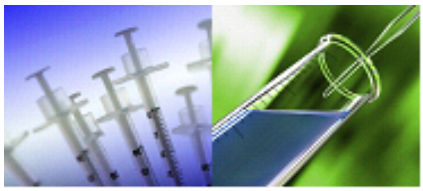

nPOD
Network for Pancreatic Organ Donors with Diabetes

ISOLATION OF PBMC

1 PURPOSE

The purpose of this Standard Operating Procedure (SOP) is to outline procedures for isolation and cryopreservation of peripheral blood mononuclear cells (PBMC).

2 SCOPE

This SOP will be applied to whole blood that is separated to cells suitable for cell culture and aliquots of plasma.

3 RESPONSIBILITIES

3.1 Managers and supervisors are responsible for making sure that technicians are properly trained and equipment and facility are maintained in good working order.

3.2 Laboratory personnel are responsible for reading and understanding this SOP and related documents and to perform these tasks in accordance with the SOPs. They are responsible for following clinical laboratory and tissue banking best practices.

4 EQUIPMENT and MATERIALS

The materials, equipment and forms listed in the following list are recommendations only and alternative products as suitable may be substituted for the site specific task or procedure.

Refrigerated table top centrifuge (Eppendorf Centrifuge 5810 R)
Cryovials with O-rings (FisherSci, Cat. No. 12-565-163N)
Cell culture freezing medium with 10% DMSO (GIBCO, Cat. No. 11101-01), aliquot 10 ml, store at -20°C
Note: Freezing medium should be thawed and kept on ice (4°C) before adding to cells.
Dulbecco's Phosphate Buffered Saline (D-PBS), Mg^{2+} Ca^{2+} free (Invitrogen, Cat. No. 10010-023), store refrigerated
Transfer pipettes, sterile 9" glass Pasteur pipettes, 5 ml serological pipettes, serological pipettor (Pipet-Aid or equivalent), pipettors (1000 ul, 200 ul, 20 ul) and sterile/barrier tips
70% ethanol (EtOH)
Ficoll-Paque Plus (GE Healthcare)
0.17M Ammonium chloride solution (Stemcell, Cat. No. 07800), store at 4°C
Ice bucket
Cell counting supplies: pipettes and tips (20P, 200P, 1000P), 0.4% Trypan blue (TB) stain (Sigma, Cat. No. T8154) (Dilute 1:5 with D-PBS to a final concentration of 0.08%), Hemacytometer with cover slip, inverted microscope
CoolCell freezing container (VWR, Cat. No. 95059-860), refrigerate before use

5 SAFETY

5.1 Use universal safety precautions when handling human samples and personal protective equipment (e.g., face mask with shield, gloves, lab coat or apron). Dispose of all solutions and supplies in contact with human blood in biohazardous waste.

6 PROCEDURE

6.1 Record number of un-coagulated blood tubes received, tube color (for anticoagulant), and blood volume in the sample database under case information (see SOP Case Processing).

6.2 Record raw data with a worksheet or notebook.

6.2.1 Use the Cell Calculator (Excel file) to determine cell viability, concentration, yield, and aliquots or otherwise document calculations.

6.3 Mix tubes by gently inverting 5 to 8 times then place on a rocker for 20 minutes at low speed.

6.4 Sterilize tubes and caps with 70% EtOH and move to biosafety hood.

6.5 Pour up to 20 ml whole blood in a 50 ml sterile conical tube. Discard tubes in biohazardous glass waste.

6.6 Add D-PBS to the conical tube to a final volume of 35 ml and mix briefly by inverting.

6.7 Place two (2) sterile 9" glass Pasteur pipettes inside the 50 ml conical tube so that the tip of each pipette rests at the bottom of the tube.

6.8 Use a 5 ml serological pipette to add Ficoll to the large end of the Pasteur pipettes. Add a total of 15 ml of Ficoll, bringing the volume up to a final of 50 ml.

6.9 Centrifuge at 450 x g for 30 minutes at room temperature, **NO BRAKE,** and medium acceleration.

6.10 Aspirate plasma layer to within ~ 1 cm of the interphase layer (buffy layer with cells) and discard.

6.11 Collect buffy coat using a transfer pipette. Slowly sweep the top of the desired layer, constantly releasing the pipette bulb and transfer to a sterile 50 ml conical tube.

