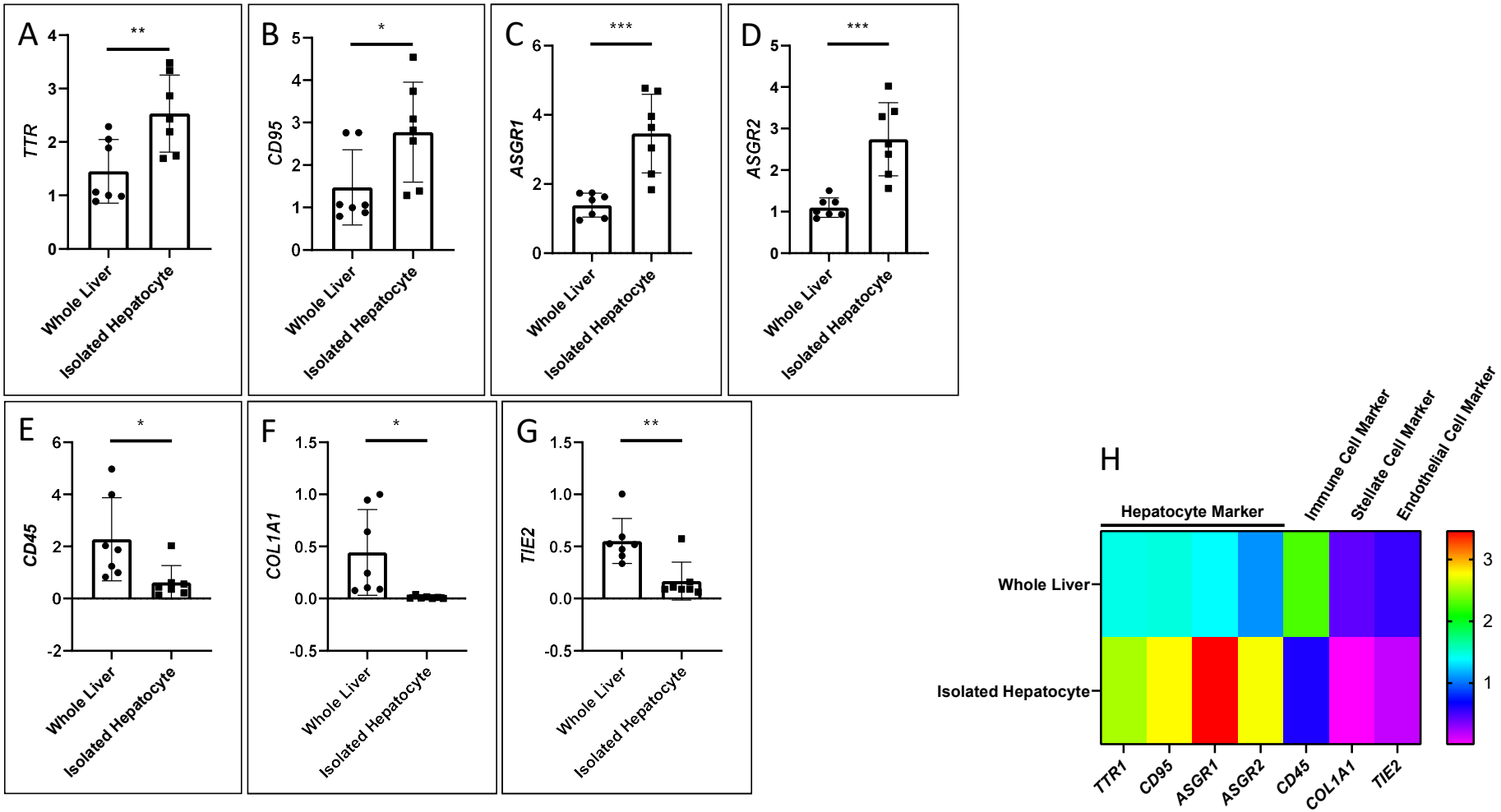

Figure 5

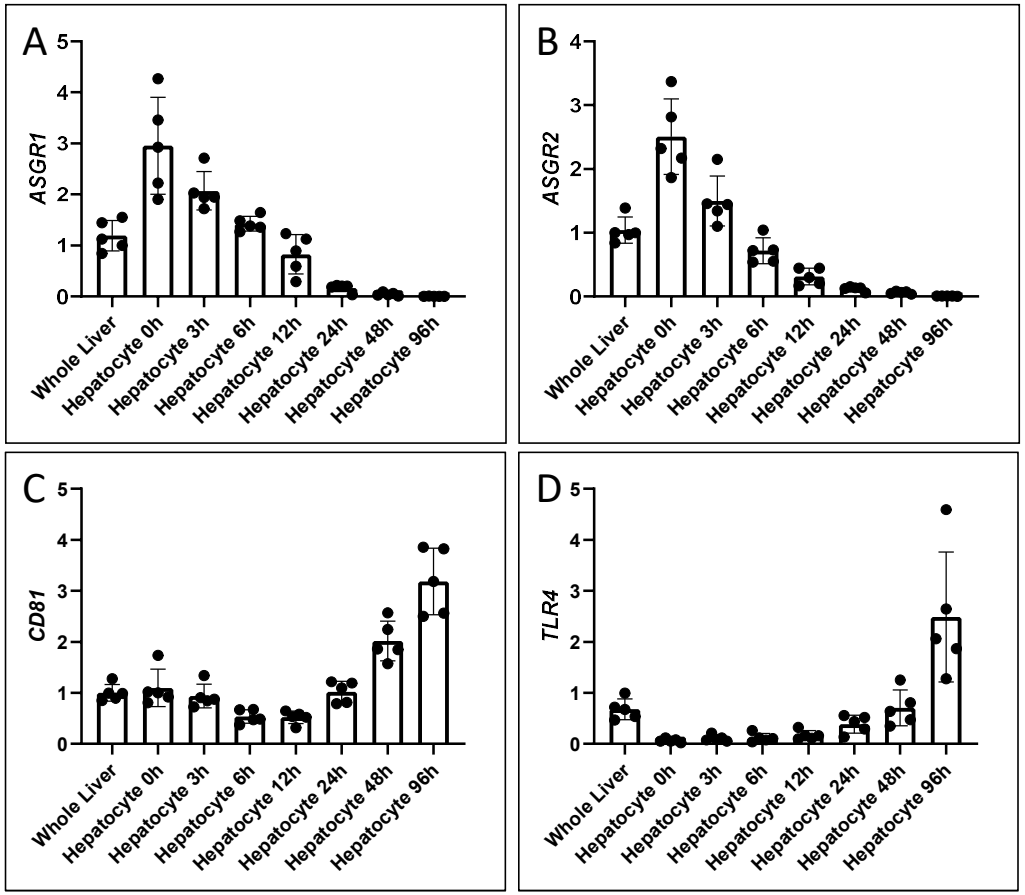

Figure 6

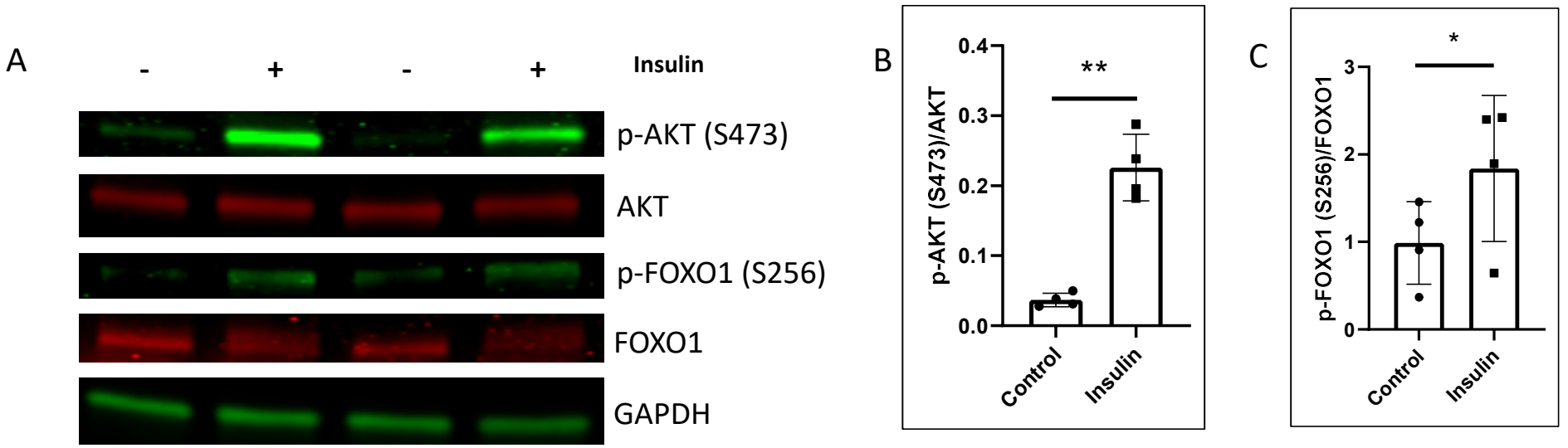

Figure 7

