BD Phosflow™ 96-Well Deep-Well Plate Protocol

Human Whole Blood or Mouse Splenocytes

Reagents Required

Full Name	Short Name	Cat. No.
Cellular stimuli		
Complete media (eg, RPMI medium containing 10% FBS) (optional)	Complete media	
Phosphate buffered saline containing $CaCl_2$ and $MgCl_2$, 1X	PBS	
Distilled or deionized water		
BD Phosflow™ Lyse/Fix Buffer, 5X	Lyse/Fix Buffer	558049
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 Pharmingen™ Stain Buffer (FBS)	Stain Buffer	554656
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.

Suggested Equipment

2.2-mL storage plate, 96 deep-well (Abgene, Cat. No. AB0932 or equivalent)

Adhesive 96-well plate sealers for 96-well plates (Costar, Cat. No. 3095 or equivalent)

Reagent reservoirs (VWR, Cat. No. 89094-676 or equivalent)

Assay plate, 96-well round-bottom (Falcon®, Cat. No. 353910)

Multichannel pipet, 100–1,200-µL capacity (Rainin, Cat. No. L12-1200 or equivalent)

Multichannel pipet, 20–200-µL capacity (Rainin, Cat. No. L12-200 or equivalent)

Benchtop aspirator with vacuum system

Aspirating manifold, 12-well for deep-well plates (V&P Scientific, Cat. No. VP 187A)**

Centrifuge with 96 deep-well plate holders

37°C water bath (provides more consistent temperature across the deep-well plate than a 37°C incubator)

** If an aspirating manifold is unavailable, supernatants may be removed from deep-well plates by inverting, flicking, and blotting the plate on an absorbent surface. Avoid contact with fixative, which contains formaldehyde.

Procedural Notes

- 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.
- Intracellular phosphoproteins and cell surface antigens can be stained simultaneously. However, if there is difficulty resolving surface marker stains, surface staining can be performed before fixation or between fixation and permeabilization. See the *Tested Surface Markers* chart, the *BD FACSelect™ Buffer Compatibility Resource*, and *BD Phosflow™ Alternative Protocol 1: Fix–Stain–Perm* or *BD Phosflow™ Alternative Protocol 2: Stain–Fix–Perm* 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.
- 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.
- Prepare the appropriate permeabilization reagent:
  - For Perm/Wash Buffer I, prepare 1X Perm/Wash Buffer I according to the TDS instructions by diluting in distilled water. Use at room temperature.
  - For Perm Buffer II or III, ensure that the buffer is chilled to between -20°C and 4°C.
  - For Perm Buffer IV, prepare 1X or 0.5X Perm Buffer IV according to the TDS instructions by diluting in 1X PBS. Use at room temperature. See Procedural Notes and the *BD FACSelect™ Buffer Compatibility Resource* for information on choosing between 1X and 0.5X Perm Buffer IV.

Procedure

1. Prepare cells:
   - 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 well to be stained will require approximately 100 µL of whole blood.
   - Prepare single-cell suspensions from mouse tissue (for example, spleen, thymus, bone marrow, etc) in PBS or complete media.
     (a) Centrifuge at 350*g* for 6 to 8 minutes and remove the supernatant.
     (b) Gently resuspend the cell pellet. Resuspend cells at 5–10 x 10^6 cells/mL in PBS or complete media and pass through a 70-µm cell strainer if needed.
     (c) (Optional) Allow cells to rest in complete media at 37°C for 2 to 4 hours (see *Suggested Stimulation Conditions for Phosphoprotein Detection*).
2. Dilute stimuli and add to the appropriate wells of a 96 deep-well plate. Add no more than 20 µL of stimulus to each well to ensure that the final volume of cells plus stimulus is no greater than 120 µL. A vehicle-alone control sample should be set up in parallel.
3. Using a multichannel pipet, add 100 µL of cells to the appropriate wells, and mix by pipetting up and down or gently vortexing. Incubate at 37°C for an appropriate length of time (1 to 30 minutes; see Procedural Notes).
4. After the stimulation period, fix the cells immediately by using a multichannel pipet to add 1.2 mL of pre-warmed Lyse/Fix Buffer to each well. Mix promptly and thoroughly by pipetting up and down three times to ensure complete erythrocyte lysis.
5. Seal the plate with a plate sealer, and incubate at 37°C for 10 to 12 minutes.
6. Centrifuge at 600*g* for 5 minutes. Remove the supernatant using an aspirating manifold, leaving no greater than 50 µL of residual volume.
7. Vortex the plate vigorously to disrupt the cell pellets.
8. Wash the cells:
   a. Add 1.2 mL of PBS (for whole blood) or Stain Buffer (for mouse splenocytes) to each well using a multichannel pipet.
   b. Centrifuge at 600*g* for 5 minutes. Remove the supernatant using an aspirating manifold, leaving no greater than 50 µL of residual volume.
   c. Vortex the plate vigorously to disrupt the cell pellets. Insufficient cell resuspension prior to permeabilization could lead to cell clumping.

