

## Technical Data Sheet

## Transcription Factor Phospho Buffer Set

## Product Information

Material Number:	<b>563239</b>
Size:	100 Tests
Component:	<b>51-9011462</b>
Description:	0.75X TFP Diluent Buffer
Size:	75 mL (1 ea)
Component:	<b>51-9011319</b>
Description:	4X TFP Fix/Perm Buffer
Size:	25 mL (1 ea)
Component:	<b>51-9011461</b>
Description:	Perm Buffer III
Size:	125 mL (1 ea)
Component:	<b>51-9011463</b>
Description:	5X TFP Perm/Wash Buffer
Size:	150 mL (1 ea)

## Description

Protective immunity depends on coordinated responses between regulatory and conventional T cells and other leucocyte subsets. High resolution flow cytometric analyses of signaling responses made by leucocyte subsets, defined by their coexpressed levels of surface differentiation antigens and specific transcription factors, provides deep insights into the cellular and molecular mechanisms that underlie immune regulation in healthy and diseased states. Historically, disparate buffer compatibility characteristics have created challenges for the simultaneous analysis of these coexpressed biomarkers. The BD Pharmingen™ Transcription Factor Phospho (TFP) Buffer Set is designed to enable robust and simultaneous flow cytometric analysis of various cell surface antigens, transcription factors, and phosphorylated signal proteins in cells derived from both human and mouse tissues. This includes fresh, fixed, and frozen human peripheral blood mononuclear cells.

The BD Pharmingen™ TFP Buffer Set has been successfully applied to the multiparameter flow cytometric analysis of phosphorylated Stat signaling responses to cytokines made by human and mouse T regulatory cell and conventional T cell subsets, and other leucocyte types defined by certain cell surface markers, transcription factors (FoxP3 and T-bet), and physical characteristics. While not all antibody compatibilities are known, cell signaling responses to different stimuli should also be amenable to analysis using this buffer set and antibodies specific for other cell surface markers, transcription factors, and types of downstream phosphorylated signaling proteins. This buffer system is backwards compatible with many BD reagents, including antibodies conjugated with new fluorescent dyes (eg, BD Horizon™ BV421, BV510, PE-CF594, V450, and V500).

4X TFP Fix/Perm Buffer - Contains 15.6% formaldehyde, 5.5% methanol

## 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 the central nervous system. Route of exposure: Oral. May cause respiratory irritation.		

## Precautionary statements

Wear protective gloves / eye protection.	Wear protective clothing.	Do not breathe mist/vapours/spray.
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. IF SWALLOWED: Call a POISON CENTER/doctor if you feel unwell		

Perm Buffer III - Contains 88% methanol

## Hazard statements

Highly flammable liquid and vapor.	Toxic if swallowed, in contact with skin or if inhaled.
Causes damage to the central nervous system. Route of exposure: Oral.	

## Precautionary statements

Keep away from heat/sparks/open flames/hot surfaces. - No smoking.		
Wear protective clothing / eye protection.	Wear protective gloves.	Do not breathe mist/vapours/spray.
IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower. IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.		

