ISOLATION AND BANKING OF PBMCS FROM HUMAN WHOLE BLOOD

Created by: Grace Teskey **Date: February 2017**
Bowdish Lab, McMaster University
Hamilton, ON, Canada
www.bowdish.ca

Purpose: To isolate peripheral blood mononuclear cells from human whole blood for freezing.

Materials and Equipment:
0.22µm Stericup Filter (Millipore Cat. # SCGPU01RE)
RPMI
hAB serum
PBS (warmed)
Ficoll
50mL Conical Tubes
Disposable Medical Examination Gloves
5mL, 10mL, and 25mL Pipets
Cell Freezer (Mr. Frosty)
Cryovials
Hemacytometer
Precision Pipettors (20µl and 1000µl)
Appropriate Sterile Pipette Tips
Blood Processing Binder
Waste Bag and Containers
Laboratory Coat
Centrifuge
Biosafety Cabinet (BSC)

Note: Centrifuge speeds vary between RCF and RPM depending on the protocol. Please be sure you are using the right one.

PBMC Isolation and Storage

Preparation of hAB serum:

a) First heat inactivate serum at 56°C in waterbath for 30 mins, swirling every 10 mins to reduce particulate formation.
b) After heat inactivation, let cool and then filter through 0.2µm filter into a sterile tube in the BSC to remove any particulates that did form. Aliquot and Store AB serum at 4°C, or long term at -20°C.

c) Prepare your 2X freezing media: 8ml hAB serum (80%) + 2ml DMSO 20% (any other 1:5 ratio is acceptable). Drip the DMSO into the hAB serum slowly while gently mixing (swirling motion).

Note: DMSO clumps will disappear with sufficient gentle mixing. Do not pipet DMSO down side of polypropylene tube; clumps will form that will not dissolve

1. Chill Mr. Frosty in refrigerator for >30 minutes.
2. Centrifuge green top tubes for 10 minutes at 1500rpm to separate plasma. *For further information, see Plasma Isolation.*
3. Remove yellow upper layer (plasma) from each green top tube. *Aliquot into cryovials (1mL/vial) for storage as described below.*
4. Using a 10mL pipet, transfer remaining blood sample into 50 mL tubes (maximum of 15 mLs / tube), record total volume of blood processed. *If there are multiple green top tubes from the same donor, these can be combined into the same 50mL tube, but only up to 15mLs*
5. Add an equal volume of PBS (up to 15mL maximum) to blood and mix gently with pipet.
6. Record information from tubes then dispose green top tubes in sharps container in BSC.
7. Slowly pipet equal volume of Ficoll under diluted blood so Ficoll layer forms below diluted blood layer (10ml blood + 10ml PBS + 20ml Ficoll). When done properly two (2) distinct layers should be observed.
8. Centrifuge for 25 minutes (**no brake)** at 1500rpm at **room temperature**.
9. A thin white interface should be observed between the top and middle layer after centrifugation. **NOTE:** This is the PBMC population to be isolated. *At this step, the separation is very sensitive. Take care not to jostle the tube. Separation shown below.*
10. Remove the top layer of plasma using a 10mL pipette, leaving approximately 5mL above the interphase (be careful not to disturb the interphase). *Since you have already harvested the plasma above, this does not need to be kept. However, if you do keep this plasma layer, make note that it has been diluted with PBS and taken from this step in the protocol.*
11. Draw off the interphase (PBMC layer) with a 10mL pipette into another 50mL tube. Do not draw up too much of the Ficoll layer below, as this may result in granulocyte contamination. **Do not** combine interphase from multiple tubes; do a 1:1 transfer. *If graulocytes are needed, **Do not** discard the Ficoll and red blood cell layer, as the RBC layer contains the granulocytes.*
12. Dilute interface by pouring PBS into the tube up to 50mL (fill tube completely). Mix by inversion.
13. Centrifuge 10 minutes at 1500rpm at room temperature (brake on).
14. After centrifugation, pour supernatant into waste bucket and resuspend cells in 20mLs PBS using a pipetman. if pellet is small add 10 – 15 mLs PBS instead of 20 mLs. Or if pellet is large add 30-35mLs PBS instead of 20mLs.
15. Remove 10µl cell suspension and add equal amount of Trypan Blue in a microcentrifuge tube, and gently mix.
16. Using hemacytometer, count mononuclear cells (large, grayish cells), **not** RBCs (small, round, shiny cells). Calculate the number of cells/mL.
17. Centrifuge samples for 5 minutes at 1500rpm at room temperature.