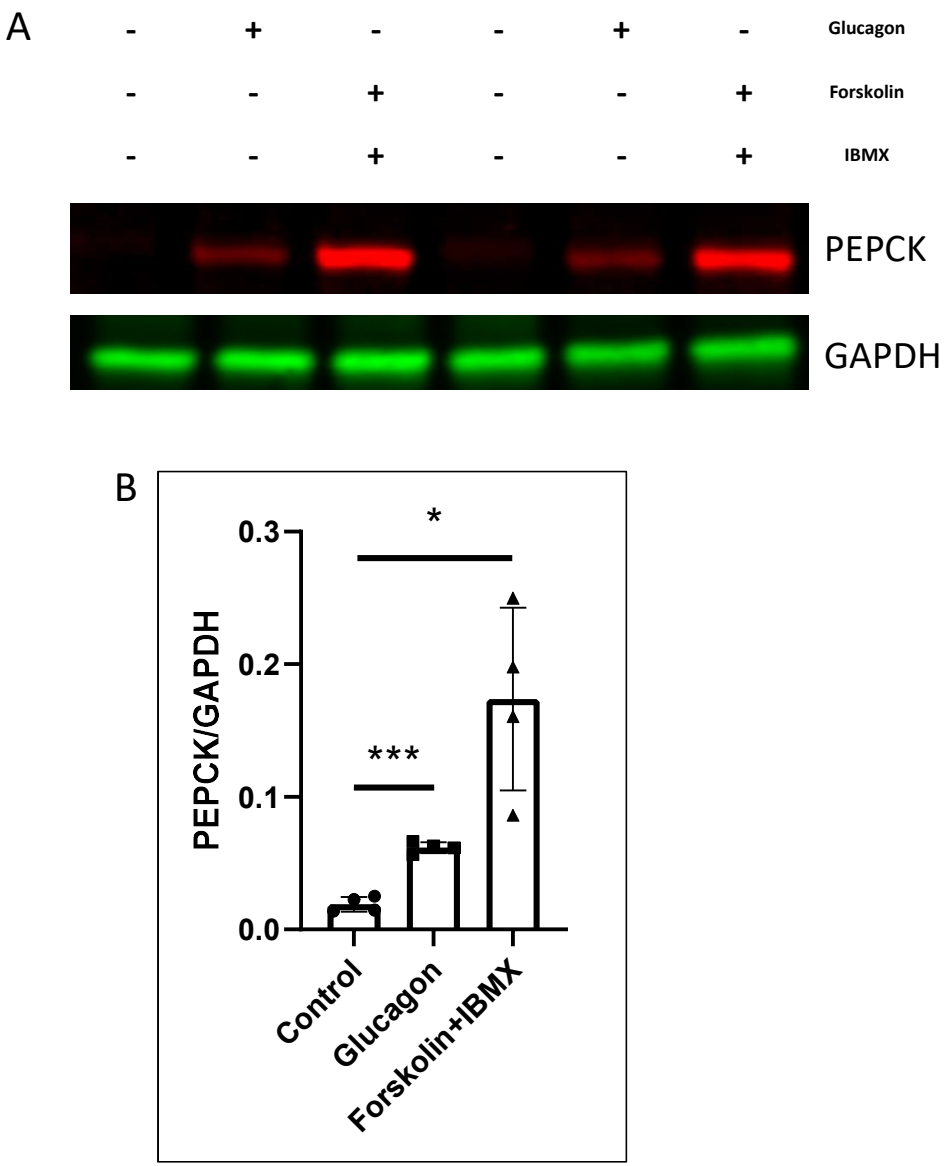

Table 1

	Centrifugation Times	Rib Cage Removal	Perfusion Buffers self-making
Present protocol	2	No	No
Severgnini et al.[2].	4	Yes	Yes
Gonçalves et al.[24].	5 or 6	No	No
Li et al.[20].	4 or 5	No	Yes
Salem et al.[21].	3	No	Yes
Cabral et al.[22].	3 or 6 (with gradient Centrifugation)	No	Yes
Korelova et al.[23].	3	No	Yes

