

Sort Protocol for Sca-1 Enriched Side Population Sca-1⁺ c-Kit⁺ Lin⁻ (Per five mice)

WBM Isolation

1. Extract Bone Marrow using Hanks+
2. Pool single cell suspension into 50mL tube. Centrifuge.
3. Resuspend the cell pellet in 20 mL of DMEM+
4. Take 5uL aliquot and mix with 95 uL of RBC lysis solution (1:20). Count cells.
5. Set cell concentration to 1×10^6 cells/mL using 37° C DMEM+. *Total volume should be around 300-400 mL.*
6. To each tube add 200X Hoechst solution. *Total Hoechst used should be around 2mL.*
7. Incubate the cell suspension at 37° C for exactly 1.5 hours with occasional shaking.
8. Spin down, pool, and resuspend in cold Hanks+ to a volume of 1×10^8 cells/mL. *If using the Automacs, set aside an aliquot for the pre-enriched control.*

-From this point on the cells must be kept on ice and spun at 4° C at all times with the exception of the Automacs separation.

Sca-1 Enrichment

9. Add anti-Sca-1-biotin antibody at 1/100 (10uL/mL) and let sit on ice for 15 min.
10. Dilute in 10-20x Hanks+. Spin down.
11. Resuspend in 80uL of Hanks+ per 1×10^7 cells and add 20uL of anti-biotin microbeads per 1×10^7 cells and let sit in the 4° C for 10 min.
12. Dilute in 10-20x Hanks+. Spin down.
13. Resuspend in 500uL per 1×10^8 cells.
14. Place on the Automacs using posselds mode of separation.
15. Add ~15mL Hanks+ to both the positive and negative fractions and spin down.

Antibody Staining

16. Resuspend the negative control in 15 mL and use 100 uL aliquots for compensation controls (Cychrome, FITC, PE, PI) and negative enrichment control.
17. Resuspend the positive fraction in 1×10^8 cells/mL. *If in a hurry, assume there is ~1/10 the number of cells counted in WBM isolation after the Sca-1 enrichment (step 4).*
18. Stain with Sca-1 FITC, c-Kit PE, and Lineage Cychrome primary antibodies at 1/100 for 15 min.
19. Dilute in 20x Hanks+ and spin down.
20. Resuspend in Hanks+/PI. *Use 300uL for compensation controls, and 1mL for sort sample.*

Solutions:

Hanks+

Hanks Balanced Salt Solution (Gibco) with 2% Fetal Calf Serum and 10 mM HEPES buffer (Gibco)

DMEM+

DMEM high glucose (Gibco) with 2% Fetal Calf Serum and 10 mM HEPES buffer (Gibco)

RBC Lysis Solution

100 uL .17 M Tris Cl and 900uL .16 M NH₄Cl

200X Hoechst Solution

1 mg/mL Bis-Benzimide (Sigma)

Hanks+/PI

Hanks+ with 2 ug/mL propidium iodide (normally from 100x stock of Hanks and PI)