

PROCEDURE FOR EXCISIONAL LYMPH NODE BIOPSY

1.0 INTRODUCTION

As our understanding of the mechanisms involved in HIV infection advances, it becomes necessary for immunologists to examine not only peripheral blood cells, but the lymph tissue of HIV-infected patients. Changes in the cellular architecture of lymph nodes may indicate stages of HIV and potential reservoirs for the virus. Differences in viral clearance, expression of immune markers, and patterns of viral expression between peripheral blood and lymph tissue may provide insight into the pathogenesis of HIV infection.

An excisional lymph node biopsy (non-inguinal), rather than a lymph node aspirate, is recommended. ACTG investigators should contact the consulting surgeon for questions or clarifications relating to excisional cervical lymph node biopsies for a given substudy before the start of the protocol. This procedure must be performed under local anesthesia. Non-palpable lymph nodes may be excised, but this is a more difficult procedure and requires intravenous sedation.

Refer to the Protocol Immunology Information Sheet (PIIS) for a given protocol and/or substudy for specific instructions regarding the use of lymph node cells.

2.0 REAGENTS

- 2.1 Operating room (OR) reagents and equipment required to perform a biopsy under local anesthesia. Consult the surgeon at your site.
- 2.2 Hank's Balanced Salt Solution (HBSS) or Dulbecco's Phosphate-Buffered Saline (D-PBS) without calcium and magnesium.
Recommended vendor: Gibco BRL Life Technologies, Grand Island, NY
Phone 1-800-828-6686
D-PBS, catalog #14190-144, 500 mL, 1X liquid
- 2.3 Ficoll-hypaque
Many brands are available, such as Ficoll-Paque Plus (Amersham Pharmacia Biotech Inc.) or Histopaque-1077 (Sigma Chemical Co.).
- 2.4 Sterile 50-mL and 15-mL conical tubes.
Many brands are available and may be purchased through Fisher, VWR, or other laboratory supply companies.
- 2.5 Trypan Blue, tissue-culture grade, for enumeration and determining viability of cells. Available through Gibco BRL Life Technologies or other companies.
- 2.6 A commercially available, sterile cell strainer for making a single-cell suspension, such as the B-D Falcon cell strainer (Fisher Scientific catalog number 08-771-1).
Optional - Nylon Mesh, such as Spectra/Mesh N size 53 μ m.
Recommended vendor: Fisher Scientific
Phone 1-800-766-7000
catalog #08-670-201

A nylon mesh filter, used for creating a single-cell suspension, can be heat-welded onto the end of a 5-mL pipette tip. First, the pipette tip end is cut with a razor blade to a diameter of about 5 mm. The cut end of the pipette tip is heated on a hot plate (be careful not to burn the end), and then immediately pressed onto a small square of the nylon mesh. After it cools, make sure there are no openings around the edges where the mesh and the tip meet. The tip/filter is then wrapped in foil and autoclaved.

- 2.7 Fetal Bovine Serum (FBS). Heat-inactivated and tissue culture tested. The ACTG reserves a lot that has been tested by ACTG labs. Contact the ACTG Operations Center for vendor and lot number.
- 2.8 Dimethyl sulfoxide (DMSO), tissue culture grade. Available through Gibco BRL Life Technologies or other companies.

3.0 INSTRUMENTATION

- 3.1 Operating room equipment, including scalpels, sutures, etc.
- 3.2 A refrigerated centrifuge capable of spinning at 200-400 x g (check the radius of the centrifuge to determine the mid-tube rpms (r_{av}) for a given g force) for Ficoll-Hypaque centrifugation, or 200 x g for washing cells. Ficoll-Hypaque spins should be done at room temperature (20°-25°C). Once cells are chilled, centrifugation should be done at 4°C.
- 3.3 Calibrated pipettes and multichannel pipettes.
- 3.4 Microscope and hemocytometer for counting cells.
- 3.5 Controlled rate freezer (i.e., Mr. Frosty), if cells are to be frozen.
- 3.6 Liquid nitrogen storage, if cells are to be frozen.

