

April 2011

BD Phosflow™ Protocols for TCR Stimulation: Human

Human Whole Blood: CD3 Cross-linking for T-Cell Stimulation

Reagents Required

Full Name	Short Name	Catalog Number
Phosphate buffered saline containing CaCl ₂ and MgCl ₂ , 1X	PBS	
Purified NA/LE Anti-Human CD3 (Clone UCHT1)	Anti-CD3	555329
Purified Polyclonal Goat Anti-Mouse Ig	Goat Anti-Mouse Ig	553998
BD Phosflow™ Lyse/Fix Buffer, 5X	Lyse/Fix Buffer	558049
Distilled water		
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**
Normal Mouse Ig	Normal Mouse Ig	Invitrogen 10400C
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.

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

Procedural Notes

- Kinetics of phosphorylation varies among cell signaling molecules and cell types. Many of the phosphorylation events induced by T-cell receptor stimulation are maximal within 1 to 15 minutes, with phosphorylation decreasing rapidly afterwards. A stimulation time course should be performed for each phosphoprotein and cell type of interest to establish the optimal stimulation time. 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.
- 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.
- The pre-stain blocking step is critical to avoid experimental artifacts that could otherwise result from binding of mouse anti-human phosphoprotein antibodies by goat anti-mouse Ig cross-linking antibody on the surface of stimulated cells.



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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.
- Chill PBS on ice for at least 15 minutes prior to use.
- Prepare Perm Buffer according to the specific product TDS instructions. See the [BD Phosflow™ Protocols for Human Whole Blood Samples](#) for detailed permeabilization protocols.

Procedure

1. Collect whole blood in the presence of anticoagulant (EDTA or sodium heparin). Each tube to be stained will require approximately 200 µL of whole blood.
2. Pre-chill blood on ice for at least 15 minutes prior to use.
3. Add anti-CD3 antibody to the blood (2 µg per 200 µL of blood). Mix and incubate on ice for 15 minutes. An untreated control sample should be set up in parallel.
4. Wash the cells:
 - a. Add at least 1 mL of cold PBS per 100 µL of blood.
 - b. Centrifuge at 4°C, 300g for 6 to 8 minutes. Excessive centrifugation speed may result in erythrocyte clumping, which can negatively impact red blood cell lysis.
 - c. Keeping the samples cold, carefully remove the supernatant, with minimal disturbance of the red blood cells.
 - d. Gently resuspend the cell pellet.
5. Resuspend the cells in a volume of cold PBS equivalent to the original blood volume.
6. Add goat anti-mouse Ig antibody to the cells (2 µg per 200 µL of blood). Mix and incubate on ice for 15 minutes.
7. Stimulate the cells by transferring the tubes to a 37°C water bath and incubating for an appropriate length of time (1 to 15 minutes; see Procedural Note).
8. 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.
9. Incubate the cells at 37°C for 10 to 12 minutes.
10. Centrifuge at 600g for 6 to 8 minutes and remove the supernatant, leaving no greater than 50 µL of residual volume.
11. Vortex to disrupt the cell pellet.
12. Wash the cells:
 - a. Add a volume of PBS equivalent to the volume of Lyse/Fix Buffer used.
 - b. Centrifuge at 600g 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.



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13. Permeabilize the cells with Perm/Wash Buffer I or Perm Buffer II, III, or IV as described in the [BD Phosflow™ Protocols for Human Whole Blood Samples](#).
 - For Perm/Wash Buffer I, add 1 mL of Perm/Wash Buffer I for 1–10 x 10⁶ cells (minimum 1 mL). Mix gently and 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 for 1–10 x 10⁶ cells (minimum 1 mL). Vortex to mix and incubate for 30 minutes on ice.
 - For Perm Buffer IV (1X or 0.5X), slowly add 1 mL of Perm Buffer IV drop by drop for 0.5–2.0 x 10⁶ cells (minimum 1 mL). Vortex to mix and incubate for 15 to 20 minutes at room temperature.
14. Following permeabilization, wash the cells as directed in the [BD Phosflow™ Protocols for Human Whole Blood Samples](#).
 - For Perm/Wash Buffer I, pellet the cells and then wash once in Perm/Wash Buffer I.
 - For Perm Buffer II or III, wash once by adding Stain Buffer and then pelleting the cells. Wash the cells two additional times in Stain Buffer.
 - For Perm Buffer IV (1X or 0.5X), pellet the cells and then wash twice in Stain Buffer.
15. After washing, pellet the cells and resuspend in 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) at a final concentration of 5–10 x 10⁶ cells/mL.
16. **[Important: see Procedural Note]** Block unbound goat anti-mouse Ig cross-linking antibody by adding 15 µg of normal mouse Ig (Invitrogen, Cat. No. 10400C) per 100 µL of cell suspension. Mix and incubate at room temperature for 15 minutes.
17. Transfer 100 µL of the cell suspension (0.5–1 x 10⁶ cells) to each 12 x 75-mm BD Falcon™ tube and add the recommended volume of BD Phosflow antibody.
18. Mix and incubate at room temperature for 60 minutes protected from light.
19. Wash the cells:
 - a. Add at least 3 mL 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 600g 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.
20. Resuspend the cells in approximately 500 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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BD Phosflow™ Protocols for TCR Stimulation: Human

