The Fleischman Lab

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Title: Heme BioBank - Processing Peripheral Blood and Bone Marrow

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 mononuclear cells intact. Cells are then split for preservation and either frozen viably in FBS + DMSO, as cell pellets for genomic DNA analysis, or as trizol pellets for RNA analysis.

Materials

1. Empty bottle with ~50-100 ml bleach or vacuum flask with ~100-200 ml 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. MicroFACS tubes
7. 100 µm cell strainers (yellow)
8. Serological pipettes and pipette-aid
9. RPMI 1640 +L-glutamine
10. Ficoll-Paque
11. 1x ACK lysis buffer
12. Trizol
13. Freezing medium (heat-inactivated FBS + 10% DMSO)
14. Swinging bucket centrifuge
15. Micro FACS tubes
16. Cytometer (BD Accuri) for counting cells

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

Protocol		*Notes*
	A. Plasma Collection	
1.	For peripheral blood, transfer blood to 50ml conicals and centrifuge tubes at 2500 rpm for 10 minutes to separate plasma.	
2.	Transfer plasma to new 1.5 ml Eppendorf tubes and flash freeze. Store in -80°C.	
3.	Dilute peripheral blood 1:1 or 1:2 with RPMI medium and proceed to MNC isolation.	
	B. MNC Isolation	
1.	Transfer blood or bone marrow 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 25 ml of diluted blood over the Ficoll.	*If clots are present, filter the sample over a 100 µl cell strainer and wash with RPMI.*

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 at 7°C.	
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 at 7°C.	*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).*
	C. Freezing Cells	
1.	Divide cells for frozen viables, genomic pellets, and trizol pellets. Fill tubes with RPMI and centrifuge at 1200 rpm (400g) for 10 minutes at 7°C. a. Freezing: Transfer cells to a 15 or 50 ml conical tube. b. Genomic pellet: Transfer cells to a 1.5 ml centrifuge tube. c. Trizol pellet: Transfer tubes to a 1.5 ml centrifuge tube.	*Freeze cells at a concentration of 5-15 million cells per ml, not to exceed more than 100 million cells per ml.* *Genomic pellets should have 1-5 million cells each.* *Trizol pellets should have 2.5-5 million cells each.*
2.	To freeze cells, resuspend the pellet in 1 ml freezing medium per cryovial. Transfer to cryovials and place in a Mr. Frosty or CoolCell. Store at -80°C for a minimum of 4 hours, then transfer to liquid nitrogen.	*Work quickly to avoid cell death due to exposure to DMSO.*
3.	For genomic pellets, aspirate the media and flash freeze. Store at -80°C.	
4.	For trizol pellets, aspirate media and resuspend in 350 µl trizol. Vortex vigorously to resuspend pellet and lyse cells. Flash freeze and store at -80°C.	