

INSTITUTE FOR CELLULAR THERAPEUTICS
University of Louisville

Protocol

Protocol: Isolation of murine Facilitating Cells from bone marrow on the Vantage Cell Sorter (revised)	S.O.P. No.: P98-13.1
	Date Prepared: March 11, 1998
	Date Edited: January 24, 2002
Prepared by: Carrie Schanie, M.A.	
Approved by:	
_____	_____
Suzanne Ildstad, M.D.	Date

I. Materials and Reagents:

Material/Reagent	Vendor/Cat No. or Serial No.
Vantage Cell Sorter/Turbo Cell Sorter	Becton Dickinson (serial no. CO322/C0319)
Pipet-aid	Drummond Scientific/ Fisher Cat# 13-681-15
p10, p20, p200, p1000 pipetmen	Rainen
Refrigerated Centrifuge	Beckman serial numbers GU93B10, GDZ93H04
Biological Hoods (The Baker Co. Model SG-600)	Serial #s: SL-44501V, SL-43150V
Microscopes (Leica Model # 020-518.000)	Serial #s: 215819, 2141520
Vortex (Genie 2)	Fisher Cat# 12-812
Forceps	General supply
Scissors	General supply
Sterile 100 micron nylon mesh	Tetko Cat# 3-100-47
Petri dishes	General supply
15 mL conical tubes (polystyrene)	Fisher Cat# 05-527-90 Falcon 2095
12 x 75 mm sterile capped flow tubes	Fisher Cat# 14-959-2A Falcon 2054
Sterile 10-200 µL yellow tips	VWR Cat# 53508-808
Sterile 200-1000 µL blue tips	VWR Cat# 53508-820
3 mL syringes	Fisher Cat# 14-823-40
18 gauge needles	Fisher Cat# 14-826-5D
22 gauge needles	Fisher Cat# 14-826-5A
Pipets, 1 mL	Falcon 7521 Fisher Cat# 13-675-15B
Pipets, 5 mL	Falcon 7543 Fisher Cat# 13-675-22
Pipets, 10 mL	Falcon 7551 Fisher Cat# 13-675-20
Pipets, 25 mL	Falcon 7525 Fisher Cat# 13-668-2
HEPES (1 M)	Gibco Cat# 15630-080
Fetal Bovine Serum	Gibco Cat# 16000-044
Hanks buffered saline solution (HBSS, 1X)	Gibco Cat # 14025-092

Medium 199	Gibco Cat# 11150-059
Gentamicin (10 mg/mL)	Gibco Cat# 15710-015
0.4% Trypan Blue	Gibco Cat# 15250-061
hemacytometer	General supply
Anti - $\alpha\beta$ TCR – FITC mAb	Pharmingen Cat# 553171
Anti - $\gamma\delta$ TCR – FITC mAb	Pharmingen Cat# 553177
Anti – CD8 α - PE mAb	Pharmingen Cat# 553033
Anti – B220 – FITC mAb	Pharmingen Cat# 553088
Anti – B220 – PE mAb	Pharmingen Cat# 553090
Rat IgG2a– FITC isotype control mAb	Pharmingen Cat# 553929
Rat IgG2a – PE isotype control mAb	Pharmingen Cat# 553930

II. Solution Preparation:

- 1. Chimera Media:** Prepare the following using sterile technique. Add 2.5 ml Gentamicin (10mg/ml; final concentration in media = 30 μ g/ml) into a 500 ml bottle of Medium 199.
- 2. Cell Sort Media:** Prepare the following using sterile technique. Add 5 ml of 1 M HEPES (final concentration = 10 mM) to a 500 ml bottle of 1X HBSS. Add 2.5 ml of Gentamicin (10 mg/ml; final concentration = 30 μ g/ml) and 10 ml of FBS (heat inactivated) (Final concentration = 2%). Store at 4° C. Expires after 7 days.

