Peripheral Blood Immunophenotyping: Sample Preparation

Protocol for isolation of plasma and peripheral blood immune populations from whole blood

REAGENTS AND BUFFERS

1. 1x DPBS (Invitrogen, Catalogue number 14190)
2. P3 Steril (Ecolab)
3. Ficoll Paque Plus (Sigma-Aldrich, Catalogue number GE17-1440-03)

MATERIALS

1. Sterile Leucosep tubes (Greiner Bio-One Ltd, Catalogue number 227290)
2. 10 ml strippettes
3. 96-well 350µl polypropylene V-bottom plates (BD Falcon, 35326)
4. 1.7 ml autoclaved Eppendorf tubes
5. 1ml, 200 ul, 20µl and 10 µl filter tips
6. 50 ml tubes
7. 15 ml tubes
8. Beakers for waste

EQUIPMENT

1. BSL3 facility
2. BSC2 safety cabinet
3. Bench top centrifuge
4. centrifuge
5. Multichannel pipette
6. P10, P20, P200 and P1000 pipettes
7. Fridge

PRIOR TO SAMPLE PROCESSING:

- Prefill leucosep tubes in advance with 15 ml of Ficoll and spin at 1006 for 3 minutes.

SAMPLE PROCESSING:

Blood samples (about 27ml/patient) are shipped in heparin tubes from St. Thomas Hospital/Guy's Hospital and New Cross Gate to KCL (approximately 2 hrs) and processed the same day.

1. Gently invert Heparin tubes to mix the blood
   - ***For plasma:*** Take 1 ml of whole blood and place it in an autoclaved Eppendorf. Centrifuge in a pre-cooled (4C) tabletop centrifuge for 10 minutes at 2,000xg. Aliquot 100 µl into 5 Eppendorfs and freeze.

   - ***For cell counts:*** Transfer 50 µl of whole blood into 3 wells of a pre-prepared 96 well-U bottom plate containing respective antibody mastermixes for whole blood staining (Panels 6, 7 and 8) and subsequent cell concentration determination by flow cytometry as described in the whole blood staining protocol.

- ***<u>For PBMC prep:</u>*** Working in a BSC2 cabinet transfer the remaining whole blood using a 10 ml pipette and evenly transfer it into two Leucosep tubes. The volume of blood must not exceed 20 ml per Leucosep tube.

2. Rinse the K2-EDTA blood tube with half the blood volume of DPBS and then add half of the PBS volume to each of the Leucosep tubes.

3. Centrifuge tubes at 800g for 15 minutes with NO brake (Acc: 1, Dec: 1) at room temperature.

4. After centrifugation, the sequence of layers from top to bottom will be:
    a. Plasma
    b. PBMC interphase (buffy coat)
    c. Porous frit membrane
    d. Ficoll-Paque Plus media
    e. Pellet of erythrocytes and granulocytes.

5. To collect the PBMC interphase, transfer all of the supernatant above the frit membrane directly into a new 50 ml Falcon tube (if two Leucosep tubes are available for each patient, transfer the supernatant from each into a new Falcon). Do not dilute the supernatant further.

6. Centrifuge at 400xg for 10 minutes at 4^{o}C with break ON.

7. Decant the supernatant from the 2 Falcon tubes corresponding to the same patient to a new, adequately labelled Falcon tube (date, DP: diluted plasma, patient code). In addition, transfer five 1ml aliquots into 5 Eppendorfs. Store at -80C.

8. Resuspend the pellet in 1ml of PBS using a p1000 pipette, then top up with 25ml of cold 1xDPBS.

9. Wash 1: Centrifuge at 1600 rpm for 6 minutes with break ON at 4^{o}C.

10. Pour off the supernatant into a waste beaker containing P3 Steril.

11. Resuspend the pellet in 1ml of PBS using a p1000 pipette, then top up with 25ml of cold 1xDPBS.

12. Wash 2: Centrifuge at 1600 rpm for 5 minutes with break ON at 4^{o}C.

13. Pour off the supernatant into a waste beaker containing P3 Steril.

14. Resuspend the pellet in 2.4 ml of cold 1xDPBS with a p1000.

- ***<u>For flow cytometric staining</u>:*** Transfer 100µl of the re-suspension into four wells of a V-bottom 96-well plate (Panels 1-4), 200 µl into one well of a different V-bottom 96-well plate (Panel 5).

- ***<u>For freezing and storage of PBMCs:</u>*** Keep the remaining 1100 µl of cells in the fridge and depending on cell counts, freeze as described in the "*Peripheral Blood: Freezing of isolated PBMCs*".