

1.0 Purpose

This procedure is used for the separation of blood mononuclear cells from human peripheral blood, cord blood or bone marrow collected in anticoagulant using Ficoll-Hypaque density gradient centrifugation.

2.0 Principle

Human Mononuclear cells and platelets have a lower density than Ficoll-Hypaque (1.077g/L) and are separated from higher density granulocytes and red blood cells when centrifuged after either under or overlaying the diluted blood on the Ficoll-Hypaque layer. To ensure optimal cell viability and recovery from cryo-preserved material, cells must be isolated as soon as they arrive in the lab. Samples older than 24 hours must have viable cell counts performed to ensure adequate viable cells are stored as cell yield and viability decreases the longer a sample stands.

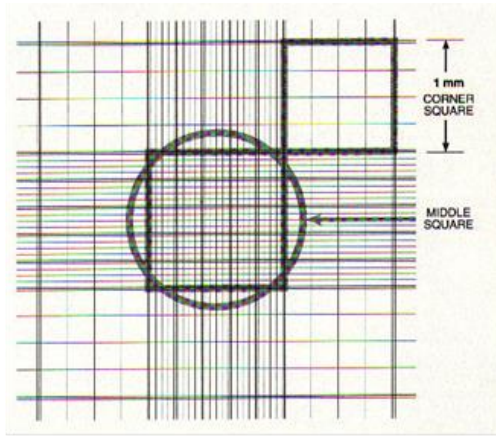
3.0 Materials & Supplies:

- 3.1. Phosphate Buffered Saline (PBS) without $\text{Ca}^{2+}/\text{Mg}^{2+}$
- 3.2. Ficoll-Hypaque (Label container with date after opening. Discard Ficoll containers open for more than 6 months).
- 3.3. FCS (or Pooled Human AB Serum)
- 3.4 Trypan Blue Stain

4.0 Equipment:

- 4.1 Laminar Flow Hood (BL2)
- 4.2 Refrigerated Centrifuge
- 4.3 Vortex
- 4.4 Personal safety equipment including gloves, lab coat, and eye protection
- 4.5 Micropipettor with disposable tips
- 5.6 Sterile conical centrifuge tubes, 15mL and 50mL
- 5.7 Disposable plastic pipettes in 2ml, 5ml and 10ml graduations
- 5.8 Pipette aid.
- 5.9 Plastic discard bucket.

- 5.10 Hemocytometer and microscope (or automated cell counter).
- 6.0 Safety procedures and precautions
- 6.1 Standard safety operating procedures are to be followed at all times
- 6.2 Treat all material as infectious,
- 6.3 All procedures (unless otherwise stated) are to be performed in a Biohazard Class II Safety Cabinet
- 6.4 White Coats and gloves must be worn at all times
- 7.0 Protocol:
- 7.1 All cell preparation will be performed in the Investigator's Lab.
- 7.2 Add 15 ml Ficoll-Hypaque to a sterile 50 ml conical tube.
- 7.3 Dilute 15 ml of blood 1:1 with sterile, endotoxin-free PBS (without Calcium and Magnesium).
- 7.4 Blood should be carefully and slowly overlaid at a ratio of parts diluted blood to parts ficoll reagent in the 50 ml sterile tubes, being careful not to disturb the interface.
- 7.5 Centrifuge the samples at 900 x g for 20 minutes at room temperature with the break off.
- 7.6 After centrifugation, remove cloudy interface (PBMC layer) into appropriately labeled 50 ml conical tubes.
- 7.7 Wash cells by filling tubes with sterile PBS and centrifuge at 250 x g for 10 minutes.
- 7.8 Decant supernatant after centrifugation, resuspend cells and fill tubes with sterile PBS to count cells.
- 7.9 Pipette 10 μ l of PBMC suspension into a 0.5 ml microcentrifuge tube. Add 90 μ l of 0.4% Trypan Blue stain, making a 1:10 dilution (final concentration of Trypan Blue is 0.36%). Mix carefully to avoid aerosol formation.
- Dilution Factor: $\frac{90 \mu\text{l Trypan Blue} + 10 \mu\text{l PBMC}}{10 \mu\text{l PBMC}} = 10$
- 7.10 Load the hemocytometer with cell mixture (Trypan Blue + PBMC's) until the area under the cover slip is sufficiently filled. Make sure to use a cover slip that is specific for the hemocytometer. Allow the cell suspension to settle in the hemocytometer for at least 10 seconds before counting. Count the 4 large corner squares (see diagram below). Viable PBMCs will be colorless; non-viable PBMCs will be blue.



Count cells in the 4 corner 1 mm squares. Include cells that touch either the top line or left vertical perimeter line of any corner square. Do NOT count any cells that touch either the bottom line or right vertical perimeter line of any corner square.

Calculate the number of PBMC/mL:

10^4 = volume conversion factor to 1 mL

10^1 = specimen dilution factor

$$\text{PBMC/mL} = \frac{\text{PBMC in all four squares}}{4} \times 10^4 \times 10^1$$

7.11 To calculate Cell Viability:

$$\% \text{ Viability} = \frac{\text{Number of Viable Cells Counted} \times 100}{\text{Total Number of Cells Counted}}$$

7.12 To determine the total number of cells, multiply the number obtained above (PBMC/mL) by the cell suspension volume (mL).

$$\text{Total Cells} = \text{PBMC} \times \text{Volume (mL) of PBMC suspension mL}$$

7.13 Proceed with SOP for freezing PBMC Cells are stored according to the Cell Storage Protocol.

Record:

1. details of the cell isolation
2. specimen distribution