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

Abdullah Lab, IMMEI, University Hospital Bonn

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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).

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Procedure

Step 1 – Preparation and setup

1. Pre-cool PBS on ice.
2. Label 50 mL conical tubes and gentleMACS C-tubes (or equivalent) for each liver.
3. For each liver, prepare:
    - 5 mL ice-cold PBS in a 50 mL tube for transport.
4. Prepare the following 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 needed for downstream steps.
5. Set a refrigerated benchtop centrifuge to 4 °C.
6. Ensure access to a gentleMACS instrument with an appropriate liver dissociation program.

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 into the pre-labelled 50 mL tube containing 5 mL ice-cold PBS.
3. Keep the tube on ice.
4. Transfer the liver to a sterile Petri dish placed on ice.

5. Using autoclaved scissors or scalpels, mince the liver into small pieces (~2–3 mm) to facilitate enzymatic digestion.
6. Transfer the minced liver pieces into a gentleMACS C-tube.

Step 4 – Enzymatic digestion and gentleMACS processing

1. For each liver C-tube, add:
   - 2 mL of 2× liver digestion buffer (BUF-MUS-LIV-DIG-001).
   - 2 mL PBS.
     Total volume: 4 mL.
2. Close the C-tube tightly and ensure the cap is well seated.

> **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.

3. Place the tube on the gentleMACS and run an appropriate liver dissociation program according to the manufacturer's instructions.
4. Immediately after mechanical dissociation, incubate the tube at 37 °C so that the total enzymatic digestion time is approximately 17 min (including any on-instrument warm steps, if applicable).
5. If compatible with the program, gently invert the tube once or twice during incubation to improve digestion.

> **Note**
>
> The specific gentleMACS program used may vary. Choose a liver-optimized program that preserves lymphocyte viability.

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 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 pre-cooled 50 mL tube without disturbing the hepatocyte pellet.
6. Adjust the volume to 50 mL with ice-cold PBS.
7. Centrifuge at 1600 rpm (approximately 547 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. Gently resuspend the cell pellet in 10 mL of 37 % Percoll (BUF-GEN-PERC-37PCT-001) prepared in PBS.

2. Mix gently by pipetting until the pellet is fully resuspended.
3. Centrifuge at 800 g for 20 min at 4 °C, using:
    - Moderate acceleration (e.g. acc 7).
    - Minimal brake (e.g. br 1) to avoid disturbing the gradient.

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

4. After centrifugation, carefully discard the supernatant.
5. Resuspend the enriched lymphocyte pellet in 20–30 mL ice-cold PBS.
6. Centrifuge at 1600 rpm (~547 g) for 10 min at 4 °C.
7. Discard the supernatant and keep the pellet on ice.

Step 7 – Red blood cell lysis and filtration

1. Gently resuspend the cell pellet in 2 mL ACK lysis buffer (BUF-GEN-ACK-LYSIS-001) at room temperature.
2. Incubate for 2 min at room temperature, gently inverting once.

> ⚠ Warning
>
> Do not exceed 2–3 min in ACK buffer. Prolonged exposure can damage lymphocytes and reduce viability.

3. Quench the lysis by adding 20 mL ice-cold PBS.
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 1500 rpm (~500–550 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 10–20 mL ice-cold PBS (depending on pellet size).
2. Centrifuge at 1500 rpm (~500–550 g) for 5–10 min at 4 °C.
3. Discard the supernatant.
4. Resuspend the final cell pellet in an appropriate volume of:
    - PBS or FACS buffer (BUF-GEN-FACS-BUF-001) for immediate staining, or
    - Culture medium (e.g. R10 or NK cell medium) for downstream functional assays.
5. Count cells and assess viability (e.g. trypan blue) as required.
6. 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
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
v1.0	2025-11-21	Dillon Corvino	First Quarto protocol version for LAL/NLC isolation from mouse liver.