

# Mouse Liver Digestion to Single-Cell Suspension

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## Purpose

This protocol describes the preparation of single-cell suspensions from mouse liver using perfusion, enzymatic digestion, mechanical dissociation, density centrifugation, and RBC lysis. The resulting cell suspension is suitable for downstream applications such as flow cytometry, FACS sorting, and single-cell omics workflows (with appropriate QC).

## Critical notes (read before starting)

- Keep all post-digestion washes cold (4 °C) where possible to preserve viability.
- Process **one liver per 70 µm strainer**; do not reuse strainers between livers.
- Liver cell pellets are often **loose**; be careful when pouring supernatants.
- Do not over-digest; limit digestion to **30 minutes at 37 °C** with agitation.
- Percoll must be at **room temperature** and properly diluted to **37%** immediately before use.
- Ensure RBC lysis is **time-limited (2-5 minutes)** to avoid damaging leukocytes.

## Approximate timing

- Preparation and setup: **10–15 min**
- Mouse euthanasia, perfusion, and liver dissection: **5–10 min per mouse**
- Liver mincing and transfer into digestion buffer: **5 min per mouse**
- Enzymatic digestion (including gentleMACS where applicable): **30–40 min**
- Mechanical dissociation, hepatocyte removal, and washes: **20–30 min**
- Percoll gradient centrifugation and leukocyte enrichment: **25–30 min**
- RBC lysis (if required), filtration, and final resuspension: **15–20 min**

**Total approximate time:** ~2–2.5 hours for 1–4 livers processed in parallel

(Effective hands-on time per liver is ~60–90 minutes, depending on batching and operator experience.)

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# Procedure

## STEP 1 – Preparation and setup

### 1. Pre-cool collection solutions

- Pre-cool **PBS or FACS buffer** on ice for liver perfusion and tissue collection.

### 2. Label collection vessels (choose one option per experiment)

- **Option A: 6-well plate collection**

- Label one well per mouse in a 6-well plate.
  - Add **3–5 mL ice-cold PBS or FACS buffer** per well.

- **Option B: 50 mL tube collection**

- Label one **50 mL conical tube** per liver.
  - Add **5 mL ice-cold PBS or FACS buffer** per tube.

### 3. Label dissociation and processing tubes

- Label **gentleMACS C-tubes** (or equivalent) for each liver.
- Label additional **50 mL tubes** as required for downstream processing steps.

### 4. Prepare buffers

- **Liver digestion buffer.**
- **37% Percoll working solution.**
- **ACK lysis buffer**, if required.
- **FACS buffer** for washes and final resuspension.

### 5. Warm digestion components

- Pre-warm a water bath to **37 °C**.
- Place an aliquot of **liver digestion buffer** in the water bath to equilibrate.
- If digestion buffer will be prepared immediately before use, pre-warm **DMEM** instead.

### 6. Equipment setup

- Pre-cool a benchtop centrifuge to **4 °C**.
- Ensure access to a **gentleMACS** instrument with an appropriate liver dissociation program.

### 7. Items to take to the animal facility

- Ice bucket with ice-cold **PBS or FACS buffer**.
- 6-well plates *and/or* pre-filled **50 mL tubes** (as selected above).
- Sterile scissors and forceps.
- **10 mL syringes** (one per mouse or per treatment group).
- Needles for liver perfusion (**25G or 26G recommended**).
- Absorbent pads and a waste container for perfusion runoff.

## STEP 2 – Liver harvest and perfusion

1. Euthanise the mouse according to institutional and ethical guidelines (e.g. **CO asphyxiation** or other approved method).
2. Place the mouse in a supine position and perform a midline incision to open the abdominal cavity.

3. Expose the liver and major vessels (**Inferior vena cava and portal vein**).
4. Load a **5 mL syringe** with **ice-cold FACS buffer** and attach a **25–27 G needle**.
5. Perfuse the liver via one of the following routes:
  - **Inferior vena cava** (preferred)
  - **Portal vein**
  - **Directly via the heart**
6. Slowly perfuse with **~5–10 mL FACS buffer per mouse** until the liver visibly blanches.

**i** Note

Efficient perfusion reduces blood content and improves hepatocyte removal, density separation, and downstream staining quality.

**⚠ Warning**

Handle needles and sharps carefully and dispose of them immediately into an approved sharps container after perfusion.

7. Excise the liver carefully, **removing the gallbladder**.
8. Place the liver briefly on **filter paper** to blot excess buffer.

