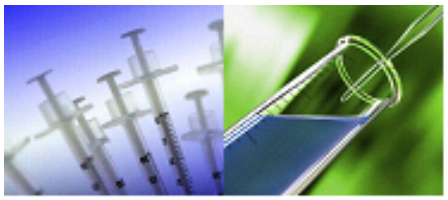

nPOD
Network for Pancreatic Organ Donors with Diabetes

STANDARD OPERATING PROCEDURE

Isolation of PBMC

OPPC-SOP-59

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ISOLATION OF PBMC

POLICY: 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.

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

SCOPE: This SOP will be applied to whole blood that is separated to cells suitable for cell culture.

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

Laboratory personnel - are responsible for reading and understanding this SOP and related documents and to perform these tasks in accordance with the SOPs.

EQUIPMENT & 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)
- Sterile Nunc Cryotubes (Thermo Sci, Cat. No. 375418)
- CryoStor CS10 cell freezing media (BioLife Solutions, Cat. No. 07930)
- Dulbecco's Phosphate Buffered Saline (D-PBS), Mg^{2+} Ca^{2+} free (Invitrogen, Cat. No. 10010-023), with antibiotic/antimycotic added prior to use at a final concentration of 1% anti/anti in D-PBS, store at 4°C. An asterisk (*) denotes that supplements have been added to the solution
- Antibiotic-Antimycotic Solution (Anti/Anti) , 10,000 I.U./ml Penicillin 10,000 ug/ml Streptomycin 25 ug/ml Amphotericin B (Corning, Cat. No. 30-004-CI), aliquot in 5 mL and store at -20^{0}C
- Sterile Leucosep Centrifuge tube with Porous Barrier, 50 mL Conical (Greiner Bio-One, Cat. No. 227290P)
- Transfer pipettes, 5 ml, 10ml, 25 ml serological pipettes, serological pipetter (Pipet-Aid or equivalent), pipettes (1000 ul, 200 ul, 20 ul) and sterile filter tips
- 70% ethanol (EtOH)
- Ficoll-Paque Plus (GE Healthcare)
- 10-50% Clorox Bleach
- 0.17M Ammonium chloride solution (Stemcell, Cat. No. 07800), store at 4°C
- Cell counting supplies: pipettes and tips (1000 ul, 200 ul, 20 ul Cellometer (Nexcelom), disposable cell counting chamber (Nexcelom), Cellometer AOPI Staining Solution in PBS (Nexcelom, Cat. No. CS2-0106-5ML), store at 4°C
- CoolCell freezing container (VWR, Cat. No. 95059-860)
- CoolCell Filler Vials, 2ml (Biocision, Cat.No. BCS-3105)

PROCEDURE:

1.0 Isolating Peripheral Blood Mononuclear Cells

1.1 Record number of un-coagulated blood tubes received and tube color (for anticoagulant), in the case worksheet (see SOP Case Processing).

1.2 Fill Leucosep tube with 15mL of Ficoll-Paque Plus and centrifuge at room temperature for 30 seconds at 1000 x g with maximum brake and acceleration. The Ficoll will migrate below the porous barrier.

1.3 Mix blood by gently inverting the tubes 5 to 8 times. Sterilize tubes and caps with 70% ethanol and move to biosafety hood.

1.4 Pour up to 15-30mL whole blood in a 50 mL sterile Leucosep conical tube. De-identify empty blood tubes and discard in biohazardous sharp waste container.

1.4.1 Dilution of the sample material with D-PBS* is not necessary, but can help improve the separation. A dilution ration of 1:2 is recommended.

1.5 Centrifuge 10 minutes at 1000 x g at room temperature for 15 minutes **WITHOUT BRAKE,** and with maximum acceleration.

1.6 Aspirate upper layer to within 5 to 10 mm of the interphase layer and discard. Collect interphase layer (buffy coat) using a transfer pipette or by pouring the supernatant above porous barrier from Leucosep tube into a labeled, sterile 50 mL conical tube.

1.7 Discard the used Leucosep tube material in the biohazardous waste container inside the hood.

1.8 Add 10 mL of D-PBS* to the collected buffy coat and centrifuge at 250 x g for 10 minutes at room temperature with maximum brake and acceleration.

1.9 Aspirate and discard the supernatant. If red blood cells are present, proceed with red blood cell lysis as described in step 1.9.1, and proceed to step 1.10.

1.9.1 Re-suspend the pellet in 1 mL D-PBS* by gently pipetting and add 9 mL 0.17M ammonium chloride per 1 mL cell pellet. Incubate the cells at room temperature for 5-7 minutes.

1.9.1.1 If the pellet is very small, re-suspend in 0.5 mL D-PBS* by gently pipetting and add 4.5 mL 0.17M ammonium chloride. Incubate the cells at room temperature for no more than 5 minutes.

1.10 Add D-PBS* to a total volume of 10-30 mL and centrifuge at 250 x g for 10minutes at room temperature with maximum brake and acceleration.

1.11 Discard the supernatant and re-suspend the pellet in 1 mL D-PBS*, then repeat step 1.10.

1.12 Aspirate supernatant completely and re-suspend in 1 mL D-PBS*. Add 9 mL D-PBS*for a final volume of 10 mL.

1.12.1 Immediately after re-suspending the cell pellet, transfer 20 µL aliquot of the cell suspension to microfuge tube and perform a cell count, as described below in step 2.0.

1.12.2 Meanwhile, centrifuge the remaining cell suspension at 250 x g 10 minutes at room temperature with maximum break and acceleration. After centrifugation, use a sterile pipette and remove the supernatant without disturbing the pellet.

1.12.3 Discard the supernatant, and re-suspend pellet in 1 mL CryoStor CS10 cell freezing media. Fill with freezing media to the appropriate final volume as determined by cell count and proceed to step 3.3.

2.0 Cell Counting

2.1 Set up a clean Cellometer disposable cell counting chamber by removing protective coating on both sides of the chamber. Add 20 µL of Cellometer AOPI Staining Solution in PBS to the 20 µL cell suspension aliquot and mix gently.

2.2 Load 20 µL of cells into the sample introduction port and place chamber in Cellometer.

2.2.1 Follow the assay protocol for "Immune cells, low RBC" and adjust bright image field focus if necessary. Save final cell count and viability data for entry in sample database.

3.0 Data Calculations

3.1 Cell viability (%) = (No. of viable cells counted) / (total cells counted) x 100

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

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

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

3.2 Calculate suitable re-suspension volume to provide 1 x 10^7 cells/ml final cell concentration (optimal range is 1x 10^7cells/ml but can be increased to up to 3x10^^{7} cells/ml).

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

3.4 Immediately place cryovials in CoolCell freezing containers and place in a -80°C freezer overnight. Inset CoolCell Filter Vials into empty wells of CoolCell freezing containers when freezing less than a full batch of vials to ensure a freezing rate of -1^{0}C/minute.

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

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

3.7 Quality control measures include periodic review of cell viabilities and yields, data calculations, and cryopreserved cell thawing and measurement of viability.

REFERENCES:

1.0 Related Documents and Procedure

1.1 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.

1.2 SOP 53 Cryotank Use and Maintenance

1.3 SOP 57 Case Processing

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

REVISION HISTORY

Version	Date	Revision
1	8/28/12	IK updated cell freezing media catalog number
2	6/15/15	MP updated equipment and reagent list, and cell counting process
3	4/15/21	EV, LE, MB updated equipment, reagents

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