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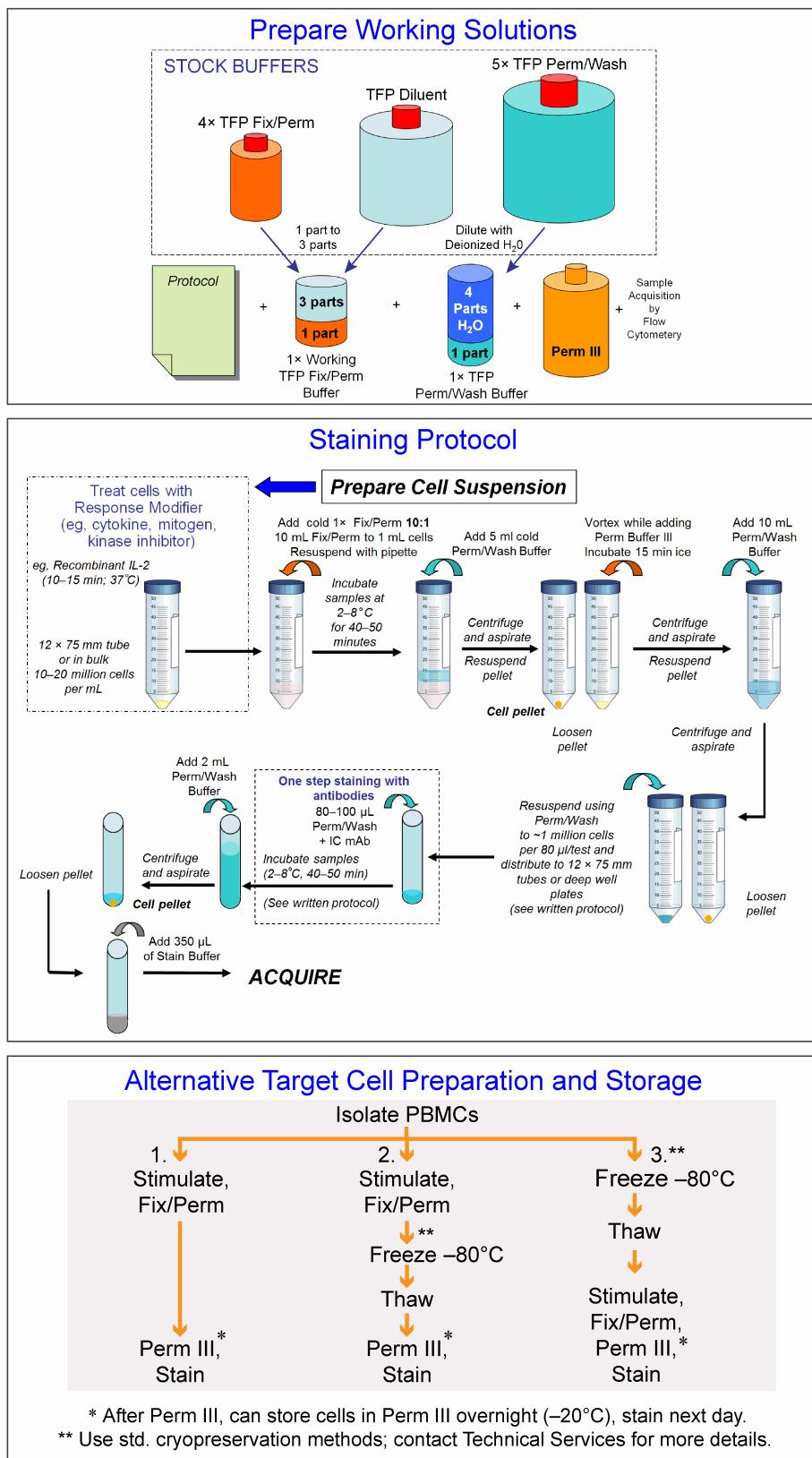
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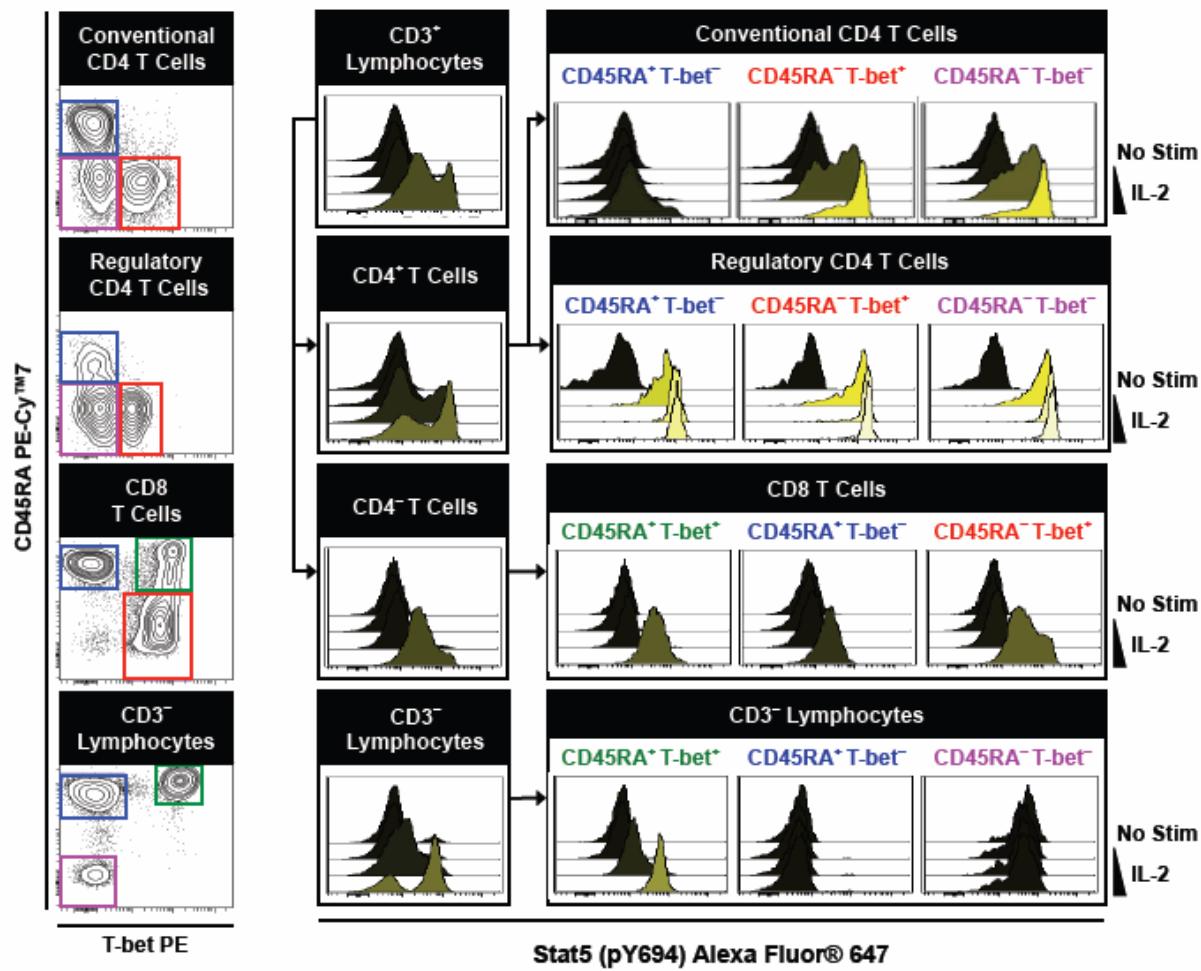
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563239 Rev. 4



