

Isolation of Resting B Lymphocytes from Sixteen Mouse Spleens
AfCS Procedure Protocol PP0000001600
Version 1, 02/19/02

This procedure describes the isolation of resting B lymphocytes (B cells) from mouse spleens by using negative selection with anti-CD43 and anti-Mac-1/CD11b monoclonal antibodies coupled to magnetic microbeads. This strategy depletes non-B cells from a mixed population of splenocytes and relies on the fact that most mature leukocytes, with the exception of resting splenic B cells, express CD43. Anti-Mac-1/CD11b microbeads are included in the negative selection to improve the removal of myeloid cells. The B-cell isolation is automated by using the AutoMACS automatic magnetic bead cell sorter (Miltenyi Biotec). As assessed by fluorescence analysis of B220+ cells, the isolation routinely yields approximately 4×10^7 B cells per spleen that are >95% pure. This method has been used for the preparation of B cells from 16 spleens (see also AfCS Protocol *Isolation of Resting B Lymphocytes from One or More Groups of Four Mouse Spleens*, PP0000000100).

Splenectomy

Note: all experiments use male C57BL/6 mice at age 6 to 8 weeks.

1. Sterilize the surgical instruments in a hot bead sterilizer or autoclave.
2. For every four mice, add 5 ml of chilled (4 °C) magnetic cell sorting buffer (MACS buffer) to a 35 x 10 mm or 100 x 15 mm petri dish. Place a sterile 70- μ m nylon mesh cell cup-shaped strainer (35 mm diameter) in each petri dish.
3. Anesthetize the mice (100% CO₂ for 45 to 60 sec in a container containing up to four mice), then sacrifice by cervical dislocation.
4. Place mice on a dissecting board on their right sides and douse the chest and abdominal fur with 70% ethanol.
5. Using surgical scissors, create an incision on the left side of each animal about 2.5 cm in length between the last rib and the hip joint, cutting the skin but not the peritoneal wall. Pull back the fur, exposing the peritoneal wall.
6. With a fresh pair of sterilized surgical scissors, create an incision approximately 2-cm long in the exposed peritoneal wall, in the same orientation as the skin incision. Grasp the spleen using sterilized medium forceps and pull it through the incision in the peritoneal wall. While holding the spleen with the medium forceps, separate the spleen from connective tissue by using fine forceps or scissors.
7. Place no more than four excised spleens on each 70- μ m nylon mesh cell strainer immersed in 5 ml of MACS buffer in a 35 x 10 mm petri dish. With sterile sharp-end scissors, cut each spleen into three to six parts.

Preparation of the Splenocyte Cell Suspension

8. Transfer the petri dishes to a tissue culture hood. Squeeze the splenocytes from the splenic capsule through the 70- μ m nylon mesh of the cell strainer into the MACS buffer to create a single cell suspension by gently mashing spleen pieces with the rubber end of a plunger from a 1-cc tuberculin syringe.
9. Pipette the 5 ml cell suspension from the petri dish (leaving the sterile mesh for a second processing, step 10). Perform a second filtration by pipetting the 5 ml suspensions of dispersed cells through a fresh 70- μ m nylon mesh cell strainer into

- 50-ml conical tubes (on ice). For 9 to 16 spleens, divide the preparation into two filtrations (into two 50-ml tubes).
10. Add 2.5 ml of chilled MACS buffer to the cell strainers in the petri dishes and further mash the remains of the splenic capsules. Filter and pool the second dispersions of 2.5 ml into the 50-ml conical tubes. Repeat this procedure with additional washes of 2.5 ml until the splenic capsules are white (approximately 5 to 6 more times).
 11. Pellet the cells at 400 x g for 8 min at 4 °C (J6 centrifuge with a JS 4.2 swingbucket rotor).
 12. Aspirate the supernatant, leaving about 0.5 ml of cells in MACS buffer in each tube. Loosen the cell pellets by stroking the tubes against a rack or by flicking the ends, then add 1 ml/spleen of red blood cell lysis buffer (RBC lysis buffer) at room temperature and gently resuspend the cells. By hand, gently roll the tubes for 2 min at room temperature and then quickly add chilled MACS buffer for a final volume of 40 ml/tube.
 13. Collect the cells by centrifugation at 400 x g for 8 min at 4 °C.
 14. Aspirate the supernatant entirely, then loosen the cell pellets as in step 12. Gently resuspend each pellet in 1 ml of iced MACS buffer. If clumps of cells are present, transfer the loosened clumps to one side of a 70- μ m nylon mesh cell strainer placed on a new 50-ml conical tube (they will not disperse). Use the washes to rinse the clumps and elute any imbedded cells.
 15. Use a 1-ml pipette to transfer the cell suspensions through a 70- μ m nylon mesh strainer into a new 50-ml conical tube. At this point, all cells can be combined into the same tube. Wash the old tubes 1 to 3 times with 1 ml MACS buffer, and transfer any remaining cells through the strainer into the same conical tube. Combine the filtrates for a final volume of approximately 8 ml.

