ARCHIVAL Isolation of Liver-Associated Lymphocytes (LALs) Including NLCs from Mouse Liver

Abdullah Lab, IMMEI, University Hospital Bonn

2025-11-21

Protocol ID: ARCHIVAL-MUS-LIV-NLC-ISO-001
Version: v1.0
Author: Dillon Corvino

Purpose

This protocol describes the isolation of liver-associated lymphocytes (LALs), including NK-like T cells (NLCs), from mouse liver. Livers are perfused, minced, enzymatically digested with collagenase IV and DNase I, and processed using low-speed centrifugation to remove hepatocytes, followed by 37% Percoll density enrichment and ACK lysis to obtain a viable single-cell suspension enriched for LALs/NLCs.

The protocol is restricted to liver processing only and does not cover spleen processing or flow cytometry staining panels.

Critical notes (read before starting)

- Keep livers and cell suspensions cold (on ice or at 4 °C) wherever possible, except during enzymatic digestion.
- Ensure thorough perfusion of the liver with ice-cold PBS to reduce blood contamination and improve Percoll separation.
- Avoid over-digestion; prolonged incubation with collagenase/DNase can damage lymphocytes and reduce NLC yield.
- The low-speed centrifugation step (50 g) is critical to remove hepatocytes without pelleting lymphocytes.
- Use gentle acceleration and minimal braking for Percoll spins to preserve the gradient.
- Perform ACK lysis only for the specified time to avoid damaging leukocytes.
- Process livers promptly after harvest to preserve cell viability.

> **Warning**
>
> Over-digestion, harsh centrifugation, or prolonged ACK lysis can markedly reduce lymphocyte viability and recovery. Strictly adhere to the indicated incubation times and centrifugation settings.

> **Tip**
>
> Prepare all buffers, label tubes, and pre-cool the centrifuge before starting animal work to minimize delays once livers are harvested.

Approximate timing

- Preparation and setup: 10–15 min
- Mouse sacrifice and liver perfusion: 10–15 min
- Liver mincing, digestion, and gentleMACS processing: 30–40 min
- Hepatocyte removal and washing: 30 min
- Percoll gradient and enrichment: 30 min
- ACK lysis, filtration, and final wash: 20–30 min

Total approximate time: 2–2.5 hours for 1–4 livers (processed in parallel).

Table of contents

Procedure

Step 1 – Preparation and setup

1. **Pre-cool solutions**
   - Pre-cool PBS on ice for liver perfusion and transport.
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 per well.
   - **Option B: 50 mL tube collection**
     - Label one 50 mL conical tube per liver.
     - Add 5 mL ice-cold PBS 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 steps.
4. **Prepare buffers** (see separate buffer protocols)
   - Liver digestion buffer (BUF-MUS-LIV-DIG-001; collagenase IV + DNase I in PBS).
   - 37% Percoll working solution (BUF-GEN-PERC-37PCT-001).
   - ACK lysis buffer (BUF-GEN-ACK-LYSIS-001).
   - FACS buffer (BUF-GEN-FACS-BUF-001), if required for downstream applications.
5. **Warm digestion components**
   - Turn on a water bath and set to **37 °C**.
   - Place an aliquot of **liver digestion buffer** in the water bath to pre-warm.
   - If digestion buffer will be prepared later, pre-warm **DMEM or PBS** instead.
6. **Equipment setup**
   - Set a refrigerated 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.
   - 6-well plates *and/or* pre-filled 50 mL tubes (as chosen above).
   - Sterile scissors and forceps.
   - 10 mL syringes (one per mouse or per treatment group).
   - Needles for liver perfusion (recommended **25G or 26G**).
   - Absorbent pads and waste container for perfusion runoff.

Step 2 – Mouse sacrifice and liver perfusion

1. Sacrifice mice according to institutional and ethical guidelines (e.g. CO asphyxiation followed by cervical dislocation, as approved).
2. Place the mouse in a supine position on a suitable surface.

3. Open the abdominal cavity to expose the liver and major vessels.
4. Perfuse the liver with ice-cold PBS via one of the following routes:
    - Inferior vena cava
    - Portal vein
    - Directly via the heart
5. Gently insert a needle into the chosen vessel and perfuse with PBS until the liver visibly blanches (typically ~5–10 mL per mouse).

> **Note**
>
> Efficient perfusion reduces blood content and improves the quality of density separation and downstream staining.

