Primary Mouse Hepatocyte Isolation

Two-step collagenase perfusion method via vena cava cannulation

Overview

This protocol describes isolation of primary hepatocytes from mouse liver using a two-step collagenase perfusion technique with retrograde perfusion through the inferior vena cava. Viable hepatocytes are purified using Percoll density gradient centrifugation.

Expected Yield:

- **30-50 × 10^6 viable hepatocytes** per mouse
- **Viability:** Typically >95%
- **Purity:** >90% hepatocytes
- **Duration:** ~2-3 hours per mouse

Applications

- Drug metabolism studies
- Hepatotoxicity assays
- Metabolic pathway analysis
- Primary hepatocyte culture
- Liver disease modeling
- Gene expression studies

Principle

- **Step 1:** Ca^{2+}-free EGTA perfusion disrupts cell-cell junctions
- **Step 2:** Liberase TM digests extracellular matrix
- **Purification:** Percoll gradient separates viable hepatocytes
- **Viability:** Trypan blue exclusion assay

Safety & Animal Care

- Ensure proper IACUC approval for all animal procedures
- Follow institutional guidelines for animal handling and euthanasia
- Wear appropriate PPE: lab coat, gloves, eye protection
- Work in biosafety cabinet for sterile isolation
- Dispose of biological waste properly
- Liberase and enzymes - handle according to SDS

Required Materials

Reagents

- **Liberase TM** (Roche, Cat# 5401127001)
- EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid)
- HEPES buffer
- NaCl, KCl, Na_2HPO_4, glucose
- $CaCl_2$ (for Liberase buffer)
- Percoll (GE Healthcare)
- Hank's Balanced Salt Solution (HBSS)
- William's Medium E or DMEM
- Fetal bovine serum (FBS)
- Penicillin/Streptomycin
- 0.4% Trypan blue solution

Equipment & Supplies

- Peristaltic pump or gravity perfusion apparatus
- Water bath (37-42°C)
- Dissection board and surgical instruments
- 23G or 24G butterfly catheter/IV catheter
- Silk suture (4-0 or 5-0)
- Sterile scissors, forceps, clamps
- 100 µm and 70 µm cell strainers
- 50 mL conical tubes (sterile)
- Centrifuge (clinical or benchtop)
- Hemocytometer or cell counter
- Biological safety cabinet

- Inverted microscope

Buffer Recipes

Perfusion Buffer 1 (Ca^{2+}-free EGTA Buffer) - 500 mL:

- NaCl: 8.3 g (142 mM)
- KCl: 0.5 g (6.7 mM)
- HEPES: 2.4 g (10 mM)
- EGTA: 0.19 g (0.5 mM)
- Adjust pH to 7.4, sterile filter

Perfusion Buffer 2 (Liberase Buffer) - 100 mL:

- Same as Buffer 1 but without EGTA
- Add $CaCl_2$: 0.056 g (5 mM)
- Add Liberase TM: 0.2-0.4 Wünsch units/mL
- Prepare fresh, keep at 37°C

Wash Buffer (HBSS + supplements):

- HBSS
- 10% FBS
- 1% Penicillin/Streptomycin
- Keep at 4°C

Percoll Solutions:

- **Stock:** 100% Percoll
- **Working (50%):** 25 mL Percoll + 25 mL 2× HBSS
- **Working (25%):** 12.5 mL Percoll + 37.5 mL 1× HBSS
- Prepare fresh, keep at 4°C

Pre-Procedure Preparation

1. Prepare Buffers (1 day before or morning of)

Mark Done

- Prepare Perfusion Buffer 1 (EGTA buffer) - sterile filter, store at 4°C
- Prepare base for Perfusion Buffer 2 (without Liberase) - sterile filter, store at 4°C
- Prepare Wash Buffer - keep at 4°C
- DO NOT add Liberase until just before use

2. Set Up Perfusion System

Mark Done

- Set up peristaltic pump with sterile tubing
- Attach 23G or 24G butterfly catheter to tubing

- Test pump flow rate: 3-5 mL/min recommended
- Fill tubing with Buffer 1 to remove air bubbles
- Clamp tubing until ready to use

3. Warm Buffers and Equipment

Mark Done

- Pre-warm Perfusion Buffer 1 to 37-42°C in water bath
- Pre-warm base of Perfusion Buffer 2 to 37-42°C
- Add Liberase TM to Buffer 2 just before use (final: 0.2-0.4 Wünsch units/mL)
- Mix gently and keep at 37°C

4. Prepare Dissection Area

Mark Done

- Autoclave dissection instruments
- Prepare dissection board
- Lay out sterile gauze, sutures, scissors, forceps
- Have 70% ethanol spray ready
- Set up biosafety cabinet with sterile cell strainers and tubes

5. Prepare Percoll Gradients

Mark Done

- Prepare 50% Percoll solution (see Materials section)
- Prepare 25% Percoll solution
- Keep on ice until use
- Will be used after hepatocyte isolation for purification

Hepatocyte Isolation Protocol

> **Timing: Allow 2-3 hours from anesthesia to final cell count**

1. Anesthesia and Surgical Preparation

Mark Done

- Anesthetize mouse according to IACUC protocol (isoflurane, ketamine/xylazine, etc.)
- Confirm deep anesthesia (no toe pinch reflex)
- Spray abdomen with 70% ethanol
- Place mouse on dissection board in supine position
- Secure limbs with tape or pins

2. Expose Inferior Vena Cava (IVC)

Mark Done

- Make midline incision from pubis to xiphoid process
- Make lateral incisions to create T-shape opening
- Reflect skin and abdominal wall to expose liver and intestines

- Gently move intestines to the left to expose the IVC
- Clear connective tissue around IVC (below liver) using cotton swabs
- Identify portal vein (will be cut later for drainage)

