

Standard Operating Procedure VI: Mononuclear Cell Isolation from Blood or Bone Marrow

**** NOTE:** The following procedure is to be performed wearing laboratory coat, gloves, eye protection, and mask. For all procedures, use a biological safety cabinet in a level 2 laboratory (room A5-126).

****Adapted from Sigma-Aldrich Procedure 1077.**

PRINCIPLE

Venous blood will be collected from patients at the indicated time points. The collection of blood should be obtained from an existing arterial or venous line, or by venipuncture, and should be performed by someone experienced in the technique and familiar with infectious precautions.

Histopaque[®]-1077 is a solution of polysucrose and sodium diatrizoate adjusted to a density of 1.077 ± 0.001 g/ml. This procedure is suitable for multiple purposes after isolation, including studying cell-mediated lympholysis and human lymphocyte antigen (HLA) typing.

Anticoagulated venous blood is layered onto Histopaque[®]-1077. During centrifugation, erythrocytes and granulocytes are aggregated by polysucrose and rapidly sediment; whereas, lymphocytes and other mononuclear cells remain at the interface. Most platelets are removed during washing steps.

Specimen Collection & Handling

1. Label all of 1 lavender-top EDTA vacutainer, 3 x 15ml polypropylene conical tubes and cryogenic freezing tubes per patient. Warm Histopaque[®]-1077 to room temperature.
2. Whole blood or bone marrow is collected into a lavender top vacutainer(s). Record whether a plastic or glass tube is used. Keep at Room Temperature.
3. Process sample as soon as possible and within 2 hours of collection.
4. If more than one tube of blood/bone marrow was collected, pool the tubes into a 15 mL conical tube and record the volume. Add an equal amount of isotonic phosphate buffered saline (PBS) and mix by gently aspiration.
5. To a 15 ml conical tube, add an equal volume of Histopaque[®]-1077 to the volume of blood recorded in step 4 and ensure it is at room temperature.
6. Using a sterile glass Pasteur pipette, carefully layer the whole blood/PBS mixture onto the Histopaque[®]-1077. Centrifuge at $400 \times g$ for 30 minutes at room temperature with brake off.
7. After centrifugation, using a cotton plugged, sterile glass Pasteur pipette, aspirate the opaque interface containing the mononuclear cells and transfer to a new labeled 15 ml conical tube.

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8. Transfer remaining plasma from above the interface to a new 15 ml conical tube and aliquot 2.0 mL into as many cryogenic freezing tubes as necessary. Store plasma on ice during remainder of procedure and then transfer to -80°C .
9. Add to the aspirate, enough isotonic phosphate buffered saline (PBS) to make 10 ml total volume. Mix by gentle aspiration.
10. Centrifuge at $250 \times g$ for 10 minutes at room temperature.
11. Aspirate the supernatant and discard.
12. Resuspend the cell pellet with 10.5 ml of PBS and mix by gentle aspiration.
13. OPTIONAL - Remove a small aliquot of cells (0.5 ml – or enough to contain about 1×10^5 to 2×10^5 cell/ml) and stain for viability using trypan blue.
 - a. Mix aliquot with 0.1 ml of 0.4% trypan blue stain. Mix thoroughly and incubate at room temperature for 3 to 5 minutes.
 - b. Count cell numbers using a hemacytometer (using 10 microliters of aliquot) and record number of stained cells and unstained cells – add together for total number (staining indicates damaged cell membrane).
 - c. Calculate the cell viability by dividing the number of stained cells by the total number of cells and multiplying by 100. To calculate the cells/ml, use the following formula: cell# from $1\text{mm}^2 \times 10,000 \times \text{dilution factor}$. In this case the dilution factor would be 1.2.
14. Centrifuge tube from step 11 at $250 \times g$ for 10 minutes at room temperature or at 4°C if cells are being frozen.
15. After centrifugation, follow a, b or c below:
 - a. For freezing cells that are to be used for culturing: resuspend the cell pellet in 1 ml of freezing media. Transfer 0.25 ml to each of 4 labeled 2.0 ml cryogenic freezing tubes. Freeze immediately at -80°C . (If a large amount of cells is obtained, a larger resuspension volume and more aliquots may be necessary).
 - b. Culturing cells immediately: resuspend the cell pellet in 10 mL of RPMI-1640 and transfer to a T75 culture media flask. Lay the flask down in a 37°C incubator with 5% CO_2 . Depending on the viability and aggressiveness of the cells, passaging may be necessary after 1 to 2 days.
 - c. If using cells for other analyses resuspend in 1.0 ml of PBS and transfer 0.25 ml to each of 4 sterile tubes. Freeze at -80°C .
16. Dispose of all handling materials as biohazardous waste.

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Special Note 1.1: Processing and handling of Blood for analysis – please remember endotoxin is ubiquitous and can change expression of all mediators being assayed. Endotoxin free precautions should be taken for handling of all procedures (sterile precautions and using endotoxin free solutions should suffice).

Special Note 1.2: 50ml conical tubes may be used for larger volumes; alter procedure by using 15 ml of Histopaque®-1077 and 15 ml of whole blood.

Special Note 1.3: If cell viability is <80%, PBS may be replaced with a tissue culture medium such as RPMI 1640 plus 10% Fetal Bovine Serum (FBS). If using this substitute, cell viability will not be able to be obtained due to a cross reaction with the serum.

Special Note 1.4: Use caution when removing the mononuclear interface to not include excess amounts of Histopaque®-1077 as this will increase the amount of granulocyte contamination. On the other hand, removing excess amount of plasma may contribute to plasma protein and platelet contamination.

Special Note 1.5: If the intended use of isolated cells is for culturing and/or animal in vivo purposes, Histopaque®-1077 should be sterile filtered prior to use.

Special Note 1.6 Type of freezing media is to be determined by individual investigators. A common media is 60% RPMI 1640 plus 30% FBS plus 10% dimethyl sulfoxide (DMSO). Alternatively, 90% FBS plus 10% DMSO may be used for increased cell survival.

Supplies

- Lavender-top EDTA glass venous blood collection tubes (for example, BD vacutainers catalog# 367654)
- 15 ml sterile polypropylene conical tubes (Falcon/Becton-Dickinson, #35-2097)
- 2ml cryogenic freezing tubes (Corning, #430289)
- Histopaque®-1077 (Sigma-Aldrich, #10771)
- Sterile, endotoxin-free 10ml serological pipets (VWR, #53283- 740)
- Sterile glass pasteur pipets (VWR, #14672-410)
- Rubber bulbs for pasteur pipets (VWR, #56311-062)
- P1000 pre-sterilized, pipet tips (VWR, # 53508-830)
- Hemacytometer (Hausser, #3100)
- Fetal Bovine Serum, Qualified (Invitrogen, #10437010)
- Dimethyl Sulfoxide (Sigma-Aldrich, #BP231-1)
- Isotonic Phosphate Buffered Saline, 1X (Invitrogen, #10010-049)

Equipment

- Clinical Centrifuge with swinging-bucket rotor. Must accommodate 15ml conical tubes [Put some dry cotton or gauge at the bottom of the 15ml tube holders in order to avoid sticking of vacutainers].
- Sterile tissue-culture hood, BSL-2 or equivalent
- Laboratory pipetter capable of delivering 1.0 ml of liquid (eg., Rainin P-I 000)
- Ultra low temperature -80°C freezer
- Access to a sterilizing autoclave