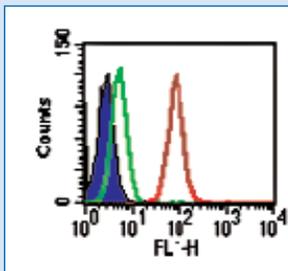


Intracellular Staining Flow Protocol

Necessary Reagents

KC-126	Fixation buffer, 1X	1 X 60 ml
KC-127	Permeabilization buffer, 10X	2 X 60 ml
KC-124	Staining buffer, 1X	2 X 60 ml
KC-128	Brefeldin A, 1000X	1 X 100 µl



Flow cytometric analysis of intracellular TLR9 (IMG-305A) in Ramos cells using IC-Flow kit to fix and permeabilize cells (**Blue**: Unstained Ramos cells, **Green**: Isotype control at 2µg /10⁶ Ramos cells, **Red**: Anti-TLR9 at 2µg /10⁶ Ramos cells).

Background information

Fixation buffer is used to “fix” the cells/cellular proteins prior to permeabilization.

Permeabilization buffer permeabilizes the cell membrane, allowing the detecting antibodies access to intracellular proteins. It is supplied as a 10X solution and can be diluted with deionized water to its final 1X working concentration.

Brefeldin A is an inhibitor of intracellular protein transport. It blocks translocation of proteins from the endoplasmic reticulum to the Golgi apparatus and can enhance detection of some intracellular proteins. Researchers are encouraged to determine whether or not Brefeldin A is needed to assay for the protein of interest. Brefeldin A is light-sensitive, store at 4°C in the dark. It should be diluted in culture media (1:1000) just prior to use. Add to cell culture and incubate for 4hr before harvesting cells.

- Determine the number of cells required for staining. For each test sample, a final concentration of 1 x 10⁶ cells in 50 µl of staining buffer will be needed. (The following controls are suggested: **a**) unstained cells [no primary or secondary antibody staining], **b**) cells with an isotype control antibody, **c**) cells with a positive control antibody.
 - Harvest the cells and spin down to a pellet at 1000 RPM for 10 minutes; decant supernatant.
 - Depending on the size of the pellet, resuspend in 2-3 ml of 1X PBS. An exact volume is not necessary at this step.
 - Count the cells with a hemocytometer. Remove 1 x 10⁶ cells for each sample (including controls) to be tested to a clean conical centrifuge tube. Add 1 ml of 1X PBS to make the decanting easier.
 - Centrifuge cells at 1000 RPM for 10 minutes and decant supernatant.
 - Tap the conical tube gently to loosen the pellet.
 - Resuspend pellet with the appropriate volume (50 µl per 1 x 10⁶ cells) of Fixation buffer. Incubate at room temperature for 30 minutes.
 - Centrifuge cells at 1000 RPM for 10 minutes and decant supernatant.
 - Resuspend pellet with the appropriate volume (50 µl per 1 x 10⁶ cells) of 1X Permeabilization buffer.
 - Dispense 1x10⁶ cells (50 µl) to the desired number of flow cytometer compatible test tubes and centrifuge cells at 1000 RPM for 10 minutes. Carefully aspirate the supernatant. During centrifugation, the primary antibodies to be used can be diluted to the required concentration in 50 µl of Permeabilization buffer.
 - Resuspend each cell pellet with the appropriate primary antibody. Pipette up and down to thoroughly mix the antibody/cell suspension.
 - Incubate at room temperature for 30 minutes (protect from light if using a fluorescent-labeled primary antibody).
 - Centrifuge at 1000 RPM for 10 minutes and carefully aspirate supernatant.
- Note:** If using a fluorescent-labeled primary antibody, skip Steps 14-17.
- Wash the cells by resuspending each cell pellet with 2 ml of Permeabilization buffer, centrifuge at 1000 RPM for 10 minutes, and decant supernatant. While centrifuging, dilute secondary antibody (FITC, PE or Biotin labeled) in 50 µl of Permeabilization buffer per sample.
 - Resuspend cells in diluted secondary antibody.
 - Incubate at room temperature (protected from light) for 30 minutes.
 - Centrifuge at 1000 RPM for 10 minutes and carefully aspirate supernatant.
 - Wash cells twice with 2 ml of Permeabilization buffer, centrifuging and decanting after each wash step.
 - After the final decanting, add 1 ml of Staining buffer to each tube.
- Note:** If not analyzing on the same day, samples can be stored overnight, in the dark, at 4°C.
- Test samples on a flow cytometer following manufacturer recommendations.

Caution Fixation buffer contains paraformaldehyde which is toxic by inhalation, skin contact, or swallowing. Permeabilization and staining buffers contain 0.05 % sodium azide. Use caution when handling. All the materials included in this kit should be treated as hazardous materials and be disposed of accordingly.

>> For Intracellular Flow Kit please see Cat. No. 10083K