Separation of Resting B Cells

16. For counting cells, the sampling volume will depend on the number of spleens. For four spleens/2 ml of buffer, take a 10- μ l aliquot of cells and dilute to 1 ml in MACS buffer. Mix thoroughly. Immediately take 25 μ l of the diluted sample, and further dilute with 25 μ l MACS buffer and 50 μ l trypan blue solution. Count cells under a phase contrast microscope, using a 40x objective, recording live (exclude trypan blue) and dead cell numbers. Count each sample twice by using both sides of the hemocytometer chamber. This presort count is used to calculate cell concentration and viability.
17. Remove and set on ice an aliquot of presorted cells (about 3 to 4 million) for subsequent analysis of cell surface proteins by flow cytometry (see AfCS Protocol *Characterization of Cells by Flow Cytometry*, PP0000001800).
18. A cell concentration of approximately 1.1×10^5 cells/ μ l (thus, approximately 10^7 cells/85 μ l) after the addition of microbeads is desired. It is important to obtain a single cell suspension for magnetic bead labeling. Using refrigerated Miltenyi beads (the 50- μ l beads are in a colloidal suspension that does not require shaking), add beads to the single-cell suspensions as follows:
 - i) 10 μ l anti-CD43 beads/ 10^7 cells

ii) 5 μ l anti-Mac-1/CD11b beads/ 10^7 cells

Thus, the final concentration, including beads, equals 10^7 cells/100 μ l.

19. Mix the cells and beads on ice for 20 min, with inversion of the tubes at 10 min (or label at 6 to 12 °C for 15 min, with inversion at 7.5 min).
20. After the incubation of cells with beads, split the bead/cell mixture evenly into new, iced 50-ml conical tubes, using a maximum of 4 ml (cells from approximately 4 spleens)/tube.
21. Add 10 to 20 times the volume of iced MACS buffer and mix by inversion.
22. Pellet the cells by centrifuging at 400 x g for 5 to 8 min at 4 °C.
23. Aspirate the supernatant, then loosen the cell pellets. Gently resuspend loosened cell pellets in 1 ml of MACS buffer. Filter cells through a 70- μ m strainer into new 50-ml conical tubes prior to loading on the AutoMACS. Rinse each tube used to pellet the cells with 1-ml aliquots of MACS buffer and pass through the 70- μ m strainer to add to the filtrates. Redistribute the filtrates (final volume approximately 16 to 18 ml) equally into four new conical tubes. These cells (approximately 4.4 ml/tube) are ready to be sorted. Store at 4 °C until diluted for sorting.
24. Increase the final volume of cells in MACS buffer to a total of 5 ml/tube (cells from 2 to 4 spleens/tube). Keep cells at 4 °C until loaded on the AutoMACS.
Note: for enrichment of B cells from 16 mouse spleens, do four individual sorts (each 50-ml tube of four spleen equivalents) interspersed with a "Rinse" program. Failure to do so may result in clogging of the machine. Maintenance of the AutoMACS tubing also includes the "SAFE" cleaning program using Coulter Clenz once per month, or every week if large numbers of cells are being processed. For each sort, add a volume of MACS buffer equivalent to the initial sample volume (about 4.5 ml) when the volume in the sample tube is reduced to approximately 100 μ l; this will ensure delivery of all of the cells. It is important to obtain a single cell suspension for loading cells onto the magnetic columns. A larger loading volume/lower cell density helps to minimize clumping, which leads to mechanical trapping of the antigen-negative population, together with antigen-positive cells, on the columns. The AutoMACS separation columns have a reported loading capacity of approximately 2×10^8 magnetically labeled cells. Thus, cells from four spleens constitute the maximum number of cells that should be loaded on a column in a single run, since approximately 50% of splenocytes express CD43 and/or Mac-1/CD11b. Miltenyi Biotec suggests that the separation columns in the autoMACS should be changed every 2 weeks, or after passing 4×10^9 cells through the column, whichever limit is reached first. For use of the autoMACS for frequent, large-scale B-cell separations, see the detailed maintenance protocol.
25. Cell separation on the AutoMACS is done at room temperature and follows the manufacturer's protocol for the AutoMACS machine. The AutoMACS is cleaned and primed (i.e., rinsed) with 70% ethanol, then purged and filled with MACS buffer to prepare for sorting. Place a tube containing labeled cells on the uptake port and choose the "Deplete S" program (flow rate 1 ml/min).
26. For each cell separation, when the sample has mostly been taken up through the uptake port, and only approximately 100 μ l remains, add another 4.5 ml of MACS buffer. This will rinse the tube and ensure delivery of all of the cells to the column.