Surgical Knot	Lamp Heating	Plate Coating	Isolated cell quantity/mouse
No	No	No	$2.5–4 \times 10^7$
Yes	Yes	Yes	$1.8–2 \times 10^7$
No	No	Yes	$1–3 \times 10^6$
Yes	No	Not Mentioned	$1–4 \times 10^7$
No	No	Not Mentioned	2×10^7
Yes	No	Yes/Recommended	N/A
Yes	No	Yes	N/A

Gradient Centrifugation	Purity Marker Genes
Yes	*TTR* , *CD95* , *ASGR1* , *ASGR2* , *CD45* , *COL1A1* , *TIE2*
No	*TTR* , *CD45* , *COL1A1* , *TIE2*
Yes	*CD45* , *CD95*
No	N/A
Yes	N/A
Optional	N/A (Purity assessed by light microscopy)
Yes	N/A

Table 2. Primers

Primer (Forward)
TTR Forward 5’-3’: AGCCCTTTGCCTCTGGGAAGAC
CD95 Forward 5’-3’: ATGCACACTCTGCGATGAAG
ASGR1 Forward 5’-3’: GAGTCGAAGCTGGAAAAACAG
ASGR2 Forward 5’-3’: CTACTGGTTTTCTCGGGATGG
CD45 Forward 5’-3’: GAACATGCTGCCAATGGTTCT
COL1A1 Forward 5’-3’: GAAGCACGTCTGGTTTGGA
TIE2 Forward 5’-3’: ATGTGGAAGTCGAGAGGCGAT
GAPDH Forward 5’-3’: CGACTTCAACAGCAACTCCCACTCTTCC
CD81 Forward 5’-3’: CCAAGGCTGTGGTGAAGACTTTC
TLR4 Forward 5’-3’: ACCTGGCTGGTTTACACGTC

Primer (Reverse)	Reference
TTR Reverse 5’-3’: TGCGATGGTGTAGTGGCGATGG	10
CD95 Reverse 5’-3’: CAGTGTTCACAGCCAGGAGA	11
ASGR1 Reverse 5’-3’: CCTTCATACTCCACCCAGTTG	12
ASGR2 Reverse 5’-3’: CAAATATGAAACTGGCTCCTGTG	13
CD45 Reverse 5’-3’: TGTCCCACATGACTCCTTTCC	14
COL1A1 Reverse 5’-3’: ACTCGAACGGGAATCCATC	15
TIE2 Reverse 5’-3’: CGAATAGCCATCCACTATTGTCC	16
GAPDH Reverse 5’-3’: TGGGTGGTCCAGGGTTTCTTACTCCTT	17
CD81 Reverse 5’-3’: GGCTGTTCCTCAGTATGGTGGTAG	18
TLR4 Reverse 5’-3’: CTGCCAGAGACATTGCAGAA	19

To Editors and Reviewers:

Thank you so much for all the efforts. It has been a hard time during pandemic, and I sincerely apologize that we have struggled with lots of difficulties in this special time. We have carefully proofread and revised the manuscript according to comments, and hope that the current version meets the publishing criteria. Please feel free to let us know if you have any further comments, and we are happy to take any suggestions to make this study better.

Thank you so much again.

Sincerely Yours,

Mingxiao Feng

Reply to Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
   Authors' reply: Thank you for the comment. We have carefully proofread thoroughly the manuscript and corrected spelling/grammar issues.

2. Please revise the Introduction to include all of the following:

a) A clear statement of the overall goal of this method

Authors' reply: Thank you for the comment. A sentence for overall goal statement has been added into Introduction: "The overall goal of this protocol is to provide a fast and labor-efficient method to isolate primary hepatocytes from mouse, without jeopardizing the isolated primary hepatocyte purity and viability."

b) The rationale behind the development and/or use of this technique

Authors' reply: Thank you for the comment. We added the stressing of the rationale behind the development and use of this technique in second paragraph of the Introduction.

c) The advantages over alternative techniques with applicable references to previous studies

Authors' reply: Thank you for your comment. We have included this information in the second paragraph of the introduction as well as the discussion.

d) A description of the context of the technique in the wider body of literature

Authors' reply: Thank you for your comment. We have included a description of wider body of literature: "Liver serves as one of the most important organs in vertebrate body, due to a vital role it plays in numerous life-supporting functions like food digestion, blood circulation, and detoxification. Usage of mouse primary hepatocyte *in vitro* culture is increasingly popular in studies of carbohydrate metabolism and hepatic carcinoma. It is therefore important to develop a convenient method for mouse primary hepatocyte isolation while maintaining its innate physiological function.", and have more deeply discussed the developed technique in context of the wider body of literature in the discussion.

