SOP: **Isolation of mouse spleen B cells**
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Summary

For isolation of highly pure B cells from mouse spleen, the Miltenyi magnetic bead purification system is utilized (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Non–B cells, i.e., T cells, NK cells, dendritic cells, macrophages, granulocytes, and erythroid cells, are indirectly magnetically labeled by using a cocktail of biotin-conjugated antibodies against CD43 (Ly-48), CD4 (L3T4), and Ter-119, and Miltenyi Anti-Biotin MicroBeads. Isolation of the highly pure B cells is achieved by depletion of magnetically labeled cells.

Materials List

1. C57BL/6 mice (8 week old)
2. 70µm Cell Strainer (BD Bioscience, Cat# 352350)
3. VersaLyse Lysing Solution (Beckman Coulter, Cat# A09777)
4. 10X PBS (Invitrogen, Cat# 14200)
5. EDTA (Fisher Scientific, Cat# BP120-1)
6. D-Sucrose (Fisher Scientific, Cat# BP220-1)
7. $MgCl_2$ 1M (100mL) (Ambion, Cat# AM9530G)
8. BSA (22%) (Sigma-Aldrich, Cat# A7034)
9. Tris-HCl 1M pH 7.5 (1 L) (Mediatech, Inc., Cat# 46-030-CM)
10. Milli-Q or Molecular Biology Grade Sterile Water
11. CD43(Ly-48) MicroBeads (mouse) (Miltenyi Biotec Inc., Cat# 130-049-801)
12. CD11b MicroBeads (mouse/human) (Miltenyi Biotec Inc., Cat# 130-049-601)
13. FITC Rat Anti-Mouse CD43 (BD Pharmingen, Cat# 553270)
14. PE Rat Anti-Mouse CD11b (BD Pharmingen, Cat# 553311)
15. PerCP Hamster Anti-Mouse CD3e (BD Pharmingen, Cat# 553067)
16. APC Rat Anti-Mouse Ly-6G and Ly-6C (BD Pharmingen, CAT# 553129)
17. APC Rat Anti-Mouse CD45R/B220 (BD Pharmingen, Cat# 553092)
18. 7-AAD (Invitrogen, Cat# A1310)
19. 500mL Corning Disposable 0.22µm Sterile Filter System (Corning, Cat# 430758)
20. 15mL Corning Polypropylene Conical Centrifuge Tubes (Corning, Cat# 430766)
21. 5mL Polystyrene Falcon tube (BD Falcon, Cat# 352003)
22. CryoTube Vials, 1.8mL (Nunc, Cat# 368632)
23. Eppendorf Refrigerated Centrifuge 5810R

Sucrose Buffer

Final concentration	*Stock concentration*	*Amount used from stock*
250mM D-Sucrose	0.5M D-Sucrose	250mL
10mM Tris-HCl, pH 7.5	1M Tris-HCl, pH 7.5	5mL
1mM $MgCl_2$	1M $MgCl_2$	0.5mL
Molecular Biology Grade sterile H_2O to 500mL		

Filter sterilize with 500mL 0.22µm Filter System. Store at 4°C.

MACS Buffer

200mL:

175mL	H_2O
20mL	10X PBS
0.8mL	0.5M EDTA
4.5mL	22% BSA

Mix all the solutions and filter sterilize.
Keep 100mL at room temperature for blowing out cells from spleens and 100mL on ice.

1% BSA in 1X PBS

50mL:

43mL	H_2O
5mL	10X PBS
2.27mL	22% BSA

Mix and keep on ice.

Preparation of B cells for Cell Sorting

1. Blow out cells from four spleen capsules, using about 10mL of MACS buffer for each one. Filter the cell suspensions through a 70µm cell strainer.
2. Pellet the cells at 1300 rpm for 10 min.
3. Aspirate off the supernatant. Resuspend the cells in 0.5mL MACS buffer and transfer the cells to a 15mL conical tube.
4. Add 4.5mL of VersaLyse lysing solution, mix gently, and incubate at room temperature for 15 min.
5. Pellet cells at 1300 rpm for 10 min. Add 500µl MACS buffer to resuspend the cells. Transfer the cells through a 70µm cell strainer into a 50mL conical tube. Wash the strainer with some MACS buffer.
6. Make a 1:50 dilution to count cells. Save 2 million cells for Pre-sort sample analysis.
7. Adjust the cell concentration to 10^7 cells/85µl with MACS buffer.
8. Add 10µl anti-CD43 beads/10^7 cells and 5µl anti-Mac-1/CD11b beads/10^7 cells. Mix and sit on ice for 20 min, with an inversion at 10 min.
9. Add 30mL of MACS buffer to each tube, mix.
10. Pellet the cells at 1300 rpm for 10 min.
11. Aspirate off the supernatant, resuspend the cells in 1mL MACS buffer, and filter the cell suspension through a 70µm strainer into a 50mL conical tube. Rinse the tube and strainer. Transfer the filtrates to a 15mL conical tube and bring the final volume to 4mL. The cells are now ready for sorting.

Cell Sorting

1. Follow the manufacturer's protocol for cell sorting using the autoMACS machine. The flow rate is 1mL/min. Choose "Depletes followed by Rinse."
2. When there is about 500µl left, add another 1mL of MACS buffer and add 1mL buffer every minute for four times to rinse the tube and ensure delivery of all the cells to the column.
3. Sorting results in two cell fractions: the flow-through fraction (negative) that contains the wanted cells, and the magnetically labeled eluted fraction (positive).
4. Count the cell numbers from both fractions. The negative fraction contains the resting B cells. Save 3 million of the B cells for flow cytometry analysis.

5. Pellet the cells at 1300 rpm for 10 min.
6. Resuspend the pellet in sucrose buffer, add DMSO to a final concentration of 10%, mixing by gentle repipeting. Aliquot into cryotube vials. Freeze at -80°C overnight and store in liquid nitrogen.

Flow analysis

1. Count cells in the presence of trypan blue and resuspend to $2x10^7$ live cells/mL in 1% BSA in PBS. Add 50µl of cells to a microfuge tube with 1µl of each appropriate antibody.
2. Stain for 30 min to 1 hr on ice in dark; mixing cells twice during the staining.
3. Wash the stained cells twice by adding 1mL 1% BSA in PBS to each microfuge tube and spin at 1300 rpm in swinging bucket rotor for 10 min.
4. Resuspend the cells in 500µl 1% BSA in PBS. Transfer the cells to a 5mL falcon tube. Add 5µl of 7AAD (1:100 dilution). Mix and put on ice until flow cytometry analysis.

Check pre-sort cells, post-sort flow through cells, and post-sort eluted cells.

Cytometry control tubes: (use the post-sort eluted fraction)

1. Blank
2. CD43 FITC only
3. CD11b PE only
4. CD3e PerCP only
5. 7AAD only
6. CD45R (B220) APC only
7. Ly-6G & Ly-6C (Gr-1) APC only

Cytometry analysis tubes:

1. CD43 FITC /CD11b PE/CD3e PerCP/Gr-1 APC
2. CD43 FITC /CD11b PE/7AAD/B220 APC