

FLOW CYTOMETRY PROTOCOL FOR INTRACELLULAR TARGETS USING DETERGENTS

The following flow cytometry protocol for staining intracellular molecules using detergents to permeabilize cell membranes has been developed and optimized by Bio-Techne. For best results, use 1×10^6 cells per 100 μ L of sample. Individual experimental designs for flow cytometry must be optimized, including antibody dilution and incubation time. For low cell density or poorly expressed intracellular targets, techniques like Single-Cell Westerns may be advantageous. [Learn more about Milo™ here.](#) Please read the protocol in its entirety before starting.

Materials

- 1 X PBS (0.137 M NaCl, 0.05 M NaH₂PO₄, pH 7.4)
- Flow Cytometry [Fixation Buffer](#) (R&D Systems, Cat No. FC-004) or an equivalent solution containing 1 - 4% paraformaldehyde
- Flow Cytometry [Permeabilization/Wash Buffer I](#) (R&D Systems, Cat No. FC-005) or an equivalent solution containing a permeabilization agent such as Saponin, Triton® X-100 or Tween® 20 (0.1-0.5% in your wash buffer).
- Flow Cytometry [Staining Buffer](#) (R&D Systems, Cat No. FC-001) or an equivalent solution containing BSA and sodium azide
- Fc Receptor Blocking Reagents (These include Fc receptor blocking antibodies or IgG solutions)
- [Primary Antibodies](#)
- [Isotype Control Antibodies](#)
- **Optional:** [Secondary Antibodies](#)
- **Recommended viability dye:** [DAPI](#), Novus Biologicals, Cat No. NBP2-31156; [Propidium Iodide](#), Novus Biologicals, Cat No. NBP2-31155; or [7-AAD](#), Novus Biologicals, Cat No. NBP2-294 46
- Trypan Blue
- FACS Tubes (5 mL round-bottom polystyrene tubes)
- Pipettes with appropriate tips
- Centrifuge
- Vortex

Sample Preparation

Sample Type	Suggestions
Cells in Suspension	After collecting suspended cells, add cold PBS to remove residual growth factors from media. After washing media remnants, use cells suspended in PBS and proceed with washing in Step 2.
Adherent Cells	After removing media from adherent cells, add cold PBS to remove residual growth factors from cell culture media. <ul style="list-style-type: none"> • Harvest cells with a 1% BSA solution in PBS and then proceed with washing in Step 2. • Adherent cell lines may require 0.5 mM EDTA to facilitate removal and then washed according to Step 2. <i>Exposure time with EDTA should be minimal.</i>
Tissue	To prepare tissues for flow cytometry, mechanical and/or enzymatic disaggregation is required. <ul style="list-style-type: none"> • First, mince the tissue into small sections that expose the cells and suspend in PBS. <i>Enzymatic digestion may be required after mincing the tissue, but digestion buffer will be tissue type dependent.</i> • Next, pass the minced tissue suspension through a fine gauge needle several times until all cells are fully in suspension. <i>If you experience resistance, exchange needle with a larger gauge to dissociate cells first.</i>

Methods

1. Harvest your cells (see Sample Preparation for guidance).
2. Add 2 mL of PBS with a pipette to wash cells. Centrifuge at 1,300 RPM (500 x g maximum) and 4 °C for 5 minutes, decanting the supernatant. Wash 3 times.
3. Using a small aliquot, count cells in a 1:1 Trypan Blue exclusion stain using a hemocytometer before starting.
TIP: Aliquot up to 1×10^6 cells per 100 μ L in Flow Cytometry Staining Buffer if performing **surface staining**. Cell viability dyes must be introduced before fixation. If only nuclear staining is performed, proceed with cell fixation.
4. Add 500 μ L of cold Fixation Buffer into FACS tubes required for your experiment. Aliquot up to 1×10^6 cells/100 μ L and vortex to mix. Incubate the cells at room temperature for 10 minutes. Gently vortex intermittently to maintain a single cell suspension. *A separate set of cells should be stained as a negative control.*
5. Centrifuge cells at 1,300 RPM and 4 °C. Decant the Fixation Buffer.
6. Wash cells ONCE using 2 mL of PBS. Centrifuge at 1,300 RPM (500 x g maximum) and 4 °C for 5 minutes. Decant the supernatant.
7. Resuspend each cell pellet in 150 μ L of Flow Cytometry Permeabilization/Wash Buffer I. Add 1 μ g blocking IgG per 1×10^6 cells and let stand for 15 minutes at room temperature. *Do NOT wash excess blocking IgG from this reaction. It is important to keep the cells in the presence of Permeabilization/Wash Buffer I during intracellular staining.*
8. Add 5-10 μ L of conjugated antibody (or a previously titrated amount) per 1×10^6 cells and vortex. *For unconjugated antibodies, be sure to check the data sheet for any appropriate concentrations validated for use in flow cytometry. 1 μ L of primary antibody per 1×10^6 cells is a good starting point.* Incubate cells for 30 minutes at room temperature in the dark.
9. Wash cells ONCE with 2mL of Flow Cytometry Permeabilization/Wash Buffer I. Centrifuge suspension at 1,300 RPM (500 x g maximum) and 4 °C for 5 minutes.
TIP: If an unconjugated primary antibody was used, incubation with an appropriate **secondary antibody** is required. After washing cells to remove the primary antibody, resuspend the cell pellet in 150 μ L of Flow Cytometry Permeabilization/Wash Buffer I. Add the recommended volume of secondary antibody and incubate for 30 minutes protected from light. Gently vortex intermittently to maintain a single cell suspension. Wash cells ONCE using Flow Cytometry Permeabilization/Wash Buffer I instead of PBS.
10. Resuspend the cell pellet in 200 – 400 μ L of Flow Cytometry Staining Buffer for flow cytometry analysis.