LYMPHOCYTE ISOLATION

Blood tubes to be processed for Lymphocyte Isolation and/or Lymphocyte Transformation should be placed in the rack located on top of the black cabinet and stored at room temperature.

A. WARM WASH MEDIUM (about 1 hour prior to beginning protocol)

1. Wash hands thoroughly with soap and water, and dry.
2. Turn on slide heater. Temperature knob should point to **2.5** (37°C).
3. Retrieve Wash Medium from refrigerator and place on the slide heater until it is warm.

B. SET UP

1. Bring all labels and blood tubes into tissue culture room.
2. Wash hands thoroughly with soap and water, and dry. Put on gloves.
3. Balance blood tubes in the centrifuge. If necessary, use a balance tube.
4. Centrifuge the blood tubes in the Sorvall Legend RT Refrigerated Table Top Centrifuge at 800 g for 15 minutes at 20°C. Press **1**. Press ►.
5. Raise sliding window of the *LABGARD NU-440-600 Class II Type A2 Biological Safety Cabinet* until you reach the line (on the left side of the hood) that indicates the correct operating height. If an alarm starts sounding, the window is too high and must be lowered. The UV light will turn off.
6. Press the touch screen LCD to activate TOUCHLINK.
   a. Press the blower icon to turn ON blower motor.
   b. Press the light bulb icon to turn ON regular light.
7. Wipe down the workspace of the hood with 70% Ethanol and paper towels. Proceed to wipe down the front grill and armrest.
8. At the sink, insert the black stopper (with vacuum line rubber tubing attached) into the top of the 500 mL Erlenmeyer flask. Bring into the hood and place on the right side of the workspace. Screw the vacuum line rubber tubing (attached to the VAC port) onto the side-arm of the flask. Turn knob of VAC port to the left, and ensure vacuum is working properly.
9. Label all conical tubes and vials per sample:
   a. Label two (2) 50 mL conical tubes and place in the first and third rows of a conical tube rack.
   b. Label two (2) 2.0 mL cryogenic vials and place both together in an orange cryogenic vial rack.
10. Bring conical tube rack, cryogenic vial rack, Wash Medium, and LSM into the hood.
11. Uncap all conical tubes but rest each cap atop its tube.
12. Uncap Wash Medium but rest cap atop the mouth of the bottle.
13. Collect 16 mL or 32 mL Wash Medium using a 25 mL Costar Stripette serological pipette and Drummond Portable Pipet Aid. Dispense 16 mL into each conical tube in the first row, resting the cap atop its tube. Place the serological pipette in its wrapper and discard in the biohazard waste container.
    REPEAT 13 FOR ALL SAMPLES.
14. Collect 10 mL, 20 mL, or 30 mL Wash Medium using a 25mL Costar Stripette serological pipette and Drummond Portable Pipet Aid. Dispense 10 mL into each conical tube in the third row, capping the conical tube. Place the serological pipette in its wrapper and discard in the biohazard waste container.
    REPEAT 14 FOR ALL SAMPLES.
15. After centrifugation, examine all blood tubes. Confirm that three fractions are distinguishable from top to bottom – plasma, buffy coat, and red blood cells.
16. In the lymphocyte notebook, note the date at the top left corner of the next blank page.
    a. List the sample names in the same order as the conical tubes and cryovials.

   b. Record the volume (mL) of each sample by comparing to the yellow top tube filled with water and marked with "mL" gradations.
17. Bring blood tubes into the hood, placing them in a tube rack in the same order as the conical tubes and cryovials.

C. ISOLATE LYMPHOCYTES

1. Confirm that the sample names on the blood tube and conical tube in the first row match.
2. Uncap blood tube.
3. Aspirate the plasma in the blood tube. While still in the wrapper, break off the top part (below the cotton plug) of a 1mL Costar Stripette serological pipette. Pull off the wrapper and collect the plasma into the waste flask. Do not remove any of the buffy coat! Place the serological pipette in its wrapper and discard in the biohazard waste container.
4. Collect 1-2 mL buffy coat using a 5mL Costar Stripette serological pipette and Drummond Portable Pipet Aid. Dispense into conical tube in the first row. Pipet up and down to mix. Place the serological pipette in its wrapper and discard in the biohazard waste container.
5. Cap the blood tube and discard in the biohazard waste container.
   REPEAT 1-5 FOR ALL SAMPLES.
6. Uncap LSM but rest cap atop the mouth of the bottle.
7. Collect 10 mL LSM using a 10 mL Costar Stripette serological pipette and Drummond Portable Pipet Aid. Rest cap of conical tube on the workspace. Lift the conical tube out of the rack, and swiftly press the tip of the pipette to the bottom of conical tube. Immediately, GENTLY dispense LSM. Eventually you will see separation of a transparent LSM bottom layer and an opaque bloody top layer. Once the meniscus goes below the interface, release the top button and carefully raise the pipette out of conical tube completely. Place conical tube back into rack. Place the serological pipette back into its wrapper and discard in the biohazard waste container. Cap the conical tube.
   REPEAT 7 FOR ALL SAMPLES.
8. Cap LSM.
9. Carefully take all conical tubes in the first row out of the hood, and balance them in the centrifuge. If necessary, use a balance tube.
10. Centrifuge conical tubes in the Sorvall Legend RT Refrigerated Table Top Centrifuge at 800 g for 40 minutes at 20°C. Press **2**. Press ►.
11. After centrifugation, examine all conical tubes. Confirm that four fractions are distinguishable from top to bottom - transparent wash medium, lymphocytes, transparent LSM, and red blood cells. Be careful not to disturb the layers.
    a. If the lymphocyte layer contains some red blood cells, record in the notebook as "bloody."
12. Carefully bring conical tubes into the hood, placing them in the first row of the conical tube rack.
13. Uncap all conical tubes but rest each cap atop its tube.
14. Aspirate the transparent wash medium layer in the conical tube. While still in the wrapper, break off the top part (below the cotton plug) of a 1mL Costar Stripette serological pipette. Pull off the wrapper and collect the top layer into the waste flask. Do not remove any of the lymphocytes! Place the serological pipette in its wrapper and discard in the biohazard waste container.
15. Collect 10-12 mL lymphocyte layer using a 10 mL Costar Stripette serological pipette and Drummond Portable Pipet Aid. Do not collect any red blood cells unless the lymphocyte layer contains some red blood cells. Dispense into corresponding conical tube in the third row. Pipet

