Direct Immunofluorescence Staining of Intracellular Antigens: Methanol plus Leucoperm Accessory Reagent Method

Protocol

FC8

Alternative protocol for cell permeabilization required prior to intracellular staining.

The detection of intracellular antigens requires a cell permeabilization step prior to staining. This method provides an alternative procedure for use when protocol FC7 "Direct Staining of Intracellular Antigens by flow cytometry using Leucoperm" does not provide the desired results. This method is particularly suitable for the detection of nuclear antigens, such as PCNA and Ki67. In some cases specific recommendations are provided on product datasheets, and these methods should always be used in conjunction with the product and batch specific information provided with each vial. A certain level of technical skill and immunological knowledge is required for the successful design and implementation of these techniques; these are guidelines only and may need to be adjusted for particular applications. Specific methodology for blood appears in [ ] brackets.

Reagents:

- Anticoagulant (**Note:** for basic staining any appropriate anticoagulant, such as heparin, EDTA, or acid citrate dextrose, may be used. In some instances specific anticoagulants may be required)
- Erythrolyse Red Blood Cell Lysing Buffer (#BUF04)
- Leucoperm Accessory Reagent (Cat. #BUF09). Includes Reagent A (cell fixation agent) and Reagent B (cell permeabilization agent)
- PBS
- Phosphate Buffered Saline (#BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- Optional: 0.5% (w/v) paraformaldehyde in PBS (**Note:** dissolve on heated stirrer and cool before use)

Method:

1. Harvest cells and determine the total number present. Adjust cell suspension to a concentration of 1 x 10^7 cells/ml with cold (4°C) PBS/BSA.
   [Whole blood samples may be used undiluted unless the cell count is high, as in leukemia samples. Use appropriate anticoagulant.]
2. Add 100 µl of cell suspension [or *whole blood*] to the appropriate number of test tubes.
3. If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at 4°C, avoiding direct light.
4. Wash cells once in 2 ml cold (4°C) PBS/BSA and discard the supernatant.
5. Resuspend cells in 100 µl cold (2-8°C) Leucoperm Reagent A (cell fixation agent) per 1 x 10^6 cells. Incubate for 10 min at 2-8°C.
6. Add 500 µl of ice cold absolute methanol, vortex and incubate for 10 min at 2-8°C.
7. Add 3 ml cold (4°C) PBS and centrifuge for 5 min at 300-400 g at 4°C.
8. Remove supernatant and add 100 µl Leucoperm Reagent B (cell permeabilization agent) per 1 x 10^6 cells. Add the directly conjugated antibody at the vendor-recommended dilution and incubate at 4°C for at least 30 min, avoiding direct light.
   [To the blood suspension add 2 ml freshly prepared erythrolyse red cell lysing buffer and mix well. Incubate for 10 min at room temperature. Centrifuge at 300-400 g for 5 min and discard the supernatant. Wash with 2 ml room temperature PBS/BSA, centrifuge at 300-400 g for 5 min at room temperature and discard the supernatant. Continue to step 9.]
9. Resuspend cells in 2 ml cold (4°C) PBS and centrifuge at 300-400 g at 4°C. Discard supernatant.
10. Resuspend cells in 200 µl cold (4°C) PBS for immediate analysis or with 200 µl 0.5% formaldehyde in PBS if required.
11. Acquire data by flow cytometry. Analyze fixed cells within 24 hr.

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Notes:

- Phycoerythrin conjugates are not suitable for the detection of cell surface antigens using this method due to damage of the RPE at low temperatures.
- To avoid unspecific binding, you also need to block Fc receptors on cell types such as spleen cells, with FcR blocking reagents e.g. Bio-Rad's Mouse Seroblock Reagent (#BUF041).
- Appropriate controls should always be carried out, for flow cytometry the following should be considered for inclusion;
  - Isotype controls used to determine if the staining is specific
  - Unstained cells should always be included in the experimental set-up to monitor autofluorescence
- For all multicolor flow cytometry experiments it is advisable to include compensation controls and Fluorescence Minus One (FMO) controls, which assist with identifying gating boundaries.

Visit **bio-rad-antibodies.com/applications** for more information about flow cytometry.

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