BD™ Phosflow Protocols for Human PBMCs

Protocol II and III (Mild or Harsh Alcohol Method)

Fix Buffer used:

BD Cytofix™ Buffer (Cat. No. 554655)

Perm Buffer used:

Depending on the surface markers and phospho antibody conjugates you are using, either BD™ Phosflow Perm Buffer II (Cat. No. 558052) or III (Cat. No. 558050) is recommended. Please refer to our phospho antibody and CD marker reference charts (pages 26–30) for details.

1. Prepare PBMCs from human blood.
2. Pre-warm the BD Cytofix™ Buffer in a 37°C water bath for 5-10 minutes before use.
3. (Optional) Culture PBMCs in RPMI with 5% human serum at 37°C in a CO_2 incubator for 2 hrs. (This step may help to improve the signal for some phospho proteins).
4. Treat cells with appropriate stimulators.

   Note: Methods of activation vary and should be determined by the researcher. Some examples tested in house are given on page 30. Visit **www.bdphosflow.com** for updates.
5. Fix cells immediately in order to maintain phosphorylation state. Rather than spinning down the cells, we recommend fixing the cells by adding an equal volume of pre-warmed BD Cytofix Buffer to the cell suspension.
6. Incubate cells at 37°C for 10 minutes.
7. (The fixed cells can be frozen in –80°C directly at this step for later usage. The cells should be thawed at 37°C, and washed immediately, to continue with the following procedure).
8. Pellet by centrifugation (300 × g) for 5–10 minutes and remove supernatant.
9. Vortex or mix to disrupt the pellet. Permeabilize the cells by adding 1ml of BD™ Phosflow Perm Buffer (for 1-10×10^6 cells) and incubating for 30 minutes on ice. Depending on the surface markers and phospho specific antibody conjugates you are using, either BD Phosflow Perm Buffer II (Cat. No. 558052) or III (Cat. No. 558050) is recommended. Please refer to our phospho antibody and CD marker reference charts (pages 26–30) for details.

   Note: Longer incubation times in this permeabilization buffer may decrease the signal intensity of surface marker staining.
10. Wash the cells twice with BD Pharmingen™ Stain Buffer (Cat. No. 554656). Centrifuge at 300 × g for 5-10 minutes and remove supernatant.

11. Resuspend the cells in BD Pharmingen™ Stain Buffer at 1×10^7 cells/ml
12. Aliquot optimal concentration of fluorochrome-conjugated antibodies to each tube, and add 100µl (1×10^6) of fixed and permeabilized cells.
13. Incubate at room temperature for 30 minutes in the dark.
14. Wash once with 2ml of BD Pharmingen™ Stain Buffer. Centrifuge at 300 × g for 5-10 minutes and remove supernatant. Resuspend the cells in 500µl of the same buffer prior to flow cytometric analysis.

BD™ Phosflow Protocols for CD3/CD28 Activation of Human PBMCs

Protocol I (Detergent Method)

Fix Buffer used:

BD™ Phosflow Fix Buffer I (Cat. No. 557870)

Perm Buffer used:

BD™ Phosflow Perm/Wash Buffer I (Cat. No. 557885)

1. Prepare PBMCs from human blood.
2. Pre-warm the BD™ Phosflow Fix Buffer I in a 37°C water bath for 5-10 minutes before use.
3. (Optional) Culture the PBMCs in RPMI with 5% human serum at 37°C in a CO_2 incubator for 2 hrs. (This step may help to improve the signal for some phospho proteins).
4. Resuspend the PBMCs in cold PBS with 1%FCS (1×10^6cells/50µl), add anti-CD3 (Cat. No. 555329) and anti-CD28 (Cat. No. 555725, 1µg each for 1×10^6 cells), and incubate on ice for 20 minutes.
5. Wash the cells with cold PBS with 1%FCS, and spin at 4°C (250 × g) for 5 minutes.
6. Resuspend the PBMCs in cold PBS with 1%FCS (1×10^6 cells/50µl), add goat anti-mouse Ig (2ug of Cat. No 553998 for 1×10^6 cells), and incubate on ice for 20 minutes.
7. Wash the cells with cold PBS with 1%FCS, spin at 4°C (250 × g) for 5 minutes.
8. Resuspend the cells in pre-warmed (37°C) PBS with 1%FCS (1×10^6 cells/50µl), incubate at 37°C for 2-5 minutes (The incubation time depends on the phospho protein tested and should be determined by the researcher).