FICOLL

Day 1

1. Obtain human blood
    - (Sheep cells are also needed for this process. Dr. Robertson had already started processing them by the time I started into the process.)
    - Numbers in parenthesis are approximately the number of tubes used. Numbers may vary according to the amount of blood obtained.
2. R5 media
    - 1-L of RPMI media
    - 50-mL of Fetal Bovine Serum (F.B.S.)
    - 5-mL of Pen-Strep
    - no amino acid is added
3. R15 media
    - 1-L of RPMI
    - ?-mL of F.B.S.
    - ?-mL of Pen-Strep
    - ?-mL of L-Glutamine
4. Using (15) 50-mL centrifuge tubes, add:
    - 20-mL of ficoll, first.
    - 20-mL of blood
    - Important: tilting the tube slightly, add the blood slowly to avoid breaking the gradient of the ficoll.
5. Centrifuge for 35 minutes at app. 1600 RPM
6. For first time donors, collect serum into a 75-cm^2 tissue culture flask. Pour off serum down to approximately 2-3-mL above the lymphocyte layer.
7. Using a pipette, collect remaining serum and lymphocyte layer into a 50-mL centrifuge tubes. Don't be too concerned with collecting from the layer below the lymphocyte layer. Go around the edge of the tube when collecting. Combine the tubes to fill each 50-mL tube to app. 35-mL.
8. Rinse lymphocytes with R5 media by filling each tube up to 50-mL. Pipette in/out to rinse.
9. Centrifuge tubes at 1600 RPM for 10 minutes. (Sheep cells) If the sheep cells have not already been processed, these can be centrifuged at this time.
10. Sheep cells:
    - Aspirate off supernatant.
    - Lightly tap cells and add 10-mL of R5 media. Pipette in/out to rinse with media.
    - Add R5 media to 45-mL and rinse with pipette.
11. Human cells:
    - Aspirate supernatant.
    - Lightly tap cells to loosen pellet.
    - Add 5-mL of R5 media to each tube.
    - Rinse by pipetting in/out.

- Combine contents of the 10 tubes into just 2 tubes.
- Add R5 media to 45-mL. Pipette in/out.

12. "Wash" sheep and human cells at 1800 RPM for 8 minutes.
13. Sheep and human cells:
    - Aspirate down to pellet
    - Add 5-mL of R5 media to all tubes.
    - Pipette in/out
    - Add 45-mL of R5 media to lymphocyte tubes and pipette in/out.
14. "Wash" cells at 1800 RPM for 8 minutes.
15. Add 5-mL of R5 media to <u>all</u> the tubes. Resuspend the human cells.
16. Add 45-mL R5 media to the human cells pipette in/out.
17. "Wash" human cells at 1800 RPM for 8 minutes.
18. Resuspend the sheep cells. Add 30-mL of R5 to sheep cells and rinse.
19. Store 1 tube of the sheep cells in the 4°C tissue culture room cooler. Prepared sheep cells are viable in this condition for 1 week.
20. Aspirate human cells and add 5-mL of R5 to each tube to resuspend.
21. Add 25-mL of R5 to each tube and rinse.
22. With the other tube of sheep cells, resuspend and add 10-mL to each tube.
23. Rinse and centrifuge at 1000 RPM for 5 minutes.
24. Place tubes on ice and in the 4°C cooler in the main lab.
25. Wipe Sorvall down with bleach.

Day 2

1. Remove tubes stored on ice overnight and resuspend.
2. Add R5 media to all tubes for a total volume of 45-mL in each. Rinse.
3. Setup new 50-mL centrifuge tubes based on the total volume of all tubes divided by 20-mL. (20-mL will be removed and added to each new tube.)
4. Once tubes are setup, add 20-mL of ficoll to each tube.
5. Next, add 20-mL of lymphocyte cells (tubes stored overnight) without breaking the gradient.
6. Centrifuge at 1800 RPM for 30 minutes.
7. Prepare a couple of new 50-mL centrifuge tubes.
8. Pipetting around the edge of the tubes, pipette the B-cells (top layer) into the 50-mL tubes. Fill the tubes to the 25-mL mark. Dispose of pellet.
9. Finish filling the tubes with R5 to the 50-mL mark
10. Rinse at least once.
11. Centrifuge at 1800 RPM for 10 minutes.
12. Aspirate supernatant.
13. Add 3-mL of NH_4Cl/Tris pH 7.4 to each tube. Resuspend.
14. Pipette contents of all the tubes into (2) 50-mL centrifuge tubes equally. Pipette in/out.
15. Into (4) new 50-mL tubes, add 5-mL each:
    - "wash" at 1800 RPM for 8 minutes
    - aspirate supernatant
    - resuspend with R5 media
    - Combine all tubes into (2) 50-mL centrifuge tubes.
16. Should only have two tubes remaining at this point.
17. Add R5 to 50-mL and rinse (pipette in/out).
18. Centrifuge at 1800 RPM for 10 minutes.
19. Aspirate supernatant. Should see clear white cells, if red then wash again with NH_4Cl (step 13-18).
20. Tap cells to loosen and add 5-mL R5 media. Resuspend.
21. Add R5 to 50-mL. Rinse couple of times.
22. Centrifuge at 1800 RPM for 10 minutes.
23. Wash two more times:
    - Aspirate supernatant
    - Add 5-mL and resuspend.
    - Add 45-mL and rinse.
    - Wash at 1800 RPM for 10 minutes.
    - Repeat one more time.
24. Aspirate supernatant if not done so already.
25. Using **R15**:
    - Add 5-mL of R15 and resuspend
    - Add 45-mL of R15 and rinse
26. Store on ice in 4°C cooler in main lab overnight.

INFECTION

Day 3

1. Remove viral samples from -20°C freezer and thaw in a 37°C circulating water bath.
2. Remove B-cell samples from ice bucket (stored at 4°C overnight) and aspirate supernatant. B-cells should have settled to the bottom.
3. Add 5-mL of R15 media to resuspend.
4. Add 45-mL R15 and rinse.
5. Using a 96-well assay plate, do a cell count (example).
    - Remove 50-µL from the tubes of B-cells and add to two wells of the assay plate.
    - Add 50-µL of Trypan Blue Stain 0.4% to two wells below B-cell samples.
    - Using a hemacytometer slide, add 20-µL of B-cell sample and stain each to the slide well.
    - Turn on the microscope and do count using the chart guideline on wall behind the microscopes.
6. This B-cell sample resulted in 1.3×10^6 cells/mL.
    - 1.3×10^6 cells/mL x 50-mL = 65 million cells/tube
    - Between the two tubes of B-cells, there is approximately 130 million B-cells
7. Using (15) 50-mL centrifuge tubes add approximately 6.5 mL of R15 media with B-cells.
8. Label each tube and inoculate with 10-mL of each viral sample:
9. Place inoculated samples in the 37°C incubation room down the hall for approximately 2-3 hours.
10. Aspirate down to ≈ 2-3-mL. (May or may not see a pellet).
11. Add enough R15 media to obtain a final volume of 30-mL. Final volume is based on two 96-well plates with 150-µL per well.
12. Incubate at 37°C and 5% CO_2 for approximately 5-6 weeks.