BD Phosflow™ Monocyte/ NK Cell Activation Kit

Instruction Manual

Catalog No. 562089

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History

Revision	Date	Change made
23-12806-00 Rev. 01	2/2011	New document
562089 Rev. 2	3/2015	Updated Warnings section

Contents

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About this kit

This section covers the following topics:

Purpose of the kit

Uses of the kit

The BD Phosflow™ Monocyte/NK Cell Activation Kit (Catalog No. 562089) provides the reagents necessary to simultaneously analyze multiple cell markers with one of several intracellular phosphoproteins for the purpose of studying monocyte and NK cell signaling events. For some studies, cells must be treated with biological response modifiers (eg, cytokines, polyclonal activators, and/or mitogens that are not included in the kit) to induce phosphorylated protein expression. The kit enables flow cytometric phosphoprotein analysis of leucocyte populations without prior purification of the monocyte and NK cell populations from fresh whole blood.

Specific antibodies

The kit includes a fluorescent anti-human CD3 antibody and a multicolor cocktail containing fluorescent anti-human CD14, CD16, CD19, and CD56 antibodies to identify $CD14^+$ monocytes, $CD16^+$ and $CD56^+$ NK cells, $CD19^+$ B cells, and $CD3^+$ T cells. Single-color Alexa Fluor® 647-conjugated fluorescent monoclonal antibodies specific for the phosphorylated cell-signaling proteins p38, ERK1/2, Stat1, Stat3, Stat5, and Stat6, are provided as individual reagents and can be used one at a time with the fluorescent CD antibodies for staining. The kit is optimized to evaluate cell-signaling events associated with monocyte and NK cell activation, including phosphorylation status information on the intracellular MAPK and JAK/STAT signaling pathway responses transduced by treated human monocytes and NK cells. Additionally, signaling events in T and B-cell populations can also be studied with the kit.

Other antibodies

Single-color Alexa Fluor® 488 anti-CD3 and PE anti-CD4 antibodies are provided to label the BD™ CompBeads that are used as compensation controls.

Fluorescence compensation control beads

Two populations of microparticles are provided.

- BD™ CompBead Anti-Mouse Ig, κ particles, which bind any mouse kappa light-chain-bearing immunoglobulin
- BD™ CompBead Negative Control particles, which have no binding capacity

When mixed together with a fluorochrome-conjugated mouse Ig, κ light-chain-containing antibody, the BD CompBeads provide distinct positive and negative (background fluorescence) stained populations that can be used to optimize fluorescence compensation settings manually or by using instrument setup software. The beads allow you to set up for the application and help conserve cells that would otherwise be used to optimize compensation.

Control cells

The kit contains two vials each of lyophilized human leucocytes:

- BD Phosflow™ Treated Human Control Cells - for MAPK: Includes monocytes and NK cells that express upregulated levels of ERK1/2 and p38 phosphoproteins and serve as positive control cells.
- BD Phosflow™ Treated Human Control Cells - for STAT: Includes monocytes and NK cells that express upregulated levels of Stat1, 3, 5, and 6 phosphoproteins and serve as positive control cells. Please note that the phosphorylated Stat3 levels may not be elevated significantly in NK cells or monocyte control cells.
- BD Phosflow™ Untreated Human Control Cells - Mono/NK Cells: Includes monocytes and NK cells that express basal levels of the kit's targeted phosphorylated proteins and serve as negative control cells.

The control cells are used as positive and negative controls for staining with fluorescent antibodies specific for the CD markers and phosphorylated cell-signaling proteins. The control cells have been prestained with fluorescent anti-CD antibodies. These cells have been fixed and permeabilized and require only immunofluorescent staining with phosphosite-specific antibodies after reconstitution and before flow cytometric evaluation. The Treated Human Control Cells - for MAPK and STAT cells are treated with multiple activators, so the activation profiles may differ from those obtained from cells treated with a single activator or biological response modifier. The negative and positive control cells in each kit are produced using buffy coat cells from the same donor. Control cells in different kits may be from different donors, so do not mix control cells from different kits. Representative data for leucocytes treated with single-response modifiers are presented in Example of single-activation profile analysis (page 43).

Buffers

The kit contains all buffers necessary for erythrocyte lysis and leucocyte fixation, permeabilization, and immunofluorescent staining. All buffers and monoclonal antibody reagents in this kit have been optimized for their combined use. Do not substitute other reagents.

Kit contents

Reagent information

The BD Phosflow Monocyte/NK Cell Activation Kit contains the following reagents.

CD marker antibody cocktail

Reagent	Details
BD Phosflow™ Human Monocyte/NK Cell (CD14/CD19/CD16/CD56) Antibody Cocktail	**Cocktail components (Clone).** Alexa Fluor® 488 anti-Human CD14 (M5E2) and anti-Human CD19 (HIB19) antibodies; PE anti-Human CD16 (3G8) and anti-CD56 (B159) antibodies **Use.** To identify $CD14^+$ monocytes, $CD16^+$ and $CD56^+$ NK cells, and $CD19^+$ B cells **Abbreviation.** Mono/NK Cell Cocktail **Quantity.** 1 vial (1 mL) **Amount for staining.** 20 µL per sample (for cells derived from 200 µL of human peripheral blood)
BD Phosflow™ PE-Cy™7 Mouse Anti-Human CD3	**Clone.** SK7 **Use.** To identify $CD3^+$ T cells **Abbreviation.** PE-Cy7 CD3 **Quantity.** 1 vial (1.2 mL) **Amount for staining.** 20 µL per sample (for cells derived from 200 µL of human peripheral blood)

Fluorescent anti-phosphoprotein antibodies

Reagent	Details
BD Phosflow™ Alexa Fluor® 647 Mouse Anti-ERK1/2 (pT202/pY204)	**Clone.** 20A **Use.** To stain ERK1 phosphorylated at threonine 202 and tyrosine 204 and ERK2 phosphorylated at threonine 184 and tyrosine 186 **Abbreviation.** Alexa Fluor® 647 ERK1/2 (pT202/pY204) **Quantity.** 1 vial (1 mL) **Amount for staining.** 20 µL per sample (for cells derived from 200 µL of human peripheral blood)
BD Phosflow™ Alexa Fluor® 647 Mouse Anti-p38 MAPK (pT180/pY182)	**Clone.** 36/p38 (pT180/pY182) **Use.** To stain p38 MAPK phosphorylated at threonine 180 and tyrosine 182 **Abbreviation.** Alexa Fluor® 647 p38 MAPK (pT180/pY182) **Quantity.** 1 vial (1 mL) **Amount for staining.** 20 µL per sample (for cells derived from 200 µL of human peripheral blood)
BD Phosflow™ Alexa Fluor® 647 Mouse Anti-Stat1 (pY701)	**Clone.** 4a **Use.** To stain Stat1 phosphorylated at tyrosine 701 **Abbreviation.** Alexa Fluor® 647 Stat1 (pY701) **Quantity.** 1 vial (1 mL) **Amount for staining.** 20 µL per sample (for cells derived from 200 µL of human peripheral blood)