## Overview of Buffer Dilution, Cell Preparation, and Staining Protocol





**Multicolor flow cytometric analysis Stat5 (pY694) expression by human T cell subsets in response to different IL-2 doses.** Human peripheral blood mononuclear cells were not cultured (No Stim) or were cultured (IL-2; 37°C for 15 min) with 1, 10, or 100 ng/mL doses of Recombinant Human IL-2 protein (Cat. No. 554603) as indicated. The cells were then fixed, permeabilized, and stained with fluorescent antibodies specific for surface (CD3, CD4, CD8, CD25, CD45RA) and intracellular (FoxP3, T-bet, pStat5 pY694) markers using the BD Pharmingen TFP Buffer Set and protocol. The fluorescence histograms showing Stat5 (pY694) expression were derived from CD3-negative or CD3-positive gated events with CD45RA+ T-bet-, CD45RA- Tbet+, or CD45RA- T-bet- phenotypes and with the forward and side light-scatter characteristics of intact lymphocytes as indicated.

## Application Notes

### Recommended Assay Procedure:

#### Protocol Outline

1. Cell Treatment: Prepare single cell suspension and pellet cells by centrifugation. Resuspend cells in 50-100 µL/test of 1× Dulbecco's PBS containing Ca++/Mg++, and then treat the cells (eg, with a response modifier).
2. Fix and permeabilize the cells with BD Pharmingen™ Transcription Factor Phospho (TFP) Buffer Set (Cat. No. 563239/565575).
3. Wash cells using 1× TFP Perm/Wash Buffer. Pellet cells by centrifugation and aspirate supernatants. Do not leave residual buffer supernatant
4. Incubate cells with BD Phosflow™ Perm Buffer III, pellet cells, and remove Perm Buffer III supernatant.
5. Wash cells with 1× TFP Perm/Wash Buffer and stain cells with fluorescent antibodies. Wash the cells with 1× TFP Perm/Wash Buffer.
6. Resuspend cells in BD Pharmingen™ Stain Buffer (BSA) (Cat. No. 554657) and analyze by flow cytometry.

#### General Comments

- The entire protocol is performed at 2-8°C with two exceptions:
  - During the treatment of fresh cells in 1× DPBS with response modifier
  - When using Perm Buffer III which has been stored at -20°C.
- Cool the centrifuge and prepare fluorescent antibody cocktails and other solutions prior to starting the protocol.
- Prepare the 1× TFP Perm/Wash Buffer from the 5× stock TFP Perm/Wash Buffer solution by diluting with filtered, deionized water.
- Also, prepare the 1× TFP Fix/Perm Buffer 10-15 minutes prior to use. Mix 4× TFP Fix/Perm Buffer stock with 0.75× TFP Diluent to make 1× TFP Perm/Wash Buffer (eg, use 10 ml of 4× TFP Fix/Perm Buffer stock and mix with 30 ml of 0.75× TFP Diluent).

- The time interval for staining cells with antibodies can likely be limited to 30 min. However, the original staining protocol was developed using at least 40 minute-incubation times.
- Keep centrifuge at 2-8°C, and keep centrifuge speeds constant (380 g prior to cellular fixation, 500 g after fixation).
- There is no permeabilization incubation time introduced using the Perm/Wash Buffer. It is not necessary.
- This protocol is not optimized for use with whole blood.

