PBMC Isolation from Whole Blood by Density Gradient Separation

Peripheral blood mononuclear cells (PBMCs) are immune cells characterized by the presence of a single round nucleus. PBMCs consist of lymphocytes (T cells, B cells, NK cells), monocytes, and dendritic cells. PBMCs derived from human blood are commonly used in preclinical and clinical research.

Key downstream applications include:

- Vaccine and drug development
- Immunophenotyping or HLA phenotyping
- Evaluation of cytotoxicity, lymphocyte recruitment and proliferation
- Cytokine and chemokine profiling
- Biomarker identification
- Disease modeling for autoimmune disorders and cancer
- Nucleic acid-based analyses and gene expression
- Creation of cell lines

Here, we explain how to isolate PBMCs from whole human blood. The technique originally developed by Bøyum[1,2] relies on the biophysical properties of the different blood components following their interaction with Ficoll – a synthetic, high molecular weight, hydrophilic polysaccharide. Our protocol, adapted from the original, consistently generates an isolated fraction in which >90% of cells are viable (**Figure 1**).

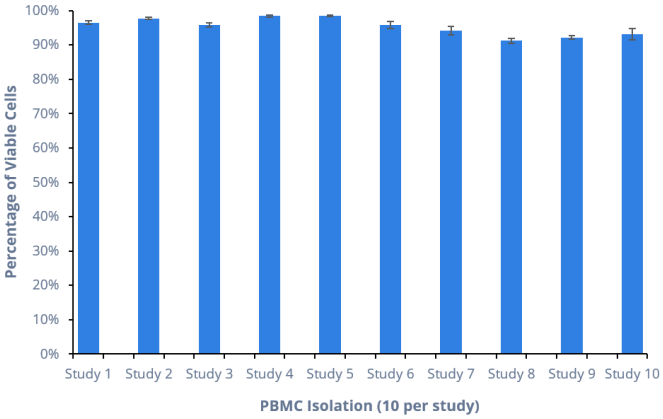

FIGURE 1. **Sample-to-sample variability from representative PBMC isolations. Shown is the average percentage of viable cells from a series of 10 PBMC isolations grouped across 10 independent studies (average >90% ± 0.022). Error bars represent standard error of mean.**

Reagents and Materials

- Density Gradient Cell-Separation Medium (e.g., Ficoll)
- 1X sterile, Ca^{2+}/Mg^{2+} free phosphate-buffered saline (PBS)
- Heat-inactivated fetal bovine serum (HI-FBS)
- 50 mL sterile conical tubes
- Sterile serological pipettes and pipetting aid
- Sterile Pasteur or transfer pipettes
- Optional: Red blood cell (RBC) lysing solution

  For cryopreservation:
  - Gibco™ Recovery™ Cell Culture Freezing Medium or freezing medium containing 50% RPMI, 40% FBS, and
  - 10% DMSO

    Freezing container (e.g. Cool Cell, Mr Frosty, Nalgene)

Equipment

- Bench-top centrifuge with swing-out rotor
- Biosafety laminar flow cabinet (Class II)
- For storage: -80 °C freezer

Procedure

- ***Preparation of reagents***
  1. Warm Ficoll density gradient medium to room temperature (18 °C to 20 °C).
  2. Make PBS wash buffer by adding 2% HI-FBS to 1X sterile Ca^{2+}/Mg^{2+} free PBS.

- ***Preparation of samples***
  1. Using aseptic technique, transfer anticoagulant-treated blood from each blood collection tube (or buffy coat unit) into 50 mL sterile conical tubes.
  2. Dilute blood with an equal volume of PBS wash buffer (1:1 dilution).
  3. Mix the blood and buffer by gently inverting the tube or pipetting up and down.

- ***Isolation of mononuclear cells***
  1. Add 15 mL of Ficoll to a fresh 50 mL conical tube to prepare for layering.
  2. Carefully overlay ~15 mL of diluted blood over the Ficoll layer by resting the pipette tip against the wall of the conical tube about 5–10 mm above the fluid meniscus. Slowly dispense the first 5 mL of blood dropwise to avoid disturbing the separation medium and blood interface.
  3. Centrifuge the tubes at 1,000 x g at room temperature (18 °C to 20 °C) for 30 minutes with the BRAKE OFF, to prevent disrupting the density gradient during deceleration.
  4. Carefully remove the tubes from the centrifuge without disturbing any of the layers. After centrifugation, multiple layers are obtained in the following order (from top to bottom, **Figure 2**):
     - Yellowish layer of dilute plasma and platelets
     - **Fluffy white layer of PBMCs at the interphase**
     - Ficoll or cell-separation medium
     - RBC and granulocyte layer

