The Fleischman Lab

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Isolation of MNCs from Peripheral Blood

Introduction

This protocol utilizes Ficoll-Paque to separate blood cells via density gradient. With this method, granulocytes and erythrocytes form the bottom layer, while mononuclear cells rest above the Ficoll layer in the buffy coat. ACK buffer is used to selectively lyse erythrocytes, leaving the cells of interest intact. If a more defined cell population is required, antibody-coated magnetic beads are bound to the cells to facilitate isolation or depletion via a magnetic column system.

Materials

1. Empty bottle with ~100 ml bleach or vacuum flask with 10% bleach
2. Beaker with 10% bleach
3. 15 ml and 50 ml conical tubes
4. 2 ml cryovials
5. 1.5 ml Eppendorf tubes
6. Serological pipettes and pipette-aid
7. RPMI 1640 +L-glutamine
8. Ficoll-Paque
9. 1x ACK lysis buffer
10. MicroFACS tubes
11. Freezing medium (heat-inactivated FBS + 10% DMSO)
12. Swinging bucket centrifuge
13. Micro FACS tubes
14. Cytometer (BD Accuri) for counting cells
15. Tissue culture plates
16. LS (MidiMACS) or MS (MiniMACS) column and magnet
17. 30 µm pre-separation filter
18. Miltenyi buffer (PBS +2 mM EDTA + 0.5% BSA) and beads
19. 37°C water bath
20. 37°C 5% CO_2 incubator with ≥95% humidity

It is crucial to always maintain good aseptic technique.
Work in the hood, wear a lab coat, and sterilize often!

Protocol	A. ACK Pellet and Plasma Collection	*Notes*
1.	Aliquot 500 µl blood per tube into 2x 1.5ml Eppendorf tubes. Centrifuge at 3500 rpm to separate plasma.	
2.	Transfer plasma to new 1.5 ml Eppendorf tubes and freeze. Store in -80C.	
3.	Fill pellet tube with ACK buffer and incubate for 15 minutes at room temperature. Centrifuge at 3500 rpm for 10 minutes and discard supernatant.	
4.	Repeat step 3 above. Discard supernatant. Wash with 1 ml PBS and centrifuge at 3500 rpm for 10 minutes.	*Final pellet should be free of red cell contamination; multiple lysis steps may be required.*
5.	Discard supernatant and freeze pellet. Store in -80C.	

	B. MNC Isolation	
1.	Transfer blood to a 50 ml conical tube and dilute with RPMI. Spit into several tubes as necessary.	*Dilute 1x minimum; 2-4x recommended*
2.	Add 12.5 ml Ficoll-Paque to a clean 50 ml conical tube. Carefully layer up to 35 ml of diluted blood over the Ficoll.	
3.	Centrifuge at 1200 rpm (400g) at room temperature for 30 minutes in a swinging bucket centrifuge without brake.	
4.	Remove the buffy coat by slowly pipetting in a sweeping motion and decant to a new 50 ml conical tube.	
5.	Fill the conical tube with RPMI and centrifuge at 1200 rpm (400g) for 10 minutes.	
6.	Discard supernatant and resuspend pellets in 10 ml 1x ACK lysis buffer. Incubate on ice for 15 minutes.	*Transfer the same 10 ml throughout all tubes to combine cells.*
7.	Fill tube with RPMI and centrifuge at 1200 rpm (400g) for 10 minutes.	*DO NOT MIX ACK BUFFER WITH BLEACH! Pour into a separate waste bottle!*
8.	Resuspend cells in 10 ml RPMI. Transfer 25 µl to a microFACS tube containing 225 µl PBS and count on the Accuri by running 20 µl on medium. Multiply events/µl output by 100,000 and record cell number.	*Be sure to gate out debris and dead cells. For volumes other than 10ml, multiply events/µl output by 10,000*(total ml).*
9.	Depending on cell count and patient type, divide cells for freezing, cell selection, macrophage production, or target cell production. Fill tubes with RPMI and centrifuge at 1200 rpm (400g) for 10 minutes. a. Freezing: Resuspend pellet in freezing medium and transfer to cryovials. Place in room-temp Mr. Frosty or CoolCell and place in -80C for a minimum of 4 hours, then transfer to liquid nitrogen. b. Macrophages: Resuspend cells in R10 + 10ng/ml hMCSF for a final concentration of ~1 million cells/ml. Plate 1 ml per well in 24-well plates.	*Freeze cells at a concentration of 5-15 million cells per ml, not to exceed more than 100 million cells per ml.*
	C. Granulocyte Isolation	
1.	Discard remaining Ficoll without disturbing red cell layer at the bottom of the tube. Fill tube with 1x ACK buffer and incubate on ice for 15 minutes.	
2.	Centrifuge at 1200 rpm (400g) for 10 minutes. Discard supernatant and fill tube with 1x ACK buffer. Resuspend cells by vortexing or pipetting up and down. Incubate on ice for 15 minutes.	*DO NOT MIX ACK BUFFER WITH BLEACH! Pour into a separate waste bottle!* *Mixing the two solutions will create a noxious gas!*

3.	Centrifuge at 1200 rpm (400g) for 10 minutes. Discard supernatant. Resuspend cells in 10 ml RPMI and transfer 25 µl to a microFACS tube containing 225 µl PBS. Count on the Accuri by running 20 µl on medium. Multiply events/µl output by 100,000 and record cell number.	*Be sure to gate out debris and dead cells. For volumes other than 10ml, multiply events/µl output by 10,000*(total ml). Granulocytes are larger cells and will have higher FSC and SSC.*
4.	To prepare target cells for phagocytosis, fill tube with RPMI and centrifuge at 1200 rpm (400g) for 10 minutes. Resuspend cells in R10 and plate.	*Cells will be apoptotic, but not necrotic, after incubation in R10 overnight.*
	D. Column Selection	
1.	Centrifuge at 1200 rpm (400g) for 10 minutes. Discard supernatant and tap tube to dislodge pellet.	
2.	Calculate the total number of remaining cells. a. CD34: for every 1 million cells, add 1 µl of blocking buffer, then 1 µl of beads. b. CD14: for every 10 million cells, add 80 µl MACS buffer and 20 µl beads.	*Wrap beads in parafilm before returning to the fridge!*
3.	Incubate cells in fridge for 30 minutes (CD34) or 15 minutes (CD14), gently mixing every 10 minutes.	
4.	Fill tube with Miltenyi buffer to 30 ml. Centrifuge at 1200 rpm (400g) for 10 minutes.	
5.	While sample is spinning, load column, pre-separation filter, and collection tube onto the magnet. Hydrate the column with 3 ml Miltenyi buffer; discard the flow-through.	
6.	Discard supernatant and resuspend cells in 2 ml Miltenyi buffer and load into column 1 ml at a time. Wash sample tube with another 2 ml Miltenyi buffer and again filter 1 ml at a time.	
7.	Wash column 3x with 3 ml Miltenyi buffer.	*If drops or splashes are present on the sides of the column, increase wash volume to 4 ml.*
8.	Toss the pre-separation filter and transfer the column to a 15 ml conical tube. Add 6 ml Miltenyi buffer to the column and force out with the plunger.	
9.	Transfer 25µl of both fractions (flow-through and selected cells) to microFACS tubes containing 225 µl PBS. Count on the Accuri by running 25 µl on medium. Multiply events/µl output by 10,000*(total volume in ml) and record cell number.	*Column cells and flowthrough cells will have different volumes, and thus will have different calculations for total cell number.*
10.	Fill collection tubes with RPMI and centrifuge at 1200 rpm (400g) for 10 minutes. Cells are now ready to freeze or culture.	