6. After perfusion, proceed immediately to liver collection.

Step 3 – Liver collection and mincing

1. Excise the perfused liver using sterile, autoclaved instruments.
2. Transfer the liver to the pre-labelled **50 mL conical tube** *or* to the appropriate **well of a 6-well plate** containing ice-cold PBS.
3. Keep the tube or plate on ice at all times.
4. *(Optional but highly recommended)* **Weigh the liver**
    - Briefly place the liver on blotting paper to wick off excess liquid.
    - Weigh the liver and record the weight in the lab book.
5. Add **4 mL of pre-warmed liver digestion buffer** to a labelled gentleMACS C-tube (one tube per liver).
6. Transfer the liver into the gentleMACS C-tube containing digestion buffer.
7. Using sterile **curved scissors**, mince the liver directly within the gentleMACS C-tube until tissue fragments are approximately ~2–3 mm.
8. Secure the C-tube onto the gentleMACS instrument.

> **Note**
>
> Ensure all tissue pieces are fully submerged in digestion buffer and not stuck to the walls or base of the tube.

9. Place the warming sleeve over the tube.
10. Run program **HH17** (17-minute digestion).

> **Warning**
>
> After starting the run, confirm that all tubes are correctly seated and rotating properly.
> If a tube is not spinning, stop the run, remove the tube, reseat it, and restart the program.

> **!** Important
>
> Do **not** leave livers in digestion buffer for prolonged periods. Over-digestion can strip surface epitopes and negatively impact downstream staining and phenotyping.
> Note: the gentleMACS instrument does **not** beep when the program finishes—set a timer or alarm before starting the run.

> ⚠ Warning
>
> Make sure the gentleMACS C-tube is firmly closed and correctly positioned. Leaks during the run can lead to sample loss and contamination of the instrument.

Step 5 – Removal of hepatocytes and washing

1. After digestion, transfer the liver cell suspension from the C-tube into a 50 mL conical tube.
2. Rinse the C-tube with ice-cold PBS and add the rinse to the same 50 mL tube to maximize yield.
3. Bring the volume up to 50 mL with ice-cold PBS.
4. Centrifuge at 50 g for 2 min at 4 °C to sediment hepatocytes.
5. Carefully transfer the supernatant (containing lymphocytes and smaller cells) to a fresh 50 mL tube without disturbing the hepatocyte pellet.
6. Adjust the volume to 50 mL with ice-cold PBS.
7. Centrifuge at 550 g for 10 min at 4 °C.
8. Discard the supernatant and keep the cell pellet on ice.

Step 6 – 37 % Percoll enrichment of liver-associated lymphocytes

1. While the liver samples are pelleting, aliquot **10 mL of 37% Percoll** (BUF-GEN-PERC-37PCT-001, prepared in PBS) into **labelled 15 mL conical tubes** (one tube per liver).
2. Carefully aspirate the supernatant from the liver cell pellet in the 50 mL tube.
3. Using a **transfer pipette, 1 mL pipette, or serological pipette**, take up approximately **1 mL of 37% Percoll** from the corresponding 15 mL tube and use it to gently resuspend the liver cell pellet in the 50 mL tube.
4. Transfer the resuspended cells back into the labelled 15 mL Percoll tube.
5. Repeat the rinse once or twice with additional small volumes of Percoll, if needed, to ensure all remaining liver cells are recovered from the 50 mL tube.
6. Cap the 15 mL tube and **invert gently 2–3 times** to ensure complete mixing.
7. Centrifuge at **800 × g for 20 min at room temperature (RT; ~20–22 °C)** using:
   - **Moderate acceleration** (e.g. acc 7)
   - **Minimal brake** (e.g. br 1) to avoid disturbing the gradient

> **!** Important
>
> Do **not** leave liver samples in Percoll for longer than necessary. Prolonged exposure can reduce cell viability and negatively impact downstream applications. Plan spins and downstream steps in advance.

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

8. After centrifugation, carefully asiprate the supernatant.
9. Resuspend the enriched lymphocyte pellet in 20–30 mL ice-cold PBS.
10. Centrifuge at 550 g) for 10 min at 4 °C.
11. Discard the supernatant and keep the pellet on ice.

STEP 7 – RBC lysis and filtration

1. Add **1 mL RBC lysis buffer per liver** to the cell pellet:
   - **HybriMax RBC Lysis Buffer:** incubate for **5 minutes at room temperature**, **or**
   - **ACK lysis buffer:** incubate for **2 minutes at room temperature**.
2. Gently resuspend the pellet by pipetting or flicking immediately after adding buffer.
3. After incubation, quench the lysis by topping up to **50 mL with FACS Buffer**.

