P01 - Sample stabilisation

Preparation phase:

Aliquot cryogenic vials[1] with **Cytodelics Stabiliser equal the planned blood sample volume**. ***Store at 5–8˚C*** *until sampling (up to* ***2 years****). Identify dispensed volume on the label.*

Protocol:

1. Bring tubes pre-filled with the Stabiliser **to room temperature** (5 – 10 minutes).
2. Transfer equal **blood** volume to the cryogenic vial (RATIO 1:1).
3. **Mix** the blood sample with the Stabiliser **by flipping** the vial 10-15 times. **Do not** vortex!
4. Incubate at room temperature for 10 minutes.
5. Store the sample until further processing at:
    a. **≤ -80˚C** (up to 1 year);
    b. **≤ -20˚C** (up to 2 months), then transfer to the ultra-low freezer (**≤ -80˚C**) as soon as possible and keep there until further processing (up to 1 year);
    c. or place on **dry ice** and transfer to to the ultra-low freezer (**≤ -80˚C**) as soon as possible and keep there until further processing (up to 1 year).

[1] Choose cryogenic vial with nominal volume of approx. 3-times of the planned blood sample.

P01 - Processing after sample thawing

1. **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
   a. Fix-concentrate
   b. Fix-Diluent
2. **Dispense** the **Fixation Buffer** (10-times volume of preserved blood; do not count on the Stabiliser volume[2]) to tubes and let to equilibrate to room temperature (approx. 5 minutes).
3. **Thaw** blood samples preserved in the Stabiliser by quickly heating on water bath or heat block set to 37˚C (approx. 1 minute).
4. **Transfer** {blood+Stabiliser} mixture to the tube with the Fixation buffer, set the timer, and **incubate** for **15 minutes** at **room temperature**. Vortex multiple times throughout.
5. **Add** 1-time concentrated **LYSIS buffer** (40-times volume of the blood sample[3]), **mix** well by inverting tube multiple times, and **incubate** at room temperature for minimum of **15 to 20 minutes** until a complete erythrocytes' lysis.
6. **Centrifuge** at **300g for 5 to 10 minutes**[4].
7. **Aspirate the supernatant,** according to the size of the sample, down to**:**
   - 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. rinse twice with 2.0 ml of the **LYSIS buffer** and transfer to the 15mL tube,
     iii. centrifuge at 300g for 5 minutes,
     iv. aspirate the remaining volume down to approx. mark 0.5 ml.

[2] E.g., for 1.0 ml of blood preserved in 1.0 ml of Stabiliser use **10.0 ml of the Fixation Buffer**, i.e., 10-times volume of the blood.
[3] E.g., for 1.0 ml of blood and 10.0 ml of Fixation Buffer use **40.0 ml of the LYSIS buffer**.
[4] 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.

- approx. mark 0.5 ml in a 15mL tube (for samples with original volume up to 250µl blood processed in the 15mL tube from the beginning),
- "ring" on the 5mL "FACS" tube, leaving a minimum of 100 µl of supernatant over the pellet.

8. Break the pellet by vortexing and **add** 1-time concentrated **WASH buffer** (40-times the volume of the blood sample[5]).
9. **Centrifuge** at **300g for 5 to 10 minutes**[6].
10. **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!
11. Proceed with:
    a. sample staining and 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. Process according to your downstream application[7].

[5] E.g., for 1.0 ml of blood, 10.0 ml of Fixation Buffer, and 40ml of LYSIS buffer use **40.0 ml of the WASH buffer**.
[6] 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.
[7] You may stain without the washing step and acquire; however, we recommend to test compatibility of your staining panel. Before the staining, centrifugate the sample at 300-400g/≈5 min and remove some of the supernatant to optimize volume of the Ab-cocktail and antibodies consumption.

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

P03 - Fresh blood fixation

1. **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
   a. Fix-concentrate
   b. Fix-Diluent
2. **Dispense** the **Fixation Buffer** (10-times volume of preserved blood) to tubes and let it equilibrate to room temperature (approx. 5 min).
3. **Transfer** blood samples to the tube with the Fixation buffer, 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[1]), **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**[2].
6. **Aspirate the supernatant** down to**:**
   a. "ring" on the 5mL "FACS" tube leaving minimum 100 µl of supernatant over the pellet,
   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 the volume of the blood sample[3]).

[1] E.g., for 1.0 ml of blood and 10.0 ml of Fixation Buffer use **40.0 ml of the LYSIS buffer**.
[2] 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.
[3] E.g., for 1.0 ml of blood, 10.0 ml of Fixation Buffer, and 40ml of LYSIS buffer use **40.0 ml of the WASH buffer**.

8. **Centrifuge** at **300g for 5 to 10 minutes**[4].
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 [5] samples or continue with staining procedure without washing[6] step.

[4] 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.
[5] Keep the volume of the CRYO#20 buffer unchanged or optimise it according to your flow cytometer specifications.
[6] Centrifugate the sample at 300-400g/≈5 min and remove some of the supernatant to optimize volume for staining with Ab-cocktail.