### STEP 3 – Liver collection, weighing, and mincing

1. Transfer the perfused liver to a **pre-labelled collection vessel** (on ice).
2. *(Recommended) Weigh the liver:*
  - Briefly blot on filter paper to remove excess liquid.
  - Record the liver weight.
3. Add **5 mL pre-warmed liver digestion buffer** (in DMEM) to a **labelled gentleMACS C-tube**.
4. Transfer the liver into the gentleMACS C-tube.
5. Using sterile scissors, mince the liver **directly in the C-tube** until fragments are approximately **2–3 mm** in size.
6. Ensure all tissue pieces are fully submerged in digestion buffer and not stuck to the tube walls.
7. Secure the C-tube onto the **gentleMACS instrument** and place the warming sleeve over the tube.
8. Run the appropriate **liver digestion program** (e.g. **HH17**, ~17 min).

**⚠ Warning**

Confirm that each C-tube is correctly seated and rotating before leaving the instrument. If a tube is not spinning, stop the run, reseat the tube, and restart the program.

**! Important**

Do **not** leave livers in digestion buffer beyond the programmed digestion time. Over-digestion can strip surface epitopes and negatively impact downstream phenotyping. Note: the gentleMACS instrument does **not** beep when the program finishes—set a timer before

starting.

## STEP 4 – Hepatocyte removal and washing

1. After digestion, transfer the entire liver cell suspension from the gentleMACS C-tube into a **50 mL conical tube**.
2. Rinse the C-tube with **ice-cold FACS buffer** and add the rinse to the same tube to maximize yield.
3. Bring the volume up to **50 mL with ice-cold FACS buffer**.
4. Centrifuge at **50 g for 2 minutes at 4 °C** to sediment hepatocytes.
5. Carefully transfer the **supernatant** (containing leukocytes) to a fresh **50 mL tube**, avoiding disturbance of the hepatocyte pellet.
6. Adjust the volume to **50 mL with ice-cold FACS buffer**.
7. Centrifuge at **400 g for 5 minutes at 4 °C**.
8. Discard the supernatant and keep the leukocyte pellet **on ice** for downstream processing.

### ⚠ Warning

Cell pellets from digested liver are often **loose and poorly compacted**.

When discarding supernatant by pouring, do so **carefully**. Avoid flicking, shaking, or abrupt movements.

## STEP 5 – 37% Percoll enrichment

1. Aliquot **10 mL of 37% Percoll** into **pre-labelled 15 mL conical tubes** (one tube per liver).
2. Using a **transfer pipette**, resuspend the cell pellet using the small remaining **dead volume**.
3. Take up approximately **1 mL of 37% Percoll** from the corresponding 15 mL tube and use it to **rinse the 50 mL tube**, resuspending any remaining cells.
4. Transfer the resuspended cells into the corresponding **15 mL tube** containing **10 mL 37% Percoll**.
5. Repeat the rinse **1–2 times** with additional small volumes of Percoll (as needed) to recover all remaining cells from the 50 mL tube.
6. Cap the 15 mL tubes and **invert gently 2–3 times** to ensure an even cell suspension.
7. Centrifuge at **800 g for 20 minutes at room temperature (20–22 °C)** using:
  - Acceleration: **moderate** (e.g. acc 7)
  - Brake: **minimal** (e.g. br 1)

### ⚠ Warning

Avoid high brake settings during Percoll centrifugation. Abrupt braking can disrupt the gradient and reduce lymphocyte enrichment.

### ❗ Important

Do not leave liver samples in Percoll longer than necessary. Prolonged exposure can reduce viability and negatively impact downstream applications. Plan spins and next steps in advance.

### Note

After the Percoll spin, a debris/hepatocyte-rich layer typically remains in the supernatant, while an enriched leukocyte pellet forms at the bottom of the tube.

9. Carefully aspirate or pour off the supernatant in one smooth motion without disturbing the pellet.
10. Keep tubes inverted briefly and gently dab the rim on paper towel to wick residual Percoll (avoid touching or dislodging the pellet).
11. Resuspend the pellet in **20–30 mL ice-cold FACS buffer**.
12. Centrifuge at **400 g for 5 minutes at 4 °C**.
13. Discard the supernatant and keep the enriched leukocyte pellet **on ice** for downstream steps.

## STEP 5 – RBC lysis

1. Add **RBC lysis buffer** to the cell pellet (**0.5–1 mL per liver**):
  - **ACK lysis buffer:** incubate for **30–60 seconds at room temperature**, or
  - **HybriMax RBC Lysis Buffer:** incubate for **2–3 minutes at room temperature**.
2. Gently resuspend immediately to distribute the buffer evenly.
3. Quench the reaction by topping up to **15 mL with FACS Buffer**.
4. Pass the suspension through a 70 µm cell strainer into a fresh 50 mL tube kept on ice.
5. Rinse the strainer with an additional 5–10 mL FACS Buffer if needed to recover remaining cells.
6. Centrifuge at **400 g for 5 minutes at 4 °C**.
7. Discard the supernatant and keep the pellet on ice.

