Isolation of PBMCs – Direct Overlay on Ficoll (EDTA)

Equipment:

- Benchtop centrifuge (Allegra X-15R, Beckman Coulter)
- Tali Image Based Cytometer (Invitrogen)
- Pipette Gun (Drummond)
- p200 micropipette (Rainin)

Materials:

- EDTA Lavender Top Tube (Fisher, #367874)
- 1.8mL Cryotube vials (Fisher, #375418)
- 50mL conical vial (Fisher, #352070)
- Sterile, filtered, p200 pipette tips (Rainin, #RT L250F)
- CoolCell (Fisher, #NC9883130) and CoolBox
- Tali Cellular Analysis Slide (Invitrogen, #110794)
- 2mL, 5mL, 10mL, 25mL, and 50mL sterile, serological pipettes (Fisher, #356507, #356543, #356551, #356525, #356550 respectively)
- Transfer pipette (Fisher, #357575)

Reagents:

- Ficoll-Paque PLUS (Fisher, #17-1440-03)
- Ca+ and Mg+ Free PBS (Invitrogen, 10010-049)
- Human Serum Type AB (Lonza, #14-490E)
- DMSO (Sigma-Aldrich, #154938)
- Freezing media (refer to **Appendix A**)

Procedure:

1. If plasma is needed prior to PBMC isolation, please refer to HIMC's Plasma Isolation SOP.
2. Pipette 15mL of Ficoll into a new 50mL conical tube.
3. Obtain whole blood from subject in EDTA green top tubes.
4. Dilute whole blood 1:1 with PBS in a new 50mL conical tube (NOTE: disregard this step if Plasma was already isolated from Step 1).
5. Add heparinized whole blood to the conical tube by slowly pipetting it down the side of the tube, layering on top of the Ficoll.
    a. Add no more than 35mL of diluted blood to the tube
        i. If necessary, split the sample into two conical tubes
6. Centrifuge the tubes at 800 x g for 20 minutes with the brake off.
7. Remove tubes carefully from centrifuge.
8. Use a transfer pipette and remove the buffy coat into a new 50mL conical vial. Take caution not to draw up the layers below the buffy coat.

   a. If a granulocyte pellet is needed, do not throw conical with ficolled blood away. Refer to HIMC's Granulocyte Isolation SOP.
9. Add PBS to the tube up to the 50mL mark
10. Centrifuge the PBMCs at 250 x g for 10 minutes.
11. Aspirate the supernatant and resuspend the cells in 48mL of PBS
12. Count the cells using the Tali Counter (or lab's preferred cell counting method)
    a. Add 25µL of the cell suspension to a Tali slide
    b. Choose the "Quick Count" selection and "Name Now"
    c. Label the data with the sample ID
    d. Insert slide into the Tali following the arrows on the slide
    e. Press the button "Press to Insert New Sample"
    f. Focus the image so that the cells can be seen clearly with definitive borders
    g. Press "Press to Run Sample"
    h. After counting, set the cell size to "5µm to 15µm" (this only has to be done the first sample of the day)
    i. Calculate the total cell count by multiplying the number of cells/mL by the total volume of cell suspension
        i. ex – 3.45×10^5 cells/mL x 48mL =165.6×10^5 cells
13. Centrifuge the conical vial at 250 x g for 10 minutes.
    a. Based off the total cell count, calculate the number of vials and volume of freezing media that will be needed (refer to Appendix B)
        i. Label the appropriate number of empty cryovials with de-identified cryogenic label and place in a CoolBox to chill for at least 10 minutes (alternatively 4C/wet ice can be used)
        ii. Pull enough Freezing Media A and Freezing Media B to create 1mL aliquots. The total mLs amount of freezing media needed is equal to the total number of aliquots needed.
14. Aspirate the supernatant
15. Resuspend the cells in Freezing Media A equal to one half of the total freezing media needed
16. Using a dropwise technique (1 drop/second) while swirling the sample, add Freezing Media B equal to the remaining half of the total volume.
17. Aliquot 1mL of cell suspension into each cryovial.
18. Place the cryovials into a CoolCell and into a -80° freezer for 24 hours (alternatively a Mr. Frosty or controlled rate freezer can be used)
19. Following this, immediately put the PBMCs cryovials into liquid nitrogen for long term storage

Appendix A - Creating Human Serum AB Freezing Media

Materials:

- 50mL conical vial (Fisher, #352070)
- 0.2μm Filter Unit (Fisher, #SCGPU02RE)
- 15mL conical vial (Fisher, #1495949B)
- 2mL, 5mL, 10mL, 25mL, and 50mL sterile, serological pipettes (Fisher, #356507, #356543, #356551, #356525, #356550 respectively)

Equipment:

- Pipette Gun (Drummond)
- Waterbath

Reagents:

- Human Serum Type AB (Lonza, #14-490E)
- DMSO (Sigma-Aldrich, #154938)

Procedure:

1. Thaw 1 bottle of Human Serum AB
2. Set the waterbath temperature to 56°C
3. Attach filter unit to the vacuum line in the biological safety cabinet, pour serum into filter unit and filter through until all the media is filtered.
    a. If unit becomes clogged, a second filter unit may be necessary.
4. In 50mL conicals, make two 30mL aliquots of serum and two aliquots of 20mL
5. Place tubes in the 56° waterbath and heat inactivate them for 30 minutes, swirling the tubes every 5-10 minutes
6. While the tubes are in the water bath, prepare 15mL conical vials, labeling them "A" or "B"
7. Remove the serum from the waterbath and allow the conicals containing 20mL to cool
8. **Freezing Media A –** 100% Human Serum AB
    a. Aliquot the serum from the 30mL tubes into the 15mL conicals, 5mL per tube – approximately 12 tubes total
9. **Freezing Media B –** 80% Human Serum AB + 20% DMSO
    a. Add 5mL of DMSO to the cooled serum
        i. Add it dropwise while swirling to prevent precipitation
        ii. Capping and inverting several times will help prevent precipitates
    b. Aliquot the serum into 15mL conicals, 5mL per tube – approximately 10 tubes total
10. Place all aliquots into a -20° freezer until use. Thaw for use in cryopreservation and do not refreeze.

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Appendix B – HIMC Aliquot Guidelines

PBMCs

	Number of Vials to Freeze for total starting blood volume of:							
PBMC Count (x 10^6 cells)	up to 10ccs	up to 20ccs	up to 30ccs	up to 40ccs	up to 50ccs	up to 60ccs	up to 70ccs	70ccs +
up to 10	1	1	2*	2*	3*	4*	5*	6*
11 - 20	2	2	2	2	3*	4*	5*	6*
21 - 30	3	3	3	3	3	4*	5*	6*
31-40	3*	4	4	4	4	4	5*	6*
41-50	3*	4*	5	5	5	5	5	6*
51-60	3*	4*	5*	6	6	6	6	6
61-70	3*	4*	5*	6*	7	7	7	7
80+	3*	4*	5*	6*	7*	8	8	8

*Re-count cells; if amount remains the same, aliquot samples per chart guidelines; provide as much observational detail on requisition form.
*Maximum number of aliquots despite cell count is 8 vials