



STANDARD OPERATING PROCEDURE

Isolation of Primary Cells from Spleen, Thymus, and Lymph Nodes

OPPC-SOP-60

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Revised by:	Leyla Evans, Ellen Verney, Erica Walters	Version Effective Date:	05/02/2019
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Network for Pancreatic Organ Donation with Diabetes (nPOD)

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JDRF nPOD Standard Operating Procedure		
SOP Number and Version: 60.1 60.0	Supersedes: nPOD	ISOLATION OF CELLS FROM SPLEEN, THYMUS, AND LYMPH NODES
Original Effective Date: 08/01/07		
Version Effective Date: 08/10/15		

- POLICY:** Use universal safety precautions when handling human samples and personal protective equipment (e.g., face mask, gloves, lab coat, and safety glasses). Use chemical and physical safety precautions when working with paraformaldehyde and sharps, respectively.
- PURPOSE:** The purpose of this Standard Operating Procedure (SOP) is to outline procedures for isolating and cryopreserving cells from spleen, thymus, and lymph nodes.
- SCOPE:** This SOP will be applied to fresh tissue processed for cell isolation.
- RESPONSIBILITIES:** Managers and supervisors - are responsible for making sure that technicians are properly trained and the 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. They are responsible for following clinical laboratory and tissue banking best practices.
- EQUIPMENT & MATERIALS:** The materials, equipment and forms in the following list are recommendations only, and alternative products may be substituted for the site-specific task or procedure.
- 70% Ethanol (EtOH)
 - 10% Clorox bleach (6% Sodium hypochlorite)
 - Virex TB Quaternary Disinfectant Spray (Medline, Cat. No. DVY04743H)
 - Super Sani-Cloth® Germicidal Disposable Wipe (Fisher, Cat. No. 23-100-124)
 - 100x Antibiotic-Antimycotic Solution (Anti/Anti) 10,000 I.U./mL Penicillin 10,000 µg/mL Streptomycin 25 µg/mL Amphotericin B (Corning, Cat. No. 30-004-CI), aliquot 5 mL and store at -20°C
 - Dulbecco's Phosphate Buffered Saline (D-PBS), Mg²⁺ Ca²⁺ free (Invitrogen, Cat. No. 10010-023), store at 4°C
 - Complete culture media [DMEM/F12 50/50 with L-Glutamine (Corning Cat. No. 10-090-CV) + 10% HyClone Fetal Bovine Serum (Fisher, Cat. No. SH3007103IH) + 1x Antibiotic-Antimycotic] (Corning Cat. No. 30-004-CI)
 - Collagenase type II (Invitrogen, Cat. No. 17101015)
 - Sterile dissecting instruments (scissors, forceps, scalpels, #10 or 11 blades)
 - TC-Treated Culture Dishes, 100 mm (Corning Cat. No. 430167)
 - Sterile cell strainer, 100 µm (BD, Cat. No. 352360)
 - Sterile syringes (5 mL), centrifuge tubes (15 or 50 mL conical), serological pipettes (i.e. 5, 10, 25), serological pipette controller (ex. Drummond Pipet-Aid)
 - Centrifuge with swinging bucket rotor
 - 0.17M Ammonium chloride solution (Stemcell Technologies, Cat. No. 07800), store at 4°C

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- Cell counting supplies: pipettes and sterile filter tips (1000 µL, 200 µL, 20 µL), Cellometer (Nexcelom), disposable cell counting chamber (Nexcelom, Cat. No. CHT4-SD100-002), AOPI Staining Solution in PBS (Nexcelom, Cat. No. CS2-0106-5ML), store at 4°C
- Cryovials with O-rings (FisherSci, Cat. No. 12-565-163N)
- CryoStor CS10 cell freezing media (Stemcell Technologies, Cat. No. 07930), store at 4°C
 - Note: Freezing medium should be kept at 4°C before adding to cells.
- CoolCell® freezing container and Filler Vials® (Biocision BCS-405, VWR Cat. No. 95059-860)

PROCEDURE:

1.0 Cell Isolation

- 1.1 Wipe down laminar flow hood and spray outside of containers with 70% ethanol. Follow aseptic techniques throughout.
- 1.2 An asterisk (*) denotes that supplements have been added to the solution.
- 1.3 Prepare D-PBS* solution, which can be used up to 3 months.
 - 1.3.1 Add 5 mL of Anti/Anti stock to 500 mL of D-PBS to make a final dilution of ~1:100.
- 1.4 Label D-PBS* containers with preparation date, additives, and preparer's initials. Store at 4°C.
- 1.5 Transfer tissue to a culture dish and mince into 3-4 mm pieces with sterile scalpel or scissors.
- 1.6 Perform an optional collagenase digestion for fibrotic tissues (i.e., thymus). Otherwise, proceed to step 1.7 below.
 - 1.6.1 To make a collagenase digestion solution, add 30 mg of type II collagenase to a 50 mL conical tube and add 30 mL D-PBS*.
 - 1.6.1.1 Swirl the tube to dissolve the enzyme and filter through a 0.22 µm filter unit.
 - 1.6.1.2 The final concentration is 1 mg/mL. Make 1 mL aliquots and store at -20°C for 3-6 months.
 - 1.6.2 Add 1-2 mL of collagenase solution to 50mL tube containing minced tissue in 40 mL of complete culture media.
 - 1.6.3 Incubate tube 30 minutes to several hours at 37°C under constant agitation.
 - 1.6.4 Filter the cell suspension through a sterile cell strainer to remove dispersed cells from tissue fragments. Proceed to step 1.11 below.
- 1.7 Fill another cell culture dish with 20-30 mL D-PBS* and place a sterile cell strainer in the dish.
- 1.8 Transfer several tissue pieces to the strainer. Ensure tissue remains moistened with D-PBS*.
 - 1.8.1 For fibrous tissue such as PLNs, use scissors to mince tissue into very small pieces before proceeding to step 1.9.
- 1.9 Remove the plunger from a sterile 5 mL syringe and press firmly on the tissue with the end of the plunger to force the fragments apart and allow cells to pass through the mesh.