up and down to mix. Place the serological pipette in its wrapper and discard in the biohazard waste container. Cap the conical tube in the third row.

16. Cap the conical tube in the first row and discard in the biohazard waste container.
    REPEAT 14-16 FOR ALL SAMPLES.
17. Take all conical tubes in the third row out of the hood, and balance them in the centrifuge. If necessary, use a balance tube.
18. Centrifuge the conical tubes in the Sorvall Legend RT Refrigerated Table Top Centrifuge at 800 g for 10 minutes at 20°C. Press 3. Press ►.
19. During centrifugation, prepare for cell count.
    a. Label one (1) 0.5mL microcentrifuge tube per sample and place in a microcentrifuge tube rack.
    b. Bring microcentrifuge tube rack, Trypan Blue, P100 micropipettor and 1-200µL pipet tips into the hood.
    c. Uncap Trypan Blue but rest cap atop the mouth of the bottle.
    d. Collect 20µL Trypan Blue using a 200µL pipet tip and P100 micropipettor. Dispense into microcentrifuge tube. Discard pipet tip in the biohazard waste container.
       REPEAT FOR ALL SAMPLES.
    e. Cap Trypan Blue.
    f. Set aside microcentrifuge tube rack on the left side of the workspace.
20. After centrifugation, examine all conical tubes. Confirm a lymphocyte pellet.
21. Bring conical tubes into the hood, placing them in the conical tube rack.
22. Uncap all conical tubes but rest each cap atop its tube.
23. Aspirate the transparent wash medium layer in the conical tube. While still in the wrapper, break off the top part (below the cotton plug) of a 1mL Costar Stripette serological pipette. Pull off the wrapper and collect the top layer into the waste flask. Do not remove any of the lymphocytes! Place the serological pipette in its wrapper and discard in the biohazard waste container.
24. Collect 2 mL Wash Medium using a 5 mL Costar Stripette serological pipette and Drummond Portable Pipet Aid. Dispense into conical tube. Pipet up and down to resuspend the lymphocyte pellet. Place the serological pipette in its wrapper and discard in the biohazard waste container. Rest cap atop its tube.
    REPEAT 23-24 FOR ALL SAMPLES.
25. Move microcentrifuge tube rack in front of conical tube rack.
26. Rinse P100 micropipettor shaft with 70% Ethanol. Make sure it is dry before continuing on to next step.
27. Collect 20 µL of the 2 mL suspension using a 200 µL pipet tip and P100 micropipettor. Dispense into corresponding microcentrifuge tube containing Trypan Blue. Slowly pipet up and down to mix. Eject tip into microcentrifuge tube.
    REPEAT 27 FOR ALL SAMPLES.
28. Collect 8 mL Wash Medium using a 10mL Costar Stripette serological pipette and Drummond Portable Pipet Aid. Dispense into conical tube. Pipet up and down to mix. Place the serological pipette in its wrapper and discard in the biohazard waste container. Cap the conical tube.
    REPEAT 28 FOR ALL SAMPLES.
29. Cap Wash Medium.
30. Take all conical tubes out of the hood, and balance them in the centrifuge. If necessary, use a balance tube.