e) Information to help readers to determine whether the method is appropriate for their application

Authors' reply: Thank you for the comment. We have added information about the characters of this protocol, and the differences with previous protocols, in order to help reader to determine whether this protocol is useful for various application in the Introduction: "In the development of present protocol, efficiency was set as priority, with all reagents ready-to-use and available from market, and as few steps as possible. The overall goal of this protocol is to provide a fast and labor-efficient method to isolate primary hepatocytes from mouse, without jeopardizing the isolated primary hepatocyte purity and viability.", and in the Discussion.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
   Author's reply: Thank you for the comments. We have revised the text to avoid the use of such pronouns.

4. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
   Authors' reply: Thank you for the comments. We have added an ethics statement before numbered protocol steps: "All procedures were approved by the Johns Hopkins Animal Care and Use Committee."

5. Please mention how animals are anesthetized and how proper anesthetization is confirmed.
   Authors' reply: Thank you for the comments. We have added the method to anesthetizing and confirming: "7. Prepare 2ml 1X Anesthetics by mixing 225µl Ketamine HCL, 93.75µl Xylazine, and 1681µl 1x PBS.
   8. Anesthetize one mouse with proper method. Here the mouse was intraperitoneally injected with 150µl 1x Anesthetics. Tests for loss of reflexes such as reaction to toe pinching need to be done to endure full anesthesia."

6. Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

Authors' reply: Thank you so much for your comment. Because this isolation protocol is fatal to mice, as it removes the entire liver, the application of vet ointment on eyes is deemed not necessary.

7. What happens to the mouse after?
   Authors' reply: Thank you for your comment. This primary hepatocyte isolation protocol is fatal to the mouse because of removed blood volume and the essential organ of the liver removed.

8. For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.
   Authors' reply: Thank you for your comment. This primary hepatocyte isolation protocol is fatal to the mouse because of removed blood volume and the essential organ of the liver removed.

9. Discuss maintenance of sterile conditions during survival surgery.
   Authors' reply: Thank you for your comment. We have added the description of maintaining sterile conditions during primary hepatocyte isolation: "The maintenance of sterile conditions also plays an important role in this step. If the condition permits, it is better to conduct perfusion in a clean hood. All the tools with direct touch to tissues should be sterile, which can be achieved by autoclave. In order to prevent any contamination from bacteria and fungi, Antibiotic Antimycotic Solution (Sigma-Aldrich, Cat. A5955) was added into culture medium" in Discussion.

10. Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.
    Authors' reply: Thank you for your comment. This primary hepatocyte isolation protocol is fatal to the mouse because of removed blood volume and the essential organ of the liver removed.

11. Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.
    Authors' reply: Thank you for your comment. This primary hepatocyte isolation protocol is fatal to the mouse because of removed blood volume and the essential organ of the liver removed.

12. If the mouse is euthanized, please specify the method.
    Authors' reply: Thank you for your comment. This primary hepatocyte isolation protocol is fatal to the mouse because of removed blood volume and the essential organ of the liver removed.

13. Please upload Table 1 as an xls/xlsx file.
    Authors' reply: Thank you so much for your comment. The Table 1 was uploaded as .xlsx file.

Reply to Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The method presented by Feng et al., is a detailed easy-to-do method for mouse hepatocytes isolation that attempts to reduce time and effort during the process of isolation. The labor is intended to be reduced mainly by purchasing commercial media for perfusion and digestion processes whereas time was shortened by reducing 2 centrifugation steps based in previous reports. The protocol is clear and well described; all material are justified and indicated. The authors compared their presented method with methods reported previously throughout the manuscript and described clearly the characterisation of primary hepatocytes at different times of culture. Characterisation encompassed from specific markers to functional assays and results are overwhelming. Finally, the authors discussed the method's efficiency by approaching perfusion and purification steps.

Major Concerns:

1. The authors mentioned that primary hepatocytes are binuclear (diploid), however, they justified binuclear hepatocytes as a signature of hepatocytes differentiation. Morales-Navarrete et al. (A versatile pipeline for the multi-scale digital reconstruction and quantitative analysis of 3D tissue architecture) in 2015, demonstrated that diploid (or polyploid) mouse hepatocytes are enriched at the middle of liver sinusoid; mononuclear hepatocytes are present at the borders where hepatocytes can proliferate and it is dependent on the stimulus. Binuclear hepatocytes can not further replicate. I suggest the authors to include discussion about this because this new method could enrich this type of hepatocytes but no quantification is shown.

