Liver Macrophages Isolation

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Reagents:

Collagenase D (Roche, Cat# 11088874103)
DNAse I (2 mg/mL of GBSS-B; Roche, Cat# 10104159001; store aliquots in -20°C)
Protease Type XIV (Sigma, Cat# P5147-1G)
Histodenz (5.81 g/20 mL of GBSS-A; Sigma, Cat# D2158-100G)
SC-1 EGTA Solution
SC-2 Enzyme Solution
GBSS-B (Sigma, Cat# G9779-6X500 ML)
MACS Buffer (0.5% BSA, 2 mM EDTA in PBS; sterile-filtered)

Solutions (for 4 mice = Max number that can be handled in a day):

1. EGTA Solution→ 200 mL; **filter sterile**
2. Collagenase D Solution→ Solution should be adjusted to 0.04 U/mL depending on lot variation (e.g., For the current lot of Collagenase D, we use 13.4 mg/75 mL of SC-2 for each mouse; Prepared 1000X by adding 5.6 mL of SC-2 to 1 g of Collagenase D; Stored at -20°C)
3. Protease Solution→ 20 mg of Protease XIV in 40 mL of SC-2; prior to adding perfused liver, add 400 uL of DNAse I (stock is 2 mg/mL) (need 40 mL for each mouse)
4. Histodenz→ Dissolve 8 g of Histodenz in 18mL of GBSS-A (on the shaker). Keep on a shaker for at least 30 min prior to filtering. Once it is dissolved, when you filtering it, pour everything (20mL) in a syringe with the filter and adjust the volume to 27.5 mL. Then press the plunger. (Need 14 mL for each mouse gradient); **filter sterile** immediately before use

- *Autoclave and filter all bottles and solutions, respectively, for sterilization.*
- *For all solutions, add enzyme into sterile bottle (on the day or day before) but then filter respective solutions into the bottle using 0.2 um bottle top filter and mix thoroughly (on the day of use).*
- *Incubate EGTA, Collagenase, Protease solutions in 40-42°C waterbath.*
- *Have the system ready for immediate EGTA infusion, i.e., 1) test flow rate and 2) have the tube full of EGTA before starting the liver perfusion procedure.*
- *Keep the area warm enough during perfusion.*
- *Make sure to flush out the line with ddH$_2$O before and after use.*

Retrograde Liver Perfusion:

1. Anesthetize mouse with Isoflurane and open peritoneal cavity. Move organs to expose IVC and hepatic portal vein.
2. Cannulate into the IVC using 22G catheter (connected to the perfusion tube). Remove needle and ensure backflow of blood to know you are in the right vessel. Stabilize the insert using a needle cap.
   (Optional) Cut a small piece of RNA from right lobe and flash freeze for RNA isolation.
3. Open chest cavity and clamp down suprahepatic IVC (Clamping is critical for avoiding any loss of liver cells during perfusion).
4. Cut portal vein to allow blood to drain.
5. Perfuse with EGTA solution only until liver gets pale (about 1 minute).
6. Turn off the pump and transfer the tube to Collagenase D solution.
7. Perfuse Collagenase D solution for 5 minutes at speed of 10 mL/min. Remove the tube from the enzyme solution and press stop when the tube becomes empty.
8. Remove liver (DO NOT REMOVE THE CLAMP!!) and place in 4 mL of ice-cold SC-2 buffer (when multiple tissue collection is needed). Use 60 mm petri dish to avoid adherence of cells.
9. Remove connective tissues with scissors, hold liver with blunt-ended forceps and then mince well using sharp-ended forceps. Mince until pipettable with a 10-mL pipette.
10. Transfer liver homogenate to 40 mL of Protease solution containing 400 uL of DNase I. Stir at 37°C on hotplate for 25 minutes. During digestion, clean up the area and flush the perfusion tubes with DDW for 3 min.
11. Filter digested liver through cell strainer (100 um) into a 50 mL Falcon tube.
12. Centrifuge at 50 x g for 3 minutes at 4°C.
13. Transfer supernatant to new 50 mL Falcon. Keep pellet on ice. Spin supernatant at 580 x g for 5 minutes at 4°C.
14. Aspirate supernatant down to 5 mL. Add 5 mL of GBSS-B and 120 uL DNAseI and resuspend using a 10-mL pipette. Fill volume up to 50 mL.
15. Spin at 580 x g for 10 minutes at 4°C. While spinning, filter Histodenz solution through syringe filter. Keep on ice.
16. Aspirate down to 5 mL of supernatant. Add 5 mL GBSS-B and 120 uL of DNase I. Resuspend and thoroughly mix to avoid any cell clumps.
17. Add 14 mL of Histodenz solution and mix well by inverting the tube.
18. Transfer 11.5-12 mL of solution into each of 2 clear 15 mL Falcon tubes.