Reagent	Details
BD Phosflow™ Alexa Fluor® 647 Mouse Anti-Stat3 (pY705)	**Clone.** 4/P-STAT3 **Use.** To stain Stat3 phosphorylated at tyrosine 705 **Abbreviation.** Alexa Fluor® 647 Stat3 (pY705) **Quantity.** 1 vial (1 mL) **Amount for staining.** 20 µL per sample (for cells derived from 200 µL of human peripheral blood)
BD Phosflow™ Alexa Fluor® 647 Mouse Anti-Stat5 (pY694)	**Clone.** 47 **Use.** To stain Stat5 phosphorylated at tyrosine 694 **Abbreviation.** Alexa Fluor® 647 Stat5 (pY694) **Quantity.** 1 vial (1 mL) **Amount for staining.** 20 µL per sample (for cells derived from 200 µL of human peripheral blood)
BD Phosflow™ Alexa Fluor® 647 Mouse Anti-Stat6 (pY641)	**Clone.** 18/P-Stat6 **Use.** To stain Stat6 phosphorylated at tyrosine 641 **Abbreviation.** Alexa Fluor® 647 Stat6 (pY641) **Quantity.** 1 vial (1 mL) **Amount for staining.** 20 µL per sample (for cells derived from 200 µL of human peripheral blood)

Fluorescent antibodies for compensation

Note: Portions of the PE-Cy7 anti-CD3 and one of the individual Alexa Fluor® 647-conjugated anti-phosphoprotein antibodies can be used to label the BD CompBeads to complete the panel of compensation controls.

Reagent	Details
BD Phosflow™ Alexa Fluor® 488 Mouse Anti-Human CD3	**Clone.** UCHT1 **Use.** To stain BD CompBeads for compensation control **Abbreviation.** Alexa Fluor® 488 CD3 **Quantity.** 1 vial (0.2 mL) **Amount for staining.** 20 µL per sample
BD Phosflow™ PE Mouse Anti-Human CD4	**Clone.** RPA-T4 **Use.** To stain BD CompBeads for compensation control **Abbreviation.** PE CD4 **Quantity.** 1 vial (0.2 mL) **Amount for staining.** 20 µL per sample

Fluorescence compensation control beads

Reagent	Details
BD™ CompBead Anti-Mouse Ig, κ	**Use.** To create control beads stained with Alexa Fluor® 488-CD3, PE-CD4, PE-Cy7 CD3, or Alexa Fluor® 647-phosphoprotein (These beads bind any mouse kappa light-chain-bearing immunoglobulin.) **Abbreviation.** Anti-mouse Ig, κ beads **Quantity.** 1 vial (6 mL)

Reagent	Details
BD™ CompBead Negative Control	**Use.** As negative control beads (These beads have no capacity to bind immunoglobulin.) **Abbreviation.** Negative beads **Quantity.** 1 vial (6 mL)

Lyophilized human control cells

Reagent	Details
BD Phosflow™ Human Control Cells Treated for MAPK - Monocyte/ NK Cells	**Use.** As a positive staining control cell **Abbreviation.** Treated Control Cells for MAPK **Quantity.** 2 vials (1 test per vial) The cells are treated in such a way that phosphorylated ERK1/2 and p38 target proteins are activated. Each vial contains sufficient cells to stain for a single phosphorylated target protein.
BD Phosflow™ Human Control Cells Treated for STAT - Monocyte/ NK Cells	**Use.** As a positive staining control cell **Abbreviation.** Treated Control Cells for STAT **Quantity.** 2 vials (1 test per vial) The cells are treated in such a way that phosphorylated Stat1, Stat3, Stat5, and Stat6 target proteins are activated. Each vial contains sufficient cells to stain for a single phosphorylated target protein.
BD Phosflow™ Untreated Human Control Cells - Mono/ NK Cells	**Use.** As a negative staining control cell **Abbreviation.** Untreated Control Cells **Quantity.** 2 vials (1 test per vial) Untreated Control Cells should be stained with the same antibodies as those for the Treated Control Cells.

Buffers

Reagent	Details
BD Phosflow™ Lyse/ Fix Buffer (5X)	**Use.** To lyse erythrocytes and fix leucocytes **Abbreviation.** 5X Lyse/Fix Buffer **Quantity.** 1 bottle (50 mL)
BD Phosflow™ Perm Buffer IV	**Use.** To permeabilize fixed leucocytes **Abbreviation.** Perm Buffer IV **Quantity.** 1 bottle (25 mL)
BD Pharmingen™ Stain Buffer (FBS)	**Use.** To wash and suspend cells for immunofluorescent staining **Abbreviation.** Stain Buffer **Quantity.** 1 bottle (500 mL)

Serum proteins

Components in this kit contain a small percentage of serum proteins. The source of all serum proteins is from USDA-inspected abattoirs located in the United States.

Storage and safe handling

Storage

Store the fluorescent antibodies, BD CompBeads, and Stain Buffer at 2 to 8°C. Do not freeze. The fluorescent antibodies and BD CompBeads should be protected from prolonged exposure to light.

Store the Perm Buffer IV, Treated and Untreated Control Cells, and the Lyse/Fix Buffer at room temperature.

Warning

Researchers are encouraged to review the listed risk and safety phrases prior to use.

Danger

BD Phosflow™ Lyse/FixBuffer (5X) (component 51-9006606) contains 20.35% formaldehyde (w/w), 15.65% diethylene glycol (w/w) and 7.15% methanol (w/w).

Hazard statements

Combustible liquid.

Harmful if swallowed or in contact with skin.

Toxic if inhaled.

Causes skin irritation.

Causes serious eye damage.

May cause an allergic skin reaction.

Suspected of causing genetic defects.

May cause cancer. Route of exposure: Inhalative.

May cause damage to organs. May cause respiratory irritafion.

May cause damage to the kidneys through prolonged or repeated exposure. Route of exposure: Oral.

Precautionary statements

Wear protective gloves / eye protection.

Wear protective clothing.

Do not breathe mist/vapours/spray.

IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.

IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do.

Continue rinsing.

IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.

Some reagents in this kit contain sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

The lyophilized human control cells contain human blood, serum, or cells, a potential biohazardous material. Use universal precautions when handling. Handle as if the product were capable of transmitting disease. Material used in this product has been tested using FDA-approved methods and found to be negative for Human Immunodeficiency Virus (HIV-1/HIV-2), Hepatitis B Surface Antigen (HBSAG), and antibody to Hepatitis C Virus (HCV). However, no known test method can offer complete assurance that specimens of human origin will not transmit infectious disease. When handling or disposing, follow precautions described in CDC and FDA recommendations and OSHA Bloodborne Pathogen recommendations.

