

SOP: Isolation of Mouse Fetal Liver Cell Subsets
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Summary

For the isolation of mouse fetal liver cell subsets that differ in their stage of erythroid maturation, the differential expression of CD117, CD71, and Ter119 antigens is exploited.

Materials List

1. Pregnant CD-1 Mice at 14.5 days post conception
2. Phosphate Buffered Saline (1X PBS) (Invitrogen/Life Technologies, Cat# 10010-023)
3. BSA (22%) (Sigma-Aldrich, Cat# A7034)
4. StemPro-34 SFM Medium (1X) with Nutrient Supplement (Invitrogen/Life Technologies, Cat# 10639-011)
5. Penicillin/Streptomycin (Pen/Strep) Solution (Cellgro Mediatech, Inc., Cat# 30-001-CI)
6. L-Glutamine, 200mM (Invitrogen/Life Technologies, Cat# 25030-081)
7. EPO (Procrit)
8. SCF/c-kit Ligand, Mouse, Recombinant (R&D Systems, Cat# 455-MC/CF)
9. Dexamethasone (Sigma-Aldrich, Cat# D4902)
10. IGF-1, Human, Recombinant (Promega, Cat# G5111)
11. Iscove's DMEM (IMDM) Medium (1X) (Cellgro Mediatech, Inc., Cat# 10-016-CM)
12. BSA (Sigma-Aldrich, Cat# A0281)
13. DMEM Medium (Cellgro Mediatech, Inc., Cat# 10-013-CV)
14. Normosol-R (Hospira, Inc., Lake Forest, IL, NDC# 0409-7967-03)
15. BSA, Fraction V (Fisher Scientific, Cat# BP1600)
16. DMSO, Hybri-Max (Sigma-Aldrich, Cat# D2650)
17. PE Rat Anti-Mouse Ter119 (BD Pharmingen, Cat# 553673)
18. FITC Rat Anti-Mouse CD71 (BD Pharmingen, Cat# 553266)
19. APC Rat Anti-Mouse CD117 (BD Pharmingen, Cat# 553356)
20. 7-AAD (Invitrogen/Life Technologies, Cat# A1310)
21. 70µm Cell Strainer (BD Bioscience, Cat# 352350)
22. 1.7mL Eppendorf Tubes
23. 1000µL Rainin Pipettor with Tips
24. 15mL Corning Polypropylene Conical Centrifuge Tubes (Corning, Cat# 430766)
25. 50mL Corning Polypropylene Conical Centrifuge Tubes (Corning, Cat# 430828)
26. 5mL Round Bottom Polystyrene Falcon Tubes (BD Falcon, Cat# 352003)
27. Eppendorf Refrigerated Centrifuge 5810R with Swinging Bucket Rotor
28. Hemocytometer with Trypan Blue Stain
29. Micropipet with P20 Tips
30. Microscope
31. FACS Aria II Cell Sorter (ARIA)
32. Cryovials, 1.8mL (Nunc, Cat# 368632)
33. Revco UltimaII -80°C Freezer
34. Thermolyne Locator 4 Liquid Nitrogen Freezer

1% BSA IN 1X PBS (PBS-BSA) BUFFER

For ~50mL:

48mL 1X PBS
2.27mL 22% BSA

Mix and keep on ice.

RESUSCITATION MEDIUM

The StemPro-34 SFM medium can be stored at 4°C for up to one year; however, our laboratory always freezes down 50 x 10mL aliquots at -80°C. Since the accompanying nutrient supplement can be stored for 12 to 18 months at -80°C, our laboratory freezes down 50 working aliquots of ~260µL.

| | |
|-----------------------|--|
| StemPro-34 SFM Medium | 10mL |
| Nutrient Supplement | 1 aliquot (~260µL) |
| Pen/Strep | 100µL |
| L-Glutamine | <u>100µL</u> |
| | 10.46mL (This can be stored at 4°C for 4 weeks in the dark.) |

Add 2X factors fresh. The 2X factor medium can be stored at 4°C in the dark for up to 4 days.

| | <u>2X</u> | <u>Stock</u> |
|------------------------------------|-----------|--|
| StemPro-34 w/Supplements | 10mL | From the noted recipe above |
| EPO (4U/mL) | 4µL | 10KU/mL (Store 4°C) |
| SCF (100ng/mL) | 10µL | 100µg/mL in PBS (Store -20°C) |
| Dexamethasone (10 ⁻⁶ M) | 100µL | 100X=10 ⁻⁴ M in IMDM (Store -20°C) |
| IGF-1 (40ng/mL) | 10µL | 1000X=40µg/mL in 0.1% BSA in PBS (Store -20°C) |

NORMOSOL FREEZE SOLUTION

For 20mL:

10mL DMEM Medium
4mL Normosol-R
4mL 25% Fraction V BSA
2mL Hybri-Max DMSO

Make fresh as needed.