Human PBMCs: CD3 and CD28 Cross-linking for T-Cell Stimulation

Reagents Required

Full Name	Short Name	Catalog Number
BD Vacutainer® CPT Cell Preparation Tube with Sodium Heparin* Ficoll-Paque™*	CPT tube	362753*
Phosphate buffered saline containing CaCl ₂ and MgCl ₂ , 1X	PBS	
Complete media (eg, RPMI media containing 10% FBS) (optional)	Complete media	
Purified NA/LE Anti-Human CD3 (Clone UCHT1)	Anti-CD3	555329
Purified NA/LE Anti-Human CD28 (Clone CD28.2)	Anti-CD28	555725
Purified Polyclonal Goat Anti-Mouse Ig	Goat Anti-Mouse Ig	553998
BD Cytotfix™ Fixation Buffer	Cytotfix 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***
Normal Mouse Ig	Normal Mouse Ig	Invitrogen 10400C
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.

Procedural Notes

- Kinetics of phosphorylation varies among cell signaling molecules and cell types. Many of the phosphorylation events induced by T-cell receptor stimulation are maximal within 1 to 15 minutes, with phosphorylation decreasing rapidly afterwards. A stimulation time course should be performed for each phosphoprotein and cell type of interest to establish the optimal stimulation time. 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.



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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.
- The pre-stain blocking step is critical to avoid experimental artifacts that could otherwise result from binding of mouse anti-human phosphoprotein antibodies by goat anti-mouse Ig cross-linking antibody on the surface of stimulated cells.

Reagent Preparation

- Warm Cytotfix Buffer to 37°C for 15 to 30 minutes prior to use.
- Chill PBS on ice for at least 15 minutes prior to use.
- Prepare Perm Buffer according to the specific product TDS instructions. See the [BD Phosflow™ Protocols for Human PBMCs](#) for detailed permeabilization protocols.

Procedure

1. Prepare PBMCs from donor blood using CPT tubes or Ficoll density gradient. Resuspend the cells at 5–10 x 10⁶ 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. Pre-chill PBMCs on ice for at least 15 minutes prior to use.
4. Add anti-CD3 and anti-CD28 antibodies to the cells (1 µg of each antibody per 100 µL of PBMCs). Mix and incubate on ice for 15 minutes. An untreated control sample should be set up in parallel.
5. Wash the cells:
 - a. Add at least 1 mL of cold PBS per 100 µL of cell suspension.
 - b. Centrifuge at 4°C, 350g for 6 to 8 minutes.
 - c. Keeping the samples cold, remove the supernatant.
 - d. Gently resuspend the cell pellet.
6. Resuspend the cells in a volume of cold PBS equivalent to the original volume (5–10 x 10⁶ cells/mL).
7. Add goat anti-mouse Ig antibody to the cells (1 µg per 100 µL of PBMC). Mix and incubate on ice for 15 minutes.
8. Stimulate the cells by transferring the tubes to a 37°C water bath and incubating for an appropriate length of time (1 to 15 minutes; see Procedural Note).
9. After the stimulation period, fix the cells immediately by adding an equal volume of pre-warmed Cytotfix Buffer. Mix well by gentle vortexing.
10. Incubate the cells at 37°C for 10 to 12 minutes.
11. Centrifuge at 600g for 6 to 8 minutes and remove the supernatant, leaving no greater than 50 µL of residual volume.
12. Vortex to disrupt the cell pellet. Insufficient cell resuspension prior to permeabilization may lead to cell clumping.



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13. Permeabilize the cells with Perm/Wash Buffer I or Perm Buffer II, III, or IV as described in the [BD Phosflow™ Protocols for Human PBMCs](#).
 - For Perm/Wash Buffer I, add 1 mL of Perm/Wash Buffer I for 1–10 x 10⁶ cells (minimum 1 mL). Mix gently and 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 for 1–10 x 10⁶ cells (minimum 1 mL). Vortex to mix and incubate for 30 minutes on ice.
 - For Perm Buffer IV (1X or 0.5X), slowly add 1 mL of Perm Buffer IV drop by drop for 0.5–2.0 x 10⁶ cells (minimum 1 mL). Vortex to mix and incubate for 15 to 20 minutes at room temperature.
14. Following permeabilization, wash the cells as directed in the [BD Phosflow™ Protocols for Human PBMCs](#).
 - For Perm/Wash Buffer I, pellet the cells and then wash once in Perm/Wash Buffer I.
 - For Perm Buffer II or III, wash once by adding Stain Buffer and then pelleting the cells. Wash the cells two additional times in Stain Buffer.
 - For Perm Buffer IV (1X or 0.5X), pellet the cells and then wash twice in Stain Buffer.
15. After washing, pellet the cells and resuspend in 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) at a final concentration of 5–10 x 10⁶ cells/mL.
16. **[Important: see Procedural Note]** Block unbound goat anti-mouse Ig cross-linking antibody by adding 15 µg of normal mouse Ig (Invitrogen, Cat. No. 10400C) per 100 µL of cell suspension. Mix and incubate at room temperature for 15 minutes.
17. Transfer 100 µL of the cell suspension (0.5–1 x 10⁶ cells) to each 12 x 75-mm BD Falcon tube and add the recommended volume of BD Phosflow antibody.
18. Mix and incubate at room temperature for 60 minutes protected from light.
19. Wash the cells:
 - a. Add at least 3 mL 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 600g 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.
20. Resuspend the cells in approximately 500 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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