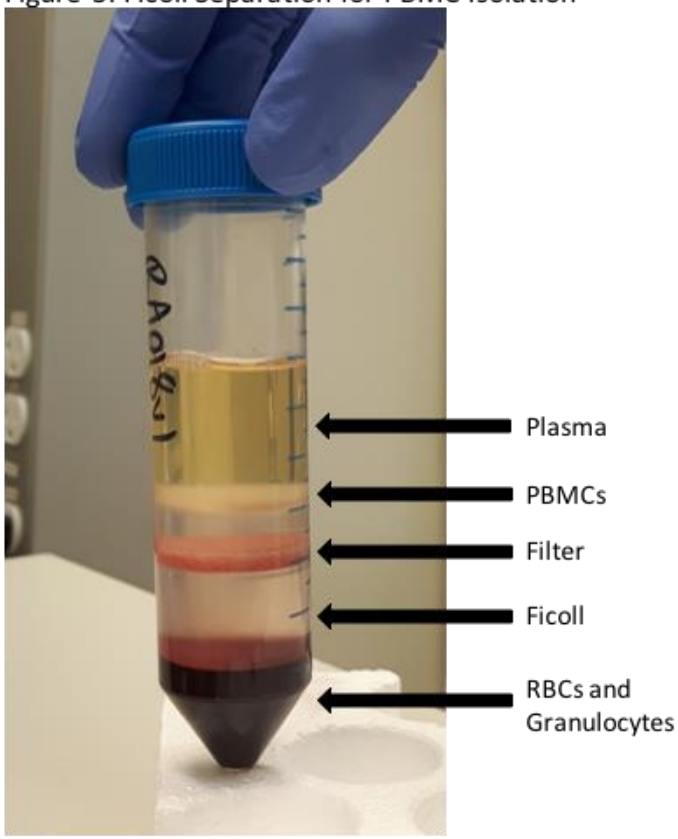
18. Calculate the number of cyrovials required as 5 -10 x 10^6 cells/mL in each cryovial is desired. Label these vials before-hand.
    Example: 1x10^6 cells/mL in 20mLs= 20x10^6 cells total. If freezing at 5x10^6 cells/mL, with 1mL/ cryovial, resuspend in 4mL freezing media and distribute into 4 cryovials.
19. Resuspend the pellet in cold hAB serum and add an equal volume of freezing media by **dripping slowly** (20%DMSO + 80% AB serum) so that the final concentration is 5 – 10 x 10^6 cells / mL (i.e. 4 mLs total = 2 mL hAB + 2 mLs freezing media), gently mix well using a swirl motion. *Work quickly from this stage forward. DMSO is toxic to cells at room temperature. Transfer cells to the freezer as quickly as possible.*
20. Transfer 1 mL cell suspension into each labeled cryovial.
21. Store freezing container (Mr. Frosty) overnight at -80^{o}C. Transfer to long-term storage the next day.

<u>Granulocyte Isolation and Storage:</u>

1. Remove RBC and granulocyte layer from the separation tube into a 50mL tube, with 5mL maximum/tube.
2. Add 1X BD Pharm Lyse buffer to each 50mL tube at a 5:1 ratio to the RBC /granulocytes. Example: 5mLs of RBC/granulocytes, add 45mLs of lysis buffer. Mix by inversion or by gently vortexing.
3. Incubate at room temperature away from the light for 15 minutes.
4. Centrifuge at 200rcf for 5 minutes.
5. Remove supernatant without disturbing the pellet.
6. Add 2.0 ml 1X PBS containing 1% heat-inactivated fetal bovine serum and 0.1% sodium azide (PBS-FBS)
7. Centrifuge at 200rcf for 5 minutes.
8. Remove supernatant and resuspend in freezing media (same as step 20 above). *You will most likely only need one cryovial, as granulocyte recovery is relatively low.*
9. Store freezing container (Mr. Frosty) overnight at -80^{o}C. Transfer to long-term storage the next day.

Figures

Figure 3: Ficoll Separation for PBMC Isolation



***Note:** This separation was done with a Leucosep tube. When done with a 50mL tube, the only difference is the filter will not be there. Everything else is the same.**