2

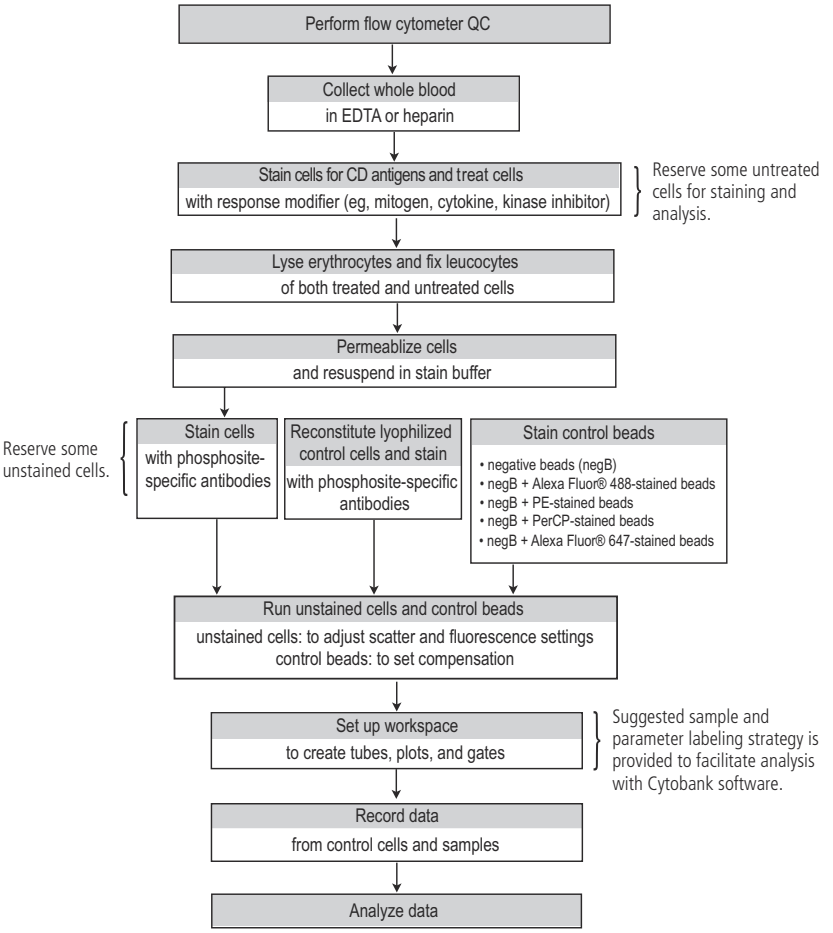
Before you begin

This section covers the following topics:

Workflow overview

Workflow

Following is an overview of the steps involved in using the BD Phosflow Monocyte/NK Cell Activation Kit to analyze cells.



Required materials

Materials list

The following reagents, consumables, and equipment are required for use with the BD Phosflow Monocyte/NK Cell Activation Kit:

- Fresh whole blood collected with EDTA or heparin anticoagulant

  Note: EDTA can potentially affect the actions of some response modifiers.

- Appropriate cellular response modifiers, such as biologically active recombinant cytokine proteins, Ionomycin, Phorbol 12-Myristate 13-Acetate (PMA), etc.

  Examples:

Response modifier	Vendor	Catalog No.
Recombinant Human Interleukin-2 (hIL-2)	BD	554603
Recombinant Human Interleukin-4 (hIL-4)	BD	554605
Recombinant Human Interleukin-6 (hIL-6)	BD	550071
Recombinant Human IFN-α (hIFN-α)	Sigma	I-4276
Lipopolysaccharides from *E. coli* O127:B8 (LPS)	Sigma	L-3137
Ionomycin	Sigma	I-0634
Phorbol 12-Myristate 13-Acetate (PMA)	Sigma	P-8139

- Distilled or deionized water
- 1X Phosphate Buffered Saline (PBS) for cell washing and PMA dilution

- 1X PBS containing 1 mg/mL of BSA for cytokine dilution
- Complete RPMI Media for LPS stock reconstitution
- DMSO for PMA and Ionomycin stock reconstitution
- Falcon® round-bottom 12 × 75-mm polystyrene tubes with caps (Catalog No. 352058), or equivalent
- Falcon® 15-mL polypropylene conical tubes (Catalog No. 352097)
- Pipets and pipet tips
- 37°C water bath
- Ice bath
- Vortex mixer
- Vacuum system and aspirator
- Centrifuge
- BD™ LSR II, BD FACSCanto™ II, BD FACSAria™ platform, BD LSRFortessa™, or BD FACSCalibur™ flow cytometer, or other flow cytometer equipped with a blue (488-nm) laser, a red (633-nm) laser, and filter configurations appropriate for the detection of Alexa Fluor® 488, PE, PE-Cy7, and Alexa Fluor® 647 (or allophycocyanin [APC]).
- Computer equipped with flow cytometric data acquisition software (eg, BD FACSDiva™, BD CellQuest™ Pro, or BD CellQuest™ software)

  Note: For flow cytometric data analysis using Cytobank software, we recommend using the Firefox web browser. Go to bdbiosciences.com/phosflow and click the Cytobank link, or go directly to cytobank.org for more details, including a list of supported web browsers and their requirements.

- For proper digital cytometer setup, BD recommends using the BD™ Cytometer Setup and Tracking Beads Kit (Catalog No. 642412) and BD FACSDiva software, version 6 or later. See the BD Cytometer Setup and Tracking Beads Kit technical data sheet and the *BD FACSDiva Software Reference Manual* for instructions.

3

Preparation of cells and beads

This section covers the following topics:

Treating and staining the cells

Purpose of the procedure

This procedure explains how to treat fresh normal human whole blood cells to induce cellular responses resulting in the phosphorylation of intracellular signaling proteins. After treating and surface staining the cells, the red blood cells are lysed, and the leucocytes are fixed and permeabilized prior to staining with one of the phospho-specific antibodies.

Before you begin

- Ensure that you have all of the necessary materials available. See Required materials (page 19) for details.
- Prepare sufficient Lyse/Fix Buffer by diluting 5X Lyse/Fix Buffer to 1X with distilled or deionized water (2.5 mL of 1X Lyse/Fix Buffer is required for every 200 µL of blood).
- Pre-warm the 1X Lyse/Fix Buffer in a 37°C water bath for 15 to 30 minutes.
- Prepare response modifier(s) immediately prior to use by diluting in the appropriate diluent(s). Dilute cytokines in 1X PBS containing 1 mg/mL of BSA. Dilute LPS in complete RPMI medium. Place on ice until use. (Diluted modifiers are less stable, and they should be diluted immediately prior to use.)
- Dilute the 10X Perm Buffer IV to 1X with PBS.

Variables that can affect results

Treatment: EDTA is the recommended anticoagulant when stimulating with PMA. PMA stimulation of heparinized blood might result in altered cellular light scatter characteristics and greater cell loss.

For response modifiers dissolved in DMSO, keep the final DMSO concentration at or below 0.1% to minimize nonspecific effects. To reduce final DMSO concentration, dilute the stock PMA in PBS containing BSA immediately before use.

Lyse/Fix: Prolonged exposure of cells to Lyse/Fix Buffer might result in poor cell surface staining. After the incubation and centrifugation steps, decant the supernatant and immediately add 1X PBS to tubes.

Permeabilization: Prolonged exposure to Perm Buffer IV might compromise surface marker staining. To avoid prolonged exposure of the cells to Perm Buffer IV, remove buffer immediately after centrifugation and proceed with wash steps.

Staining: Shorter antibody incubation times might result in dimmer staining of some markers. Stain cells for 60 minutes at room temperature, in the dark.

Procedure

Perform *all* of the procedures in this section in one continuous flow.

Treating the cells

1. Label four 12 × 75-mm tubes:
   - Untreated Unstained
   - Untreated Stained
   - Treated Unstained
   - Treated Stained
2. Add 20 µL of the CD cocktail (containing Alexa Fluor® 488 anti-Human CD14, Alexa Fluor® 488 anti-Human CD19, PE anti-Human CD16, and PE anti-Human CD56 antibodies) for every 200 µL of blood. Mix tube contents by brief, gentle vortexing.

