Bone Marrow Immunophenotyping: Sample Preparation

Protocol for isolation of bone marrow and processing into single cell suspension

Reagents & Buffers

1. HBSS (Invitrogen14170-138)
2. Fetal Calf Serum (FCS)
3. RBC lysis solution (eBiosciences 00-4300-54, made up to 1× with ddH_2O)
4. FACS buffer (PBS (-Mg/-Ca), 0.5% FCS, 2 mM EDTA)

Materials

1. Forceps & scissors
2. Paper tissues
3. 200 µl pipette tips
4. 1.7 ml microfuge tubes
5. 30 µm CellTrics filters (Partec, 04-0042-2316)
6. Dispensing troughs for multichannel pipetting
7. 96-well 350 µl polypropylene V-bottom plates (BD Falcon, 353263)

Equipment

1. Table top centrifuge

Samples are shipped as whole legs in 50 ml tubes containing HBSS on ice from WTSI to KCL (approximately 2 hours by courier) and processed on the same day.

1. Prepare buffers and antibody master mix (see staining protocol) beforehand. Label plates for staining.
2. Prepare bone marrow extraction tubes – see instructions below protocol.
3. Remove leg from 50 ml tube and remove muscle from the tibia using forceps and scissors. Residual muscle can be removed using paper tissues.
4. Put the leg back into the tube to prevent drying out. Continue until all samples have been prepared up to this step.

Note: Once legs have been cleaned; batches are processed (step 5 onwards) in groups of 6 to prevent drying out.

5. One at time, remove leg from tubes again, and cut at the ends of the tibia using bone scissors. The cut points are just below the knee and just above the ankle. Ensure you can see red marrow through the cross section of the cut.
6. Place the rest of the leg back into the 50ml tube for contingency in case insufficient marrow is obtained from the tibia.
7. Insert the bone into the bone marrow extraction tube with the widest end on the bone at the bottom.
8. Six at a time, place microfuge tubes into microfuge and spin at 800×g for 30 seconds.
9. Inspect tubes to ensure bone marrow has been extracted. Otherwise cut the end off the bone to increase the opening and repeat. If no bone marrow can be extracted, repeat protocol with femur after all other samples have been processed.
10. Discard the pipette tips containing the bone from the microfuge.
11. Resuspend the bone marrow pellet in 50 μl of 1× RBC lysis buffer at room temperature. Pipette several times to break up pellet.
12. Incubate for 1 minute at room temperature.
13. Add 200 μl FACS buffer.

Note: Doing six at a time, allocating 10 seconds to each tube, 1 minute is just enough to do 6 tubes and to go back to the 1st tube to add FACS buffer.

14. Centrifuge at 500×g for 30 seconds.
15. Remove supernatant and resuspend in 200 μl FACS buffer.
16. Repeat steps 4-14, six samples at a time, until all the bones have been processed
17. Filter cells into 1.7ml microfuge tubes using 30 μm filters. Rinse tube and filter using 200 μl FACS buffer.
18. Pipette 200 μl of each sample into a 96-well plate.
19. Centrifuge plate at 800×g for 1 minute at 8^{o}C.
20. Cells are now in single cell suspension on plates and ready for staining (see staining protocol).

Preparation of bone marrow extraction tubes

1. Cut a 200 ml pipette tip at the 10 µl line and just below the line where the tip of the pipette would end (see picture, colour for illustration only).
2. Insert the middle section of the cut tip into the top section
3. Place into a 1.7 ml tube.