

## Surface Staining (Thymus-Spleen)

Perform all the steps on ice, if possible.

Centrifugation steps: 1400rpm at 4°C during 5 minutes.

1. Cut the tissue in pieces. For spleen, cut in 2 pieces
2. Place in a small petri dish a piece of nylon mesh (3cmx3cm), put the tissue on the mesh and covered it with another piece of nylon mesh
3. Add 2ml of complete medium 10% FCS, to the petri dish
4. Thymus: Take a plunger from a 5ml syringe and press the thymus gently in order to disgregated the tissue and extract the cells  
Spleen: Use the forceps to press the nylon mesh, until the spleen turn white
5. Recover and filter the cells: Transfer the cell suspension into a 15 ml falcon tube, passing the cells through a piece of nylon mesh or cell strainer (40µm)
6. Spin the cells.
7. Thymus: check the colour of the pellet. If the pellet is red, add red cell lysis buffer following the indications of the buffer producer company. When using home-made ACT Buffer, add 1ml of buffer to resusped the pellet and incubate during 3 minutes at room temperature (RT)  
Spleen: When using home-made ACT Buffer, resuspend the pellet in 1ml of buffer pipetting up and down, add another ml of buffer and incubate during 5 minutes at room temperature (RT)
8. Fill up the tube with staining buffer and spin the cells
9. Resuspend the pellet in 1ml of staining buffer and count the cells using trypan blue to determine viability
10. Resuspend the cells in staining buffer at  $10 \times 10^6$ /ml
11. Distribute in eppendorf tubes 100µl of cell suspension equivalent to  $1 \times 10^6$  cells, spin the cells and remove the supernatant.
12. Resuspend the pellet in Fc block, following the indications of the producer company, incubate accordingly.
13. Add 1ml of staining buffer and spin the cells.
14. Resuspend the cell pellet in 100µl of antibody mix and incubate 20 minutes on ice, protecting the samples from light with foil.
15. Add 1ml of staining buffer and spin the cells.

16. Resuspend the cell pellet in 200µl of PBS 1X, transfer the cell suspension to a facs tube suitable for acquisition in the cytometer. For the LSRFortessa use: Polystyrene Round-Bottom tubes, Ref. 352058 Falcon.
17. Acquire the samples: add a viability dye just before acquisition, vortex the cells and acquire.

**Staining Buffer:** PBS 1X-2% FCS-2mM EDTA

1000 ml PBS 1X

Take out 24 ml and add:

20ml FCS

4ml EDTA 0.5M

**Antibody Mix:**

In order to avoid precipitates from the antibodies, prepare your antibody mix in a 1.5ml eppendorf tube and spin during 10min at 4°C, 13.000rpm. Recover the supernatant and use it for staining.

**Red lysis buffer : Tris-Buffered Ammonium Chloride (ACT)**

Solution A: 4.5 gr Amoniun Chloride (NH<sub>4</sub>Cl) + 500ml of distilled water

Solution B: 10.3gr Tris –Base + 500ml of distilled water

Dissolve the Tris base in 450ml of distilled water. Adjust to pH 7.6 using 2N HCl.

Working solution: Mix 450ml of solution A + 50ml of solution B

Adjust to pH 7.2 using 2N HCl.