3. Add 20 µL of PE-Cy7 anti-Human CD3 for every 200 µL of blood. Mix by brief, gentle vortexing.

   Note: Minimize exposure to light from this step on.

4. Immediately aliquot 200 µL of blood to each of the tubes.

5. Add the appropriate amount of response modifier (eg, cytokine, mitogen, kinase inhibitor, etc) to the tubes labeled "Treated," and add an equal volume of matching activator diluent buffer to the tubes labeled "Untreated." Gently vortex the tubes to mix.

   The following table provides human experimental model system information.

   Note: The incubation times and temperatures for all examples were 15 minutes at 37°C.

Response modifier	Final concentration	Induced target phosphorylation
hIL-2	100 ng/mL	Stat5
hIL-4	100 ng/mL	Stat6
hIL-6	100 ng/mL	Stat3
hIFN-α	40,000 units/mL	Stat1
Ionomycin	250 ng/mL	No induction by Ionomycin only
PMA	250 ng/mL	ERK1/2, p38 MAPK
LPS	10 µg/mL	p38 MAPK

6. Incubate all of the tubes in a 37°C water bath for the appropriate length of time for your experiment.

7. Lyse erythrocytes and fix leucocytes from treated and untreated blood samples immediately by adding 2.5 mL of pre-warmed 1X Lyse/Fix Buffer to each of the 12 × 75-mm tubes.

8. Mix the contents well by gently vortexing or inverting 5 to 10 times, and incubate the samples in a 37°C water bath for 10 to 12 minutes in the dark.
9. Centrifuge at 600*g* for 6 minutes. Remove the supernatant by decanting, followed by dabbing.
10. Quickly vortex each tube to loosen the cell pellet, and add 3 mL of 1X PBS to each tube.
11. Centrifuge at 600*g* for 6 minutes. Remove the supernatant by decanting, followed by dabbing.

Note: For convenience, bulk surface CD staining and treatment of cells followed by erythrocyte lysis and leucocyte fixation can be done first. To do this, add the amount of blood needed for each group plus 20% extra to a 15-mL conical tube labeled "Stained." After similarly processing the cells as described in step 1 through step 11, aliquot the cells into 12 × 75-mm tubes. Then permeabilize and stain cells with individual Alexa Fluor® 647 phosphosite-specific antibodies as described in step 1 through step 4.

Permeabilizing and staining the cells

1. Vortex each tube to loosen the cell pellet. While vortexing, permeabilize the cells by adding 1 mL of room temperature 1X Perm Buffer IV to the tube.
2. Cap the tubes and incubate at room temperature for 15 to 20 minutes in the dark.
3. Add 3 mL of Stain Buffer to each tube and immediately centrifuge at 600*g* for 6 minutes. Remove the supernatant by decanting, followed by dabbing.
4. Vortex each tube to loosen the cell pellet.
5. Add 3 mL of Stain Buffer to each tube and centrifuge at 600*g* for 6 minutes. Remove the supernatant by decanting, followed by dabbing.

6. Vortex each tube to loosen the cell pellet.
7. Add 50 µL of Stain Buffer to each tube. (The final volume, including the residual volume, will be approximately 100 µL.) Mix well and keep on ice, in the dark, until ready to stain.

Preparing lyophilized human control cells

1. Open the vials containing the lyophilized human control cells (treated and untreated) and reconstitute the cells in 120 µL of Stain Buffer.
2. Gently vortex for 1 to 2 seconds and let stand at room temperature for 5 minutes.
3. Transfer 100 µL of control cells to appropriately labeled 12 × 75-mm tubes. Keep cells on ice, in the dark, until ready to stain.

Staining cell samples and control cells

1. Add the following to the prepared 12 × 75-mm sample and control cells tubes that are being kept on ice, in the dark.

   Determine which phosphorylated target protein you want to stain in the lyophilized control samples.

   Note: Unstained cells are used to assess autofluorescence.

The following table provides an example of the general setup for staining cells for phosphorylated signaling molecules.

Component	Unstained blood samples		Stained blood samples		Lyophilized control samples	
	Untreated cells	Treated cells	Untreated cells	Treated cells	Untreated cells	Treated cells
Prepared cells	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
One Alexa Fluor® 647 anti-phospho-protein Ab	—	—	20 µL	20 µL	20 µL	20 µL

2. Vortex all tubes and incubate at room temperature for 1 hour, protected from light.

   Note: Compensation beads can be stained during this incubation period. See Staining BD CompBeads (page 30).

3. Wash cells with Stain Buffer by adding 3 mL of Stain Buffer to each tube and immediately centrifuging at 600*g* for 6 minutes. Remove the supernatant by decanting, followed by dabbing.

4. Suspend the stained cells in 300 to 500 µL of Stain Buffer.

Next step

The samples are ready to be run on the flow cytometer. Acquire samples immediately (optimal) or no longer than 4 hours after preparation. Keep cell samples at 2 to 8°C and protected from light prior to data acquisition.

If you have not already stained the BD CompBeads, proceed to Staining BD CompBeads (page 30).

If you have stained the BD CompBeads, proceed to Cytometer procedures (page 33) for details on setting up the cytometer.

Staining BD CompBeads

Before you begin

This procedure can be performed during the incubation step (step 2) of Staining cell samples and control cells (page 28).

Procedure

To stain the BD Comp Beads:

1. Set up the Anti-mouse Ig, κ and Negative beads for fluorescence compensation. Label five 12 × 75-mm polystyrene tubes as follows:
   - Negative
   - Alexa Fluor® 488
   - PE
   - PE-Cy7
   - Alexa Fluor® 647

2. Add the following to each tube in the order shown. Vortex the beads thoroughly immediately before dispensing drops.

Component	Negative	Alexa Fluor® 488	PE	PE-Cy7	Alexa Fluor® 647
Stain Buffer	100 µL	100 µL	100 µL	100 µL	100 µL
Negative beads	1 drop	1 drop	1 drop	1 drop	1 drop
Anti-mouse Ig, κ beads	—	1 drop	1 drop	1 drop	1 drop
Alexa Fluor® 488 CD3	—	20 µL	—	—	—
PE CD4	—	—	20 µL	—	—
PE-Cy7 CD3	—	—	—	20 µL	—
Alexa Fluor® 647 anti-phosphoprotein Ab	—	—	—	—	20 µL

3. Vortex all tubes and incubate at room temperature for 30 to 60 minutes, protected from light.
4. Wash the beads by adding 3 mL of Stain Buffer to each tube and immediately centrifuging at 600*g* for 6 minutes. Remove the supernatant by aspirating.

   Note: Aspirate the BD CompBeads supernatant. Do not decant. Decanting the supernatant of the beads can cause loss of beads.
5. Resuspend the beads in 300 to 500 µL of Stain Buffer. Keep beads at 2 to 8°C, protected from light, prior to data acquisition.
6. Use the stained beads to set up compensation prior to acquiring the stained samples.

Next step

Proceed to Cytometer procedures (page 33).

4

Cytometer procedures

This section covers the following topics:

Running the beads

Purpose of the procedure

The stained BD CompBeads are run to calculate and establish fluorescence compensation.

Before you begin

The guidelines and examples in this section use a BD FACSCanto II flow cytometer, BD FACSDiva software for acquisition, and the optional Cytobank software for data analysis. However, the fundamental approach to cytometer setup, acquisition, and analysis can be adapted to other flow cytometers and data analysis software applications.