4.0 DETAILED PROTOCOL

- 4.1 Suggested Procedure for Excisional Lymph Node Biopsy.
 - 4.11 The protocol should give the name and contact information of a consulting surgeon for questions and clarifications. The subject should be evaluated by an Ear, Nose, and Throat consultant prior to the initial lymph node biopsy in the office whereby the procedure and associated risks (e.g., bleeding, infection, scarring, injury to spinal accessory and/or greater auricular nerve), are described in detail. The medical history should be reviewed to confirm that no aspirin, NSAIDs, or similar medications have been taken within TWO WEEKS prior to the procedure, which otherwise may affect coagulation. Subject should also receive evaluations of PT and PTT before procedures are performed.
 - 4.12 A head and neck examination is performed with careful attention paid to the cervical lymphatics. Shoulder function should be documented.
 - 4.13 At surgery, the largest, most accessible lymph node (occipital or posterior cervical triangle lymph node chains are preferable; however, inguinal nodes SHOULD NOT be sampled) is selected, a planned incision is outlined (~3 cm) with a surgical marker (preferably in a skin crease) and infiltrated with 1% lidocaine with

- 1:100,000 epinephrine. The subject is prepped and draped in the usual fashion.
- 4.14 The skin incision is made with a 15 blade along the surgical mark and carried through the platysma, or by electro-cautery. At this point, the 15 blade is taken off the surgical field to minimize the risk of accidental injury. Potts forceps with teeth are used to grasp the cervical fascia by the surgeon and first assistant. The fascia is divided with Stevens' scissors in the direction of the spinal accessory and great auricular nerves to reduce the incidence of iatrogenic injury.
- 4.15 Once the fascia is opened, the lymph node becomes visible. **It is essential to maintain the architecture and integrity of the lymph node.** Extreme care should be taken so that the lymph node is not grasped directly or "tugged upon", avoiding any crush or pull artifact. The use of cautery is minimized until the lymph node is removed to avoid trauma to the node. Blunt surgical retractors may also be used to reduce risk to the surgeon.
- 4.16 Hemostasis should be obtained with electrocautery; the wound is irrigated with saline and closed with interrupted 4-0 undyed vicryl and subcuticular 5-0 PDS sutures.
- 4.17 The surgeon closes the wound without assistance, again, to avoid an inadvertent needle stick.
- 4.18 A single steristrip is placed, and the patient is escorted back to the preoperative holding area. Shoulder function should be documented prior to discharge.
- 4.2. Preparation of frozen tissue blocks.
Refer to specific instructions in the protocol or the PIIS.
The preparation of frozen tissue is crucial to allow evaluation of the patient's lymphoid tissue by immunohistochemistry. If the tissue is not obtained immediately after surgery, or if frozen blocks are not prepared correctly, then the block may not be suitable for analysis. It is important that the technician responsible for the preparation of frozen block contact Dr. R. Pat Bucy (University of Alabama at Birmingham) directly for step-by-step instructions for this procedure.
- 4.3 Single Cell Suspension (Lymph Node Biopsy).

As previously stated, a portion of lymph node biopsy that is of sufficient size will be separated and teased into a single-cell suspension. In the OR, the tissue should be placed in a 15-mL conical tube in approximately 6 mL of cold HBSS or D-PBS supplemented with 5% FBS and then placed on ice. The tissue should then be transported immediately to the biocontainment lab on ice for further processing. Lymph node tissue should be processed for single-cell suspension aseptically under a laminar flow tissue culture hood in an appropriate biocontainment facility. Approximately 5 to 40 x 10⁶ cells can be obtained, depending on the size of the node.

NOTE: SUBSEQUENT PROCEDURES SHOULD BE PERFORMED IN A CLASS 2 BIOSAFETY LAMINAR FLOW HOOD USING STERILE TECHNIQUE AND ADHERING TO CDC/NIH STANDARDS (INCLUDING THE USE OF GLOVES AND LAB COATS).