   Author's reply: Thank you for your comment. We have updated this part to include this information: "Based on the nucleus morphology of mouse hepatocytes, within liver, mononuclear hepatocytes were enriched at borders, while binuclear/polynuclear hepatocytes, signature of terminal differentiation, were in the middle. A significant amount of cells imaged displayed typical dual-nucleus (diploid) morphology, indicating the success of isolating and purifying of live primary hepatocytes.."

2. At the same paragraph the authors mentioned that there are not evident differences at the attachment of isolated hepatocytes between collagen-coated and collagen-free plates. I consider if the authors mentioned this data they should show a representative comparison. At the end the authors discussed properly that the plates they used could promote adhesion due to their fabrication features regardless of a deposition of a ECM protein.

   Authors' reply: Thank you for your comment. We have revised this part to conclude that "coating plates with collagen is not a requirement for primary hepatocyte attachment", instead

of directly comparing collagen-pre-coated plates with non-coated ones. Given the performance of our primary culture in our representative experiments, direct comparison is not necessary.

3. Characterisation of primary hepatocytes was based on RT-PCR method for positive and negative markers as for pharmaceutical biomarkers. Nevertheless, the authors did not discussed about the likelihood of that transcripts are reflected as translated proteins. Authors may discuss whether in other methods (ASGR1 protein levels are shown in reference 2) or reports, these proteins were detected or indicate that further protein characterisation is needed (immunoblotting).

   Authors' reply: Thank you for your comments. We have added discussion about protein level checking in the Results, section "Activity of pharmaceutical biomarkers was preserved": "It is worth noting that RNA and protein levels may be inconsistent, because of influences from post-transcriptional activities like signal peptide-induced RNA migration, posttranslational modification and/or protein degradation. Therefore, protein level and bioactivity verification of pharmaceutical biomarkers identified by mRNA may be necessary if required by the experiment paradigm."

4. At the section of glucose production, it is mentioned "gluconeogenesis/glycogenolysis studies" but only glucose production and phosphorylation of PEPCK (gluconeogenesis) are evaluated. It is likely that glycogenolysis is promoted but no evidence was presented so I suggest that authors should mention this as a high possibility. Gluconeogenesis, glycogenolysis and glycolysis are zonated in liver and it is dependent strongly on the activation of HNF4alpha and beta-catenin transcriptional factors among others (Colnot and Perret. Liver Zonation. Molecular Pathology of Liver Diseases 2011; Jungermann. Metabolic Zonation of Liver Parenchyma: Significance for the Regulation of Glycogen Metabolism, Gluconeogenesis, and Glycolysis 1987), I consider that it is important to discuss because the metabolic phenotype of primary hepatocytes is focused in glucose metabolism and important functional markers were not evaluated or discussed such as HNF4alpha.

   Authors' reply: Thank you for your pointing this out. We have added a disclaimer that we did not specifically investigate the cells' performance in experiments of glycogenolysis in the representative results, and have also added this as a limitation in the discussion this part in glucose production section.

Minor Concerns:

1. The authors should compare overall times between their method and the previous methods to highlight the reduction in time as it is mentioned in the title and highlighted along the manuscript.

Authors' reply: Thank you for your comment. We considered discussion comparing the times. However, because of different equipment logistics and experience of each user, the estimated times may vary. Instead, we emphasized that present protocol can be done within ~35min, and with fewest steps of published protocols, indicating it is of high possibility that present protocol takes the shortest time to complete.

2. The authors discussed bath temperature settings from 40 to 42ºC and decided to use an optimised temperature of 45ºC arguing the laboratory conditions and perfusion set-up, however, the authors should mention about the particular features of their set-up (room temperature, length of tubing, temperature in the outlet, etc.) that could help much for establishing conditions in new lab groups.

   Authors' reply: Thank you for your comments. We have added the room temperature information into the "Preparation" section of the manuscript: "Results are most reliable if the room temperature is at 25 °C.".

Reviewer #2:

Manuscript Summary:

Primary mouse hepatocyte isolation is a fundamental technique to investigate the hepatocyte physiology and function ex vivo. This study shows a relatively convenient way to isolate the primary hepatocytes and compare the method with already reported protocols. This proposed protocol is not so innovative, but there are some improvements compared to other protocols. Therefore, this study would provide informative information to the hepatocyte research community. However, the current manuscript needs some revisions.