## Materials and Equipment

- 1× Dulbecco's PBS (DPBS) containing Ca<sup>++</sup>/Mg<sup>++</sup>
- 1× Dulbecco's PBS (DPBS) containing without Ca<sup>++</sup>/Mg<sup>++</sup>
- BD Pharmingen™ Transcription Factor Phospho Buffer Set (Cat. No. 563239/565575)
- BD Pharmingen™ Stain Buffer (BSA) (Cat. No. 554657)
- 50 or 15 mL sterile polypropylene conical centrifuge tubes
- 37°C incubator or water bath
- Temperature controlled centrifuge capable of 200-600 g and accommodating flow cytometry tubes, 96 deep-well plates, and 15 and 50 mL conical tubes
- Samples: Peripheral blood mononuclear cells (PBMC) 1-2 million cells per test for bulk processing and flow cytometry tube. PBMC 0.75-to-1.0 million cells per test for HTS in deep-well plates and 96-well round bottom plates for HTS on the flow cytometer
- Heat-inactivated FBS with 7.5% DMSO freezing solution
- Flow cytometry tubes or 2.2 mL Storage Plate, MARK II (96 Deep Well; Abgene Cat. No. AB0932) and 96-well tissue culture plates
- Fluorescent antibodies specific for intracellular and cell surface antigens
- Multichannel pipettes that can deliver 20 µL, 200 µL or 1,000 µL volumes

## Staining Cells in Tubes

1. **Treat cell preparations** (human or mouse) with a response modifier, such as, a cytokine at suboptimal or saturating (eg, 50-100 units per mL) doses of interest, using a **1 mL** suspension of 10-20 million cells (10 tests) in 1× DPBS with Ca<sup>++</sup>/Mg<sup>++</sup>. *Note: Keep this volume of DPBS low, it is important to the intended effect of the 1× TFP Fix/Perm Buffer. Use a 50 mL polypropylene conical centrifuge tube for bulk cell treatment.*
2. **Incubate 15 min at 37 °C**, exactly, or use another specific treatment condition as desired.
3. **Stop cell treatment** by adding **1× TFP Fix/Perm Buffer** to the cells. *Note: Slowly add **10 mL** of 1× TFP Fix/Perm Buffer (at 2-8°C). Cap and invert tubes several times.*
4. Incubate for 50 min at 2-8°C to fix and permeabilize the cells.
5. Add 5 mL of 1× TFP Perm/Wash Buffer.
6. Centrifuge cells at 500 g (15 min at 2-8 °C).
7. Aspirate supernatants away from pelleted cell samples, wash cells once with 10 mL **1× TFP Perm/Wash Buffer**.
8. Centrifuge cells at 500 g (10 min); aspirate all residual supernatant volume away from pelleted cells.
- Long Term Storage Option:** *After step 8, can resuspend cells in 200-500 µL FBS with 7.5% DMSO and freeze at -80°C in cryopreservation tubes. Thaw by removing cells from freezer, adding equal volumes of cold TFP Perm/Wash Buffer, and thaw in cold water bath. Wash once with TFP Perm/Wash Buffer, and then proceed with the protocol from step 9.*
9. Add **1.0 mL Perm Buffer III** (or other desired volume if doing in bulk) **per 1-2 million cells**  
*Notes: i) Perm Buffer III is taken from a -20°C freezer. ii) Cell pellets become translucent during this step.*
10. Incubate for **20 min on ice** to allow exposure of the phosphorylated protein epitopes.
- Short Term Storage Option #2:** *Store overnight at -20°C. Cover tubes with Parafilm® and protect from moisture. Continue with protocol next day. If choosing to store samples in this manner, remove to 2-8 °C and allow to warm for 5-10 minutes and proceed to step 11.*
11. Centrifuge at 500 g (8 min, at 2-8°C) and carefully aspirate Perm Buffer III being sure to remove all residual volume.
12. Wash once with **1× TFP Perm/Wash Buffer**. Use 10 mL when using 50 mL conical tubes in bulk or 2 mL if using flow cytometry tubes; remove all residual buffer from the cell pellet after centrifugation. Add **80-90 µL 1× TFP Perm/Wash Buffer** per test and resuspend the cell pellets in preparation for staining. *Optional: Repeat washing step to be sure to remove all Perm Buffer III. Failing to wash the methanol out well enough prior to staining can lead to destruction of protein fluorochromes and tandem dyes (eg, PerCP, PE, PE-Cy™).*
13. **Staining with fluorescent antibodies:** *At this time it may be necessary or desirable to aliquot the cell samples into flow cytometry tubes for staining with different panels of fluorescent antibodies.*
  - a. **Intracellular Staining:** Add antibodies specific for intracellular target antigens such as FoxP3, T-bet, phosphorylated Stat proteins, or other intracellular molecules as desired.  
*Note: It is known that when staining cells in TFP Perm/Wash Buffer, lower concentrations of antibodies are needed for intracellular staining, eg, 0.5× or 0.25× test size (usually 2.5 µL of a 5 µL test size). However, fluorescent antibodies specific for phosphorylated Stat proteins should be used at the stated test size on the vial.*  
*Note: When staining Human FoxP3, antibody clone 236A is recommended for best results.*
  - b. **Staining Surface Molecules:** Prepare a cocktail of fluorescent antibodies specific for staining cell surface antigens in 1× TFP Perm/Wash Buffer or Stain Buffer (BSA). For example, dilute fluorescent surface antibodies at a 10 µL:1000 µL dilution to make a cocktail of 50 tests at 20 µL per test.
14. Incubate for 40-50 min at 2-8°C
15. Wash cells once with 2 mL **1× TFP Perm/Wash Buffer**, centrifuge cell suspensions at **500 g** for 5 min at 2-8°C, and aspirate supernatants away from the pelleted cells.
16. Resuspend cells in 350 µL Stain Buffer (Cat. No. 554657). *Note: do not resuspend cells in 1× TFP Perm/Wash Buffer!*

