



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)

10-50% Clorox bleach (6% Sodium hypochlorite)

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% Fetal Bovine Serum (Corning, Cat. No. MT35016CV + 1x Anti/Anti)

Collagenase type II (Invitrogen, Cat. No. 17101015)

Sterile dissecting instruments (scissors, forceps, scalpels, #10 or 11 blades)

Sterile cell culture dish (100 mm)

Sterile cell strainer, 100 µm (BD, Cat. No. 352360)

Sterile syringes (3 or 5 ml), centrifuge tubes (15 or 50 ml conical), serological pipettes (i.e. 5, 10, 25), serological pipette (Pipet-Aid or equivalent)

Centrifuge (swinging bucket rotor)

0.1M Ammonium chloride solution (Stemcell, Cat. No. 07800), store at 4°C

Cell counting supplies: pipettes and sterile filter tips (1000 µl, 200 µl, 20 µl), Cellometer (Nexcelom), disposable cell counting chamber (Nexcelom), Cellometer 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 (BioLife Solutions, 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)

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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 that come in contact with human blood and tissues in liquid or solid 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 Anti/Anti stock to 500 ml of D-PBS 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 to a culture dish and mince into 3-4 mm pieces with sterile scalpel or scissors.
- 6.6 Perform an optional collagenase digestion for fibrotic tissues (i.e., thymus). Otherwise, proceed to step 6.11 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 µm filter unit.
 - 6.7.2 The final concentration is 1 mg/ml. Make 1 ml aliquots and store at -20°C.
- 6.8 Add 1-2 ml of collagenase solution to 50ml tube containing minced tissue in 40 ml of complete culture media.
- 6.9 Incubate tube 30 minutes to several hours at 37°C under constant agitation.
- 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 Fill another cell culture dish with ~5-10 ml D-PBS* and place a sterile cell strainer in the dish.
- 6.12 Transfer several tissue pieces to the strainer and keep moistened with D-PBS*.
- 6.13 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.
 - 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 using a serological pipette. Rinse the dish and strainer and add to tube.
- 6.15 Centrifuge the cell suspension at 400-500 x g for 5 minutes at room temperature.
- 6.16 If the pellet contains red blood cells, aspirate and discard the supernatant.
 - 6.16.1 Add 1 mL D-PBS* and slowly pipette up and down to re-suspend pellet.
 - 6.16.2 Add 9 ml 0.17M ammonium chloride per 1 ml cell pellet.
 - 6.16.3 Incubate the tube at room temperature for 5-10 minutes.
 - 6.16.4 Add D-PBS* to a total volume of 30-35 ml.
 - 6.16.5 Centrifuge at 400-500 x g for 5 minutes at room temperature.
- 6.17 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 minutes.
- 6.18 Repeat step 6.17.
 - 6.18.1 Filter the cell suspension through another cell strainer after re-suspending.

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- 6.19 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*. Immediately after re-suspending the cell pellet, transfer a 20 µl aliquot of the cell suspension to a microfuge tube and perform a cell count, as described below in 6.23.
- 6.20 Meanwhile, centrifuge the cell suspension at 400-500 x g for 5 minutes. Use a sterile pipette to carefully remove the supernatant without disturbing the cell pellet. Re-suspend pellet in 1 ml CryoStor CS10 cell freezing media. Fill to the appropriate final volume as determined by cell count.
- 6.21 Cell Counting
- 6.21.1 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. Load 20 µl of cell-stain mix into the sample introduction port and place chamber in Cellometer.
- 6.21.2 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.
- 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 10⁴
- 6.22.3 Total cells: (cells/ml) x suspension volume (ml)
- 6.23 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).
- 6.24 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.
- 6.25 Immediately place cryovials in CoolCell® freezing containers. Fill all (if any) empty slots of CoolCell with Filler Vials and place CoolCell in a -80°C freezer overnight.
- 6.26 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).
- 6.27 Record cryovial storage location, aliquot volume, and cell concentration in sample inventory database.
- 6.28 Quality control measures include review of cell viabilities and yields and data calculations.

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

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