9. Permeabilize the cells with Perm/Wash Buffer I or Perm Buffer II, III, or IV.
   - For Perm/Wash Buffer I, add 1 mL to each well using a multichannel pipet. Mix thoroughly by pipetting up and down three times. Incubate for 15 to 30 minutes at room temperature.
   - For Perm Buffer II or III, add 1 mL of pre-chilled Perm Buffer II or III to each well using a multichannel pipet. Mix thoroughly by pipetting up and down three times. Seal the plate with a plate sealer, and incubate for 30 minutes on ice or at 4°C.
   - For Perm Buffer IV (1X or 0.5X), slowly add 1 mL to each well using a multichannel pipet. Mix thoroughly by pipetting up and down three times. Incubate for 15 to 20 minutes at room temperature.
10. Following permeabilization, centrifuge at 600*g* for 5 minutes. *Immediately* remove the supernatant using an aspirating manifold, leaving no greater than 50 µL of residual volume. **Important**: Delayed removal of permeabilization buffer could lead to poor surface marker staining or reduced cell yield.
11. Vortex the plate vigorously to disrupt the cell pellets.
12. Wash the cells twice:
    a. Add 1.2 mL of Perm/Wash Buffer I (for cells permeabilized with Perm/Wash Buffer I) or Stain Buffer (for cells permeabilized with Perm Buffer II, III, or IV) to each well using a multichannel pipet.
    b. Centrifuge at 600*g* for 5 minutes. Remove the supernatant using an aspirating manifold, leaving no greater than 50 µL of residual volume.
    c. Vortex the plate vigorously to disrupt the cell pellets.
    d. Repeat steps a–c.
13. Add 50–100 µL of Perm/Wash Buffer I (for cells permeabilized in Perm/Wash Buffer I) or Stain Buffer (for cells permeabilized in Perm Buffer II, III, or IV) to each well using a multichannel pipet. Pipette up and down or vortex to resuspend.
14. Transfer 100 µL from each well to a round-bottom 96-well plate, and add the recommended volume of BD Phosflow antibody.
15. Mix and incubate at room temperature for 60 minutes protected from light.
16. Centrifuge at 600*g* for 5 minutes and remove the supernatant, leaving no greater than 50 µL of residual volume.
17. Vortex the plate vigorously to disrupt the cell pellets.
18. Wash the cells twice:
    a. Add 200 µL of Perm/Wash Buffer I (for cells permeabilized in Perm/Wash Buffer I) or Stain Buffer (for cells permeabilized in Perm Buffer II, III, or IV).
    b. Centrifuge at 600*g* for 5 minutes and remove the supernatant by inverting, flicking, and blotting the plate on an absorbent surface.
    c. Vortex the plate vigorously to disrupt the cell pellets.
    d. Repeat steps a–c.
19. Resuspend the cells in approximately 200 µL of Perm/Wash Buffer I (for cells permeabilized in Perm/Wash Buffer I) or Stain Buffer (for cells permeabilized in Perm Buffer II, III, or IV) prior to flow cytometric analysis.