17. Acquire a sufficient number of events by flow cytometry to obtain statistically significant results, eg,  $\geq 15,000$  CD4+ or  $\geq 150$  FoxP3+ events, as the case may be. *Due to the fixation and permeabilization procedure, forward and side light-scatter signals can be slightly different than those of live cells.*

### Staining Cells in 96-Well Plates

*Use 2.2 mL Storage Plate, MARK II (96 Deep Well; Abgene Cat. No. AB0932) and 96-well tissue culture plates*

1. Treat cell preparations (human or mouse) with response modifiers (as above) in 96 deep-well plate using cells at 0.5-1.0 million cells/well in 1× DPBS with Ca++/Mg++. For example: start with 50  $\mu$ L of cells at 10-20 million cells/mL (0.5-1 million cells/well) and add 20  $\mu$ L of response modifier (diluted in DPBS + 1 mg/mL BSA), for a final cell + response modifier volume of 70  $\mu$ L.
2. Incubate 15 min at 37 °C, exactly, or use another specific treatment condition as desired.
3. **Stop cell treatment by adding 1× TFP Fix/Perm Buffer** to the cells. Use a 1.2 mL multichannel pipette to add 1.0 mL of cold 1× TFP Fix/Perm Buffer. Mix thoroughly by pipetting up and down several times.
4. Incubate for 50 min at 2-8°C to fix and permeabilize the cells.
5. Add 350  $\mu$ L of 1× TFP Perm/Wash Buffer to each well.
6. Centrifuge cells at **500 g** for 8 min at 2-8°C.
7. Aspirate supernatants from samples and wash cells once with 700  $\mu$ L of 1× TFP Perm/Wash Buffer.
8. Centrifuge cells at **500 g** for 8 min and aspirate all residual supernatant volume away from pelleted cells.

**Long Term Storage Option:** *After step 8, resuspend cells in each well with 200-500  $\mu$ L FBS with 7.5% DMSO and freeze at -80°C. Thaw by removing cells from freezer, adding 1 mL cold TFP Perm/Wash Buffer, and thawing plate in a cold water bath. Wash cells with TFP Perm/Wash Buffer one additional time, and then proceed with the protocol from step 9.*

9. Before adding any buffer to the plate, vortex to resuspend the cells. Add **500  $\mu$ L of Perm Buffer III** per well, and pipette up and down to mix.  
*Notes: i) Perm Buffer III is taken from -20 °C freezer prior to use. ii) Cell pellets turn translucent during this step.*
10. Incubate for **20 min on ice** to allow exposure of the phosphorylated epitopes.

**Short Term Storage Option:** *Store overnight at -20°C. Cover plate with lid, secure it with Parafilm® and protect from moisture. Continue with protocol next day. If choosing to store samples in this manner, remove plate to 2-8 °C and allow to warm for 5-10 minutes and proceed to step 11.*

11. Centrifuge cells at **500 g** for 8 min at 2-8 °C, carefully aspirate Perm Buffer III supernatant away from pelleted cells.
12. Wash once with **1× TFP Perm/Wash Buffer**, 1 mL each per well and centrifuge; remove all residual buffer.  
*Optional: Repeat washing step to be sure to remove all Perm Buffer III. Failing to wash the methanol out well enough prior to staining can lead to destruction of the protein fluorochrome and tandem dyes (eg, PE, PerCP, PE-Cy™7, etc.). Resuspend cells in 100  $\mu$ L **1× TFP Perm/Wash Buffer**.*

### 13. Staining with fluorescent antibodies:

- a. **Intracellular Staining:** Add antibodies specific for intracellular target antigens such as FoxP3, T-bet, phosphorylated Stat proteins or other intracellular molecules as desired.