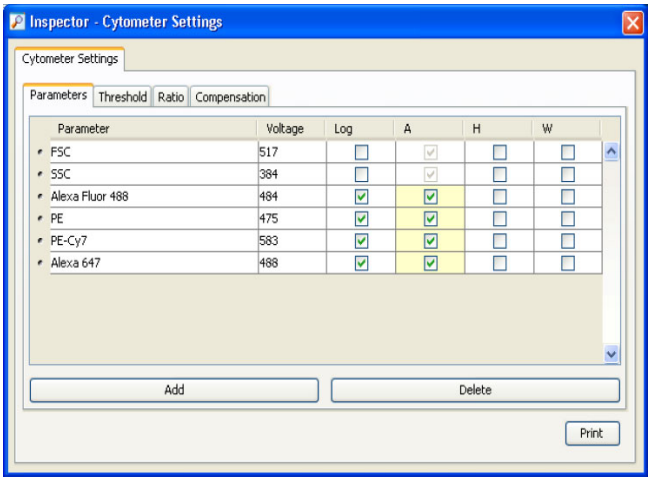
Run the appropriate instrument setup and QC procedures for your flow cytometer. We recommend the use of the BD Cytometer Setup and Tracking Beads and BD FACSDiva software, version 6.0 or later. Ensure that your instrument configuration is appropriate for this assay. See your instrument user's guide for more information.

Complete the steps in Preparation of cells and beads (page 23).

Procedure

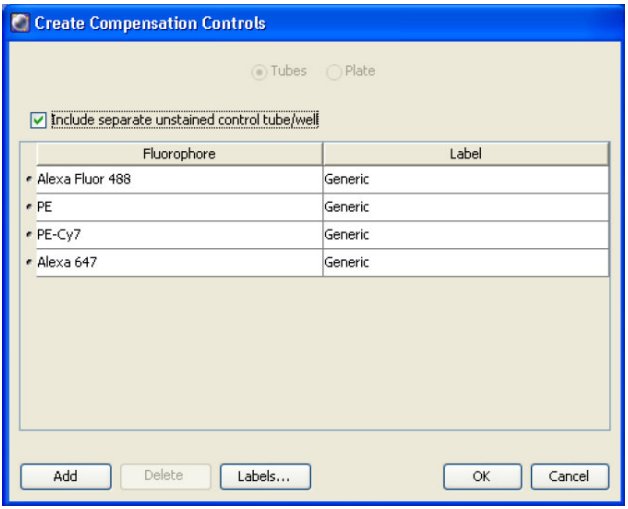
To run the prepared compensation control beads:

1. Create a new experiment in BD FACSDiva software.
2. If you have saved application settings for use with this kit, apply the application settings. If no application settings exist, proceed to the next step.
3. In the **Cytometer Settings** dialog, select the **Parameters** tab and delete all parameters except FSC, SSC, Alexa Fluor® 488, PE, PE-Cy7, and Alexa Fluor® 647.



Note: Click the **Threshold** tab and ensure that the threshold value is appropriate to display BD CompBeads.

4. Create compensation controls using the Compensation Setup feature in BD FACSDiva software. Use the generic rather than the antibody-specific labels as indicated in the following figure.



5. Prior to running BD CompBeads, ensure that fluorescence PMT voltages are appropriate for cell analysis.

   a. Place a tube of unstained cells on the cytometer and acquire data using the Unstained Control worksheet. Adjust FSC and SSC voltages so that all cell populations are on scale. Record voltages for future use in Running the cells (page 42).

   b. Adjust the P1 gate to include the lymphocyte population. Ensure that the fluorescence signals measured from the cells are on scale for all fluorescence parameters. Adjust PMT voltages, if necessary.

   c. Place a tube of stained cells, positive for each fluorochrome, on the cytometer and acquire data using the Unstained Control worksheet. Ensure that the fluorescence signals measured from the cells are on scale for all fluorescence parameters. Adjust PMT voltages, if necessary.

6. Save the application settings for future use.

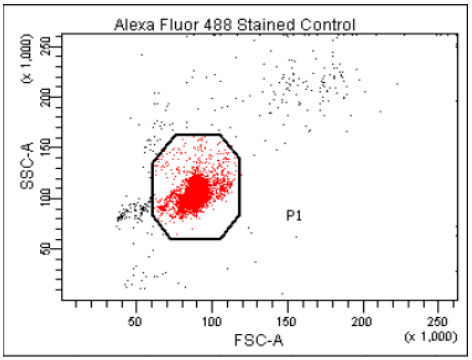
   a. In the Browser, right-click **Cytometer Settings** and select **Application Settings > Save**.

   b. Name the application, then click **OK**.

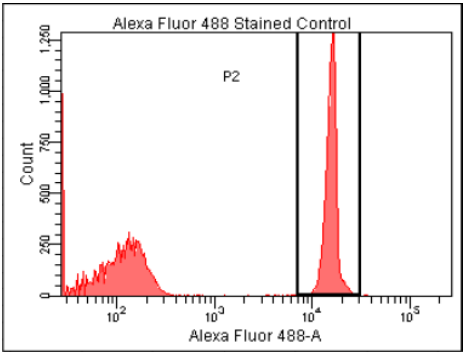
7. Place the tube of Negative beads on the cytometer and acquire data using the Unstained Control worksheet. Adjust FSC and SSC voltages such that the bead population is on scale.

   Note: BD CompBeads require higher FSC voltage settings than cells.

8. Set the P1 gate around the singlet bead population.



9. Record data for the Negative beads and each of the stained BD CompBeads. Make sure to adjust the P2 histogram regions to fit the positive populations.



10. Calculate compensation.

    a. From the **Experiment** menu, select **Compensation Setup > Calculate Compensation.**

    b. Name the compensation setup, then click **Link and Save.**

Note: Do not adjust the fluorescence settings. Adjusting the settings now will invalidate the compensation calculations.

Next step

Proceed to Setting up the workspace for running cells (page 39).

Related documents

See *Getting Started with BD FACSDiva Software* for information about creating and working with experiments.

See the *BD FACSDiva Software Reference Manual* for information about creating compensation controls, creating statistics views, acquiring data, and calculating compensation.

See the *BD Cytometer Setup and Tracking Application Guide* for information about applying application settings.

Setting up the workspace for running cells

Before you begin

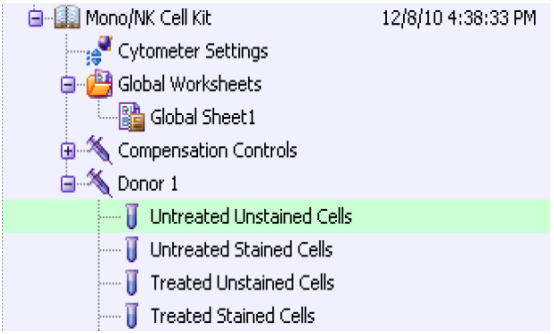
Complete the steps in Running the beads (page 34).

If you have previously saved a template for use with this kit, import the template and proceed directly to Running the cells (page 42).

