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## Flow Cytometry ICS Nuclear Antigens V.3

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**We use this protocol and it's working**

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## Abstract

High-parameter flow cytometry enables identification and characterization of a wide range of cell populations within a biological sample. A combined analysis of extracellular epitope staining (ECS) and intra-cellular epitope staining (ICS) using a collection of fluorophore-labeled antibodies sufficiently identifies discrete cell populations and their respective phenotypes. Importantly, ECS/ICS can be applied to cryo-preserved cell suspensions recovered in tissue culture media, enabling samples to be conveniently analyzed after collection and proper cryopreservation. However, consistent cryopreserved sample recovery and ECS procedures are critical to data comparison across multiple experiments. Herein, we describe a standardized protocol for cryopreserved sample recovery and ECS/ICS procedures for cell-surface and intra-cellular epitope labeling.




## Materials


### Materials Required

1.  1x phosphate saline buffer (PBS) **Corning Catalog #21-031-CM**
2.  Bovine serum albumin (BSA) **Gemini Bioproducts Catalog #700-101P**
3.  Sodium Azide **Fisher Scientific Catalog #S2271-500**
4. FACS buffer (1xPBS, 10g/L BSA, 1 g/L sodium azide)
5.  RPMI **Corning Catalog #10-040-CM**
6.  Fetal calf serum (FCS) **Gemini Bioproducts Catalog #900-108**
7.  Penicillin/streptomycin 10000 U/mL penicillin 10000 µg/mL streptomycin **Lonza Catalog #17-602E**  
Lonza ([RRID:SCR\\_000377](https://pubchem.ncbi.nlm.nih.gov/compound/SCR_000377))
8. "R10" medium (RPMI, 10% FCS, 1% penicillin/streptomycin)
9.  DNase I **Roche Catalog #04716728001**
10.  Live/Dead fixable Aqua Dead Cell Stain kit **Invitrogen - Thermo Fisher Catalog #L34966**  
diluted 1:60 in 1x PBS (prepare fresh each day from DMSO stock)
11.  
 e-Bioscience Foxp3 / Transcription Factor Staining Buffer Set **Invitrogen - Thermo Fisher Catalog # 00-5523-00**
12.  Paraformaldehyde (PFA) **Electron Microscopy Sciences Catalog #15712-S** diluted to 1% in PBS
13. Fluorophore-labelled antibodies of choice
14. Hemocytometer

## Procedure


### 1 Thawing and Resting


a. Pre-warm R10 media in a  37 °C water bath.





b. Thaw samples in-vial using a  37 °C water bath.

c. Add thawed cells to  14 mL of R10, then spin cells at 500 xg for 5 min.


d. Resuspend cell pellet in  3 mL of R10 and count cells.

e. Rest cells at least 3 hours (up to overnight) at  $2 \times 10^6$  cells/mL in R10 medium + 1  $\mu$ L/mL DNase I at  37 °C , 5% CO<sub>2</sub>.

**Note:** during resting, prepare antibody cocktail master mix. Adjust volume of ECS for  50  $\mu$ L per test with FACS buffer.

f. After resting, add PBS up to  15 mL or  50 mL (whichever is closer, rounding up) to cells and transfer to  15 mL or  50 mL conical tube.


g. Spin cells at 500 xg for 5 minutes at room temperature (RT).

h. Resuspend cells in PBS to  $1 \times 10^7$  cells/mL and count. If cells are too dilute, re-spin cells and resuspend at  $1 \times 10^7$  cells/mL. Transfer  200 mL of cells ( $2 \times 10^6$  cells) into each well of a V-bottom 96 well plate.

### 2 Viability and extracellular staining (ECS)



a. Spin plate at 500 xg for 5 minutes at RT

b. Using a multichannel pipette, carefully remove the supernatant.







c. Add  5  $\mu$ L of 1:60 Aqua viability dye directly to cell pellet and resuspend cells.

d. Incubate for 10 minutes at RT in the dark.



- e. Add  50  $\mu\text{L}$  of ECS antibody cocktail to cells and incubate for 20 minutes at RT in the dark (prepare 1% PFA fixation buffer in the meantime).
- f. Add  100  $\mu\text{L}$  of FACS buffer to each well and spin plate at 500 xg for 5 minutes. Remove supernatant.

### 3 Fixation, Permeabilization, and ICS Staining

- a. Fix cells with  100  $\mu\text{L}$  of 1xFixation/permeabilization buffer (1 part concentrate + 3 parts diluent) for 30 minutes at RT in the dark.  
**Note:** Make ICS abs here
- b. Add  100  $\mu\text{L}$  of 1x perm/wash buffer and centrifuge 800 xg for 5 min.
- c. Remove supernatant and add  100  $\mu\text{L}$  ICS cocktail (made in perm/wash buffer, made with  $\text{dH}_2\text{O}$ ) to the cells.
- d. Incubate for 1h at RT in the dark.
- e. Add  100  $\mu\text{L}$  perm/wash buffer. Centrifuge at 800 xg for 5 min.
- f. Discard supernatant and resuspend pellet in  200  $\mu\text{L}$  1% PFA and transfer to FACS tubes.
- g. Wrap in aluminum foil and store at  4  $^{\circ}\text{C}$  until flow cytometry.