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- 1.9.1 Grind the tissue in the strainer and rinse in culture dish until it no longer retains its original texture and color.
- 1.10 Transfer cells from culture dish to a 50 mL conical tube using a serological pipette. Rinse the dish and strainer with ~10-20 mL D-PBS* and add to tube, up to a total volume of 40 mL.
- 1.10.1 Optional: When processing large amounts of tissue or tissue with high cell yield (i.e. splenocytes, thymus), it is recommended to distribute the cell suspension between 2-4 50 mL conical tubes.
- 1.11 Centrifuge the cell suspension at 400-500 x g for 5-7 minutes at room temperature.
- 1.12 Aspirate and discard the supernatant. Proceed to 1.13. If the pellet contains red blood cells, perform the following steps:
- 1.12.1 Add 1 mL D-PBS* and slowly pipette up and down to re-suspend pellet.
- 1.12.2 Add 9 mL 0.17M ammonium chloride per 1 mL cell pellet and slowly pipette up and down to mix.
- 1.12.3 Incubate at room temperature for 5-10 minutes.
- 1.12.4 Add D-PBS* to a total volume of 30-35 mL.
- 1.12.5 Centrifuge at 400-500 x g for 5-7 minutes at room temperature.
- 1.12.6 Note: For splenocytes or suspensions with many red blood cells, an additional application of ammonium chloride is sometimes warranted (maximum application x2).
- 1.13 Discard the supernatant and re-suspend cell pellet in 1 mL D-PBS*. Fill to a total volume of 30-45 mL with D-PBS*, then centrifuge at 400-500 x g for 5-7 minutes.
- 1.13.1 Optional: If there are clumps of cells that cannot be re-suspended, filter the suspension through a new cell strainer into a new 50 mL tube before centrifugation.
- 1.13.2 Optional: If pellet is small, re-suspend in 1mL D-PBS* and transfer cell suspension to 15mL tube to avoid cell loss. Fill to a total volume of 10mL with D-PBS* and centrifuge as described above.
- 1.14 Repeat step 1.13.
- 1.15 Discard the supernatant and gently re-suspend cell pellet in 1 mL D-PBS*. Fill to a total volume 10-30 mL with D-PBS*. Transfer a 20 µL aliquot of the cell suspension to a microcentrifuge tube and perform a cell count, as described below in section 2.0.
- 1.16 Meanwhile, centrifuge the cell suspension at 400-500 x g for 5-7 minutes. Carefully remove the supernatant without disturbing the cell pellet.
- 1.17 Optional: If cell count revealed viability below expected value and cell total is high, re-suspend pellet and centrifuge again as described in step 1.13. Perform another cell count as in section 2.0, record re-count data, and proceed to step 1.18 based on re-count values.
- 1.18 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 Cellometer disposable cell counting chamber by removing protective coating on both sides of the chamber. Avoid touching clear portions of chamber. Place the cell counting chamber on a flat surface. Add 20 µL of Cellometer AOPI Staining Solution in PBS to the 20 µL cell suspension aliquot and mix gently. Load 20 µL of cell-stain mix into the sample introduction port and insert counting chamber in the Cellometer. To ensure assay accuracy, the chamber should be completely full of sample and free of smudges prior to insertion in the Cellometer.

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- 2.2 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(%): (total live cells)/(total live cells + total dead cells)
- 3.1.1 Expected cell viability is > 75%.
- 3.1.2 Cell concentration: cells/mL = (total live cells/4) x dilution factor (DF) x 10⁴
- 3.1.3 Total cells: (cells/mL) x suspension volume (mL)
- 3.2 Calculate suitable re-suspension volume to provide at least 1 x 10⁷ cells/mL final cell concentration (optimal range is 1-3 x 10⁷ cells/mL).
- 3.3 Aliquot the cell suspension by 1 mL into labeled cryovials (see SOP Case Processing). 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 room temperature CoolCell® freezing containers. Fill all (if any) empty slots with CoolCell Filler Vials and place CoolCell in a -80°C freezer overnight.
- 3.4.1 Note: Minimum freeze time is 4 hours.
- 3.5 The following day, transfer the vials from CoolCell® to cryogenic storage boxes. Store at vapor phase (See SOP Cryotank Use and Maintenance).
- 3.6 Record storage location, aliquot volume, and cell concentration for each sample in inventory databases.
- 3.7 Quality control measures include review of cell viabilities, yields, data calculations, and appropriate corrective action if necessary.

REFERENCES:

4.0 Related Documents and Procedure

- 4.1 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.
- 4.2 SOP 53 Cryotank Use and Maintenance.
- 4.3 SOP 57 Case Processing
- 4.4 SOP 59 Isolation of PBMC

REVISION HISTORY

Version	Date	Revision	
60.1	08/10/15	Added cellometer, CoolCell, revised washes	
60.2	05/02/19	Added details, reformatted	
	Name	Signature	Date
Prepared by:	Emily Montgomery		08/01/2007
Edited by:	Maria Beery Leyla Evans	Ellen Verney Erica Walters	05/02/2019
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