BD Phosflow™ Protocol for Human PBMCs

Protocol I (Detergent Method)

Reagents Required

Full Name	Short Name	Catalog Number
BD Vacutainer® CPT Cell Preparation Tube with Sodium Heparin*	CPT tube	362753*
Ficoll-Paque™*		
Phosphate buffered saline containing $CaCl_2$ and $MgCl_2$, 1X, or complete media (eg, RPMI media containing 10% FBS)	PBS or complete media	
Cellular stimuli		
BD Cytofix™ Fixation Buffer	Cytofix Buffer	554655
BD Phosflow™ Perm/Wash Buffer I, 10X	Perm/Wash Buffer I	557885
Distilled water		
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.**

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. Refer to 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.
- Allowing peripheral blood mononuclear cells (PBMCs) to recover in complete media at 37°C for 2 to 4 hours following PBMC preparation might reduce basal levels of phosphorylation within unstimulated cells and/or affect the ability of cells to respond to some stimuli.
- 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 Cytofix Buffer to 37°C for 15 to 30 minutes prior to use.
- Prepare 1X Perm/Wash Buffer I according to the Technical Data Sheet (TDS) instructions by diluting in distilled water. Use at room temperature.

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Procedure

1. Prepare PBMCs from donor blood using CPT tubes or Ficoll density gradient. Resuspend the cells at 1–10 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 Procedural Note).
3. 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.
4. After the stimulation period, fix the cells immediately by adding an equal volume of pre-warmed Cytofix Buffer. Mix well by gentle vortexing.
5. Incubate the cells at 37°C for 10 to 12 minutes.
6. Centrifuge at 600*g* for 6 to 8 minutes and remove the supernatant, leaving no greater than 50 µL of residual volume.
7. Vortex to disrupt the cell pellet.
8. Permeabilize the cells by adding 1 mL of Perm/Wash Buffer I for 1–10 x 10^6 cells (minimum 1 mL). Mix gently and incubate for 15 to 30 minutes at room temperature.
9. Centrifuge at 600*g* for 6 to 8 minutes and remove the supernatant, leaving no greater than 50 µL of residual volume.
10. Vortex to disrupt the cell pellet.
11. Wash the cells:
    a. Add a volume of Perm/Wash Buffer I equivalent to the volume used for permeabilization.
    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.
12. Resuspend the cells in Perm/Wash Buffer I at a final concentration of 5–10 x 10^6 cells/mL.
13. 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 the recommended volume of BD Phosflow antibody.
14. Mix and incubate at room temperature for 60 minutes protected from light.
15. Wash the cells:
    a. Add at least 3 mL of Perm/Wash Buffer I.
    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.
16. Resuspend the cells in approximately 500 µL of Perm/Wash Buffer I prior to flow cytometric analysis.

BD Phosflow™ Protocol for Human PBMCs

Protocol II and III (Mild or Harsh Alcohol Method)

Reagents Required

Full Name	Short Name	Catalog Number
BD Vacutainer® CPT Cell Preparation Tube with Sodium Heparin*	CPT tube	362753*
Ficoll-Paque™*		
Phosphate buffered saline containing $CaCl_2$ and $MgCl_2$, 1X, or complete media (eg, RPMI media containing 10% FBS)	PBS or complete media	
Cellular stimuli		
BD Cytofix™ Fixation Buffer	Cytofix Buffer	554655
BD Phosflow™ Perm Buffer II**	Perm Buffer II	558052**
BD Phosflow™ Perm Buffer III**	Perm Buffer III	558050**
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 either Perm Buffer II or III 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

- 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. Refer to 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.
- Allowing peripheral blood mononuclear cells (PBMCs) to recover in complete media at 37°C for 2 to 4 hours following PBMC preparation might reduce basal levels of phosphorylation within unstimulated cells and/or affect the ability of cells to respond to some stimuli.
- 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 Cytofix Buffer to 37°C for 15 to 30 minutes prior to use.
- Ensure that Perm Buffer II or III is chilled to between -20°C and 4°C.