Preparation of Fetal Liver Cells for Cell Sorting

1. Dissect uterus and wash in 1X PBS.
2. Move fetuses into fresh 1X PBS.
3. Dissect fetal livers and place all into fresh 1X PBS.
4. Pool ~8 fetal livers into a 1.7mL Eppendorf tube in ~1mL 1X PBS.
5. Disrupt fetal livers with a 1000µL Rainin Pipettor (~10 passes).

6. Pool all material and filter the cell suspension through a 70 μ m cell strainer into a 15mL conical tube.
7. Pellet the cells at 1100 rpm for 5 min.
8. Aspirate off the supernatant. Resuspend the cells in PBS-BSA buffer (1-5mL, depending on pellet size).

Staining of Fetal Liver Cells for Flow Cytometry

1. Count cells with Trypan Blue (dilute in Trypan as needed for counting).
2. Resuspend at a concentration of 2×10^7 live cells/mL in PBS-BSA buffer. For 10 million cells or less, stain the cells in 1.7mL Eppendorf tubes. For larger amounts of cells, stain in 15mL Corning tubes or 50mL Corning tubes, if needed.

STAINING (*scale up as needed*):

| | | <u>Cells in PBS-BSA</u> | <u>TER119-PE</u> | <u>CD71-FITC</u> | <u>CD117-APC</u> |
|---|-------------------|-------------------------|------------------|------------------|------------------|
| A | Blank | 50 μ L | | | |
| B | Ter119-PE | 50 μ L | 1 μ L | | |
| C | CD71-FITC | 50 μ L | | 1 μ L | |
| D | CD117-APC | 50 μ L | | | 1 μ L |
| E | 7-AAD | 50 μ L | | | |
| F | Ter119/CD71/CD117 | 1000 μ L | 20 μ L | 20 μ L | 20 μ L |

3. Stain for 30min to 1hr on ice in dark. Mix the cells a couple of times during the staining process.
4. Wash 2x by filling the tubes with PBS-BSA buffer.
5. Spin at 1100 rpm in swinging bucket centrifuge for 5 minutes. Larger volumes may require a longer spin. Avoid using fixed rotor centrifuges because many cells stuck to the side of the tube will be lost.
6. Resuspend cell pellet in PBS-BSA buffer. For the control sample, resuspend in 500 μ L. For samples to be sorted, resuspend at 10 to 20 million cells per mL.
7. For live versus dead cell staining, add 7-AAD to a final dilution of 1:1000.

Cell Sorting

1. Follow the manufacturer's protocol for cell sorting using the ARIA. Appropriate cytometry gates are determined from A-E-stained cells.
2. For sorting, use F-stained cells plus 7-AAD and gate on live cells.
3. From live cells, gate on CD-117+ and - populations.
4. For CD117+ population, gate on three subpopulations: F1(+ - -)=CD71-/Ter119-, F2(+ + -)=CD71+/Ter119-, and F5(+ + +)=CD71+/Ter119+.
5. For CD117- population, gate on two subpopulations: F3(- + +)=CD71+/Ter119+ and F4(- - +)=CD71-/Ter119+.
6. Collect populations of interest in PBS-BSA buffer.
7. Spin at 1100 rpm for 10 min.

8. Resuspend cell pellet in 1mL Resuscitation Medium and leave in a 37°C, 5% CO₂ incubator for 45-60 minutes.
9. Pellet the cells at 1100 rpm for 10 min.
10. Resuspend the pellet in Normosol Freeze Solution, mixing by gentle pipeting. Aliquot into cryovials. Freeze at -80°C overnight and store in liquid nitrogen for long-term storage. Alternatively, one can make up 2X DMSO freezing solution and mix 1mL with cells at step #7, followed by freezing and storing as previously stated.