Isolation of Human PBMCs from Whole Blood by Ficoll Gradient

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Purpose

This protocol describes the isolation of peripheral blood mononuclear cells (PBMCs) from whole human blood using Ficoll density gradient centrifugation. Whole blood is diluted with serum-free medium (R0), layered onto Ficoll, centrifuged to generate a mononuclear cell interface, and PBMCs are harvested, washed, and optionally cryopreserved in freezing medium.

Critical notes (read before starting)

- Handle human blood and PBMCs as potentially infectious material under appropriate biosafety conditions (e.g. BSL2).
- Carefully layer diluted blood onto Ficoll to avoid mixing; a sharp interface is essential for good yield and purity.
- Use **rcf (g)** where possible rather than rpm; if rpm is used, ensure it corresponds to the correct g-force for your rotor.
- Use low brake (or reduced deceleration) during the Ficoll spin to avoid disturbing the gradient.
- Avoid aspirating Ficoll when harvesting the PBMC layer; take only the "fluffy" interface.
- Keep cells at room temperature during the Ficoll separation and at room temperature or 4 °C during washes, as appropriate.
- For cryopreservation, use controlled-rate freezing (e.g. Mr Frosty) and avoid leaving cells at room temperature with DMSO for prolonged periods.

> ⚠ Warning
>
> Mixing of diluted blood with Ficoll or using high brake during the gradient centrifugation can collapse the interface and dramatically reduce PBMC yield and purity.

> **Tip**
>
> Prepare all R0 and R10 media, Ficoll tubes, and pre-labelled cryovials before starting. This simplifies handling once blood is collected and reduces the time PBMCs spend at room temperature.

Approximate timing

- Setup and blood dilution: 10–15 min
- Ficoll gradient and spin: 30 min
- First PBMC wash: 15–20 min
- Second wash and resuspension in R10: 15–20 min
- Counting and freezing (if applicable): 20–30 min

Total approximate time: 1.5–2 hours.

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Procedure

Step 1 – Tube labelling and blood dilution

1. Label 50 mL conical tubes for each blood sample.
2. For each sample, add whole blood to a 50 mL tube:
    - Transfer up to **17.5 mL whole blood** per 50 mL tube.
3. Dilute blood **1:2** with R0 medium (RPMI without FCS; `BUF-R0-MED`):
    - Add **17.5 mL R0** to 17.5 mL blood (total 35 mL).
    - If using less than 17.5 mL blood, top up to **35 mL** with R0.

> **Note**
>
> Dilution with R0 reduces viscosity, improves layering on Ficoll, and can improve separation quality.

Step 2 – Ficoll layering and gradient centrifugation

1. In a fresh 50 mL tube, add **10 mL Ficoll** (density gradient medium).
2. Using a 10 mL serological pipette, **slowly overlay** the **35 mL diluted blood** onto the Ficoll:
    - Hold the Ficoll tube at a gentle angle.
    - Carefully dispense the diluted blood onto the Ficoll surface, avoiding mixing.
    - Maximum volume of diluted blood per tube: **35 mL**.
3. Prepare all Ficoll gradients required and ensure they are balanced.
4. Centrifuge at **450 g (rcf)** for **30 min** at room temperature with:
    - Acceleration: 2
    - Deceleration: 1 (minimal brake)
5. During centrifugation, prepare **10 mL tubes** containing **3 mL R0** (2 tubes per Ficoll gradient) for PBMC collection.

Step 3 – Harvest of PBMC layer and first wash

1. After centrifugation, identify:
    - Plasma / diluted medium layer on top.
    - A white, “fluffy” PBMC layer at the plasma/Ficoll interface.
    - Ficoll layer.
    - Pellet of erythrocytes and granulocytes at the bottom.
2. Using a sterile transfer pipette, gently harvest the PBMC layer:
    - Carefully aspirate the interface and transfer to a 10 mL tube containing **3 mL R0**.
    - Continue until the visible fluffy layer is removed.
3. For each gradient, distribute the collected PBMCs across **two 10 mL tubes** (each with 3 mL R0).

