Whole Blood Stimulation protocol

P07 - Stimulation experiment

1. **Collect** fresh **blood** into a **heparinised tube** and maintain the sample on a tube rotator until processing begins.
2. **Combine** the blood with **WB-STIM** buffer containing the necessary stimulation reagents at a **1:1 volume ratio**.
3. **Incubate** the mixture in a humidified incubator **at 37°C**.
4. After incubation, proceed with either:
    a. **P06 – Plasma collection and whole blood stabilisation**
    b. **P03 – Fresh blood fixation**
    OR
    c. the complete **P01 – Sample stabilisation/Processing after sample thawing** protocol

Notes:

- We recommend optimising experimental conditions for every new experimental scheme, even if it was established with a similar model, e.g., whole blood vs. PBMC samples.

Whole Blood Stimulation protocol

Stimulation experiment optimisation

1. **Collect** fresh **blood** into a **heparinised tube** and maintain the sample on a tube rotator until processing begins.
2. **Mix** the collected blood with **WB-STIM** buffer supplemented with the appropriate stimulation reagents at a **1:1 volume ratio**.
3. **Incubate** the mixture in a humidified incubator **at 37°C**.
4. At multiple time points within the first 24 hours, and subsequently daily, withdraw small aliquots from each experimental condition for viability analysis. **Evaluate viability** using an assay of your preference. For example:
    i. Fluorescence-based cell counter
    ii. Flow cytometry:
    a. No Lyse/No Wash
    b. Lyse/No Wash
    c. MACS-based RBC-depletion

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Recommendations:

- Avoid determining the optimal incubation time solely based on previously published data, particularly when those studies utilised different experimental models (e.g., PBMCs). Fresh whole blood may display markedly different responses, necessitating tailored optimisation.
- Always include unstimulated controls to distinguish between background activation and true stimulus-induced responses.
- Verify that the incubator is accurately calibrated for both temperature (37 ℃) and humidity, as fluctuations can influence cellular responses. **For short-term experiments lasting only a few minutes, the use of pre-warmed heat blocks or water baths may be preferable.**
- Implement a simple viability evaluation to optimise experimental conditions. For your further experiments, use only conditions with an insignificant impact on survival. Non-physiological experimental conditions can give misleading results.

- For long-term experiments, consider using a tube rotator or other gentle mixing method to maintain sample homogeneity.

Whole Blood Stimulation protocol

P03 – Fresh blood fixation

1. **Calculate** the required volume of the **Fixation Buffer** (total volume of blood processed within one batch multiplied by a factor of 10)[1] and **prepare** it by mixing equal volumes of
   a. Fix-concentrate
   b. Fix-Diluent
2. Choose one alternative according to the actual need:
   a. **Dispense** the **Fixation Buffer** (10-times the volume of preserved blood) to tubes and let it equilibrate to room temperature (approx. 5 min). **Transfer** blood samples to the tube/plate with the Fixation buffer and set the timer to 15 minutes.
   b. **Dispense** the **Fixation Buffer** (10 times the volume of preserved blood) over the blood samples, resuspend properly, and set the timer to 15 minutes.
3. **Incubate** for **15 minutes** at **room temperature**. Vortex (tubes) or shake (plates) multiple times throughout.
4. **Add** 1-times concentrated **LYSIS buffer** (40-times volume of the blood sample[2]), **mix** well by:
   a. inverting the tube multiple times,
   b. resuspending the samples in the plate multiple times, and **incubate** at room temperature for a minimum of 15 to 20 minutes until complete erythrocyte' lysis (please see the guide below).
5. **Centrifuge** at **300g for 5 to 10 minutes**[3].
6. **Aspirate the supernatant** down to**:**
   a. the rim at the top of the "V" in the V-shaped bottom plate.
   b. "ring" on the 5mL "FACS" tube, leaving a minimum of 100 µl of supernatant over the pellet,

[1] Recalculated based on the volume of the blood sample; do not count the volume of the WB-STIM buffer (or another buffer) unless it exceeds the volume of blood 3 times.
[2] E.g., for 1.0 ml of blood and 10.0 ml of Fixation Buffer use **40.0 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.

c. 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),
d. approx. mark 5.0-7.5 ml in a 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 or plate shaking and **add** 1-time concentrated **WASH buffer** (40 times the volume of the blood sample[4]).
8. **Centrifuge** at **300g for 5 to 10 minutes**[5].
9. **Gently aspirate** the supernatant down to:
    a. the rim at the top of the "V" in the V-shaped bottom plate.
    b. approx. mark 0.25 ml (15mL tube)
    c. ring" on the 5mL "FACS" tube, leaving approx. 100 µl of supernatant over the pellet.

    Do not decant!
10. Proceed with:
    a. **Staining** and **acquisition (step 11)**[6]

    OR

    b. **Cryogenic preservation** in CRYO#20 buffer at ≤ -80℃. For thawing, proceed as follows:
        i. Thaw the sample in a range from 20℃ to 37℃ (ideally on a heat block),
        ii. Acquire[7] samples or continue with the staining procedure (step 11) without the washing step.

[4] 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**.
[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] Permeabilisation step addition should be considered in the optimisation phase of each assay.
[7] Keep the volume of the CRYO#20 buffer unchanged or optimise it according to your flow cytometer specifications.

