# Whole Blood PBMC Isolation

PROTOCOL

## Materials

- Collection tube (e.g. Vacutainer)
- SepMate™ Tubes, 50mL
- Serological pipettes, 5-20 mL
- Conical tubes, 50 mL
- Internally threaded cryovials, 2 mL
- Media bottle, 500 mL
- Microcentrifuge tube
- Single channel pipette with sterile tips
- Cell counting slide

## Reagents

- 70% Ethanol
- Density gradient medium
- 1X red blood cell (RBC) lysis buffer
- Heat-inactivated fetal bovine serum (HI-FBS)
- Wash buffer (2% HI-FBS + 1X sterile DPBS or serum-free)
- Cell cryopreservation medium, 10% DMSO

## Equipment

- Biological safety cabinet (BSL-2)
- Temperature-controlled swinging bucket centrifuge
- Controlled-rate freezing container (-1°C/min)
- Automated dual fluorescence cell counter
- -80°C freezer
- Liquid nitrogen storage system

## Procedure

### Considerations

Fresh whole blood samples are processed within ~24 hours of collection unless otherwise stated.

All procedures are performed with Universal Safety Precautions using appropriate PPE, aseptic technique, and under sterile conditions in a BSL-2 cabinet.

All samples are considered potentially infectious (BSL-2 or higher).

Samples and pertinent patient information are accessioned and stored on a LIMS system.

An automated cell counting system with dual fluorescence capabilities should be used to assess cell viability and nucleated cell count.

### Preparation

1. Warm density gradient medium to room temperature (20°C to 25°C).
2. Prepare the wash buffer by supplementing 1X sterile $Ca^{2+}$/$Mg^{2+}$-free PBS with 2% HI-FBS.

### Sample Processing

3. Turn on the biosafety cabinet and ensure a sterile environment, spraying all materials, equipment, and reagents entering the cabinet with 70% ethanol.
4. Using aseptic technique, transfer up to 15 mL of anticoagulant-treated blood from each blood collection tube into 50 mL sterile conical tubes.
5. Dilute the blood 1:1 (30 mL total) with wash buffer. Mix gently by inversion or pipetting and avoid foaming.

### PBMC Isolation

6. Add 15 mL of density gradient medium to a fresh 50 mL SepMate™ Tube.
7. Carefully overlay the diluted specimen over the density gradient medium layer.
8. Centrifuge the tubes at 1,200 x g at room temperature (20°C to 25°C) for 10 minutes with the brake on.
9. Remove the tubes from the centrifuge without disturbing the layers. Multiple layers should be visible (top to bottom, **Figure 1**):
   - Light yellow layer of diluted plasma and platelets
   - Cloudy white layer of peripheral blood mononuclear cells (PBMCs) at the interphase
   - Density gradient medium layer
   - Red blood cells (RBCs) and granulocytes layer
10. Slowly remove and discard the top 10mL of the diluted plasma layer without disturbing the PBMC layer.
11. Carefully transfer all of the PBMCs to a 50 mL conical tube while minimizing the amount of density gradient medium collected.

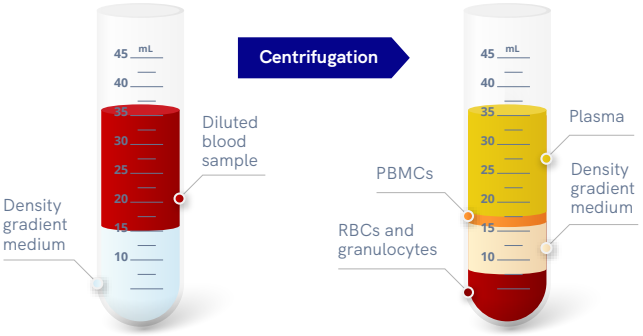


**FIGURE 1.** Density gradient separation of whole blood

### Cell Wash 1

12. Top off the PBMCs to 50 mL with wash buffer, gently mix by inverting the tubes.
13. Centrifuge the collection tubes at 120 x g at room temperature (20°C to 25°C) for 10 minutes with the brake off.

### RBC Lysis (optional)

14. Remove the collection tubes with cell pellets from the centrifuge. Carefully aspirate and discard the supernatant, leaving 1-2 mL of wash buffer.
15. Resuspend the PBMC pellet in the remaining wash buffer.
16. Add 9 mL RBC Lysis Buffer per 1 mL of the PBMC suspension. Mix gently.

17. Incubate at room temperature (20°C to 25°C) for 5 minutes.
18. Top off the cell suspension in each tube to 50 mL with wash buffer.
19. Centrifuge the collection tubes at 400 x g at room temperature (20°C to 25°C) for 5 minutes with the low brake on.

### Cell Wash 2

20. Remove the collection tubes with cell pellets from the centrifuge. Carefully aspirate and discard the supernatant, leaving 1-2 mL of wash buffer.
21. Resuspend the pellet in the remaining wash buffer.
22. Top off the PBMC suspension in each tube to 50 mL with wash buffer. Invert the tube gently to mix.
23. Centrifuge the collection tubes at 300 x g at room temperature (20°C to 25°C) for 8 minutes with the brake on.
24. Remove the collection tubes with cell pellets from the centrifuge. Carefully aspirate and discard the supernatant, leaving 1-2 mL of wash buffer.
25. Combine all of the individual cell suspensions from the same specimen into a single tube, if necessary.

### Cell Counting

26. Bring the PBMC suspension to a fixed volume with wash buffer. Invert the tube gently to mix.
27. Perform a cell count and viability assessment using an automated cell counter.

### Cryopreservation and Storage

28. Centrifuge the collection tubes at 300 x g at room temperature (20°C to 25°C) for 10 minutes with the low brake on.
29. After isolation, PBMCs may be used immediately or cryopreserved. Sanguine uses a standard PBMC concentration of $1x10^7$ cells/mL in cryopreservation medium.
30. Discard the supernatant and resuspend the pellet in an appropriate amount of cryopreservation medium.
31. Quickly aliquot 1 mL of PBMC cell suspension into an appropriate number of cryovials.
32. Immediately place the cryovials in a controlled-rate freezing container in a -80°C freezer for at least 4 hours or overnight.
33. Transfer the vials to the vapor phase of liquid nitrogen for long-term storage.