III. Procedure:

NOTE: all procedures are conducted using sterile technique

1. Harvest Bone marrow Cells

- Remove the femurs and tibias from 5-8 week old donors and place bones in Chimera Media, on ice. The bones should be free of muscle and fatty tissue. Cut the bones just before flushing to eliminate a loss of BMC. Keep bones on ice at all times until processing.
- Flush the tibias and femurs with a 22 gauge needle using a 3 cc syringe filled with Chimera Media. (Depending on the number of donors used, try not to use more than 15 ml of Chimera Media when flushing bones so that all of your sample will fit into one 15 ml conical tube.) Resuspend the BMC using the 18 gauge needle and 3 cc syringe by flushing the suspension up and down. Flush forcefully enough to break up clumps, but not so forcefully that you damage cells. Filter the cell suspension through 100 micron nylon mesh into a 15 ml conical tube using the same 18 gauge needle and syringe. Keep the sample and the media on ice at all possible times.
- After processing and filtering, spin the BMC suspension at 1000 rpm, brake on, 4°C, for 10 minutes in a refrigerated centrifuge. Note: bone marrow cells are sensitive to speeds greater than 100 rpm. Pour off the supernatant and gently resuspend the cells by ratcheting. **DO NOT ACK LYSE.** Add approximately 1 ml of CSM. Pipet up and down to make sure that the cells are adequately resuspended.
- Determine the number of cells harvested and their viability by using a hemacytometer. Take 10 μ l of cells and stain with 990 μ l of 0.1% trypan blue. (Dilute 0.4% stock trypan blue to 0.1%, or 1:4 dilution, with D-PBS.) For a clearer field of view, you may lyse the red blood cells by mixing the 10 μ l aliquot of cells with 10 μ l ACK lysing solution and incubating for 2 minutes; then add 980 μ l trypan blue and count. Viability should be greater than 85%. After the count is completed, adjust the concentration to approximately 100 x 10⁶ cells/ml. Note: each donor mouse will yield approximately 30 x 10⁶ BMC.

2. Staining

- A. For control tubes, add 20 μ l cells (approximately 1×10^6 cells) to each of the three control tubes outlined below. To each of these control tubes, add 20 μ l of the appropriate diluted antibody.

TUBE 1: FC IC (Isotype control)

TUBE 2: B220 FITC

TUBE 3: B220 PE

- B. To the main sample (for each 100×10^6 cells), add:

anti-CD8 α -PE: 25 μ l straight

anti- $\alpha\beta$ TCR-FITC: 10 μ l straight

anti- $\gamma\delta$ TCR-FITC: 10 μ l straight

- C. Incubate all stained tubes for 45 minutes at 4°C in the dark (covered and on ice). Gently mix tubes every 15 minutes during incubation.
- D. Wash twice with cold CSM: add 10 ml to the main sample and 3 ml to each control tube and centrifuge at 1000 rpm at 4°C for 10 minutes. Decant, blot and ratchet to break up the pellet.
- E. Resuspend the cells with CSM to a concentration of 10×10^6 cells/ml. (So, if you have stained 70×10^6 cells, resuspend your sample in 7 ml; if you have 90×10^6 cells, resuspend in 9 ml, etc.)
- F. Filter the resuspended cells once more through sterile 100 micron nylon mesh.
- G. Changing pipets, further dilute the cells by aliquoting 1 ml amounts into sterile snap cap flow tubes containing 3 ml cold CSM. (Each 1 ml aliquot contains 10×10^6 cells. After aliquoting 1 ml into 3 ml of CSM, each sort tube will have a final concentration of 2.5×10^6 cells/ml, or 10×10^6 cells total in the tube.)
- H. Resuspend each control tube in 1 ml CSM. (No need to filter controls.)
- I. Prepare unmarked collection tubes by adding 1 ml cold CSM to each tube, returning the snap cap. Vortex or swirl the tubes to “coat” the sides of the tube with media so as to minimize cells sticking to the sides during the sort. Generally, prepare about double the amount of collection tubes as sample tubes.
- J. Take all tubes on ice, with a lid, to the flow cytometry room.

3. Collection of FC by Vantage Flow Cytometers

- A. The sort operator will gate on the lymphoid region, collect the cells, and analyze their purity at the end of the sort.

B. FC ARE CD8⁺/TCR⁻

4. Injection preparation

- A. Centrifuge sorted cells at 1000 rpm at 4°C for 10 minutes. Decant, blot and ratchet.
- B. Using Chimera Media, pool the cell types together (ensuring uniformity in transplants) and then divide into appropriate injections.