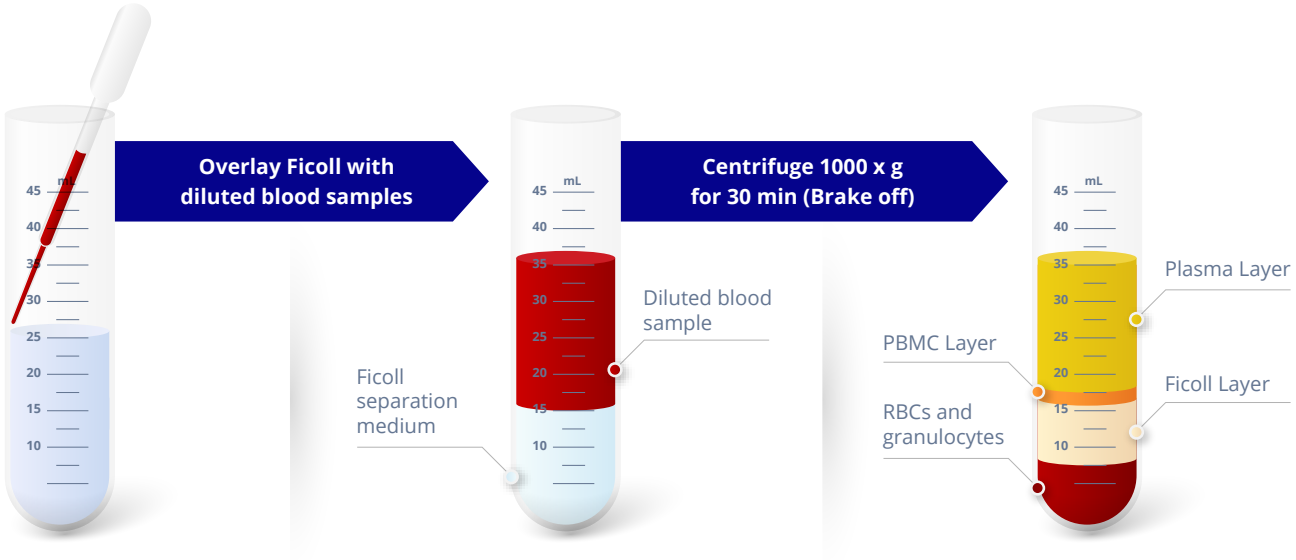

FIGURE 2. Separation of whole blood using Ficoll.

5. Slowly remove the top diluted plasma layer (~15 mL) using a plastic Pasteur pipette or a serological pipette
6. Carefully collect the PBMC layer using a sterile Pasteur pipette, starting at the periphery, and slowly moving the pipette tip over the entire cross-sectional area of the tube. Avoid aspirating too much Ficoll medium as this can be toxic to the cells.
7. Transfer PBMCs to a fresh 50 mL conical tube. Discard the remaining Ficoll and RBC layers.

- ***Removal of contaminants***

1. Wash PBMCs by bringing up the volume in the conical tube to 50 mL using PBS wash buffer.
2. Centrifuge at 300 x g for 10 minutes at room temperature (18 °C to 20 °C) with the BRAKE ON.
3. Carefully discard the supernatant and loosen the cell pellet in the remaining solution, using a transfer pipette to mix large clumps.
4. Optional step: Add 9 mL of RBC lysing solution per 1 mL of cell suspension. Mix gently and incubate on ice for 5 minutes.

   *Note: Longer incubation may reduce cell viability and recovery.
5. Repeat steps 1–3 from this section.

- ***Cell counting***

1. Re-suspend the cells in an appropriate amount of PBS wash buffer for subsequent assays or cell counting.
2. Count cells using an automated cell counter or manually using a Neubauer chamber hemocytometer.
3. Determine cell viability using Trypan blue stain.

- ***Cryopreservation and storage***

1. After isolation, PBMCs may be used immediately or cryopreserved to maintain sample integrity for future use. Gibco™ Recovery™ Cell Culture Freezing Medium or freezing medium containing 50% RPMI, 40% FBS, and 10% DMSO is used for cryopreservation.
2. To obtain a cell pellet, centrifuge the PBMC cell suspension at 300 x g for 10 minutes at room temperature (18 °C to 20 °C) with the BRAKE ON.
3. Discard the supernatant and re-suspend the pellet in cold freezing medium to obtain a cell density of 1–10 x 10^6 cells/mL.
4. Quickly aliquot 1 mL of PBMC cell suspension in each cryovial.
5. Place PBMC-containing cryovials in a freezing container (e.g., Cool Cell, Mr. Frosty) in a -80 °C freezer overnight.
6. Transfer the vials to liquid nitrogen for long-term storage the next day. Store samples in liquid nitrogen till they are ready to be shipped.

- ***Shipment***

1. Use World Courier to ship PBMCs to clients.
2. Ship PBMCs on dry ice (unless the client requests liquid nitrogen dry shippers).
3. World Courier maintains the temperature during transport by
   - Replenishing dry ice if it is a dry ice shipment
   - Replenishing liquid nitrogen if a dry shipper is requested

Troubleshooting

Problem	Potential Solutions
Lack of distinct layers after centrifugation	▪ Always perform centrifugation with brakes off. Deceleration disrupts the formation of distinct layers. ▪ Weigh tubes to ensure rotors are appropriately balanced. This avoids excessive shaking during centrifugation.
Low yield and/or viability of PBMCs	▪ Ensure that Ficoll temperature is between 18 °C to 20 °C. Ficoll is less dense at higher temperatures. This may allow lymphocytes to enter the Ficoll layer instead of collecting at the interphase. ▪ Avoid aspirating excessive Ficoll while collecting the PBMC layer. Exposure to Ficoll for long durations may be toxic to cells. Wash PBMCs with PBS two to three times. ▪ If you use RBC lysing solution, perform the incubation on ice and reduce the duration of exposure to cells.
RBC and granulocyte contamination of PBMCs	▪ Ensure that Ficoll temperature is between 18 °C to 20 °C. Ficoll is denser at low temperatures. The higher density impairs aggregation of RBCs and granulocytes, preventing them from settling down to the bottom of the tube. ▪ Ensure centrifugation speed is not too slow, nor time too short. Adequate time and g-force is essential for complete sedimentation of RBCs and granulocytes.

REFERENCES

1. Bøyum A. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand J Clin Lab Invest Suppl*. 1968; 97:77–89.
2. Bøyum A. Isolation of lymphocytes, granulocytes and macrophages. *Scand J Immunol*. 1976; Suppl 5:9–15.