*Note: It is known that when staining cells in TFP Perm/Wash Buffer, lower concentrations of antibodies are needed for intracellular staining, eg, 0.5× or 0.25× test size (usually 2.5  $\mu$ L of a 5  $\mu$ L test size). However, fluorescent antibodies specific for phosphorylated Stat proteins should be used at the stated test size on the vial.*

*Note: When staining Human FoxP3, antibody clone 236A is recommended for better results*

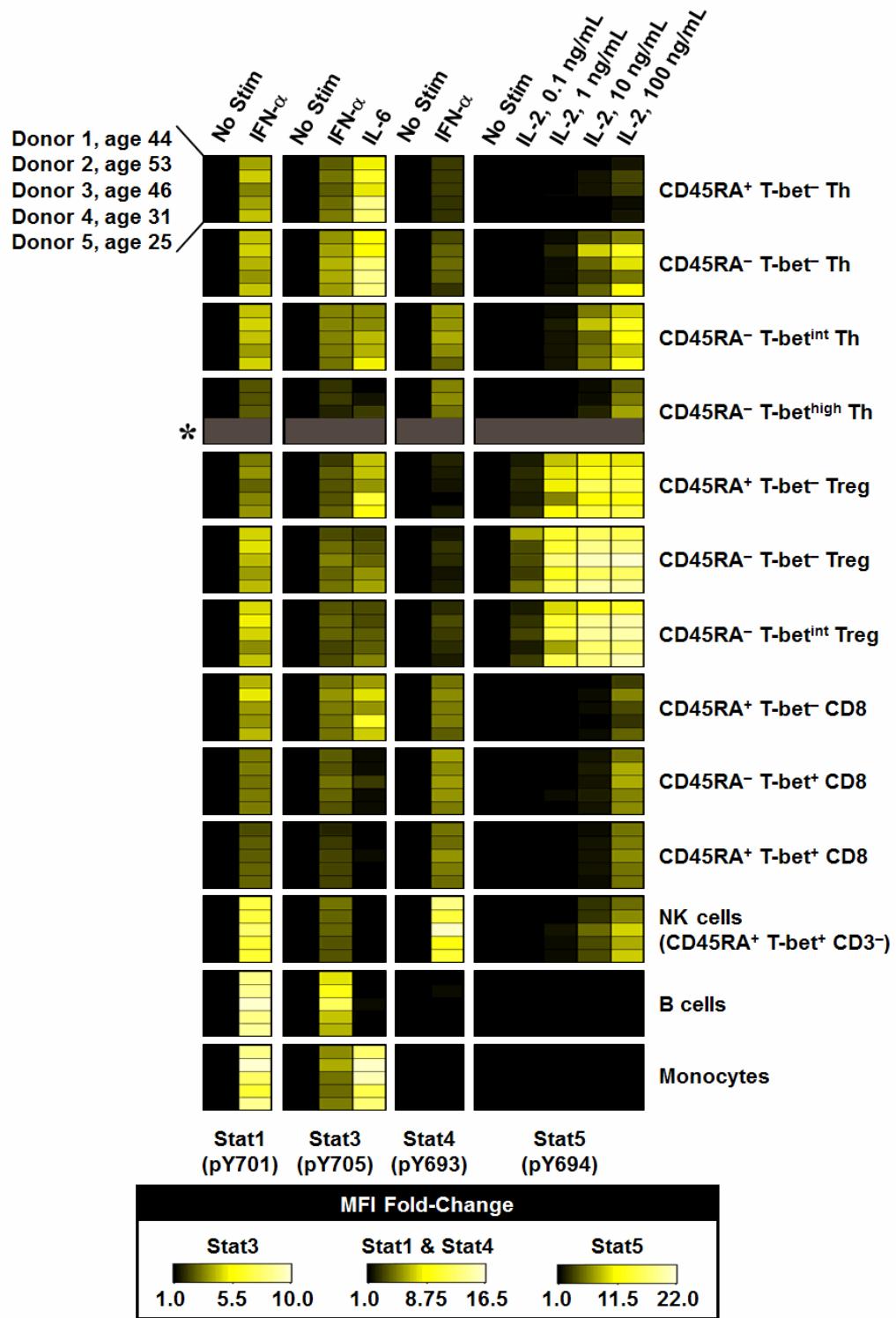
- b. **Staining Surface Molecules:** Prepare a cocktail of fluorescent antibodies specific for staining cell surface antigens in **1× TFP Perm Wash Buffer** or **Stain Buffer (BSA)**. *For example, dilute fluorescent surface antibodies at a 10  $\mu$ L:1000  $\mu$ L dilution to make a cocktail of 50 tests at 20  $\mu$ L per test. Add this test volume to the tube after adding the intracellular antibodies.*

14. Incubate for 40-50 min at 2-8°C
15. Wash cells once or twice with 200  $\mu$ L TFP Perm/Wash Buffer, centrifuge at **500 g** for 5 min at 2-8°C; carefully aspirate sample supernatants.  
*Transfer cells to standard, round-bottom 96-well plates for acquisition.*
16. Resuspend pelleted cells in 200  $\mu$ L Stain Buffer (BSA) (Cat. No. 554657). *Note: do not resuspend in 1× TFP Perm/Wash Buffer!*
17. Acquire a sufficient number of events in order to obtain statistically significant results, eg,  $\geq 15,000$  CD4+ or  $\geq 150$  FoxP3+ events, as the case may be.

