

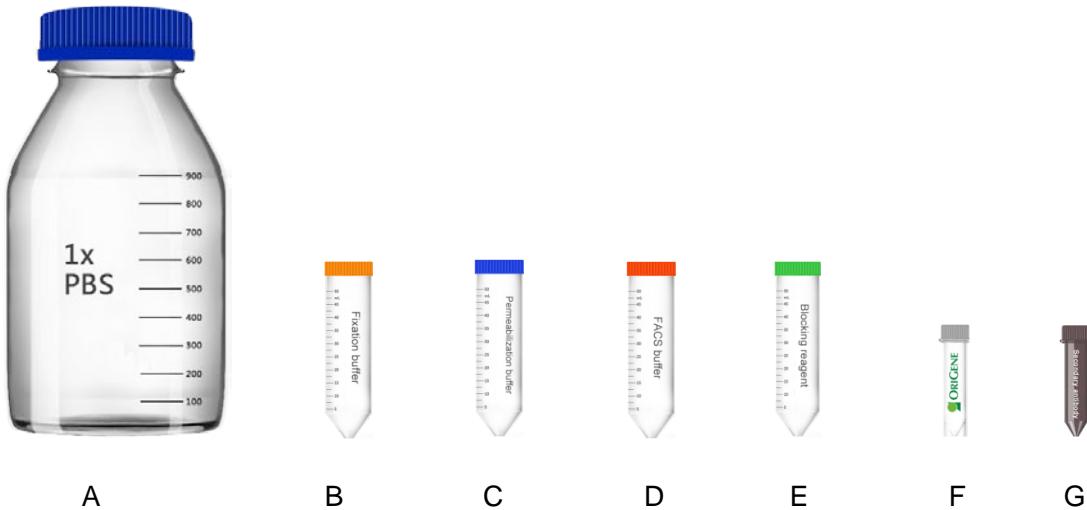
## Flow Cytometry Protocol

Solutions and reagents

Protocol

Troubleshooting

**Solutions and reagents**

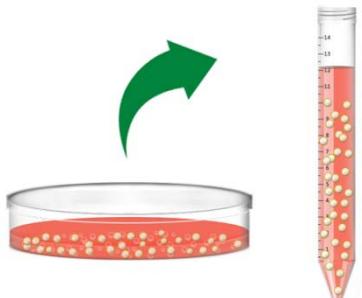


- A. 1X Phosphate Buffered Saline (PBS): Dissolve 8 g NaCl, 0.2 g KCl, 1.15 g Na<sub>2</sub>HPO<sub>4</sub> and 0.2 g KH<sub>2</sub>PO<sub>4</sub> in 800 mL distilled water (dH<sub>2</sub>O). Adjust the pH to 7.4 and the final volume to 1 liter.
- B. Fixation buffer: 2 % paraformaldehyde in 1x PBS
- C. Permeabilization buffer : 0.1 % Triton X-100 in 1xPBS
- D. FACS buffer: 0.5 % BSA and 0.05 % Sodium Azide(NaN<sub>3</sub>) in 1x PBS
- E. Blocking reagent: 0.5 % BSA and 2 % normal fetal bovine serum in 1X PBS
- F. Primary antibody
- G. Fluorescent dye conjugated secondary antibody

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## Protocol

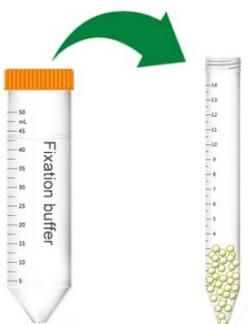
### A. Prepare samples



1. Detach cells from culture dish or flask (5 mM EDTA in 1X PBS without calcium and magnesium if cells are attached).
2. Collect cells by centrifugation and aspirate supernatant.
3. Wash the cells 2 X with 1X PBS at 4°C.

(Note: Skip step B-C if you test live cells)

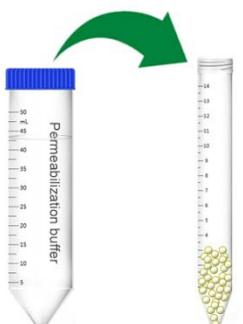
### B. Fixation



4. Centrifuge for 5 min at 300 g, discard the supernatant, and then apply cold fixation buffer to the cells, vortex briefly.
5. Incubate at room temperature for at least 30 min or overnight at 4°C.
6. Centrifuge for 5 min at 300 g, remove the supernatant.
7. Wash the cells 3 X with 1X PBS at 4°C.