6.12 Discard the remaining material in the appropriate biohazardous waste container inside the hood.

6.13 Add D-PBS up to 50 ml to the collected buffy coat for the first wash.

6.14 Centrifuge at 450 x g for 10 minutes at room temperature with maximum brake and acceleration.

6.15 If the pellet contains red blood cells, aspirate and discard the supernatant.

6.15.1 If red blood cells are present, the pellet will be red.

6.15.2 Add 9 ml 0.17M ammonium chloride per 1 ml cell pellet. Re-suspend the pellet by shaking the tube. Let the cells sit at $4^{o}C$ for 10 minutes.

6.16 Add D-PBS to a total volume of 30 ml and centrifuge at 450 x g for 10 minutes at room temperature, maximum brake and acceleration.

6.17 Discard the supernatant and wash again.

6.18 Aspirate supernatant completely and re-suspend in D-PBS according to the pellet size. The total D-PBS volume depends on the cell pellet size, with a 1 ml pellet generally requiring about 10 ml D-PBS. Completely re-suspend cells by gentle agitation.

6.18.1 Immediately after re-suspending the cell pellet, transfer ~100 ul aliquot of the cell suspension to microfuge tube and perform a cell count, as described below in 6.20.

6.19 Meanwhile, centrifuge the remaining cell suspension at 430 x g for 10 minutes at room temperature without brake. After centrifuging, use a sterile pipette and remove the supernatant without disturbing the pellet. Discard the supernatant into biohazardous liquid waste container.

6.19.1 A suitable re-suspension volume is detailed below in 6.23 after obtaining a cell count.

6.20 Cell Counting

6.20.1 Set up a clean, dry hemocytometer chamber with coverslip.

6.20.2 Transfer 10 ul of cells to another microcentrifuge tube. Add 90 ul of diluted TB solution. Dead cells will stain blue. Count within 5 minutes to avoid false cell staining.

6.20.3 Load ~10 ul of cells by capillary action into each side of the hemocytometer.

6.20.4 Count the number of live and dead cells in each of the 4 corner squares (counting 50-100 cells per square provides the best accuracy, dilute further if too concentrated). Record raw data. If variation is high or there is clumping, repeat counts on a cleaned hemocytometer.

6.21 Enter cell count data into the nPOD Cell Calculator (Excel file) or manually calculate.

6.21.1 Use the Cell Calculator to determine cell viability, concentration, yield, and aliquots or otherwise document calculations.

6.22 Data Calculations

6.22.1 Cell viability(%): (total live cells)/(total live cells + total dead cells)

6.22.1.1 Expected cell viability is >75% and is usually > 90%.

6.22.2 Cell concentration: cells/ml = (total live cells/4) x dilution factor (DF) x 10^4

6.22.3 Total cells: (cells/ml) x suspension volume (ml)

6.23 Calculate suitable re-suspension volume to provide 1 x 10^7 cells/ml final cell concentration (optimal range is 0.5-3 x 10^7cells/ml).

6.24 Work quickly and keep tube on ice. Gently flick the tube with finger several times and resuspend the pellet by adding the previously thawed freezing media drop by drop, very slowly, while rapidly rotating the 50 mL conical tube in a circular motion.

6.25 Aliquot the cell suspension into labeled cryotubes (see SOP Case Processing) on ice in 200 ul-1 ml volumes. Avoid exposure of cells to freezing media longer than 15 minutes before starting the cryopreservation to improve post-thaw cell viability.

6.26 Immediately place cryovials in CoolCell freezing containers and place in a -80°C freezer overnight.

6.27 The following day, transfer the vials from CoolCell to storage boxes for the liquid nitrogen cryotank. Store at vapor phase. See SOP Cryotank Use and Maintenance.

6.28 Record cryovial storage location, aliquot volume, and cell concentration in sample inventory database.

6.29 Quality control measures include review of cell viabilities and yields and data calculations when performed manually.

7 REFERENCES

7.1 Mallone R., et al. *Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses*: position statement of the T-Cell Workshop Committee of the Immunology of Diabetes Society. Clinical and Experimental Immunology. 2010.

7.2 SOP 53 Cryotank Use and Maintenance

7.3 SOP 57 Case Processing

7.4 SOP 60 Isolation of Cells from Spleen, Thymus, and Lymph Nodes

8 REVISION HISTORY

Version	Date	Revision

Prepared by	Maria Martino		
Approved by	Martha Campbell-Thompson		
	Name	Signature	Date