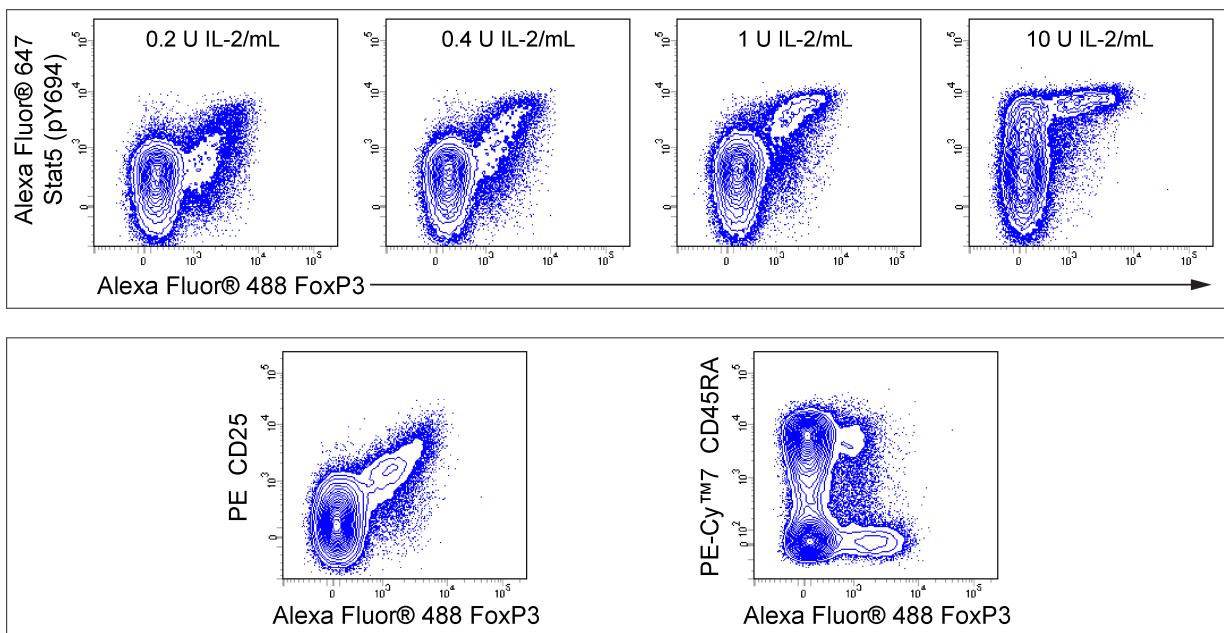
**Antibodies Tested as Compatible for Staining using the BD Pharmingen™ Transcription Factor Phospho Buffer Set**

<b>Description</b>	<b>Clone</b>	<b>Reactivity</b>	<b>Formats Tested</b>	<b>Cat. No.</b>
CD3	SK7	Hu	PE	340662
CD3	UCHT1	Hu	PerCP-Cy5.5, BD Horizon™ BV421 and V450	560835, 562427, 560365
CD4	SK3	Hu	BV510, PerCP-Cy5.5	562970, 562971, 560650
CD20	HI (FB1)	Hu	Alexa Fluor® 700	561171
CD21	B-ly4	Hu	APC	559867
CD25	2A3, MA251	Hu	PE, BD Horizon™ BV421	564033, 557138, 341010, 564033, 562442
CD45RA	HI100	Hu	PE-Cy™7	560675
CD3	17A2	Ms	APC-Cy™7, BV421	560590, 564008
CD4	RM4-5	Ms	PerCP-Cy5.5	553050
CD25	3C7	Ms	BV421	564370
Stat1 (pY701)	4a	Hu, Ms	Alexa Fluor® 647	612597
Stat3 (pY705)	4/P-STAT3	Hu, Ms	Alexa Fluor® 647	557815
Stat4 (pY693)	38/p-Stat4	Hu, Ms	Alexa Fluor® 647	558137
Stat5 (pY694)	47/Stat5(pY694)	Hu, Ms	Alexa Fluor® 647	562076, 612599
Stat6 (pY641)	23/Stat6, 18/P-Stat6	Hu, Ms	Alexa Fluor® 647	560000, 612601
CD247, CD3 Zeta (pY142)	K25-407.69	Hu	Alexa Fluor® 647	558489
ZAP-70 (PY319)/Syk (PY352)	17A/P-ZAP70	Hu	Alexa Fluor® 647	557817
SLP-76 (pY128)	H3	Hu	Alexa Fluor® 647	560057
AKT (pS473)	M8961	Hu	Alexa Fluor® 647	560343
LAT (pY171)	158-1169	Hu	Alexa Fluor® 647	559518
ERK 1/2 (pT202/pY204)	20A	Hu	Alexa Fluor® 647	561992
Ki-67	B56	Hu	Alexa Fluor® 647	561126
FOXP3	259D/C7	Hu	Alexa Fluor® 488, PE	560047, 560046
FOXP3	236A/E7	Hu	Alexa Fluor® 488, PE	561181, 560852
FOXP3	MF23	Ms	Alexa Fluor® 488, PE	560407, 561181
T-bet	O4-46	Hu, Ms	PE, PE-CF594	561268, 562467
GATA3	L50-823	Hu	PE	560074
EZH2 PE	11/EZH2	Hu	PE	562478
BMI-1	P51-311	Hu	PE	562528
Pax-5	1H9	Hu	PE	562688
Fixable Viability Stain 450	N/A	Hu	BD Horizon™ V450	562247

