LABORATORY PROCEDURE: **Ficoll**: Lymphocyte Collection from heparin tubes

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PURPOSE: To obtain lymphocytes (PBMC) for flow cytometry from Peripheral blood or Bone Marrow (BM) aspirate

SCOPE: This procedure applies to all normal and autoimmune bone marrow processed in the Anolik laboratory.

PRINCIPLE: Blood or Bone marrow (BM) aspirate is collected into green top sodium heparin tubes. The blood or BM 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 20min before being poured down the drain.

NOTE: Pay particular attention to “\(^{\text{HOT SPOT}}\)” steps. These are crucial to optimize cell yield and viability.

MATERIALS AND REAGENTS:

<table>
<thead>
<tr>
<th>Supplies+Equipment</th>
<th>Reagents</th>
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<tbody>
<tr>
<td>50ml conical <em>(Falcon 352070)</em></td>
<td>1X PBS <em>(Cellgro 21-040-CV)</em></td>
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<tr>
<td>15ml conical <em>(Falcon 352097)</em></td>
<td>0.4% Trypan Blue <em>(Invitrogen 15250-061)</em></td>
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<tr>
<td>5 ml pipet <em>(VWR 89130-896)</em></td>
<td>Ficoll LSM <em>(MP 50494)</em></td>
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<tr>
<td>10 ml pipet <em>(VWR 89130-898)</em></td>
<td>Trypan/PBS</td>
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<tr>
<td>25 ml pipet <em>(VWR 89130-900)</em></td>
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<tr>
<td>Pipet aid</td>
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<tr>
<td>Centrifuge</td>
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<tr>
<td>P-20, 200,1000 + Tips</td>
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<tr>
<td>Refrigerator 4°C or Ice/bucket</td>
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<tr>
<td>Hemocytometer/Microscope</td>
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<tr>
<td>Waste container for liquid</td>
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</table>
REAGENT PREPARATION:  a. Trypan/PBS: 3 of trypan + 5ml of 1XPBS

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

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

QUALITY CONTROL: Ensure that the LSM is prior to expiration date.

PROCEDURE:

^HOT SPOT Before starting ensure that the centrifuge is at room temperature

- 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
  - 10mls of BM to a 50 ml conical
- Rinse all vacutainers with 10ml of 1X PBS (keep reusing the same 10ml of PBS) and add to 1 of the 50 ml conicols already containing blood or BM
- Add 1X PBS and mix up and down with a 25 ml pipet
  - Blood: add 1:1 1X PBS
  - BM: add 2:1 1X PBS
- ^HOT SPOT Slowly add 12ml of Ficoll LSM underneath the blood with a 10ml pipet
- Centrifuge at room temperature 400 RCF/g for 30 min ^HOT SPOT NO BRAKE
- Return the centrifuge temp to 4C and turn BRAKE on
- 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 4-8°C 350 RCF/g for 5 minutes
- Invert to discard liquid in waste container
- Resuspend the cells in 1ml of 1X PBS and transfer cells to a 15ml conical
- Add 9 ml of 1X PBS and invert 1X to mix
- Centrifuge at 4-8°C 350 RCF/g for 5 minutes
- Invert to discard liquid in waste container
- Resuspend in up to 10 ml of 1X PBS
- Count cells on hemocytometer (see counting SOP)
• Place the tube at 4°C or on ice until use

- PBS/PLASMA
- PBMC
- Ficoll
- RBC and Debris

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

CALCULATIONS: Calculations can be obtained in the counting SOP.

INTERPRETATION: The average cell number from blood is 1X10^6 cell/ml of blood. In a lymphopenia patient you might expect as low as 0.5X10^6 cells/ml and in a robust patient up to 2X10^6 cell/ml. If number deviates from the range, consider error in counting, RBC interference or inadequate harvest of layer. The average cell number from bone marrow varies greatly from 0.5X10^6 cell/ml in a lymphopenia patient to 200X10^6 cell/ml in a robust patient.

RESULTS REPORTING: The results are reported with Sample ID, Date, MLS of Blood, and Ficoll cell#, cell #.

TRAINING: Personnel will be trained by staff. Up to one-time visual shadowing of staff member AND up to one time hands on training with staff member AND one or more times independent performance with successful completion of SOP.