University Research Facility in Life Sciences
The Hong Kong Polytechnic University

Antibody staining protocol

Reagents and equipment required

1. Falcon 5 mL tubes
2. Micropipettor with tips
3. Antibodies
4. Centrifuge
5. FACS Staining Buffer
6. Brilliant Staining Buffer Plus
7. BD Cytofix™ Solution

Additional items needed for the intracellular staining of transcription factors:

8. Transcription Factor Buffer Set

Procedures

For Surface Marker Staining:

1. Label each tube with the antibody/fluorochrome name and antibody titre level
2. Harvest, wash the cells, and then adjust the cell concentration to 1-5 × 10^6 cells/mL with sodium azide- and protein-free 1× DPBS.
3. Add 1 µl of BD Horizon™ Fixable Viability Stain 440UV Stock Solution for each 1 ml of cell suspension (1:1000) and vortex immediately. (Note: We recommend titrating the dye for optimal performance, as different cell types and different applications can result in a wide degree of variability in staining.)
4. Incubate the mixture for 10-15 minutes at room temperature protected from light (Optional: Alternatively, incubate mixtures at 37°C for 5-7 minutes or 2-8°C for 30-60 minutes).
5. Wash cells twice with 2 ml of Stain Buffer.
6. Decant the supernatant and gently mix to disrupt the cell pellet.
7. Resuspend the cells in 100 uL Stain Buffer.
8. Add 10 uL of brilliant staining buffer plus (BSB plus) to each tube
9. Preincubate cell suspension with Mouse BD Fc Block™ purified anti-mouse CD16/CD32 mAb 2.4G2 (eg, ≤ 1 µg/million cells in 100 µl) at 4˚C for 5 minutes. Skip this step if you don't have Fc Block, but highly recommend to use.

10. Add the antibody of interest in the correct titre volume into each tube, and then leave to stain at 4 °C in the dark for 30 minutes.
11. Add 2 mL of Stain Buffer into the tubes, and then centrifuge at 350 g for 3 minutes at 2-8 °C.
12. Decant the supernatant in one smooth motion, and then gently vortex the cells gently to break up the pellet quickly (2-3 seconds).
13. Repeat the washing steps 7-8 for 2 more times (wash a total of 3 times).
14. Resuspend the cells in a final volume of 500 uL Stain Buffer.
    Take note that usually after the decanting of the supernatant from the previous washing step, there will usually still be some residual stain buffer (~200-300 uL). Therefore, to make up to a final volume of 500 uL, usually only 200-300 uL of additional FACS Staining Buffer needs to be added at this point.
15. Vortex cells gently for 2-3 seconds. If fixation is not needed, you may take the tube for analysis and skip the below steps. Otherwise, proceed with below steps. Fixation after staining is recommended
16. Add 250 uL of BD Cytofix™ solution (or equivalent) to each tube and allow the cells to incubate in the fixation solution for 15 to 30 minutes at 4˚C protected from light.
17. Wash the cells twice using steps 7-8.
18. Resuspend the cells into a total volume of 500 uL with Stain Buffer.
    Take note that usually after the decanting of the supernatant from the previous washing step, there will usually still be some residual stain buffer (~200-300 uL). Therefore, to make up to a final volume of 500 uL, usually only 200-300 uL of additional Stain Buffer needs to be added at this point.
19. Vortex gently, and then you may take the cells for analysis. Stained and fixed cells should be analyzed as soon as possible.

For Intracellular Marker Staining with the Transcription Factor Buffer Kit (please see kit instructions for how to make the stock solution of the Fix/Perm and the Perm/Wash Buffer):

1. Label each tube with the antibody/fluorochrome name and antibody titre level
2. Harvest, wash the cells, and adjust the cell concentration to 1-5 × 10^6 cells/mL with Stain Buffer.
3. Broadcast 100 uL of the cell suspension into FALCON 5 mL tubes.
4. Preincubate cell suspension with Mouse BD Fc Block™ purified anti-mouse CD16/CD32 mAb 2.4G2 (eg, ⩽ 1 µg/million cells in 100 µl) at 4˚C for 5 minutes.
5. Wash cells twice with Stain Buffer.
6. Add 1 mL of freshly prepared 1x Fix/Perm Buffer working solution to each tube and resuspend cell pellets by vortexing for approximately 3 seconds. Incubate samples at 2-8°C for 40-50 minutes protected from light.
7. Perm/Wash: Add 1 ml of 1x Perm/Wash Buffer directly to the fixed and permeabilized cells suspended in the 1x Fix/Perm Buffer. Pellet the cells by centrifugation. (Note: All centrifugation steps post Fix/Perm are at 350g and at 2-8 °C for 6 minutes). Decant the supernatants.

8. Perm/Wash: Add 2 ml of 1x Perm/Wash Buffer to the pelleted cells followed by centrifugation. Decant or aspirate wash buffer.
9. Intracellular Staining: Add 100 µl of 1x Perm/Wash Buffer to cell samples, 10 uL of Brilliant Staining Buffer Plus and the fluorescent antibodies specific for intracellular proteins (eg, FoxP3, IL-17A). Vortex tube or rack for 10 seconds and incubate at 2-8°C for 40-50 minutes protected from light.
10. Perm/Wash: Briefly vortex samples prior to washing. Wash cells with 2 ml of 1x Perm/Wash Buffer. Centrifuge cells. Decant or aspirate the wash buffer.
11. Perm/Wash: Wash cells with 2 ml 1x Perm/Wash. Centrifuge cells. Decant or aspirate wash buffer.
12. Sample preparation for flow cytometry: Resuspend cell pellet in 500 µl of stain buffer. Analyze the cells and acquire data using a flow cytometer.