4. Centrifuge the 10 mL tubes at **400 g for 10 min** at room temperature:
    - Acceleration: 9
    - Deceleration: 9
5. Carefully remove and discard the supernatant without disturbing the cell pellets.

Step 4 – Second wash and resuspension in R10

1. Resuspend each PBMC pellet in **3 mL R0**.
2. Combine **two pellets into one tube** and top up to **10 mL R0**.
3. Centrifuge at **400 g for 5 min** at room temperature:
    - Acceleration: 9
    - Deceleration: 9
4. Remove and discard the supernatant.
5. Resuspend the pellet in **10 mL R10** (complete RPMI with 10 % FCS; `BUF-R10-MED`).
6. If multiple gradients were processed for the same sample, combine their PBMC pellets into a single tube in R10.
7. Centrifuge at **400 g for 5 min** at room temperature.
8. Remove and discard the supernatant.
9. Resuspend the pellet in **1–10 mL R10**, depending on pellet size and planned downstream use.

Step 5 – Counting and cryopreservation (optional)

1. Mix the cell suspension gently and take a small aliquot for counting with trypan blue or an equivalent viability dye.
2. Calculate the total number of viable PBMCs.
3. For cryopreservation, aim for a final concentration of **10 × 10 cells/mL** per cryovial.

5.1 Preparation of 2× freezing medium (`BUF-FREEZE-MIX`)

1. Prepare 2× freezing medium by mixing:
    - 7 mL R0
    - 1 mL FCS
    - 2 mL DMSO
      (Final: R0 + 10 % FCS + 20 % DMSO; 2× relative to standard 10 % DMSO freezing medium.)
2. Keep the 2× freezing medium on ice and protected from light.

5.2 Adjust cell concentration and freeze

1. Adjust PBMCs in R10 to **20 × 10 cells/mL**:
    - If only one cryovial is needed, pellet cells at **200 g for 5 min**, remove supernatant, and resuspend so that the cell pellet is in **500 µL R10** (20×10 cells/mL).
2. Add an **equal volume** of 2× freezing medium (`BUF-FREEZE-MIX`) to the cells:
    - Final concentration: **10 × 10 cells/mL** in **10 % DMSO**.
3. Gently mix to avoid bubbles.
4. Dispense **1 mL** of cell suspension into **pre-cooled cryovials**, labelled with:
    - Patient name or anonymised ID.
    - Sample type (PBMC).
    - Date.
5. Place cryovials into a **pre-cooled controlled-rate freezing container** (e.g. Mr Frosty) and transfer to a **−70 °C or −80 °C** freezer.
6. After at least **overnight freezing**, move cryovials to **liquid nitrogen** for long-term storage.

Buffers Required

Full composition, preparation, storage, and safety information for each buffer lives in its own linked buffer document — only a concise per-buffer summary is reproduced here, generated automatically from that document.

R0 Medium (Serum-free RPMI)

Component	Final concentration / amount
RPMI 1640	to 500 mL (serum-free, no additives)

- **Final volume:** 500 mL
- **Preparation note:** Optional: add 1% (v/v) Pen/Strep (100x stock) only if sterility support is explicitly required.
- **Storage:** 4C, protected from light; 4-6 weeks unopened, 2-4 weeks after opening; do not freeze.
- **Caveat:** Serum-free by design - do not add FCS; serum interferes with Ficoll density gradient separation and interface formation.

Full composition, preparation steps, storage, and safety information: `BUF-R0-MED`.

R10 Medium (RPMI + 10% FCS)

Component	Final concentration / amount
RPMI 1640	to 500 mL
Fetal calf serum (FCS)	10% (v/v)
Penicillin/Streptomycin	1% (v/v)

- **Final volume:** 500 mL
- **Preparation note:** Gently invert to mix; do not vortex.
- **Storage:** 4C, protected from light; use within 4 weeks; do not freeze.

Full composition, preparation steps, storage, and safety information: `BUF-R10-MED`.

2x Freezing Medium

Component	Final concentration / amount
RPMI 1640 (R0)	base medium (7 mL per 10 mL batch)
Fetal calf serum	10% (v/v)
DMSO (cell-grade)	20% (v/v)

- **Final volume:** 10 mL (2x concentrate; example batch, scale as needed)
- **Preparation note:** Mix gently by inversion; do not vortex. Keep on ice until use.
- **Storage:** Use fresh on day of preparation; do not store; discard any unused buffer.