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Procedure

1. Prepare PBMCs from donor blood using CPT tubes or Ficoll density gradient. Resuspend the cells at 1–10 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 Procedural Note).
3. 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.
4. After the stimulation period, fix the cells immediately by adding an equal volume of pre-warmed Cytofix Buffer. Mix well by gentle vortexing.
5. Incubate the cells at 37°C for 10 to 12 minutes.
6. Centrifuge at 600*g* for 6 to 8 minutes and remove the supernatant, leaving no greater than 50 µL of residual volume.
7. Vortex to disrupt the cell pellet. Insufficient cell resuspension prior to permeabilization may lead to cell clumping.
8. Permeabilize the cells by adding 1 mL of chilled Perm Buffer II or III for 1–10 x 10^6 cells (minimum 1 mL). Vortex to mix and incubate for 30 minutes on ice.
9. Wash the cells:
    a. Before pelleting the cells, add at least 3 mL of Stain Buffer for every 1 mL of Perm 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.
10. Wash the cells two additional times:
    a. Add a volume of Stain Buffer equivalent to that used in Step 9.
    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. Repeat steps a–c.
11. Resuspend the cells in Stain Buffer at a final concentration of 5–10 x 10^6 cells/mL.
12. 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 the recommended volume of BD Phosflow antibody.
13. Mix and incubate at room temperature for 60 minutes protected from light.
14. 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.
15. Resuspend the cells in approximately 500 µL of Stain Buffer prior to flow cytometric analysis.

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Protocol IV (Harsh Detergent Method)

Reagents Required

Full Name	Short Name	Catalog Number
BD Vacutainer® CPT Cell Preparation Tube with Sodium Heparin*	CPT tube	362753*
Ficoll-Paque™*		
Phosphate buffered saline containing $CaCl_2$ and $MgCl_2$, 1X	PBS	
Complete media (eg, RPMI media containing 10% FBS) (optional)	Complete media	
Cellular stimuli		
BD Cytofix™ Fixation Buffer	Cytofix Buffer	554655
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.**

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. Refer to 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.
- Allowing peripheral blood mononuclear cells (PBMCs) to recover in complete media at 37°C for 2 to 4 hours following PBMC preparation might reduce basal levels of phosphorylation within unstimulated cells and/or affect the ability of cells to respond to some stimuli.
- 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.
- Permeabilization with Perm Buffer IV might result in decreased cell recovery. For maximal cell recovery, avoid aspirating the supernatant during all post-permeabilization washes. Instead, decant the supernatant and gently blot the tube edge on an absorbent surface to minimize residual volume.
- Longer permeabilization time or using a ratio of cell to buffer volume outside the recommended ratio might result in increased cell loss and poorer fluorescent surface marker and/or phosphoprotein-specific antibody staining and detection.
- Perm Buffer IV may be used at a 1X or 0.5X concentration. The 1X concentration might result in increased cell loss and decreased ability to stain certain cell surface markers, but it provides optimal resolution of certain intracellular phosphoprotein stains. The 0.5X concentration results in less optimal staining of some intracellular phosphoproteins, but it may provide improved cell recovery and improved compatibility for staining cell surface CD markers. See the BD FACSelect™ Buffer Compatibility Resource for more information.

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- 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 Cytofix Buffer to 37°C for 15 to 30 minutes prior to use.
- 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 donor blood using CPT tubes or Ficoll density gradient. Resuspend the cells at 1–10 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 Procedural Note).
3. 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.
4. After the stimulation period, fix the cells immediately by adding an equal volume of pre-warmed Cytofix Buffer. Mix well by gentle vortexing.
5. Incubate the cells at 37°C for 10 to 12 minutes.
6. Centrifuge at 600*g* for 6 to 8 minutes and remove the supernatant, leaving no greater than 50 µL of residual volume.
7. Vortex to disrupt the cell pellet.
8. Permeabilize the cells by slowly adding 1 mL of Perm Buffer IV drop by drop for 0.5–2.0 x 10^6 cells (minimum 1 mL). Vortex to mix and incubate for 15 to 20 minutes at room temperature.
9. Centrifuge at 600*g* for 6 to 8 minutes. Decant the supernatant and gently blot the tube edge on an absorbent surface, leaving no greater than 50 µL of residual volume.
10. Vortex to disrupt the cell pellet.
11. Wash the cells twice:
    a. Add at least 3 mL of Stain Buffer.
    b. Centrifuge at 600*g* for 6 to 8 minutes. Decant the supernatant and gently blot the tube edge on an absorbent surface, leaving no greater than 50 µL of residual volume.
    c. Vortex to disrupt the cell pellet.
    d. Repeat steps a–c.
12. Resuspend the cells in Stain Buffer at a final concentration of 5–10 x 10^6 cells/mL.
13. 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 the recommended volume of BD Phosflow antibody.
14. Mix and incubate at room temperature for 60 minutes protected from light.

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15. Wash the cells:
    a. Add at least 3 mL of Stain Buffer.
    b. Centrifuge at 600*g* for 6 to 8 minutes. Decant the supernatant and gently blot the tube edge on an absorbent surface, leaving no greater than 50 µL of residual volume.
    c. Vortex to disrupt the cell pellet.
16. Resuspend the cells in approximately 500 µL of Stain Buffer prior to flow cytometric analysis.

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