31. Centrifuge the conical tubes in the Sorvall Legend RT Refrigerated Table Top Centrifuge at 800 g for 10 minutes at 20°C. Press 3. Press ►.
32. During centrifugation, perform cell count.
    a. Take the microcentrifuge tube rack out of the hood, and place by the laptop.
    b. Open HP Laptop. If off, power on. Select *Administrator*.
    c. Open *Cellometer Auto* program on the desktop.
        - SETUP:
            - Test Viability: ✓
            - Cell type: lymphocytes_human
            - Dilution factor: 2.0
    d. Power on *Cellometer Auto T4*.
    e. On a Nexcelom Cellometer Cell Counting Chamber slide (Nexcelom *CHT4-PD100-003*), use a marker to label a counting chamber for each sample. There are 2 counting chambers on each slide.
    f. Slowly pipet up and down to mix the Trypan Blue suspension in the microcentrifuge tube. Collect 20 µL of the 40 µL suspension using a 200 µL pipet tip and P100 micropipettor. Dispense into the sample introduction port (closest to base) of the corresponding counting chamber. Eject tip into microcentrifuge tube.
    REPEAT F FOR ALL SAMPLES.
    g. Insert slide into the *Cellometer*, with the chamber you wish to count sliding in first.
    h. Click *Display Image*. Compare image to reference sheet. Lymphocyte cells should be in focus. If blurry, use knobs on back of machine and on the right side of the machine to adjust.
    i. Click *Count*. Record the following in the notebook:
        - *Live Cell Concentration (cells/mL)* – #.## x 10^6
        - *Viability (%)*

    REPEAT G-I FOR ALL SAMPLES.

D. FREEZE LYMPHOCYTES AT -80°C

1. Bring Freeze Medium (non-transformed) into the hood, placing it in the conical tube rack. Uncap Freeze Medium but rest cap atop its tube.
2. After centrifugation, examine all conical tubes. Confirm a lymphocyte pellet.
3. Bring conical tubes into the hood, placing them in the conical tube rack.
4. Uncap all conical tubes but rest each cap atop its tube.
5. Aspirate the transparent wash medium layer in the conical tube. While still in the wrapper, break off the top part (below the cotton plug) of a 1mL Costar Stripette serological pipette. Pull off the wrapper and collect the top layer into the waste flask. Do not remove any of the lymphocytes! Place the serological pipette in its wrapper and discard in the biohazard waste container.
   REPEAT 5 FOR ALL SAMPLES.
6. Move cryogenic vial rack in front of conical tube rack.
7. Collect 2 mL Freeze Medium (non-transformed) using a 5 mL Costar Stripette serological pipette and Drummond Portable Pipet Aid. Dispense into conical tube. Pipet up and down to resuspend the lymphocyte pellet. Collect entire lymphocyte suspension. Dispense about 1 mL into each cryogenic vial, capping the vial immediately. Place the serological pipette in its wrapper and discard in the biohazard waste container. Cap the conical tube and discard in the biohazard waste container.

REPEAT 7 FOR ALL SAMPLES.

8. Cap Freeze Medium.
9. Take cryogenic vial rack out of the hood. Retrieve a blue cryo freezing container (Nalgene 5100-0001) filled with isopropanol. Open container and transfer all cryogenic vials, beginning with spot 1. Use multiple containers if necessary. Close container(s).
10. Note final lymphocyte locations on the lymphocyte box printouts in the Lymphocyte Locations binder.
11. Place container(s) in available -80°C freezer until ready to store in in final locations in a liquid nitrogen freezer.

E. CLEAN UP

1. Discard counting chambers and microcentrifuge tubes in the biohazard waste container.
2. Shut laptop and power off Cellometer Auto T4.
3. Clear out the hood.
   a. Put Wash Medium and Freeze Medium (non-transformed) back to original refrigerator locations.
   b. Put LSM and Trypan Blue back to original room temperature locations.
   c. Put all racks back to original locations.
   d. Put pipet tips and P100 micropipettor back to original locations.
   e. Plug in Drummond Portable Pipet Aid.
   f. Turn VAC knob to the right and unscrew the vacuum line rubber tubing from the side-arm of the flask. Take flask to sink area.
      i. Pull out the black stopper (with vacuum line rubber tubing attached) from the top of the flask. Spray 10% bleach through opening of one end of this rubber tubing. Run tap water through this same opening. Hang tubing around sink faucet.
      ii. Pour 100% bleach (below sink) for a few seconds into the flask. Swirl for about 10 seconds. With tap water running, pour all contents of flask down the drain. Rinse flask with tap water. Set flask by sink to dry.
   g. Change into a new pair of gloves, and clean up any spills on the workspace with 10% bleach and paper towels. Allow to dry before wiping entire workspace with 70% Ethanol and paper towels. Proceed to wipe down the front grill and armrest.
   h. Lower sliding window all the way down to the armrest.
   i. Press the touch screen LCD to activate TOUCHLINK.
      i. Press the light bulb icon to turn OFF regular light.
      ii. Press the blower icon to turn OFF blower motor.
      iii. Press the UV light icon to turn ON UV light.
4. Use a twist tie to tie up the biohazard bag and pull out of container. Replace with new biohazard bag. Bring biohazard waste bag into NA2.118 and set by front lab sink for Bruce to collect.

F. TRANSFER LYMPHOCYTES TO LIQUID NITROGEN

1. When all blue cryo freezing containers are full, it is time to transfer the lymphocytes to their final locations in a liquid nitrogen freezer.
2. Use the lymphocyte box printouts as a reference to quickly transfer all lymphocytes from containers to their final locations.
3. Thaw the containers at room temperature overnight.
4. Note another round of use on the label of each container. (Replace isopropanol every 5^{th} use.)

5. Close the containers and transfer back to tissue culture room.