> ⚠ Warning
>
> Do not exceed the indicated incubation times. Over-lysis (particularly >5 minutes with HybriMax or >2 minutes with ACK) can reduce leukocyte viability.

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 PBS if needed to recover remaining cells.
6. Centrifuge at 550 g for 10 min at 4 °C.
7. Discard the supernatant and keep the pellet on ice.

Step 8 – Final wash and resuspension

1. Resuspend the cell pellet in 10 mL ice-cold PBS.
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).

Materials

Reagents

Reagent	Supplier	Cat. #	Notes
PBS, sterile, 1×	Various	–	Perfusion, washes, resuspension

Reagent	Supplier	Cat. #	Notes
Collagenase IV	Various	–	Component of BUF-MUS-LIV-DIG-001
DNase I	Various	–	Component of BUF-MUS-LIV-DIG-001
Percoll	Cytiva/GE or similar	–	Component of BUF-GEN-PERC-37PCT-001
ACK lysis buffer	Various / in-house	–	BUF-GEN-ACK-LYSIS-001
Fetal calf serum (FCS), heat-inactivated	Various	–	For BUF-GEN-FACS-BUF-001 or culture medium
EDTA, 0.5 M stock	Various	–	For BUF-GEN-FACS-BUF-001
Appropriate culture medium (e.g. RPMI 1640)	Various	–	For downstream cultures, if required

Disposables

Item	Specification
50 mL conical tubes	Sterile
GentleMACS C-tubes or equivalent	For tissue dissociation
Sterile Petri dishes	For liver mincing
70 µm cell strainers	For filtration
Pipette tips (10 µL, 200 µL, 1000 µL)	Sterile
Syringes and needles	For liver perfusion
Waste containers and sharps bins	According to local regulations

Equipment

Equipment	Specification / Notes
CO chamber and euthanasia setup	As per institutional animal welfare guidelines
Dissection instruments	Scissors, forceps, autoclaved
GentleMACS dissociator (or equivalent)	With liver-optimized program
Refrigerated centrifuge	4 °C, swing-out rotor for 50 mL tubes
Biological safety cabinet	For aseptic handling of tissues and cell suspensions
Ice bucket	To maintain samples on ice
Cell counter or hemocytometer	For cell counting

Buffers used

- **BUF-MUS-LIV-DIG-001** – Liver digestion buffer (collagenase IV + DNase I in PBS).
- **BUF-GEN-PERC-37PCT-001** – 37 % Percoll working solution in PBS.

- **BUF-GEN-ACK-LYSIS-001** – ACK red blood cell lysis buffer.
- **BUF-GEN-FACS-BUF-001** – FACS buffer (PBS + 2 % FCS + 2 mM EDTA), if used for downstream staining.

Troubleshooting

Problem	Possible cause	Suggested solution
Low lymphocyte yield	Incomplete perfusion or digestion	Confirm efficient perfusion; ensure thorough mincing and full 17 min digestion.
High red blood cell contamination	Insufficient or ineffective ACK lysis	Repeat ACK lysis briefly (2 min) and quench immediately with excess PBS.
Poor Percoll separation or indistinct pellet	Incorrect Percoll concentration or harsh braking	Prepare fresh 37 % Percoll; confirm centrifuge settings; reduce brake.
Low cell viability	Over-digestion, prolonged ACK exposure, or warm handling	Shorten digestion and ACK steps; keep samples on ice; process promptly.
Excessive debris or clumps	Inadequate filtration or DNase activity	Confirm DNase in digestion buffer; pass suspension through 70 µm strainer.

Safety (brief)

- Perform all animal procedures in accordance with approved animal licences and institutional animal welfare regulations.
- Wear appropriate PPE (lab coat, gloves, eye protection) when handling animals, tissues, and reagents.
- Use caution when handling sharps (needles, scalpels); dispose of them in designated sharps containers.
- Work in a biological safety cabinet when handling tissues and cell suspensions to maintain sterility and operator safety.
- Dispose of animal tissues, cell suspensions, and contaminated consumables as biological waste according to local guidelines.

Version history

Version	Date	Author	Changes
v1.1	2025-12-12	Dillon Corvino	Updated liver collection to allow 6-well plate or 50 mL tube handling; added liver weighing, refined gentleMACS digestion workflow, and clarified Percoll resuspension and centrifugation steps.
v1.0	2025-11-21	Dillon Corvino	First Quarto protocol version for LAL/NLC isolation from mouse liver.