- **Caveat:** DMSO is toxic at room temperature - keep the 2x mix on ice and mix with cells immediately after preparation. Final working freezing medium (after 1:1 mix with cells) contains 10% DMSO.

Full composition, preparation steps, storage, and safety information: `BUF-FREEZE-MIX`.

Materials

Reagents

Reagent	Supplier	Cat. #	Notes
Ficoll-Paque or equivalent density medium	Cytiva / other	–	Density gradient for PBMC isolation
RPMI 1640	Various	–	Base for R0 and R10 media
Fetal calf serum (FCS), heat-inactivated	Various	–	10 % v/v in R10, 10 % v/v in freezing medium
Penicillin/Streptomycin	Various	–	1 % v/v in R10
DMSO, cell culture grade	Various	–	20 % in 2× freezing medium; 10 % final in cell suspension
PBS, sterile, 1× (optional)	Various	–	For additional washes if required
Trypan blue or viability dye	Various	–	For counting and assessing viability

Disposables

Item	Specification
50 mL conical tubes	Sterile, for blood and Ficoll
10 mL round-bottom tubes	Sterile, for PBMC collection
Cryovials	Suitable for liquid nitrogen storage
Serological pipettes (5–25 mL)	For layering and media handling
Pipette tips	Sterile (10 µL, 200 µL, 1000 µL)
Transfer pipettes	For harvesting PBMC interface
Biohazard waste containers	For blood-contaminated disposables

Equipment

Equipment	Specification / Notes
Benchtop centrifuge	For 50 mL and 10 mL tubes; adjustable rcf
Class II biological safety cabinet	For handling human blood and PBMCs
Controlled-rate freezing container	e.g. Mr Frosty, for −70/−80 °C freezing
−70 °C or −80 °C freezer	For initial freezing
Liquid nitrogen storage system	For long-term storage
Hemocytometer or automated cell counter	For PBMC counting

Troubleshooting

Problem	Possible cause	Suggested solution
Poor PBMC layer formation	Blood and Ficoll mixed; incorrect centrifuge settings	Carefully overlay blood; verify 450 g; reduce brake to minimal
Low PBMC yield	Interface harvested too shallow or too deep	Practice aspirating only the fluffy interface; avoid Ficoll and pellet
High granulocyte or RBC contamination	Interface disturbed; Ficoll aspiration	Harvest more gently; leave a thin Ficoll layer above the pellet
PBMC clumping	High cell concentration; extended room temperature time	Gently resuspend; consider DNase during processing; shorten handling time
Reduced viability after thawing	Inadequate freezing rate or DMSO handling	Use controlled-rate freezing; minimize time with DMSO at room temperature
Loss of cells during washes	Pellets disturbed when decanting	Leave a small volume above the pellet; decant carefully; use pipette to remove residual supernatant

Safety (brief)

- Treat all human blood and PBMCs as potentially infectious material.
- Work in a Class II biological safety cabinet and follow institutional BSL2 procedures.
- Wear appropriate PPE (lab coat, gloves, eye protection) at all times when handling blood and PBMCs.
- Dispose of blood-contaminated materials (tips, tubes, pipettes, Ficoll waste) as biohazardous waste.
- Handle DMSO and cryogenic materials (e.g. liquid nitrogen) according to institutional chemical and cryogen safety guidelines.

Version history

Version	Date	Author	Changes
v1.0	2025-11-21	Dillon Corvino	First Quarto protocol version for PBMC isolation by Ficoll gradient.
v1.1	2026-09-18	Dillon Corvino	Replaced `# Buffers used` (bare `library(tidyverse)`, leaked R package-startup warnings) with an auto-generated `# Buffers Required` section; moved it from after Materials to before Materials. Fixed stale buffer-ID references `BUF-GEN-R0-MED-001` -> `BUF-R0-MED`, `BUF-GEN-R10-MED-001` -> `BUF-R10-MED`, `BUF-GEN-FREEZE-MIX-001` -> `BUF-FREEZE-MIX`