P02 - Staining of fixation-sensitive epitopes

1. **Prepare staining cocktail**:
   a. complete Ab-panel,
   b. containing antibodies targeting fixation-sensitive epitopes.
2. Mix **fresh blood** sample with the **Ab-cocktail** and incubate for 30-60 minutes at temperatures ranging from 5˚C to 37˚C according to your preference and conditions recommended by the antibodies' producers.
   Meanwhile:
   a. **Calculate** the required volume of the **Fixation Buffer** (total volume of blood processed within one batch multiplied by factor 10) and **prepare** it by mixing equal volumes of
      i. Fix-concentrate
      ii. Fix-Diluent
      and let to equilibrate to room temperature (approx. 5 min).
3. **Dispense** the **Fixation Buffer** (10-times volume of stained blood[1]) to tubes with stained blood samples, set the timer, and **incubate** for **15 minutes** at **room temperature**. Vortex multiple times throughout.
4. **Add** 1-times concentrated **LYSIS buffer** (40-times volume of the blood sample[2]), **mix** well by inverting tube multiple times, and **incubate** at room temperature for minimum **15 to 20 minutes** until a complete erythrocytes lysis.
5. **Centrifuge** at **300g for 5 to 10 minutes**[3].
6. **Aspirate supernatant** down to**:**
   a. "ring" on the 5mL "FACS" tube, leaving a minimum of 100 µl of supernatant over the pellet,

[1] E.g., for 100 µl of blood use **1.0 ml of the Fixation Buffer**.
[2] E.g., for 100 µl of blood and 1.0 ml of Fixation Buffer use **4.0 ml of the LYSIS buffer**.
[3] Set the centrifugation timing according to the liquid column height in the tube and rotor size. The timing should increase with shorter rotor diameter and/or more liquid (higher liquid column) per tube.

b. approx. mark 0.5 ml in 15mL tube (for samples with original volume up to 250µl blood processed in the 15mL tube from beginning),
c. approx. mark 5.0-7.5 ml in 50mL tube, and before the next step, proceed as follows:
    i. transfer to a 15mL tube
    ii. centrifuge at 300g for 5 minutes,
    iii. aspirate the remaining volume down to approx. mark 0.5 ml.

7. Break the pellet by vortexing and **add** 1-time concentrated **WASH buffer** (40-times volume of the blood sample[4]).
8. **Centrifuge** at **300g for 5 to 10 minutes**[5].
9. **Gently aspirate** the supernatant down to:
    a. approx. mark 0.25 ml (15mL tube)
    b. ring" on the 5mL "FACS" tube leaving approx. 100 µl of supernatant over the pellet.

    Do not decant!
10. Proceed with:
    a. acquisition
    b. **cryogenic preservation** in CRYO#20 buffer at ≤ -80˚C. For thawing, proceed as follows:
        i. Thaw the sample in a range from 20˚C to 37˚C (ideally on a heat block),
        ii. Acquire [6] samples or continue with staining procedure without washing[7] step.

[4] E.g., for 100 µl, 1.0 ml of Fixation Buffer, and 4.0 ml of LYSIS buffer use **4.0 ml of the WASH buffer**.
[5] Set the centrifugation timing according to the liquid column height in the tube and rotor size. The timing should increase with shorter rotor diameter and/or more liquid (higher liquid column) per tube.
[6] Keep the volume of the CRYO#20 buffer unchanged or optimise it according to your flow cytometer specifications.
[7] Centrifugate the sample at 300-400g/≈5 min and remove some of the supernatant to optimize volume for staining with Ab-cocktail

Scaling ratios		
Blood	1 : 10	Fixation buffer
Blood	1 : 40	LYSIS buffer
Blood	1 : 40	WASH buffer
Blood	1 : 0.5 - 1	CRYO#20

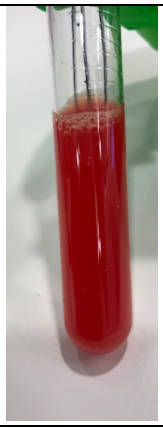
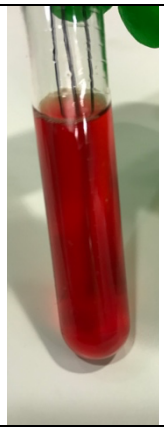
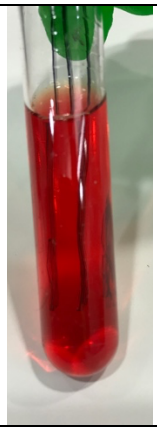
Examples of recommended processing conditions

Blood sample volume	**Fix** buffer volume	**LYSIS** buffer volume	**WASH** buffer volume	**Recommended processing tube size**		**CRYO#20** volume
				First wash	All consequent washes	
100 µl	1.0 ml	4.0 ml	4.0 ml	10 – 15 ml	5-15 ml	100 µl
250 µl	2.5 ml	10 ml	10 ml	15 ml	15 ml	100 µl
500 µl	5.0 ml	20.0 ml	20.0 ml †	50 ml *	15 ml	250 µl
1.0 ml	10.0 ml	39.0 ml	40.0 ml †	50 ml *	15 ml	500 µl

* To achieve optimal cell yields execute only first spin after Fix&Lyse step in 50 ml tube, aspirate to mark 5 ml or above (not below 5 ml mark), transfer to 15 ml tube and continue with altered WASH buffer volumes.

† In case you decide to run all consequent processing steps in 15 ml tube, use 14 ml of WASH buffer and adjust number of washing steps based on pellet color. Typically, only one more washing step is required.

Guide on decision of RBCs lysis step duration:

Time after addition of LYSIS buffer						
	0 min	5-6 min	10 min	15 min	20 min	35 min
	No	No	No	OK	OK	OK

Conditions: Fresh whole blood fixed for 15 min at RT followed by addition of LYSIS buffer for indicated time