PROCEDURE	**PBMC/CBMC Isolation Procedure**	
PREPARED BY	GAPPS staff	
DATE ADOPTED		
REVIEWED BY	**SIGNATURE**	**REVIEWED DATE**

REVISED BY	SIGNATURE	REVISED DATE

SUMMARY OF CHANGES TO THIS SOP
Version 1.0

PURPOSE
This Standard Operating Procedure (**SOP**) describes a procedure for PBMC/CBMC isolation for 1 blood draw using a Leucosep tube.

SCOPE
This procedure covers the isolation of PBMC/CBMC for 1 blood draw using a Leucosep tube.

Authority and Responsibility for SOP's
1. The GAPPS Medical Director (or his/her designee) and Laboratory Manager have the authority to establish this procedure.
2. The GAPPS Laboratory and the QA monitors are responsible for the implementation of SOP documentation at participating sites.

Supplies
Ensure everything is at room temperature (15-25°C)
1. 12mL Leucosep tube
2. Mr. Frosty (hold at 4°C)
3. Box of dry ice
4. Human Serum
5. PBS + 2% Human Serum (example- 75mL PBS and 1.5mL HS)
6. Human Serum + 10% DMSO (Dimethyl sulphoxide) (example- 900µL HS and 100µL DMSO)
7. 190uL Trypan Blue in small tube
8. Hemacytometer for cell counting and microscope
9. 10mL and 25mL pipets
10. 50mL conical tubes
11. Sterile plastic transfer pipets
12. 10% Bleach waste cup
13. PBMC worksheet
14. Labeled cryovials for whole blood, serum, buffy coat and plasma from blood collection kit

Supply Preparation
1. Add 1.5 mL Human Serum to 1X PBS to obtain working solution of PBS + 2% HS.
2. Add 100µL DMSO to 900µL Human Serum to obtain working solution of cell freezing medium.

Safety
All technicians are expected to be trained and follow universal precautions when handling biological or hazardous materials prior to completing this protocol.

Limitations of the Procedure
Handle the isolated PBMCs/CBMCs gently during the washing and resuspending steps to avoid cell loss. The procedure should be done in a biological safety hood and the technician should wear gloves and a lab coat.

PBMC/CBMC Isolation Procedure:

Spinning the Blood and Collection of Blood Products
1) Gather all supplies and make reagents before staring procedure.
2) If there is **only one** purple top vacutainer of blood (8 mL), pipet 0.5 mL of whole blood (purple vacutainer) into one labeled whole blood cryovial. If there is **more than one** purple top vacutainer of blood, pipet 1mL whole blood into 2 labeled whole blood cryovials. **Immediately keep on dry ice.**

3) From the purple top vacutainer, slowly pour the whole blood into 1 or 2 Leucosep tubes until it reaches the 8mL or 9mL mark. *(Do not fill the Leucosep tube completely)* The blood should layer above the porous white barrier in the tube.
4) Centrifuge both the Leucosep tube and red top vacutainer at **800 x *g*** (*about 2316 rpm @ r_{ave}*) for **15 minutes** at room temperature (20- 23°C).
5) From the red top vacutainer, pull off the **serum** and aliquot **1mL** into each labeled serum cryovial. **Immediately keep on dry ice.**
6) From the Leucosep tubes, pull off the **plasma** layer and aliquot **1mL** into each labeled plasma cryovial. **Immediately keep on dry ice.**
7) Also from the Leucosep tubes, use a transfer pipet to pull off the white PBMC layer and put it into 1 fresh 50mL conical vial. You can also pour off the supernatant containing the PBMC layer into the 50mL conical vial.

Washing the PBMCs/CBMCs

8) Add PBS+2% Human Serum to the conical vial until it reaches the 40mL mark. Close the lid tightly and invert the conical vial several times.
9) Centrifuge at **250 x *g*** (*about 1295 rpm @ r_{ave}*) for **10 minutes** at room temperature (20- 23°C), with the brake on or off, depending on the centrifuge. *Note: For samples older than 24 hours, a centrifugation time of 20 minutes is recommended.* There should be a small white/pink pellet at the bottom of the conical vial.
10) Use a 25mL pipet to pipet off the PBS+2% Human Serum to the 10% bleach waste container while leaving about 1 mL in the conical vial.
11) Resuspend the PBMCs by raking the tube across a rough surface or pipetting up and down.
12) Wash PBMCs again with PBS + 2% HS (about 30-40mL) and pipet up and down. Spin at **250 x *g*** (*about 1295 rpm @ r_{ave}*) for **10 minutes** at room temperature (20- 23°C).
13) After final wash and spin, pipet all liquid off the top and resuspend the PBMCs in 1mL HS by gently pipetting up and down.

Counting the Cells

14) In another tube, add 10uL of the resuspended PBMCs to 190uL Trypan Blue. Let it sit for 5 min.
15) Fill a hemacytometer with 10µL of the cells/Trypan Blue mixture for cell counting.
16) Under a microscope, observe if non-viable cells are stained and viable cells excluded the stain. Count the number of dead and live cells and record results on the worksheet. Calculate the total number of live cells.

Cryopreserving the Cells

17) Determine the proportion of DMSO to human serum and final volume to give an appropriate number of cells per ml (usually $5x10^6$/ml).
18) Dilute the PBMCs in the 50mL conical drop wise with HS+DMSO to get a concentration of 5 or 10 million cells/mL (*depends on the total live cell number*) and a final concentration of 10% DMSO. For example, if the final volume is 5ml, 0.5 ml DMSO would be added to 3.5ml HS, mixed, and then this would be added to the 1.0ml of cells in HS. Calculate the number of 2mL tubes needed to get 5 million cells per tube.
19) Label 2.0 mL cyrovials with the PBMC Labels. In a sequential order use letter A to Z to for each aliquot from the same collection.
20) Add the resuspended PBMCs to the labeled PBMC cryovials (5 million cells per tube) and note the ID codes and concentrations on the PBMC worksheet.
21) Place the PBMC tubes in the 4°C Mr. Frosty and put in -80°C overnight (or at least 4 hrs), then transfer to liquid nitrogen. Place the whole blood, serum, and plasma tubes at -80°C while noting locations on the worksheet.
22) After processing, digitally record the data, and file a paper copy.

$$RPM = \sqrt{\frac{RCF}{(1.118 \times 10^{-5}) \times (Radius)}}$$