Human Peripheral Blood Mononuclear Cells (PBMCs)

Reagents Required

Full Name	Short Name	Cat. No.
BD Vacutainer® CPT Cell Preparation Tube with Sodium Heparin*	CPT tube	362753*
Ficoll-Paque™*	Density gradient	
Cellular stimuli		
Phosphate buffered saline containing $CaCl_2$ and $MgCl_2$, 1X	PBS	
Complete media (eg, RPMI medium containing 10% FBS) (optional)	Complete media	
BD Cytofix™ Fixation Buffer	Fixation Buffer	554655
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 Pharmingen™ Stain Buffer (FBS)***	Stain Buffer	554656***
BD Phosflow™ fluorochrome-conjugated antibodies to phosphoproteins		
BD™ fluorochrome-conjugated antibodies to cell surface antigens (optional)		

* PBMC preparation may be performed using either CPT tubes or Ficoll density gradient.

** 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.

*** Stain Buffer is not needed if using Perm/Wash Buffer I.

Suggested Equipment

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Adhesive 96-well plate sealers for 96-well plates (Costar, Cat. No. 3095 or equivalent)

Reagent reservoirs (VWR, Cat. No. 89094-676 or equivalent)

Assay plate, 96-well round-bottom (Falcon®, Cat. No. 353910)

Multichannel pipet, 100–1,200-µL capacity (Rainin, Cat. No. L12-1200 or equivalent)

Multichannel pipet, 20–200-µL capacity (Rainin, Cat. No. L12-200 or equivalent)

Benchtop aspirator with vacuum system

Aspirating manifold, 12-well for deep-well plates (V&P Scientific, Cat. No. VP 187A)****

Centrifuge with 96 deep-well plate holders

37°C water bath (provides more consistent temperature across the deep-well plate than a 37°C incubator)

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Procedural Notes

- 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.
- Intracellular phosphoproteins and cell surface antigens can be stained simultaneously. However, if there is difficulty resolving surface marker stains, surface staining can be performed before fixation or between fixation and permeabilization. See the *Tested Surface Markers* chart, the *BD FACSelect™ Buffer Compatibility Resource*, and *BD Phosflow™ Alternative Protocol 1: Fix–Stain–Perm* or *BD Phosflow™ Alternative Protocol 2: Stain–Fix–Perm* for more information.
- Use of freshly prepared PBMCs is recommended. If frozen PBMCs must be used, conditions for post-thaw cell recovery should be optimized to ensure appropriate basal levels of phosphorylation within unstimulated cells and appropriate cellular responsiveness to stimuli.
- Strict adherence to time and temperature recommendations for fixation, permeabilization, and staining is necessary for optimal resolution of phosphoprotein and cell surface marker stains.
- 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

- Warm Fixation Buffer to 37°C for 15 to 30 minutes prior to use.
- Prepare the appropriate permeabilization reagent:
  - For Perm/Wash Buffer I, prepare 1X Perm/Wash Buffer I according to the TDS instructions by diluting in distilled water. Use at room temperature.
  - For Perm Buffer II or III, ensure that the buffer is chilled to between -20°C and 4°C.
  - For Perm Buffer IV, prepare 1X or 0.5X Perm Buffer IV according to the TDS instructions by diluting in 1X PBS. Use at room temperature. See Procedural Notes and the *BD FACSelect™ Buffer Compatibility Resource* for information on choosing between 1X and 0.5X Perm Buffer IV.

