LABORATORY PROCEDURE: PBMC isolation by density centrifugation from heparin tubes

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PURPOSE: To obtain lymphocytes (peripheral blood mononuclear cells, PBMC) for flow cytometry from peripheral blood

SCOPE: This procedure applies to all normal and autoimmune blood processed in the Barnas laboratory.

PRINCIPLE: Blood is collected into green top sodium heparin tubes. The blood is diluted and layered over Lymphocyte separation media (LSM) to obtain lymphocytes.

SAFETY PRECAUTIONS: All work should be performed under the biological safety cabinet observing safety regulations and using sterile technique. Personal protective equipment such as lab coat, gloves and glasses should be used during the procedure. Specimens should be handles as if capable of transmitting infection. All contaminated supplies should be properly disposed of in biohazard or sharps containers and liquid waste should be decontaminated with bleach for 20 minutes before being poured down the drain.

NOTE: Pay particular attention to **"^HOT SPOT**" steps. These are crucial to optimize cell yield and viability.

MATERIALS AND REAGENTS:

Supplies+Equipment

50ml conical (*Falcon 352070*) 15ml conical (*Falcon 352097*) 5 ml pipet (*VWR 89130-896*) 10 ml pipet (*VWR 89130-898*) 25 ml pipet (*VWR 89130-900*)) Pipet aid Centrifuge P-20, 200,1000 + Tips Refrigerator 4°C or Ice/bucket Hemocytometer/Microscope Waste container for liquid

Reagents

1X PBS (phosphate buffered saline, *Cellgro 21-040-CV*) 0.4% Trypan Blue (*Invitrogen 15250-061*) Lymphoprep LSM (*Stemcell 07861*)) Trypan Blue/PBS Bleach REAGENT PREPARATION: a. <u>Trypan/PBS</u>: 3 ml of trypan + 5 ml of 1X PBS

REAGENT STORAGE: Room Temperature: 1X PBS, Trypan blue, Trypan/PBS, LSM.

SPECIMAN STORAGE: The blood should be at room temperature while doing the procedure. After preparation, cells should be in 1X PBS at 4-8°C or on ice.

QUALITY CONTROL: Ensure that the LSM is before the expiration date.

PROCEDURE:

^HOT SPOT^ Before starting, ensure that the centrifuge is at room temperature Obtain a sample worksheet from the lab manager (see Appendix A for example)

- Remove the green caps from the vacutainers and discard
- With a 10 ml pipet, add no more than 20mls of Blood to a 50ml conical
- Rinse all vacutainers with 10ml of 1X PBS (keep reusing the same 10ml of PBS) and add to 1 of the 50 ml conical already containing blood.
- Add 1:1 1X PBS and mix up and down with a 25 ml pipet
- **^HOT SPOT** Slowly add 12ml of LSM underneath the blood with a 10ml pipet
- Centrifuge at room temperature 800 RCF/g for 20 min **^HOT SPOT^** <u>NO BRAKE (set the</u> <u>brake to the lowest value on the centrifuge)</u>
- Remove the lymphocyte layer with a 5ml pipet, being careful not to aspirate any RBC, and place lymphocytes in a 50ml conical. (Do not exceed 25ml per tube to allow for PBS)
- Top off the conical with 1X PBS and **invert** 1X to mix
- Centrifuge at 350 RCF/g for 6 minutes
- Invert to discard liquid in waste container
- Resuspend the cells in 1 ml of 1X PBS per one 50 ml conical, and transfer cells to a 15 ml conical. Pool cells from each 50 ml conical to one 15 ml conical.
- Add 1X PBS to 10 ml total volume and **invert** 1X to mix.
- Count cells on a hemocytometer (see counting SOP).
- Place the tube at 4°C or on ice until use
- Fill out the sample worksheet and give it to the Lab manager.



LIMITATIONS: This SOP is for a minimum of 10 ml of Blood and 5 ml of Bone marrow.

CALCULATONS: Calculations can be obtained in the counting SOP

INTERPRETATION: The average cell number from blood is 1×10^6 cells/ ml of blood. In a lymphopenia patient, you might expect as low as 0.5×10^6 cells/ml and in a robust patient up to 2×10^6 cells/ml. If the number deviates from the range, consider an error in counting, RBC interference, or inadequate harvest of the layer. The average cell number from bone marrow varies greatly from 0.5×10^6 cells/ml in a lymphopenia patient to 200×10^6 cells/ml in a robust patient.

RESULTS REPORTING: The results are reported with Sample ID, Date, Volume (ml) of Blood, and Cell # (see Appendix A for worksheet). Hand over the filled worksheet to the Lab manager to input the sample to the Biological Specimen Inventory (BSI).

APPENDIX A: Sample worksheet (obtain a copy from the Lab manager)

PATIENT SAMPLE LOG WORKSHEET

Study Name:	-	Date:	
Sample ID:	-	Processor's Initials:	
# Tubes Received: Red Tops:	Green Tops:		Purple Tops:
mls of Blood:	-	PaxGene:	
Total Cell #:	-	Serum:	
OR		Plasma:	
Purified Cell #:	-	Kit Used:	
Cell # Frozen:		Media Used:	
Assay Type:			

PBMC Isolation by Density Centrifugation