All formats of the antibodies listed were tested and found to work in staining cells using the BD Pharmingen™ Transcription Factor Phospho Buffer Set. This buffer is likely to work with other formats of the same clones as well as other antibodies. However, they have not been tested.



**Multicolor flow cytometric analysis of cytokine signaling responses in human T-cell and other leucocyte subsets defined by the correlated expression of FOXP3+ and T-bet+ or other markers.** Human peripheral blood mononuclear cells were treated with 100 ng/mL of recombinant human  $\text{IFN-}\alpha$ ; 100 ng/mL  $\text{IL-6}$ ; or 0.1, 1, 10, or 100 ng/mL of  $\text{IL-2}$  for 15 minutes at 37°C and then fixed, permeabilized, and stained using the BD Pharmingen TFP Buffer Set as described in the protocol. Leucocyte subsets were identified by the correlated expression of FoxP3 and T-bet, and additional phenotypic markers specific for Th, Treg, NK, CD8 T cells, B cells, and monocytes using surface antibodies (Anti-Human CD45RA, CD3, CD4, CD20, and/or CD25). Patterns of Stat1, Stat 3, Stat 4, Stat 5 phosphorylation in response to cytokine stimulation are shown across leucocyte subsets in five donors. Heat maps display fold-change in phosphorylated STAT staining in response to stimulation. \* Not detected, <50 events acquired.



**Multicolor flow cytometric analysis of surface and intracellular markers coexpressed by previously-frozen and thawed human CD4+ T cells.**

**Top Panel - IL-2-induced pSTAT5 (pY694) signaling responses by human CD4 T cell subsets defined by FoxP3.** Frozen human peripheral blood mononuclear cells were thawed and cultured (37°C, 15 min) with 0.2, 0.4, 1 and 10 U/mL (or 12, 24, 61, 611 pg/mL, respectively) of recombinant human IL-2 protein. The cells were then fixed, permeabilized, and stained with fluorescent antibodies specific for CD3, CD4, FoxP3, and phosphorylated Stat5 (pY694) using the BD Pharmingen TFP Buffer Set and protocol. Two-color flow cytometric contour plots showing the correlated expression of FoxP3 versus phosphorylated Stat5 (pY694) were derived from CD3+ CD4+ gated events with the forward and side light-scatter characteristics of intact lymphocytes.

**Bottom Panel - CD4 T cell subsets defined by surface and intracellular markers.** Frozen human peripheral blood mononuclear cells were thawed and then fixed and permeabilized using the BD Pharmingen TFP Buffer Set and protocol. Cells were labeled with surface (Anti-human CD4, CD45RA, and CD25) and intracellular (Anti-FoxP3) antibodies after treatment with Perm III. Two-color flow cytometric contour plots showing the correlated expression of FoxP3 versus CD25 (Left Plot) or CD45RA (Right Plot) were derived from CD3+ CD4+ gated events with the forward and side light-scatter characteristics of intact lymphocytes.

Data courtesy of Yang, Hsiao-Mien, King College London, UK.

## Product Notices

1. Please refer to [www.bdbiosciences.com/pharmingen/protocols](http://www.bdbiosciences.com/pharmingen/protocols) for technical protocols.
2. Alexa Fluor® is a registered trademark of Molecular Probes, Inc., Eugene, OR.
3. Source of all serum proteins is from USDA inspected abattoirs located in the United States.
4. Caution: 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.
5. Cy is a trademark of GE Healthcare.

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