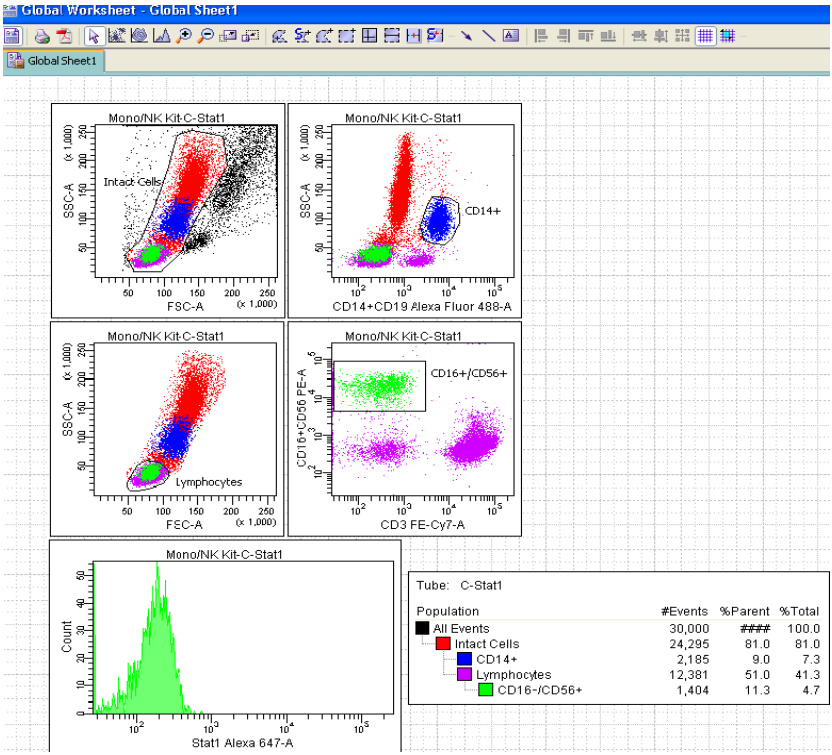
Procedure

To set up the workspace for running cells:

1. Create tubes and label them appropriately as shown in the following figure.



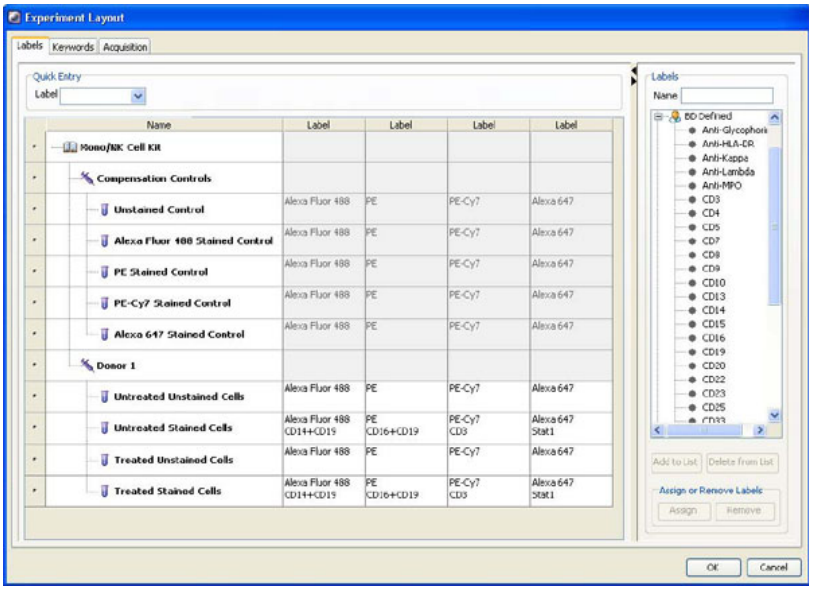
2. Enter the detector voltages for FSC and SSC from step 5 in Running the beads.
3. Toggle to the Global Worksheet and create the plots shown in the following figure.



4. Place a tube of cells stained with the BD Phosflow Human Monocyte/NK Cell (CD14/CD19/CD16/CD56) Antibody Cocktail and BD Phosflow PE-Cy7 Mouse Anti-Human CD3 antibody and Alexa Fluor® 647 anti-phosphoprotein on the cytometer. Acquire sufficient cells to create the gates shown in the preceding global worksheet figure. Label the gates as shown.

5. On the **Labels** tab of the **Experiment Layout** window, enter parameter labels for each marker in the experiment as shown in the following figure.

   Note: For more complex experiments, see Sample and parameter labels (page 50).



6. Save this worksheet as a template for use in future experiments.

Next step

Proceed to Running the cells (page 42).

Related documents

See *Getting Started with BD FACSDiva Software* for information about working in the BD FACSDiva workspace.

See the *BD FACSDiva Software Reference Manual* for information about how to import analysis templates.

Running the cells

Purpose of the procedure

This procedure will instruct you on how to:

- Detect autofluorescence by acquiring data from the unstained cells
- Record data from the Control Cells and the treated and untreated stained cells

Before you begin

Complete the steps in Staining BD CompBeads (page 30) and Setting up the workspace for running cells (page 39).

Procedure

To run the cells:

1. Acquire a sufficient number of events to ensure statistically meaningful data of all cell subpopulations being studied.
2. On the **Dashboard**, ensure that **Storage Gate** is set to record **All Events**.
3. Record data for all tubes.
4. You can use BD FACSDiva software for the analysis, or you can export FCS data files in the appropriate format for your third-party analysis software, such as Cytobank software.

   Note: Cytobank software can open both FCS 2.0 and FCS 3.0 data files.

Data analysis examples

Cytobank software

The experiments shown in the following examples were prepared and acquired as outlined in this manual and analyzed using Cytobank software.

Analysis using Cytobank requires access to the internet and a recommended web browser. Detailed instructions on uploading data and analyzing it using Cytobank software, as well as the appropriate browser recommendation, can be found by clicking the Cytobank link at bdbiosciences.com/phosflow, or going directly to cytobank.org/manual.

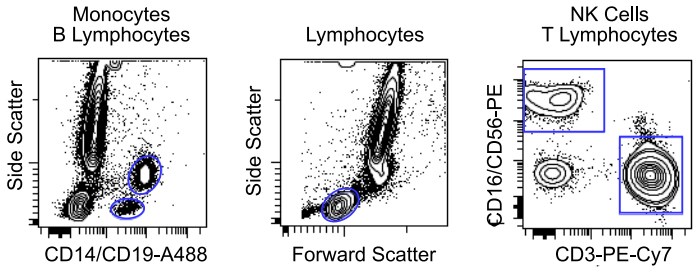
Example of single-activation profile analysis

The following example shows activated $CD14^+$ monocytes and $CD16^+CD56^+$ NK cell signaling profiles for each phosphoprotein marker included in the BD Phosflow Monocyte/NK Cell Activation Kit. The signaling profiles for $CD3^+$ T cells and $CD19^+$ B cells are shown for comparison. The whole blood samples were from healthy human donors.

Note: Phosphoprotein staining may vary depending on the cell sample source and treatment.

