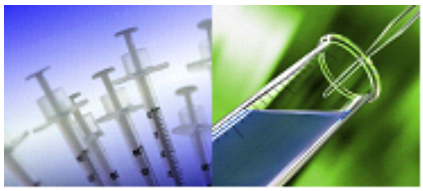

nPOD
Network for Pancreatic Organ Donors with Diabetes

ISOLATION OF CELLS FROM SPLEEN, THYMUS, AND LYMPH NODES

1 PURPOSE

The purpose of this Standard Operating Procedure (SOP) is to outline procedures for isolating and cryopreserving cells from spleen, thymus, and lymph nodes.

2 SCOPE

This SOP will be applied to fresh tissue processed for cell isolation.

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.

70% Ethanol (EtOH)
100x Antibiotic/Antimycotic solution (Sigma, Cat. No. A9909), aliquot 10 ml and store at -20°C
Dulbecco's Phosphate Buffered Saline (D-PBS), Mg^{2+} Ca^{2+} free (Invitrogen, Cat. No. 10010-023), store refrigerated
Collagenase type II (Invitrogen, Cat. No. 17101015)
Dissecting instruments (scissors, forceps, scalpels, #10 or 11 blades)
Cell culture dish (100 mm)
Cell strainer, 100 um (BD, Cat. No. 352360)
Syringes (3 or 5 ml), centrifuge tubes (15 or 50 ml conical), graduated pipettes (i.e., 2, 5, 10, 25, 50 ml), transfer pipettes
Centrifuge (swinging bucket rotor), capable of maintaining 18-26°C, 250-800 g-force spins with brake (on/off)
0.17M Ammonium chloride solution (Stemcell, Cat. No. 07800), store at 4°C
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
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.
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 Spray outside of containers with 70% ethanol and follow aseptic techniques throughout.
6.2 An asterisk (*) denotes that supplements have been added to the solution.
6.3 Prepare D-PBS* solution, which can be used up to three months.
    6.3.1 Add 5 ml of 100x antibiotic/antimycotic stock to make a final dilution of 1:100.
6.4 Label D-PBS* containers with preparation date, additives, and preparer's initials and store at 4°C.
6.5 Transfer tissue pieces to a culture dish and mince into about 3-4 mm pieces with sterile scalpel or scissors.
6.6 Perform an optional collagenase digestion for fibrotic tissues (i.e., thymus). Otherwise, skip to step 6.12 below.
6.7 To make a collagenase digestion solution, weigh out 30 mg of type II collagenase on an analytical balance. Pour into a 50 ml conical tube and add 30 ml D-PBS*.
    6.7.1 Swirl the tube to dissolve the enzyme and filter through a 0.22 uM filter unit.
    6.7.2 The final concentration is 1 mg/ml. Make 10 ml aliquots and store at -20°C.
6.8 Add the collagenase solution (50-200 U/ml) to the fresh tissue.
    6.8.1 Note: 2 mM $CaCl_2$ increases efficiency of digestion.
6.9 Incubate from about 30 minutes to several hours at room temperature or 37°C.
6.10 Filter the cell suspension through a sterile cell strainer to remove dispersed cells from tissue fragments. Continue at step 6.15 below.
6.11 Set up another cell culture dish with ~5-10 ml D-PBS*.
6.12 Transfer several tissue pieces to strainer and keep moistened with D-PBS*.
6.13 Open a sterile 3 or 5 ml syringe. Remove the plunger and press firmly on the tissue with the end of the plunger to force the fragments apart and allow cells to pass through the wire mesh.
    6.13.1 Gentle grinding of the fragments across the mesh with the plunger will hasten the breaking apart of the fragments. Continue to grind tissue and rinse cells from strainer.
6.14 Transfer cells from dish to a centrifuge tube. Rinse the dish and strainer and add to tube.
6.15 Centrifuge the cell suspension at 250 x g for about 5 minutes at room temperature.
6.16 If the pellet contains red blood cells, aspirate and discard the supernatant.
    6.16.1 If red blood cells are present, the pellet will be red.
    6.16.2 Add 9 ml 0.17M ammonium chloride per 1 ml cell pellet. Re-suspend the pellet by shaking the tube.
    6.16.3 Incubate the tube at room temperature for about 5 minutes. Centrifuge at 250 x g for about 5 minutes.
6.17 Discard the supernatant and re-suspend cell pellet in D-PBS* to wash. Centrifuge at 250 x g for about 5 minutes.
6.18 Repeat the wash step twice. Discard supernatant each time.
    6.18.1 If there are cell clumps, filter the cell suspension through another cell strainer.
6.19 Re-suspend the pellet in volume of D-PBS*, adding the first 0.5 ml slowly and gently re-suspending cells by swirling. The total D-PBS* volume depends on the cell pellet size, with a 1 ml pellet generally requiring about 10 ml D-PBS*.
    6.19.1 Completely re-suspend cells by gentle agitation.

6.20 Immediately after re-suspending the cell pellet, transfer ~100 ul aliquot of the cell suspension to a microfuge tube and perform a cell count, as described below in 6.23.

6.21 Meanwhile, centrifuge the cell suspension at 250 x g for about 5 minutes. Use a sterile pipette and carefully remove the supernatant without disturbing the cell pellet. Discard the supernatant into a biohazardous liquid waste container.

6.21.1 A suitable re-suspension volume is detailed below in 6.26 after obtaining a cell count.

6.22 Cell Counting

6.22.1 Set up clean, dry hemocytometer chamber with coverslip.

6.22.2 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.

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

6.22.4 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 the variation is high or there is clumping, repeat counts on a cleaned hemocytometer.

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

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

6.24 Data Calculations

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

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

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

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

6.25 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).

6.26 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.

6.27 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.

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

6.29 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.

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

6.31 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 59 Isolation of PBMC

8 REVISION HISTORY

Version	Date	Revision

Prepared by	Emily Montgomery		
Approved by	Martha Campbell-Thompson		
	Name	Signature	Date