3. Cannulate Inferior Vena Cava

Mark Done

- Use 23G or 24G butterfly catheter connected to perfusion tubing
- Make small nick in IVC wall (below liver, above renal veins)
- Insert catheter tip into IVC lumen, advance toward liver
- Secure catheter with suture around IVC and catheter
- Tie suture gently to hold catheter in place without collapsing vessel

4. Perfusion - Step 1 (EGTA Buffer)

Mark Done

- Cut portal vein to allow outflow (creates open circulation)
- Immediately start perfusion with warm Buffer 1 (EGTA buffer, 37-42°C)
- Flow rate: 3-5 mL/min
- Duration: 3-5 minutes
- Liver should blanch and become pale as blood is cleared
- Observe fluid draining from portal vein cut

5. Perfusion - Step 2 (Liberase Buffer)

Mark Done

- Switch perfusion to Buffer 2 (Liberase + Ca^{2+}, 37-42°C)
- Continue perfusion at same flow rate
- Duration: 5-8 minutes (or until liver becomes soft)
- Liver should appear "mushy" and develop mottled appearance
- Gently touch liver surface - should feel very soft
- Stop perfusion when digestion is complete

6. Liver Excision and Cell Release

Mark Done

- Carefully remove liver from mouse using scissors and forceps
- Transfer liver to sterile Petri dish containing cold Wash Buffer
- Move to biosafety cabinet
- Remove gall bladder if still attached
- Tear open liver capsule gently with forceps
- Use sterile spatula or forceps to gently agitate liver and release cells
- Pipette suspension gently to help dissociate tissue

7. Filter Cell Suspension

Mark Done

- Pass cell suspension through 100 µm cell strainer into 50 mL conical tube
- Use cold Wash Buffer to rinse all cells through strainer
- Optional: Filter again through 70 µm strainer for cleaner preparation

- Collect filtrate in sterile 50 mL tubes on ice

8. Low-Speed Centrifugation

Mark Done

- Centrifuge at **50 × g for 3 minutes at 4°C**
- Hepatocytes pellet at low speed due to large size
- Carefully aspirate supernatant (contains dead cells, debris, non-parenchymal cells)
- Resuspend pellet in cold Wash Buffer
- Repeat centrifugation 2-3 times until supernatant is clear

9. Percoll Gradient Purification

Mark Done

- Resuspend washed hepatocytes in 25% Percoll solution
- Layer onto 50% Percoll cushion in 50 mL tube
- Centrifuge at **100 × g for 10 minutes at 4°C** (no brake)
- Viable hepatocytes pellet at bottom
- Dead cells and debris remain at interface and in supernatant
- Carefully aspirate supernatant and interface layer
- Resuspend pellet in cold Wash Buffer

10. Final Wash and Resuspension

Mark Done

- Centrifuge at 50 × g for 3 minutes at 4°C
- Aspirate supernatant
- Resuspend in appropriate culture medium or assay buffer
- Common media: William's Medium E + 10% FBS + 1% Pen/Strep

11. Cell Counting and Viability Assessment

Mark Done

- Take small aliquot for cell counting
- Mix 1:1 with 0.4% trypan blue
- Count using hemocytometer or automated counter
- Calculate: **Viability (%) = (Viable cells / Total cells) × 100**
- Expected: **30-50 × 10^6 cells per mouse, >95% viability**
- Hepatocytes are large (20-30 µm diameter) and polygonal

12. Plating or Storage

Mark Done

- **For immediate use:** Plate on collagen-coated dishes at 5-10 × 10^4 cells/cm^2
- Allow to attach for 2-4 hours before experiments
- Change medium after attachment to remove dead cells
- **For cryopreservation:** Freeze slowly in freezing medium (FBS + 10% DMSO)
- Note: Primary hepatocytes do not passage well; use within 3-5 days

Troubleshooting

Problem	Possible Cause	Solution
Low cell yield	Insufficient digestion Liberase activity low Poor perfusion	Increase Liberase concentration (0.3-0.4 units/mL) Extend perfusion time by 1-2 min Check catheter placement Use fresh Liberase
Low viability (<80%)	Over-digestion Buffers too warm Rough handling	Reduce Liberase time Monitor liver softness Keep buffers at 37-40°C max Handle cells gently, avoid vortexing
Liver did not blanch	Catheter not in IVC lumen Portal vein not cut Blood clot blocking flow	Check catheter position, adjust if needed Ensure portal vein is fully cut Increase flow rate slightly
Liver not soft after Liberase	Liberase inactive Insufficient perfusion time No Ca^{2+} in Buffer 2	Use fresh Liberase Extend perfusion 2-3 more minutes Verify $CaCl_2$ added to Buffer 2
High debris in prep	Over-digestion Incomplete filtering	Reduce Liberase time Filter through 70 µm and 100 µm sequentially Perform additional washes
Cells not attaching to culture dish	Dish not coated Low viability Wrong plating density	Pre-coat dishes with collagen type I (50 µg/mL, 1hr) Check viability before plating Plate at 5-10 × $10^4/cm^2$

Quality Control Tips

- Successful isolation: Hepatocytes should be large (20-30 µm), round to polygonal, with clear cytoplasm and distinct nucleus
- Viability >95% is ideal; 85-95% is acceptable; <85% indicates problems
- Purity can be assessed by cell morphology and size (hepatocytes much larger than NPCs)
- Functional tests: Albumin secretion, urea synthesis, CYP450 activity

- Keep cells cold (4°C) throughout isolation until plating to preserve viability
- Primary hepatocytes are post-mitotic - do not passage, use within 3-5 days

Protocol adapted from published methods and laboratory procedures

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