Procedure

1. Prepare PBMCs from sample blood using CPT tubes or density gradient. Resuspend the cells at 5–10 x 10^6 cells/mL in PBS or complete media.
2. (Optional) Allow PBMCs to rest in complete media at 37°C for 2 to 4 hours (see *Suggested Stimulation Conditions for Phosphoprotein Detection*).
3. Dilute stimuli and add to the appropriate wells of a 96 deep-well plate. A vehicle-alone control sample should be set up in parallel.
4. Using a multichannel pipet, add 0.1 to 0.6 mL of cells to the appropriate wells, and mix by pipetting up and down or gently vortexing. Incubate at 37°C for an appropriate length of time (1 to 30 minutes; see Procedural Note).
5. After the stimulation period, fix the cells immediately by using a multichannel pipet to add a volume of pre-warmed Fixation Buffer equivalent to the volume of cell suspension in each well. Mix thoroughly by pipetting up and down three times.
6. Seal the plate with a plate sealer, and incubate at 37°C for 10 to 12 minutes.
7. Centrifuge at 600*g* for 5 minutes. Remove the supernatant using an aspirating manifold, leaving no greater than 50 µL of residual volume.
8. Vortex the plate vigorously to disrupt the cell pellets. Insufficient cell resuspension prior to permeabilization could lead to cell clumping.
9. Permeabilize the cells with Perm/Wash Buffer I or Perm Buffer II, III, or IV.
   - For Perm/Wash Buffer I, add 1 mL to each well using a multichannel pipet. Mix thoroughly by pipetting up and down three times. Incubate for 15 to 30 minutes at room temperature.
   - For Perm Buffer II or III, add 1 mL of pre-chilled Perm Buffer II or III to each well using a multichannel pipet. Mix thoroughly by pipetting up and down three times. Seal the plate with a plate sealer, and incubate for 30 minutes on ice or at 4°C.
   - For Perm Buffer IV (1X or 0.5X), slowly add 1 mL to each well using a multichannel pipet. Mix thoroughly by pipetting up and down three times. Incubate for 15 to 20 minutes at room temperature.

10. Following permeabilization, centrifuge at 600*g* for 5 minutes. Immediately remove the supernatant using an aspirating manifold, leaving no greater than 50 µL of residual volume. Important: Delayed removal of permeabilization buffer could lead to poor surface marker staining or reduced cell yield.
11. Vortex the plate vigorously to disrupt the cell pellets.
12. Wash the cells twice:
    a. Add 1.2 mL of Perm/Wash Buffer I (for cells permeabilized with Perm/Wash Buffer I) or Stain Buffer (for cells permeabilized with Perm Buffer II, III, or IV) to each well using a multichannel pipet.
    b. Centrifuge at 600*g* for 5 minutes. Remove the supernatant using an aspirating manifold, leaving no greater than 50 µL of residual volume.
    c. Vortex the plate vigorously to disrupt the cell pellets.
    d. Repeat steps a–c.
13. Add 50–100 µL of Perm/Wash Buffer I (for cells permeabilized in Perm/Wash Buffer I) or Stain Buffer (for cells permeabilized in Perm Buffer II, III, or IV) to each well using a multichannel pipet. Pipette up and down or vortex to resuspend.
14. Transfer 100 µL from each well to a round-bottom 96-well plate, and add the recommended volume of BD Phosflow antibody.
15. Mix and incubate at room temperature for 60 minutes protected from light.
16. Centrifuge at 600*g* for 5 minutes and remove the supernatant, leaving no greater than 50 µL of residual volume.
17. Vortex the plate vigorously to disrupt the cell pellets.
18. Wash the cells twice:
    a. Add 200 µL of Perm/Wash Buffer I (for cells permeabilized in Perm/Wash Buffer I) or Stain Buffer (for cells permeabilized in Perm Buffer II, III, or IV).
    b. Centrifuge at 600*g* for 5 minutes and remove the supernatant by inverting, flicking, and blotting the plate on an absorbent surface.
    c. Vortex the plate vigorously to disrupt the cell pellets.
    d. Repeat steps a–c.
19. Resuspend the cells in approximately 200 µL of Perm/Wash Buffer I (for cells permeabilized in Perm/Wash Buffer I) or Stain Buffer (for cells permeabilized in Perm Buffer II, III, or IV) prior to flow cytometric analysis.

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