Note: when processing multiple batches of cell/bead preparations, run a “Rinse” program after each sort (Deplete S) program. Failure to do so may result in clogging of the machine.

27. Each sort yields two fractions:
 - i) From the autoMACS “NEG 1” port, nonadherent CD43(-)/Mac-1(-) flow-through cells. The volume of this sample equals the loading volume plus 2 ml.
 - ii) From the autoMACS “POS 1” port, the CD43(+)/Mac-1(+) cells labeled with magnetic beads, retained on the column then eluted. The volume of this sample equals 2 ml.Fractions from multiple sorts of spleens processed at the same time are combined into separate pools of CD43(-)/Mac-1(-) cells and CD43(+)/Mac-1(+) cells and stored on ice.
28. Remove an aliquot of cells from each fraction to determine the total number of viable cells by trypan blue dye exclusion. Dilute the cells sufficiently to obtain between 100 to 200 cells/quadrant of a hemocytometer grid. Count duplicate samples.
29. Remove an aliquot containing about 1×10^6 cells from both the CD43(-)/Mac-1(-) cells and the CD43(+)/Mac-1(+) cells to use for analysis of cell surface proteins by flow cytometry (see AfCS Protocol *Characterization of Cells by Flow Cytometry*, PP0000001800).
30. Pellet the cells at $400 \times g$ for 8 min at 4 °C. Gently resuspend the cells in culture medium for use in experiments.

Reagents and Materials

Mice (6- to 8-week-old males): Charles River Lab; catalog no. C57BL/6 (C57 Black)

Hot bead sterilizer: Fine Science Tools; catalog no. 18000-45

Magnetic cell sorting buffer (MACS buffer): AfCS Solution Protocol ID PS0000000100

Petri dish (polystyrene), 35 x 10 mm: Fisher Scientific; catalog no. 08-757-11YZ

Petri dish (polystyrene), 100 x 15 mm: Fisher Scientific; catalog no. 08-757-103A

Nylon mesh cell strainer, 70 μ m: Falcon; catalog no. 352350

Ethanol, 70%: AfCS Solution Protocol ID PS0000001100

Surgical scissors: Fine Science Tools; catalog no. 14060-11

Medium forceps (straight): Fine Science Tools; catalog no. 13008-12

Fine forceps (straight): Fine Science Tools; catalog no. 11253-20

Plunger from 1-cc tuberculin syringe: Monoject; catalog no. 501400

Conical tubes, 50 ml: Greiner; catalog no. 4943
J6 centrifuge with a JS 4.2 swinging bucket rotor: Beckman Coulter; catalog no. 360271
Red blood cell lysis buffer (RBC lysis buffer): AfCS Solution Protocol ID PS0000000200
Trypan blue solution: Sigma-Aldrich; catalog no. T8154
Phase contrast microscope, Zeiss Axiovert 25: Brinkmann Instruments; catalog no. 517046
Hemocytometer: Fisher Scientific; catalog no. 02-671-5
Anti-CD43 [Ly-48] microbeads: Miltenyi Biotec; catalog no. 130-049-801
Anti-Mac-1/CD11b microbeads: Miltenyi Biotec; catalog no. 130-049-601
AutoMACS: Miltenyi Biotec; catalog no. 201-01
AutoMACS separation columns: Miltenyi Biotec; catalog no. 130-021-101
Coulter Clenz: Beckman Coulter; catalog no. 8546930

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