Concerns:

1. Please include information about methods of anesthesia and euthanasia appropriately.

   Authors' reply: Thank you for your comment. We have added anesthesia information in the "Preparation" of "Protocol" section: "Anesthetize one mouse with proper method. Here the mouse was intraperitoneally injected with 150µl 1x Anesthetics. Tests for loss of reflexes such as reaction to toe pinching need to be done to ensure full anesthesia.

2. The type of collagenase was not clearly stated. Please add this information to the method, discussion, and/or Table 1. In addition, the pH of the perfusion buffer is also important. Please add the pH information.

   Authors' reply: Thank you for your comment. Both the collagenase and perfusion buffer were purchased. The Liver Digest Medium contains collagenase, and is available at Gibco with Cat.

No.: 17703-034. The Liver Perfusion Medium is available with “Gibco, Cat. No. 17701-038”, and there catalog numbers were provided in manuscript so that users can access the pH and type of collagenase provided by the manufacturer.

3. The authors use Liver Digest Medium, Gibco (500 mL). Does the medium have to be used freshly? (do the authors open a new bottle in every isolation experiment?). Or can the unused medium be used in the future? If so, how long of a period can the unused medium in the bottle be maintained in a refrigerator and be used for the hepatocyte isolation? This information would be helpful for the readers.

   Authors’ reply: Thank you for your comment. The Liver Digest Medium was aliquoted to 25mL each and keep in -20 degree freezer. It can be used until the expiring date marked by manufacturer. Each time 25ml Liver Digest Medium was shawled and used. We have included this information in Materials part : ”Aliquot within tissue culture hood to 25 mL each in 50 ml tube, and keep in -20 oC freezer”.

4. How many seconds and how many times did authors press portal vein during perfusion? Do the authors peel the liver membrane after the perfusion?

   Authors’ reply: Thank you for your comment. Every minute the portal vein was pressed throughout the perfusion and digestion process. The liver membrane was not peeled. However, in the step of filtering through 70 um filter, most of undigested liver membrane was filtered out.

5. The authors mix cell-containing media and percoll buffer, which may result in contamination of cells. In many protocols, cell-containing media is loaded on the percoll buffer, and then centrifuged, to avoid the mix. Do authors try the gradient method? Please include information (either in the method/discussion or in Table. 1).

   Authors’ reply: Thank you for your comment. To prevent this, we have included the method of preparation of 1 x Percoll-HBSS. The 1 x Percoll-HBSS used in present protocol was prepared in clean hood free of pathogens with purchased Percoll and 10 x HBSS. Percoll (Cat. 12-0891-01, GE Healthcare) is sterile (Ref.: http://www.ebiotrade.com/custom/GE/120220tw/images/D3.pdf, page 77), and so is 10x HBSS (Cat. 14065-056, Gibco) (Ref.: https://www.thermofisher.com/order/catalog/product/14065056#/14065056, on label). In this case, well-prepared 1 x Percoll-HBSS should not be the cause of contamination.

6. In Table 1, please include additional information about experiments done with the primary hepatocyte and/or applications used in each study. For instance, in this current protocol, glucose metabolism (pAKT, pFOXO1, glucose production) was investigated with the isolated

hepatocytes. Similarly, please add specific experiments/applications done in each study to the Table 1.

Authors' reply: Thank you for your comment. Since not all protocols compared in this study tested isolated primary hepatocytes at protein and metabolism level, and primary hepatocytes isolated in each protocol were best fit for usage of various purposes, we compared purity marker genes in Table 1 to assess the quality of isolated primary hepatocytes.

Reviewer #3:

Manuscript Summary:

The authors present a 2-step collagenase-based in vivo liver perfusion method for the isolation of primary mouse hepatocytes that is similar to many other published methods. The authors promote the efficiency of their method to isolate primary mouse hepatocytes that are suitable for conducting metabolism studies.

Major Concerns:

-The paper is lacking a number of important details that would need to be addressed in the video including: the required surgical equipment, means by which the mouse is secured for the procedure, mention of type of surface the mouse is adhered to, and steps taken to minimize bacterial contamination (e.g., conducting the procedure in a hood, preparing the mouse abdomen with an alcohol-based disinfectant, preparation of surgical instruments and other equipment prior to procedure).

Authors' reply: Thank you for your comment. We have included such information in manuscript: In Preparation section, "Sterilized forceps and scissors are prepared for dissection. To avoid possible contamination, it is also recommended to conduct all steps within sterile hood.", "Secure mouse on its back onto dissection pad by four limbs, by either pining or using water-proof tape or other methods approved by institution's Animal Care and Use Committee (or equivalents)." and in "Procedure" section, "Disinfect mouse abdomen with 70% EtOH.".