- 4.31 Place tissue and original HBSS into a sterile 60 mm tissue culture plate.
- 4.32 Tease tissue apart using sterile forceps to hold tissue and scalpel to tease and slice into a homogenate.
- 4.33 Pipet the cells back into the original 15-mL conical tube using a small bore pipette, leaving larger pieces of tissue in the plate.
- 4.34 Allow any small pieces of tissue that came through the pipette to settle out to the bottom of the tube by allowing the tube to sit undisturbed for about 5 minutes.
- 4.35 Create a single-cell suspension by passing the cell suspension aseptically through a nylon mesh filter. Carefully pipette the cells from the 15-mL tube, being careful not to disturb the larger tissue that has settled out at the bottom of the tube. Pipet the cells through a sterile nylon mesh sieve created from a 5-mL pipette tip (see Section 2.6, optional), so that the cells and buffer drip into a new 50-mL tube. Be careful not to handle the tip of the sieve so that it remains sterile.
- 4.36 Resuspend cells adequately by pipetting up and down and determine the concentration of cells and total cell yield by counting on a hemacytometer. Usually a 1:10 or 1:50 dilution of the single cell suspension works well for counting.
- 4.37 Determine the cell viability by trypan blue dye exclusion.
- 4.38 If the cells are greater than 75% viable, proceed with preparation.
- 4.39 If the cells are less than 75% viable, separate the viable lymphocytes by standard ficoll density centrifugation and then recount the cells on hemacytometer.
- 4.40 The cell concentration can be adjusted by pelleting cells at 200 x g for 10 minutes and resuspending in an appropriate volume of HBSS or D-PBS containing 5% FBS.
- 4.41 Keep cells at 4° C throughout procedure by using ice cold HBSS and a refrigerated (4°C) centrifuge.

Freeze cells in DMSO cryoprotective medium (see below) or use immediately for advanced flow cytometry, lymphoproliferative assays, or other assays (refer to the PIIS).

4.4. Freeze Viable Frozen Cells

If cells are to be stored as viable frozen cells, freeze in medium consisting of 90% FBS with 10% DMSO (final):

- 4.41. Pellet the cells in appropriate tubes by centrifugation at 200 x g for 10 minutes.
- 4.42. Determine the final volume required to produce as many aliquots as indicated at the cell number indicated in the PIIS. For example, if cells are to be cryopreserved at a final cell concentration of 5 x

- 10⁶ cells/mL, and you have 20 x 10⁶ cells total, then you would save four 1-mL aliquots at 5 x 10⁶ cells/mL.
- 4.43. Resuspend the cell pellet in FBS to 0.9X the final volume. For example, if you need 4 mLs final volume, resuspend in 3.6 mLs FBS.
 - 4.44. Add 100% DMSO to a final concentration of 10% by dropwise addition and gentle swirling. For example, add 0.4 mL DMSO to 3.6 mL of cells in FBS.
 - 4.45. Immediately aliquot the cells into 1.8 mL cryovials at the indicated cell concentration (usually 5 x 10⁶ cells/mL, 1 mL per vial).
 - 4.46. Place cells in a Mr. Frosty at -70° C for a minimum of 4 hours. If using a controlled-rate freezer hooked up to a liquid nitrogen tank, follow manufacturer's instructions.
 - 4.47. Transfer cells to liquid nitrogen storage within 24 hours of preparation.
 - 4.48. Make sure to log-in the storage location of vials in the tank for future retrieval of cells.

5.0 SPECIMEN LABELING

All tubes should be labeled with PID, study number, unique identifier, VID, draw date, collection time (24-hour time clock), and LYM/CLN.

6.0 AUTHORS

Dr. Pat Bucy, with modifications by Drs. Mostafa Nokta and XiaoDong Li, and contributions by ACTG Immunologists and ACTG Laboratory Technologists.

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