Gating strategy

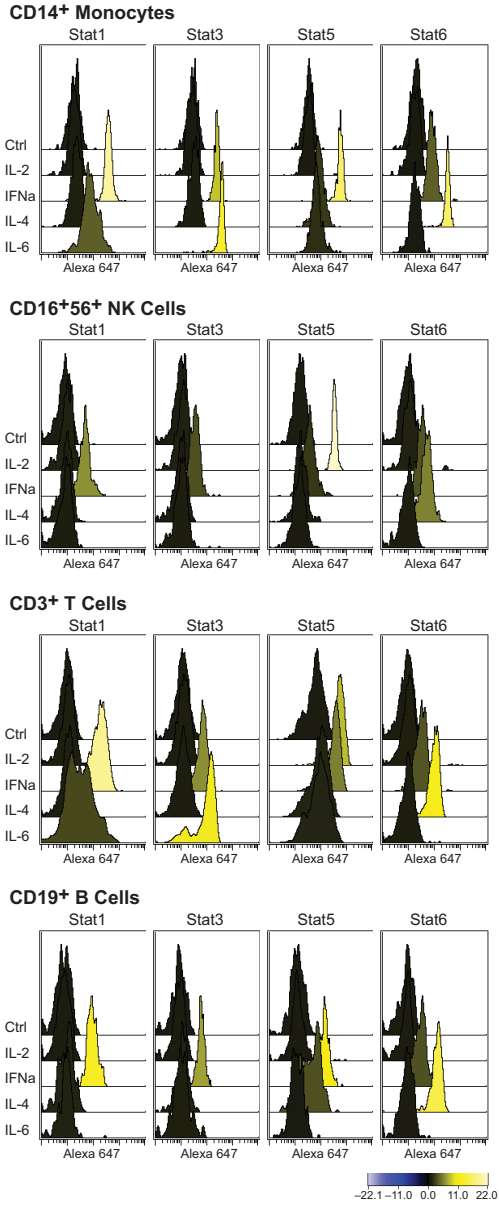
The first plot shows gates for CD14$^+$ monocytes and CD19$^+$ B cells using side-scattered light signals to identify peripheral blood cell populations (left). Next, a lymphocyte gate based on forward- and side-light scatter (center) was used in conjunction with CD markers to identify intact CD16$^+$CD56$^+$ NK cells and CD3$^+$ T cells (right).



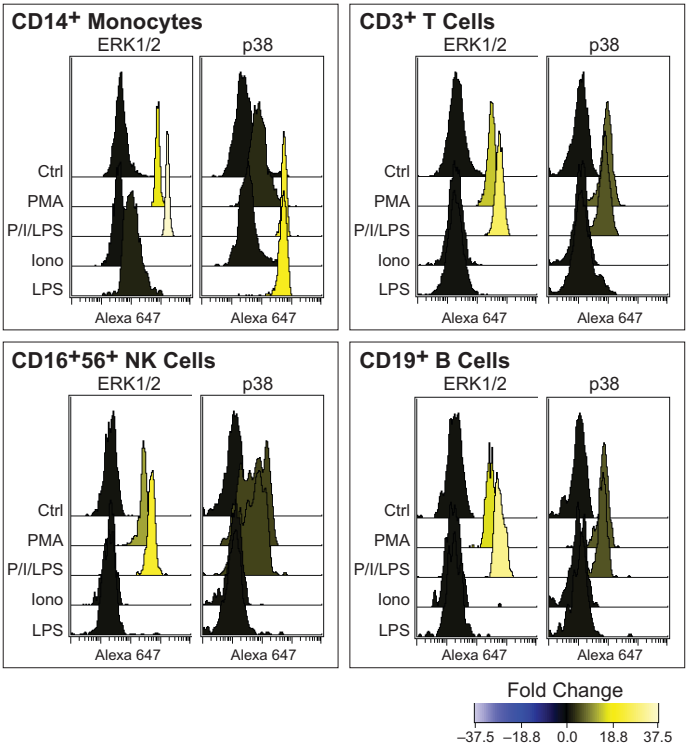
Activation profiles

The following figure shows the phosphorylated STAT activation profiles for CD14$^+$ monocytes, CD16$^+$CD56$^+$ NK cells, CD3$^+$ T cells, and CD19$^+$ B cells. The data are displayed as histogram overlays using Cytobank software. These histograms show the signaling response of each phosphorylation marker induced by the corresponding response modifier as listed in the following table.

Phosphorylation marker	Response modifier
Stat1 (pY701)	Human IFN-α
Stat3 (pY705)	Human IL-6
Stat5 (pY694)	Human IL-2
Stat6 (pY641)	Human IL-4



The next figure shows the phosphorylated ERK1/2 and p38 MAPK activation profiles for $CD14^+$ monocytes, $CD16^+CD56^+$ NK cells, $CD3^+$ T cells, and $CD19^+$ B cells. The data are displayed using the described gating strategy to show histogram overlays using Cytobank software. These histograms show the signaling response of each phosphorylation marker induced by the corresponding response modifiers: PMA, PMA+Ionomycin+LPS, Ionomycin, and LPS.



Example of dose-response analysis

The following example shows a phosphorylated Stat5 signaling response to various doses of IL-2 in $CD14^+$ monocytes, $CD16^+CD56^+$ NK cells, lymphocytes, and $CD3^+$ T cells. Data were collected using a BD FACSCanto II flow cytometer and analyzed and displayed in a variety of ways using Cytobank software.

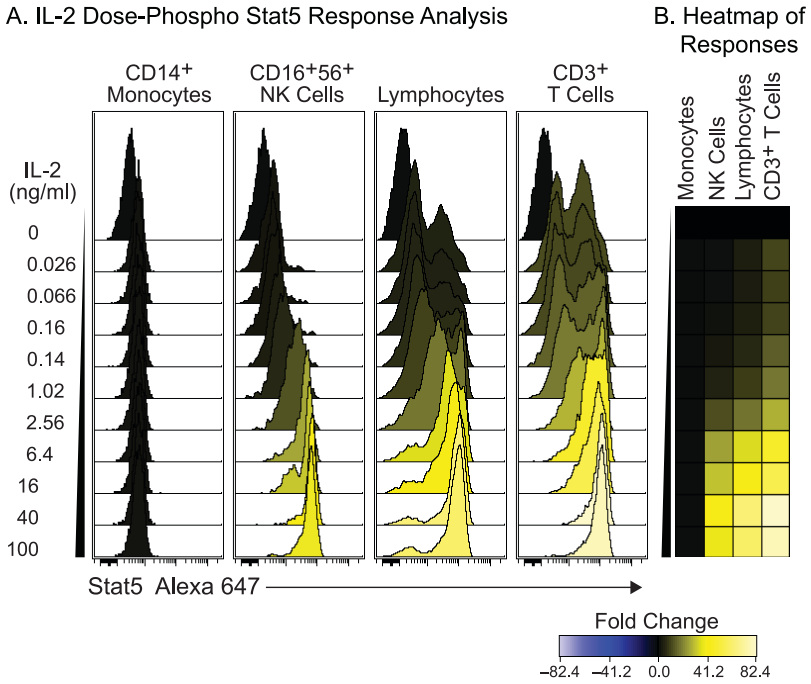
Note: The fluorescence intensity frequency distributions of phosphoprotein-positive and -negative cell populations in a sample may vary depending on the cell sample source and treatment.

Gating strategy

The gating strategy used is the same as shown in Gating strategy (page 44).

Dose-response analysis

The following series of plots shows IL-2 dose-phosphorylated Stat5 response analysis of the various leucocyte subsets. The histograms (A) and heatmap (B) depict a comparison of phosphorylated Stat5 staining to show that the various leucocyte subsets have a differential sensitivity to IL-2. The heatmap shows the increase in phosphorylated Stat5 response to varying doses of IL-2, as represented by the change in color.



