

ZAP-70 staining, SBZAP-PE

Membrane Staining buffer :

(Tick the box after the step is completed)

For 5 ml
-FCS 50 μ l
-EDTA 25 μ l (100 μ M final concentration)
-PBS 1X 4.925 ml

Washing buffer :

For 2 tubes (25 ml)
-FCS 250 μ l
-EDTA 125 μ l (100 μ M final concentration)
-Triton X100 10% 250 μ l
-PBS 1X 24.4 ml

Permeabilization buffer :

For 2 tubes : (250 μ l)
- **Washing buffer** 175 μ l
- Triton X100 10% 50 μ l
- Mouse Serum 25 μ l

Using WBC count, prepare a dilution with membrane staining buffer to obtain 550.000 lymphocytes in 200 μ l : (the ratio **550 / lymphocyte count in Giga/l** gives the volume of blood required in μ l)

Tube 1 = CTRL : prepare the dilution in this tube.

- Tube 2** = ZAP-70 staining : transfer 100 μ l of diluted blood from tube 1
(If lymphocyte count is below 2.75 Giga/l, use 100 μ l of undiluted whole blood per tube.)

Antibody premix : for 2 tubes
-40 μ l CD2 FITC
-40 μ l CD5 PerCP-Cy5.5
-10 μ l CD19 APC

- Add 45 μ l of **antibody premix in each** tube, vortex, incubate for 15 minutes at room temperature in the dark.
- Vortex, add 100 μ l of fixation reagent « Reagent A » Fix&Perm (Caltag), vortex and incubate for 10 minutes at room temperature in the dark.
- Wash with 3 ml of **washing buffer**, centrifuge at 200g for 3 min.
Discard the supernatant using aspiration, add 1 ml of **washing buffer**, **do not vortex** and carefully resuspend the pellet by pipeting 15 times avoiding the formation of foam.
- Then add 2 ml of **washing buffer** and vortex before centrifugation at 200g for 3 min.

Discard all the supernatant using aspiration. Resuspend the pellet by vortexing.

- Add 50 μ l of **Permeabilization buffer** in each tube, vortex, incubate for 10 minutes at room temperature in the dark.

Prepare the **labeling solution** :
-66 μ l of **Permeabilization buffer**
-4 μ l of SBZAP-PE antibody

In **tube 1** (control) : -42 μ l of **Permeabilization buffer**
-2.7 μ l of PE-conjugated isotype CTRL
-5 μ l of **labeling solution**

In **tube 2** (ZAP-70 staining) : add -50 μ l of **labeling solution**.
Vortex thoroughly

- Incubate for 20 minutes at room temperature in the dark.
- Wash with 3 ml of **washing buffer**, centrifuge at 200g for 3 min. Discard the supernatant, add 200 μ l of **washing buffer** and acquire data on cytometer.