Direct Immunofluorescence Staining of Intracellular Cytokines in Blood

Protocol

FC9

For the staining of intracellular antigens in whole blood using directly conjugated antibodies.

This is a rapid and simple approach to the analysis of intracellular cytokines in whole blood. It permits the analysis of small samples and avoids generating artefacts due to the separation of peripheral blood cells by density gradient centrifugation. All blood samples must be collected into heparin anticoagulant. EDTA interferes with the cell stimulation process and therefore must be avoided.

The detection of intracellular antigens requires a cell permeabilization step prior to staining. The method described below has been found to provide excellent results in our hands; however other permeabilization techniques have been published and may also be successfully used in this application. This method provides a general procedure for use with the majority of Bio-Rad reagents. 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.

Note: Resting cells often require stimulation in vitro prior to the detection of intracellular cytokines.

Reagents:

- Leucoperm™ Accessory Reagent (BUF09). Includes Reagent A (cell fixation agent) and Reagent B (cell permeabilization agent)
- Phosphate buffered saline (BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- Erythrolyse red blood cell lysing buffer (BUF04)
- Cell culture medium
- Monensin
- Ionomycin
- PMA
- Optional: 0.5% (w/v) paraformaldehyde in PBS (**Note:** dissolve on heated stirrer and cool before use)

Method:

1. Aliquot 500 µl of blood into as many tubes as required, including 2 extra control tubes, then add 500 µl of cell culture medium (without any additives) to each sample.
2. To one tube (the resting population), add monensin to a final concentration of 3 µM.
3. To another tube (activated cells), add PMA to a final concentration of 10 ng/ml, ionomycin (2 µM) and monensin (3 µM).
4. To the rest of the tubes (experimental samples) add monensin (3 µM) and treat as required by the experiment.
5. Incubate for 2-4 hours at 37°C in a 5% CO_2 atmosphere.
6. If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at 4°C, avoiding direct light.
7. Wash cells once with PBS/BSA and discard supernatant.
8. Add 100 µl of Leucoperm Reagent A (cell fixation agent) and incubate for 10 minutes at 2-8°C.
9. Add 2 ml cold (4°C) PBS/BSA and centrifuge for 5 minutes at 300-400 g at room temperature.
10. 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 for at least 30 minutes at 4°C, avoiding direct light.
11. Add 2 ml freshly prepared erythrolyse red blood cell lysing buffer to the blood suspension and mix well.
12. Incubate for 10 minutes at room temperature.
13. Centrifuge at 300-400 g for 5 minutes and discard the supernatant.
14. Wash once in PBS/BSA, and then resuspend in 200 µl PBS for immediate analysis or with 200 µl of 0.5% formaldehyde in PBS if required.
15. Acquire data by flow cytometry. Analyze fixed cells within 24 hours.

Reference

Sewell WA et al. (1997). Determination of intracellular cytokines by flow cytometry following whole-blood culture.
J Imm Meths. 209: 67-74

For more information on flow cytometry resources visit **bio-rad-antibodies.com/applications**

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