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Permeabilisation, staining and acquisition

11. Prepare blocking buffer[8], add it to cells, and incubate for 10-15 minutes.
12. Spin 300-400g/5-10 min.
13. Aspirate supernatant without disturbing cell pellets.
14. Break cell pellets by vortexing/plate shaking.
15. Add Ab-cocktail[9] and incubate for:
    a. 30-45 min at RT
    b. Overnight in the fridge at 5-8 ℃.
16. Add blocking buffer, vortex and spin 300-400g/5-10 min.
17. Aspirate supernatant without disturbing cell pellets.
18. Break cell pellets by vortexing/plate shaking.
19. Repeat washing with blocking buffer and spin 300-400g/5-10 min.
20. Aspirate supernatant without disturbing cell pellets.
21. Break cell pellets by vortexing/plate shaking.
22. Acquire.

Recommendations:

- Overnight staining might dramatically increase antibody binding efficiency. Maintaining the optimal "signal-to-noise" ratio might require titration toward significantly lower antibody volumes.
- Each antibody should be titrated separately. New batches or products from new vendors should be evaluated carefully.
- Please consider involving extremely low titration points to ensure optimal testing conditions. Some antibodies might work optimally in a range from as low as 0.001 to 0.1 µl antibody per 1 million cells.

[8] BD Horizon™ Brilliant Stain Buffer can be added when Brilliant Violet dyes are used and the antibodies are added directly to the samples.
[9] BD Horizon™ Brilliant Stain Buffer should be added when Brilliant Violet dyes are used, and the Ab-cocktail is prepared in bulk.

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P06 – Plasma collection and whole blood stabilisation

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Protocol:

1. Centrifuge a whole blood sample in the original collection tube at 1.000 – 2.000g/10-15 minutes.

   Note: Meanwhile, bring tubes pre-filled with the Stabiliser **to room temperature** (5 – 10 minutes).

2. Collect separated plasma without disturbing the separated white blood cells and erythrocytes (approx. 50-75% of the column) and aliquot it according to your downstream application requirements.

   Note: If you suspect your plasma samples are contaminated with cells, transfer them to a fresh tube and repeat the centrifugation. Separate and aliquot plasma without disturbing the pellet.

3. Replenish the volume of plasma removed from the tube with blood with PBS and gently resuspend.
4. Transfer equal blood volume to the cryogenic vial containing the Stabiliser reagent (RATIO 1:1).
5. **Mix** the blood sample with the Stabiliser **by flipping** the vial 10-15 times. **Do not** vortex!
6. Incubate at room temperature for 10 minutes.
7. Store the sample until further processing at:
   a. **≤ -80˚C** (long-term storage; whole blood stored for up to 2.5 years kept a stable immune profile in an independent study[1]);
   b. **≤ -20˚C** (short term, up to 3 weeks), then transfer to the ultra-low freezer (**≤ -80˚C**) as soon as possible and keep there until further processing;
   c. or place on **dry ice** and transfer to the ultra-low freezer (**≤ -80˚C**) as soon as possible and keep there until further processing.

[1] *Parthasarathy V. et al., Cytometry Part A (2026), doi: 10.1002/cyto.a.70059.*

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Note: After thawing, follow the instructions from P01 protocol:
"P01 - Processing after sample thawing."

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

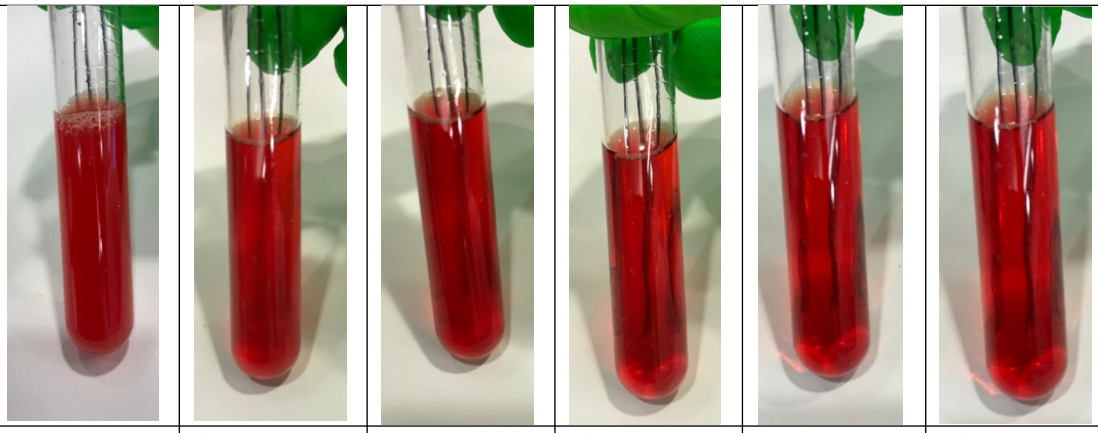
Blood sample volume	**Fix** buffer volume	**LYSIS** buffer volume	**WASH** buffer volume	**Recommended processing tube size**		**CRYO#20** volume
				First wash	All consequent washes	
100 µl	1.0 ml	4.0 ml	4.0 ml	10 – 15 ml	5-15 ml	100 µl
250 µl	2.5 ml	10 ml	10 ml	15 ml	15 ml	100 µl
500 µl	5.0 ml	20.0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

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