(Note: Skip step C if the target is extracellular)

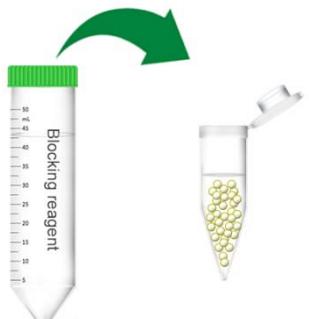
### C. Permeabilization



8. Re-suspend cells with permeabilization buffer and incubate for 10-20 min at room temperature.
9. Centrifuge briefly and aspirate supernatant, then wash with 1X PBS 3 X.

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#### D. Staining



10. Aliquot  $1-2 \times 10^6$  cells into each tube.
11. Add 1 mL blocking buffer to each tube, vortex briefly and incubate on ice for 30 min.

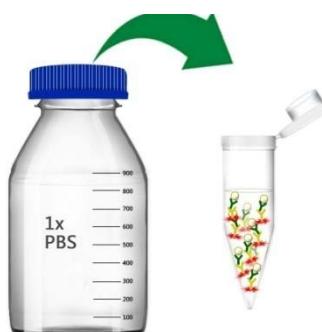


12. Centrifuge briefly and aspirate supernatant, resuspend cell pellet with 125  $\mu$ L FACS buffer containing diluted primary antibody per manufacturer's recommendations, vortex and incubate on ice for 30 min.
13. Rinse the cells 3 X in FACS buffer by centrifugation.

**(Note: Skip step 14-16 if using primary antibody directly conjugated to fluorescent dyes)**



14. Resuspend cells in fluorescent dye conjugated secondary antibody, diluted in FACS buffer per manufacturer's recommendations.
15. Incubate for 30 min on ice in the dark.
16. Rinse the cells 3 X in FACS buffer by centrifugation with minimum light exposure.



17. Resuspend cells in 0.5 mL 1X PBS in the dark.

#### E. Analyze with a flow cytometer.

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## Troubleshooting

[No signal / weak fluorescence intensity](#)

[High fluorescence intensity](#)

[Low event rate](#)

[High event rate](#)

### **No signal / weak fluorescence intensity**

#### **1. Intracellular target not accessible**

>Check if target protein is intracellular. Extend permeabilization incubation time to 20-30 min. Make sure to have the permeabilization on ice with cold reagents in order to stop all reactions.

#### **2. Insufficient antibody present for detection**

>Increase amount/concentration of antibody.

#### **3. Intracellular staining-fluorochromeconjugates too large**

>Fluorochromes for intracellular staining should have low molecular weight. Fluorochromes with large molecular weight can reduce antibody motility and possibly halt its entry into the cell.

#### **4. Primary and secondary are not compatible**

>Secondary antibody should be raised against the host species of the primary antibody.

#### **5. Target protein not present or expressed at low level**

>Ensure tissue or cell type expresses target protein and that it is present high enough to be detected.

### **High fluorescence intensity**

#### **6. Antibody concentration too high**

> Reduce the amount of antibody added to each sample. Too much antibody will result in high non-specific binding.

#### **7. Excess antibody trapped**

>This can be a particular problem for intracellular staining where large fluorochrome molecules on the antibody can be trapped. Ensure adequate washing steps and may include Tween-20 or Triton X-100 in washing buffers.

#### **8. Inadequate blocking**

>Dilute antibodies in blocking solution.

### **Low event rate**

#### **9. Low number of cells**

>Optimal cell concentration for each sample should be around  $1 \times 10^6$  cells/mL. Ensure cells are gently mixed. Adjust the flow rate if applicable.

#### **10. Cells clumped/blocking tubing**

>Pipet the samples gently before staining and again before running the cytometer.

### **High event rate**

#### **11. High number of cells**

>Dilute sample to  $1 \times 10^6$  cells/mL. Adjust the flow rate if applicable.

#### **12. Contamination**

>Repeat the staining procedures.

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