### Warning

Liver leukocytes are particularly sensitive to over-lysis. Do not exceed the stated incubation times, especially after enzymatic digestion or Percoll separation.

### Note

RBC lysis is often unnecessary for liver samples when a Percoll gradient is used. If lysis is required, HybriMax is generally preferred due to its gentler action compared to ACK.

## STEP 6 – Final resuspension

1. Resuspend the cell pellet in 1-5 mL ice-cold FACS Buffer
2. Count cells and assess viability (e.g. trypan blue) as required.
3. Proceed with downstream applications (e.g. flow cytometry, sorting, or culture of NLCs).

 Tip

For single-cell sequencing applications, minimise the time from tissue harvest to final resuspension, keep samples on ice whenever possible after digestion, and consider additional debris/dead cell removal steps if yields permit.

# Materials

## Reagents

Reagent	Supplier	Notes
FACS buffer	In-house / various	Used for perfusion, washes, and final resuspension; kept cold
Liver digestion buffer	In-house	DMEM-based; contains collagenase and DNase I; pre-warmed to 37 °C
Collagenase (e.g. Collagenase IV or D)	Various	Component of liver digestion buffer
DNase I	Various	Component of liver digestion buffer
Percoll	Cytiva/GE or similar	Prepared as a <b>37% working solution</b> on day of use
ACK lysis buffer	Commercial or in-house	Optional; for RBC removal if required
Fetal calf serum (FCS), heat-inactivated	Various	Component of FACS buffer
EDTA	Various	Component of FACS buffer
CO <sub>2</sub> or approved euthanasia agent	Facility supply	For mouse sacrifice

## Disposables

Item	Notes
50 mL conical tubes	Liver collection and processing
15 mL conical tubes	Percoll enrichment (one per liver)
gentleMACS C-tubes (or equivalent)	Enzymatic and mechanical dissociation
Sterile Petri dishes (10 cm)	Backup mincing / straining if required
70 µm cell strainers	Filtration of digested tissue
Syringes (5–10 mL)	Liver perfusion
Needles (25–27 G)	Inferior vena cava or portal vein perfusion
Transfer pipettes	Resuspension and Percoll transfer
Filter paper	Blotting liver prior to weighing
Paper towels / bench wipes	Wicking supernatants after Percoll
Gloves and sharps container	Standard laboratory safety

## Equipment

Equipment	Notes
CO <sub>2</sub> chamber or euthanasia setup	As per animal ethics approval
Dissection instruments	Scissors and forceps
gentleMACS dissociator	With liver-appropriate digestion program
Refrigerated centrifuge	Compatible with 15 mL and 50 mL tubes; adjustable brake
Benchtop incubator or heating sleeve	37 °C for enzymatic digestion

Equipment	Notes
Analytical balance	For weighing mice and livers
Biological safety cabinet (Class II)	Recommended for tissue and cell handling
Ice bucket	To keep samples cold between steps
Timer	Critical for digestion, Percoll, and lysis steps

## Troubleshooting

Problem	Possible cause	Suggested solution
Low lymphocyte yield	Incomplete perfusion or digestion	Ensure liver blanches during perfusion; mince thoroughly; avoid under-digestion
High red blood cell contamination	Insufficient or skipped RBC lysis	Perform brief ACK lysis ( 2 min) and quench immediately
Poor Percoll separation or weak pellet	Incorrect Percoll concentration or harsh braking	Use fresh <b>37% Percoll</b> ; confirm centrifuge settings; minimise brake
Loose pellets and cell loss	Aggressive pouring of supernatant	Pour slowly in one smooth motion; wick residual liquid gently
Low viability	Over-digestion or prolonged handling at RT	Limit digestion time; keep washes cold; process promptly
Excess debris or clumping	Inadequate DNase activity or filtration	Confirm DNase in digestion buffer; filter through 70 µm mesh

## Safety (brief)

- All animal procedures must comply with approved animal licences and institutional regulations.
- Wear appropriate PPE (lab coat, gloves, eye protection) when handling animals, tissues, and reagents.
- Handle sharps (needles, scalpels) with care and dispose of immediately in approved sharps containers.
- Perform tissue and cell handling in a biological safety cabinet where appropriate.
- Percoll and RBC lysis buffers are chemical hazards; avoid skin and eye contact and dispose of waste according to local safety guidelines.

## Version history

Version	Date	Author	Change summary
v1.0	2025-11-20	Dillon Corvino	Initial Quarto version based on SOP_001 (liver) from IEO
v1.1	2025-11-26	Dillon Corvino	Manual check and update protocol
v1.2	2025-12-29	Dillon Corvino	Unified with Abdullah lab protocol