BD Phosflow™ Alternative Protocol 1: Fix–Stain–Perm

Human Whole Blood

Reagents Required

Full Name	Short Name	Catalog Number
Cellular stimuli		
BD Phosflow™ Lyse/Fix Buffer, 5X	Lyse/Fix Buffer	558049
Distilled water		
Phosphate buffered saline containing $CaCl_2$ and $MgCl_2$, 1X	PBS	
BD Pharmingen™ Stain Buffer (FBS)	Stain Buffer	554656
BD Phosflow™ Perm/Wash Buffer I, 10X*	Perm/Wash Buffer I	557885*
BD Phosflow™ Perm Buffer II*	Perm Buffer II	558052*
BD Phosflow™ Perm Buffer III*	Perm Buffer III	558050*
BD Phosflow™ Perm Buffer IV, 10X*	Perm Buffer IV	560746*
BD Phosflow™ fluorochrome-conjugated antibodies to phosphoproteins		
BD™ fluorochrome-conjugated antibodies to cell surface antigens (optional)		

***Select Perm Buffer I, II, III, or IV based on the surface markers and phosphospecific antibodies used. See the Tested Surface Markers chart and the BD FACSelect™ Buffer Compatibility Resource for more information.**

Procedural Notes

- Alternative staining protocols may allow resolution of surface marker stains that cannot be resolved when staining after permeabilization. Many surface marker epitopes are maintained during fixation but are damaged during permeabilization with harsh buffers such as Perm Buffer III or IV. For these surface markers, stains can be performed after cells have been stimulated and fixed but before they are permeabilized (Alternative Protocol 1). For epitopes that are not stable under fixation conditions, surface stains can be performed prior to fixation (Alternative Protocol 2).

  Alternative Protocol 1: Stimulate cells → Fix → Stain permeabilization-unstable surface markers → Permeabilize → Stain intracellular proteins and additional surface markers

  Alternative Protocol 2: Stimulate cells and stain fixation/permeabilization-unstable surface markers → Fix → Permeabilize → Stain intracellular proteins and additional surface markers
- Some fluorophores may be denatured by exposure to Perm Buffer II, III, or IV, resulting in loss of fluorescence. Non-protein fluorophores (eg, BD Horizon™, Alexa Fluor®, Pacific Blue™, and FITC dyes) are recommended for surface marker stains performed prior to permeabilization with Perm Buffer II or III. Both protein and non-protein fluorophores can be damaged by exposure to Perm Buffer IV, particularly at the 1X concentration. Staining resolution is influenced by staining protocol, permeabilization buffer, antibody clone, and fluorophore choice. Careful testing is recommended to confirm acceptable resolution of surface markers. See the BD FACSelect™ Buffer Compatibility Resource for more information.
- As with surface marker stains performed after permeabilization, surface marker stains performed after fixation may require lower antibody concentrations than those recommended for live cell staining. Antibodies should be titrated for optimal performance in the context of an Alternative Protocol 1 stain.
- Methods and kinetics of activation vary for each phosphorylated cell signaling molecule. Select appropriate stimuli and stimulation times before beginning the protocol. See the Suggested Stimulation Conditions for Phosphoprotein Detection chart for more information.
- Strict adherence to time and temperature recommendations for fixation, permeabilization, and staining is necessary for optimal resolution of phosphoprotein and cell surface marker stains.

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- Be sure to remove the majority of the supernatant after each centrifugation step. High residual volumes of supernatant will dilute buffers in subsequent steps, which could result in poor staining.

Reagent Preparation

- Prepare 1X Lyse/Fix Buffer according to the Technical Data Sheet (TDS) instructions by diluting in distilled or deionized water. Warm to 37°C for 15 to 30 minutes prior to use.

Procedure

1. Collect whole blood in the presence of anticoagulant (EDTA or sodium heparin). EDTA is preferred for preserving light scatter properties when PMA is used as an activator. Each tube to be stained will require approximately 200 µL of whole blood.
2. Treat the cells with appropriate stimuli, and incubate at 37°C for an appropriate length of time (1 to 30 minutes; see Procedural Note). An untreated control sample should be set up in parallel.
3. After the stimulation period, fix the cells immediately by adding 10 volumes of pre-warmed Lyse/Fix Buffer to the samples. Mix well by inverting 5 to 10 times or by vortexing. Samples should be mixed promptly and thoroughly to ensure complete erythrocyte lysis.
4. Incubate the cells at 37°C for 10 to 12 minutes.
5. Centrifuge at 600*g* for 6 to 8 minutes and remove the supernatant, leaving no greater than 50 µL of residual volume.
6. Vortex to disrupt the cell pellet.
7. Wash the cells twice:
    a. Add a volume of PBS equivalent to the volume of Lyse/Fix Buffer used.
    b. Centrifuge at 600*g* for 6 to 8 minutes and remove the supernatant, leaving no greater than 50 µL of residual volume.
    c. Vortex to disrupt the cell pellet.
    d. Add a volume of Stain Buffer equivalent to the volume of Lyse/Fix Buffer used.
    e. Repeat steps b and c.
8. Resuspend the cells in Stain Buffer at a final concentration of 5–10 x 10^6 cells/mL.
9. Transfer 100 µL of the cell suspension (0.5–1 x 10^6 cells) to each 12 x 75-mm BD Falcon™ tube and add appropriate amounts of surface marker antibodies. Antibodies should be titrated for optimal performance for staining fixed cells.
10. Mix and incubate at room temperature for 60 minutes protected from light.
11. Wash the cells:
    a. Add at least 3 mL of Stain Buffer.
    b. Centrifuge at 600*g* for 6 to 8 minutes and remove the supernatant, leaving no greater than 50 µL of residual volume.
    c. Vortex to disrupt the cell pellet. Insufficient cell resuspension prior to permeabilization may lead to cell clumping.
12. Permeabilize the cells using Perm Buffer I, II, III, or IV, and stain intracellular proteins and any additional surface markers as described in the BD Phosflow™ Protocols for Human Whole Blood Samples.