19. Carefully overlay 1.5 mL of GBSS-B onto the solution using a 21G syringe (2.5 mL) to avoid any turbulence.
20. Centrifuge at 1380 x g for 15 minutes at 4°C WITH **NO BRAKE**!!! While gradient centrifugation, degass the MACS buffer (stored at 4°C) for 15-20 min with the bottle top filter used during buffer preparation. Seal the gaps with parafilm before turning the vacuum on.
21. The white/brownish layer under the clear layer of GBSS-B solution and above the Histodenz gradient phase contains endothelial cells, Kupffer cells, small hepatocytes, HSCs, etc. Collect this layer using a 5-ML pipette at slow rate and transfer into new 50 mL tube. Need not to be too careful with the purity of collection as MACS will be conducted afterwards.
22. Fill up with GBSS-B to 50 mL to wash and centrifuge at 50 x g for 2 minutes at 4°C to pellet out hepatocytes.
23. Collect supernatant and transfer to fresh tube. Centrifuge at 700 x g for 5 minutes at 4°C.
24. Discard supernatant and resuspend pellet in 10 mL of MACS buffer. Transfer into fresh 15 mL tube.
25. Count cells and pellet at 700 x g for 5 minutes at 4°C.
26. Resuspend in volume that gives density of 10^7 cells per 1 mL.
27. Proceed with antibody labeling procedure for MACS.

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http://www.miltenyibiotec.com/download/datasheets_en/8/DS130_041_301_042_201.pdf

1. Add Biotin anti-F4/80 (eBioscience; stock conc = 0.5 ug/uL): 2.5 uL/10^7 cells.
2. Mix well by flicking the tube and incubate on ice for 30-40 min (roll the tube every 10 min).
3. Add MACS buffer 1 mL/10^7 cells and spin at 700 x g for 5 min.
4. Resuspend in MACS buffer again: 80 uL/10^7cells.
5. Add 9 uL of anti-biotin micro beads (Miltenyi) with 80 uL of MACS buffer (ratio 1:9).
6. Mix well and incubate for 30 min at 4°C in dark.
7. Wash in 1.0 mL MACS buffer and spin at 500 x g for 5 min. Meanwhile, prime the MS column with 500 uL of buffer.
8. Discard the supernatant and resuspend up to 10^8 cells in 500 uL of MACS buffer.
9. Load the cell suspension into the column and let it run through.
10. Collect the flow-through containing F4/80 unlabeled cells.
11. Wash for 3 times with 500 uL of MACS buffer (each time wait until everything has run through but be sure not to make the column dry).
12. Remove the column from the magnet and add 1 mL MACS buffer, expel the retained cells into a fresh 1.5 mL tube with an ejector.
13. Count cells and centrifuge at 500 x g for 5min.
14. Treat cells with Laemmli Buffer w/ BME (100 uL/10^6 cells) for total protein or 600 uL of RLT Buffer for RNA isolation.

MACS Buffer (0.5% BSA, 2mM EDTA in PBS, sterile filtered)

	EGTA solution SC-1		**Collagenase solution SC-2**
NaCl	8000mg/L		8000mg/L
Kcl	400mg/L		400mg/L
NaH2PO4. H2O	88.17mg/L		88.17mg/L
Na2HPO4	120.45mg/L		120.45mg/L
HEPES	2380mg/L		2380mg/L
NaHCO3	350mg/L		350mg/L
EGTA	190mg/L		(-)
Glucose	900mg/L		(-)
CaCl2. 2H2O	(-)		560mg/L add slowly after stirring 30min!

pH: 7.35~7.4 by 10N NaOH

GBSS (Gey's balanced salt solution):

	GBSS/A SC-3		**GBSS/B SC-4**
NaCl	(-)		8000mg/L
Kcl	370mg/L		370mg/L
MgCl2. 6H2O	210mg/L		210mg/L
MgSO4. 7H2O	70mg/L		70mg/L
Na2HPO4.	59.6mg/L		59.6mg/L
KH2PO4	30mg/L		30mg/L
Glucose	991mg/L		991mg/L
NaHCO3	227mg/L		227mg/L
CaCl2. 2H2O	225mg/L		225mg/L

pH: 7.35

Histodenz solution: 5.81g / 20 mL of GBSS/A. Filtered through 0.22 μm (hard to filter).