Leukopak PBMC Isolation

PROTOCOL

Materials

- Collection tube (e.g., Vacutainer)
- Conical tubes, 50 mL
- Serological pipettes, 5–20 mL
- Internally threaded cryovials, 2 mL & 5 mL
- Syringe, 60 mL
- Media bottle, 500 mL or 1 L
- Microcentrifuge tube
- Plastic tubing clamps
- Luer lock connector
- Connecting set (e.g., OnGuard)
- Single channel pipette with sterile tips
- Cell counting slide

Reagents

- 70% Ethanol
- Wash buffer (2% FBS + 1X sterile DPBS or serum-free)
- AO/PI staining solution
- CryoStor CS10 freezing media (10% DMSO)
- Density gradient medium (e.g., Ficoll)

Equipment

- Biological safety cabinet (BSL-2)
- Temperature-controlled swinging bucket centrifuge
- Freezing container, 2-mL & 5-mL (e.g., Mr. Frosty)
- Automated dual fluorescence cell counter
- Liquid nitrogen storage system

Procedure

Considerations

Fresh Leukopaks 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 the CloudLIMS system.

Sample Processing

1. Turn on the biosafety cabinet and ensure a sterile environment, spraying all materials, equipment, and reagents entering the cabinet with 70% ethanol.
2. Prepare a freezing container.
3. Transfer the leukapheresis product to a sterile media bottle using the connecting set and a syringe via the luer lock connector. Note the total volume of fluid transferred.
4. Dilute the leukapheresis product 2:1 with wash buffer. Mix gently by swirling and avoid foaming.
5. Prepare 50-mL conical tubes containing 15 mL of the density gradient medium, one per 35-mL of diluted specimen in step 4.
6. Slowly pipette the diluted specimen on top of the density gradient medium with the pipette tip angled at 45 degrees against the side of the conical tube to ensure the medium/sample interphase remains intact. Distribute the diluted specimen amongst the conical tubes equally.
7. Centrifuge the tubes at 400 x g at room temperature for 30 min with the brake off.
8. Ensure the plasma, buffy coat, and RBC layers are visible.
9. Remove and discard the top plasma layer with a pipette until approximately 3–4 mL remains above the buffy coat.
10. Transfer the buffy coat layer containing the PBMCs to a fresh 50 mL conical tube for each centrifuged tube.

Washing

11. Top up each PBMC-containing tube with wash buffer and mix by inversion 3–4 times.
12. Centrifuge at 300 x g at room temperature for 10 min with the low brake on.
13. Carefully remove the supernatant without disturbing the cell pellet. Resuspend the cell pellet in the remaining solution.
14. If RBCs are visible, incubate 9:1 with lysis buffer for 10 min, top up with wash buffer, and centrifuge at 400 x g at room temperature for 5 minutes with the low brake on.
15. Resuspend each pellet in 5 mL of wash buffer. Combine up to two conical tubes, top up each tube to 50 mL with wash buffer, and mix by inversion 3–4 times.
16. Centrifuge at 300 x g at room temperature for 10 minutes with the low brake on.
17. Carefully remove the supernatant without disturbing the cell pellet. Resuspend the cell pellet in the remaining solution.
18. Add 4 mL of wash buffer to each tube and transfer the suspension from each tube to a sterile media bottle.
19. Rinse each tube with 5 mL of wash buffer and transfer the remaining suspension to the media bottle.
20. Add wash buffer to a final volume of 200 mL and gently mix.

Cell Counting

21. Serially dilute a 10 µL sample 100:1 with wash buffer in a microcentrifuge tube.
22. Prepare a 1:1 mixture of 20 µL 100:1 diluted sample with 20 µL of room temperature AO/PI staining solution.
23. Apply 20 µL of the 1:1 mixture to a clean cell counting slide and load it into the automated cell counter.
24. Use the average of two separate cell count and viability measurements and determine the maximum number of cells to transfer:
    a. If $\geq 2x10^7$ live cells per conical tube, max = $8x10^8$ cells
    b. If $<2x10^7$ live cells per conical tube, max = $5x10^8$ cells

PBMC Aliquoting and Freezing

25. Transfer the appropriate volume of sample to 50-mL conical tubes and top up each with wash buffer.
26. Centrifuge at 300 x g at room temperature for 10 minutes with the low brake on. Discard the supernatant.
27. Slowly dispense the appropriate amount of CryoStor CS10 freezing media into each conical tube and slowly pipette up and down to resuspend the cells.
28. Aliquot the appropriate amount of cell suspension into cryovials.
29. Immediately place the cryovials into a freezing container at 2–8°C and freeze at -80°C for at least 4 hours.
30. The next day, transfer the cryovials to a liquid nitrogen storage system in the vapor phase (≤-130°C).