The fold-change values of the p-Stat5 response to IL-2 in the various leucocyte subsets are also generated in tabular form by Cytobank software. These values can be exported to a third-party spreadsheet program for further analysis, eg, exported fold-change values for the various leucocyte subsets can be plotted against varying doses of IL-2 to determine other metrics such as EC_{50} values.

5

Reference

This section covers the following topics:

Sample and parameter labels

Before you begin

A labeling strategy for parameters and samples will expedite data analysis using Cytobank software. We recommend identifying individual parameters, specimens, and tubes with unique, descriptive labels.

Parameter labels

Label each fluorescence parameter with the name of the antibody specificity.

Note: The phosphoprotein should be specifically identified by the parameter label (for example, p-ERK-Alexa Fluor 647, not p-protein-Alexa Fluor 647).

Specimen and tube labels

Label each specimen and tube with text indicating how it is unique within the experiment. Labels should be at least three characters long and should include enough experimental information to uniquely identify each tube. Include one or more of the following:

Condition. Stimuli and inhibitors represent different conditions (eg, IL-2, IL-6, PMA, inhibitor14, drug35). Growth media and states of cells (eg, fresh cells, frozen cells) also represent conditions.

Timepoint. Timepoints are specified durations used in the experiment. Most commonly, these are times that samples were allowed to signal before being fixed (eg, 30 s, 5 min, 4 h).

Dose. Dosage titrations are various specified concentrations used in the experiment (eg, 0.1 ng/mL, 10 ng/mL, or 5 µM).

Individual. Individuals can be different donors or animals (eg, donor3, BALB/c5 for a mouse).

Sample (specimen) type. Types can include major classifications of the samples tested (eg, wild type vs knockout mouse, tumor vs normal, cell lines vs whole blood, spleen, or lymph node).

A good example of a tube label:

01 healthy whole blood donor1 IL-2 10 ng-mL 15 min

Number	Sample type	Individual	Condition	Dose	Timepoint
01	healthy whole blood	donor1	IL-2	10 ng/mL	15 min

Note: Use the **Specimen** and **Tube Name** fields if using BD FACSDiva software or the **Sample Name** field if using BD CellQuest Pro software.

Compensation and control labels

Label samples used for compensation and controls with text indicating how the sample is unique within the experiment. For example:

Compensation samples. Include "comp" in the sample name (eg, comp_A488 or PE).

Lyophilized control cells. Include "lyo" in the name of all controls derived from lyophilized human cells (eg, lyocell control).

Other controls. If other controls or beads are used, include the word "control" or "bead" in the sample name.

Troubleshooting

Recommended actions These are the actions we recommend taking if you encounter the following problems.

Problem	Possible cause	Recommended action
No or incomplete red blood cell lysis	Lyse/Fix buffer diluted in 1X PBS	Use water to dilute the 5X Lyse/Fix Buffer to 1X.
	Inadequate mixing with blood	Immediately after adding the Lyse/Fix Buffer to blood samples, mix thoroughly by inverting the tubes at least 5 to 10 times.
	Inadequate incubation time	After mixing the Lyse/Fix Buffer with blood samples, incubate them for 10 to 12 minutes at 37°C.
	Improper whole blood to Lyse/Fix ratio	Use 10 times the volume of whole blood for the Lyse/Fix step.
No or poor activation	No biological response modifier added	Add the appropriate amount of biological response modifier.
	Biological response modifier too old, expired, or lost activity	Prepare fresh biological response modifier and store as aliquots. Use new aliquots for all experiments. Avoid repeated freeze/thaw cycles.
	No carrier protein in the dilution buffer for cytokines	Use a buffer (such as PBS) containing at least 1 mg/mL of BSA to dilute mitogens and cytokines, and keep the diluted stimulant on ice until use.
	Diluted biological response modifier kept too long before use	We recommend using stimulants within 30 minutes of dilution.
	Inappropriate biological response modifier selected	As a positive control, use the suggested biological response modifier at the recommended concentration, incubation time, and temperature.

Problem	Possible cause	Recommended action
No or poor surface CD marker staining	Cells are exposed to Lyse/Fix Buffer for too long	Incubation time in the Lyse/Fix Buffer should not exceed 15 minutes at 37°C.
	Delay in centrifugation	After the Lyse/Fix step, the Lyse/Fix Buffer should be removed immediately by centrifuging the cells, removing the supernatant, and washing the cells once with 1X PBS.
	Cells are exposed to Perm Buffer IV for too long	Do not exceed the recommended permeabilization time—15 to 20 minutes at room temperature (<5 minutes causes insufficient permeabilization; >20 minutes can cause compromised CD staining and greater cell loss). After the permeabilization step, immediately remove the Perm Buffer IV by centrifuging the cells, removing the supernatant, and washing the cells with the Stain Buffer.
	Inadequate washing	After permeabilization, immediately add the recommended amount of Stain Buffer to the tubes, centrifuge, and follow with an additional wash.
	Residual volume too large after Lyse/Fix and permeabilization steps	Residual volume should be no more than 50 µL after each wash.

References

Related publications

1. Perez OD, Mitchell D, Campos R, Gao GJ, Li L, Nolan GP. Multiparameter analysis of intracellular phosphoepitopes in immunophenotyped cell populations by flow cytometry. *Curr Protoc Cytom*. 2005;Chapter 6:Unit 6.20.
2. Montag DT, Lotze MT. Successful simultaneous measurement of cell membrane and cytokine induced phosphorylation pathways [CIPP] in human peripheral blood mononuclear cells. *J Immunol Methods*. 2006;313:48–60.
3. Montag DT, Lotze MT. Rapid flow cytometric measurement of cytokine-induced phosphorylation pathways [CIPP] in human peripheral blood leukocytes. *Clin Immunol*. 2006;121:215–226.
4. Covey TM, Putta S, Cesano A. Single cell network profiling (SCNP): mapping drug and target interactions. *Assay Drug Dev Technol*. 2010;8:321–343.
5. Critchley-Thorne RJ, Simons DL, Yan N, et al. Impaired interferon signaling is a common immune defect in human cancer. *Proc Natl Acad Sci U S A*. 2009;106:9010–9015.
6. Irish JM, Kotecha N, Nolan GP. Mapping normal and cancer cell signalling networks: towards single-cell proteomics. *Nat Rev Cancer*. 2006;6:146-155.
7. Krutzik PO, Crane JM, Clutter MR, Nolan GP. High-content single-cell drug screening with phosphospecific flow cytometry. *Nat Chem Biol*. 2008;4:132–142.

8. Lee AW, Sharp ER, O'Mahony A, et al. Single-cell, phosphoepitope-specific analysis demonstrates cell type- and pathway-specific dysregulation of Jak/STAT and MAPK signaling associated with in vivo human immunodeficiency virus type 1 infection. *J Virol*. 2008;82:3702–3712.

9. Perez OD, Krutzik PO, Nolan GP. Flow cytometric analysis of kinase signaling cascades. *Methods Mol Biol*. 2004;263:67–94.

10. Schulz KR, Danna EA, Krutzik PO, Nolan GP. Single-cell phospho-protein analysis by flow cytometry. *Curr Protoc Immunol*. 2007;Chapter 8:Unit 8.17.

Notes

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