-Based on the provided data, the purity and survival of the isolated hepatocytes is questionable. The authors do not describe how survival was determined and the light microscopy images suggest both contamination and poor survival. Transcriptional and metabolic analyses of contaminated, dying, or dead hepatocytes may lead to erroneous conclusions.

Authors reply: Thank you for your comment. The live hepatocytes were purified using Percoll gradigent centrifugation. The survival rate was determined by Trypan blue staining, and we have added "measured with Trypan Blue staining according to manufacturer's protocol." in section "Discussion".

According to the activity analysis of pharmaceutical biomarkers and glucose metabolism, the isolated hepatocytes retained strong bioactivity for transcriptional and metabolic analysis.

-Maintaining the differentiated and in vivo metabolic state of primary mouse hepatocytes is very challenging. The authors should acknowledge the rapid decline in 'hepatocyte-like' gene expression. Here, is it important to note that the authors specifically discuss that the method does not use collagen coated plates or some type of attachment matrix. The authors may want to note that the use of these substrates is less to promote attachment as it is to promote and maintain hepatocyte function.

Authors reply: Thank you for your comment. We have included this information in manuscript in section of Representative Results: “After plating, their mRNA level decreased considerably with time, but levels still remained decent after 12 h, especially for ASGR1” in “Activity of pharmaceutical biomarkers was preserved”. Thank you also for the pointing out the usage of coating substrates is to promote and maintain hepatocyte function. Based on the purity marker gene tests, marker gene tests and metabolism tests in this study, the function of isolated hepatocytes should be maintained healthy.

Minor Concerns:

-Although temperature is highlighted as an important aspect of digestion, it does not appear that the solutions are not directly tested for temperature. It is recommended that a mock solution tube (of water) is stored under the same conditions as the coldest buffer and a thermometer is placed directly into that tub to ensure buffers at at the desired temperature.

Authors’ reply: Thank you for your comment and consideration. The mock solution tube should be of the same temperature as other tubes, as they are kept in same condition.

-No direct comparison against other protocols or collagenase-based buffers is made to demonstrate the claimed superiority of the method/commercial reagent.

Authors’ reply: Thank you for your comment. We have included the comparison of steps and method differences in discussion and Table 1. There is indeed no direct comparisons to other protocols. However, Figure 4-8 carefully tested the level of hepatocyte-related genes and activities, which showed comparable results with other similar protocols and proper functions, indicating the healthiness and activities of primary hepatocytes isolated with present protocol are of no considerable difference.

-The authors do not cite relatively recent similar methods published in JoVE.

https://www.jove.com/t/58323/isolation-primary-mouse-hepatocytes-for-nascent-protein-synthesis

https://www.jove.com/t/60507/isolation-3d-collagen-sandwich-culture-primary-mouse-hepatocytes-to

https://www.jove.com/v/56993/purification-hepatocytes-sinusoidal-endothelial-cells-from-mouse

Authors' reply: Thank you for your comment. We have cited and included the paper 58323 (Salem et al.) as reference 21, 60507 (Korelova et al.) as reference 23, and 56993 (Cabral et al.) as reference 22 in Table 1 for comparion.

Reply to comments:

Thank you so much for the important comments. The manuscript has been revised according to comments, and please feel free to let us know if further revision is required.

Thank you so much again.

Editorial comments:

1. Please note that the manuscript file has been formatted to fit the journal standard. Comments to be addressed are included within the manuscript file. Please review and revise.

   Reply: Thank you so much for your comments. The commented parts have been revised accordingly.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. The commercial term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language. All commercial products should be sufficiently referenced in the Table of Materials.
   Example: Percoll, Liver Digest Medium, Liver Perfusion Medium, etc.

   Reply: Thank you so much for your comments. The manuscript has been checked thoroughly to make sure that there are no commercial languages. All commercial products used in this protocol are referenced in the Table of Materials.

3. Please include the primers used in the study in the form of a table and cite the table in the appropriate sections of the manuscript.

   Reply: Thank you so much for your comment. Table 2. Primers has been created and contains all primers used in this study.

4. Please highlight up to 3 pages 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

   Reply: Thank you so much for your comment. The essential steps are highlighted in manuscript.

5. Please title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

   Reply: Thank you so much for your comment. No journal titles or book titles were cited in manuscript, except in References section, which has been formatted according to the reference format of JoVE.