

**BD Pharmingen™**

# Intracellular Cytokine Staining Starter Kit - Human Instruction Manual

Catalog No. 559302



*Falcon® is a registered trademark of Corning Incorporated.  
Ficoll-Paque is a trademark of GE Healthcare.*

*BD flow cytometers are Class 1 laser products.*

*For Research Use Only. Not for use in diagnostic or therapeutic procedures.*

*© 2014 Becton, Dickinson and Company. All rights reserved. No part of this publication may be reproduced, transmitted, transcribed, stored in retrieval systems, or translated into any language or computer language, in any form or by any means: electronic, mechanical, magnetic, optical, chemical, manual, or otherwise, without prior written permission from BD Biosciences.*

*Purchase does not include or carry any right to resell or transfer this product either as a stand-alone product or as a component of another product. Any use of this product other than the permitted use without the express written authorization of Becton, Dickinson and Company is strictly prohibited. BD, BD Logo and all other trademarks are property of Becton, Dickinson and Company. © 2014 BD*

## Kit Contents

**Note:** Store the following items at 4°C.

- **BD Pharmingen™ Stain Buffer:** 1 vial, 125 ml
- **BD Cytofix/Cytoperm™ Buffer:** 1 vial, 25 ml
- **BD Perm/Wash™ Buffer:** 1 vial, 25 ml
- **PE-conjugated anti-human IL-2:** 1 vial (25 tests)
- **PE-conjugated anti-human IFN-γ:** 1 vial (25 tests)
- **PE-conjugated anti-human TNF:** 1 vial (25 tests)
- **PE-conjugated isotype control cocktail:** 1 vial (25 tests)
- **Purified blocking antibody cocktail:** 1 vial (15 tests)
- **Staining Kit Manual**

**Note:** These items are shipped separately and should be stored at -80°C.

- **Leukocyte Activation Cocktail:** 2 vials, 100 µl each
- **HiCK-1 Cytokine Positive Control Cells:** 1 vial

### Disclaimer:

Use of these products to measure activation antigens expressed on mononuclear cell subsets for the purpose of monitoring immunoregulatory status can fall under one or more claims of the following patents: US Patent Nos. 5,445,939, 5,656,446, 5,843,689; European Patent No. 319,543; Canadian Patent No. 1,296,622; Australian Patent No. 615,880; and Japanese Patent No. 2,769,156

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

# Table Of Contents

Introduction.....5

Starter Kit Contents.....6

    Cell Activation Reagents and Controls.....6

    Buffers for Intracellular Staining.....7

    Antibodies for Intracellular Staining.....7

Warnings and Precautions.....8

Cell Preparation and Activation.....9

    Isolation of human PBMCs.....10

    Activation of human PBMCs.....11

    Appendix A.....12

Intracellular Staining Procedure.....13

    Resuspension of the HiCK-1 Positive Control Cells.....13

    Intracellular Staining Protocol.....14

    Appendix B.....16

    Helpful Tips on Intracellular Staining.....17

Flow Cytometric Acquisition and Analysis of Data.....17

    Technical Tips.....18

    Appendix C.....19

Intracellular Staining Possibilities (Going beyond this Kit) .....22

References.....24

# Introduction

Improved methods have continually been sought to analyze cytokine-producing cells at the single cell level. Techniques for analyzing individual cytokine-producing cells include immunohistochemistry, immunocytochemistry, ELISPOT, *in-situ* hybridization, limiting dilution analysis, and single cell PCR.<sup>1</sup> All of these techniques have their respective advantages, but also significant drawbacks including the requirement for either technical proficiency or tedious data collection and analysis. Flow cytometry, however, is a powerful analytical technique in which individual cells can be simultaneously analyzed for several parameters, including size and granularity, as well as the expression of surface and intracellular markers defined by fluorescent antibodies.<sup>1-5</sup>

Fluorescent anti-cytokine and anti-chemokine monoclonal antibodies have been used extensively for the intracellular staining and multiparameter flow cytometric analysis of individual cytokine-producing cells within purified and mixed cell populations.<sup>1,3-8</sup> Multicolor immunofluorescent staining with antibodies specific for intracellular cytokines and cell surface markers provides a high resolution method to identify the nature and frequency of cells that express cytokines in either a restricted (eg, Th1- versus Th2-like cells) or unrestricted (eg, Th0-like cells) pattern.<sup>9,10</sup> While enabling highly specific and sensitive measurements of several parameters for individual cells simultaneously, this method also has the capacity for rapid analysis of large numbers of cells required for making statistically significant measurements.<sup>2</sup>

Staining of intracellular cytokines depends on the identification of cytokine-specific monoclonal antibodies that are compatible with a fixation-permeabilization procedure.<sup>11-13</sup> Optimal intracellular cytokine staining has been reported using a combination of fixation with paraformaldehyde and subsequent permeabilization of cell membranes with the detergent saponin. Paraformaldehyde fixation allows for the preservation of cell morphology and intracellular antigenicity, while also enabling the cells to withstand permeabilization by detergent.<sup>14</sup> Membrane permeabilization by saponin allows the fluorochrome-conjugated cytokine-specific monoclonal antibody to penetrate the cell membrane, cytosol, and membranes of the endoplasmic reticulum and Golgi apparatus. Critical parameters for intracellular cytokine staining include the following: cell type; activation protocol and cellular response kinetics (important for determining when to harvest cells); the inclusion of a protein transport inhibitor during cell activation; and the choice of an anti-cytokine antibody. Lack of information concerning these parameters has often precluded researchers from utilizing the intracellular staining technique.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

The BD Pharmingen™ Intracellular Cytokine Staining Starter Kit serves as an introduction to the intracellular staining technique. This kit includes all of the reagents and protocols necessary for the researcher to activate human cells in culture and stain these activated cells for intracellular accumulations of IL-2, IFN- $\gamma$  and TNF. These cytokines are representative of the cytokine response profile elicited by short-term activation of T-cell populations. Individually, IL-2 is a powerful cell growth and differentiation factor while IFN- $\gamma$  and TNF both mediate pro-inflammatory cellular responses. Each serves a key role in short- and long-term immune responses to pathogens and are critical mediators of immune function. (Longer or different activation methods may be required to elicit production of other cytokines and chemokines.) A detailed listing of the reagents included in the starter kit and the descriptions of the protocols involved in cell activation and intracellular cytokine staining are included in this manual. Finally, this manual outlines several suggestions for further investigation using the wide variety of reagents available for intracellular staining of cytokines from BD Biosciences.

## Starter Kit Contents

This kit contains the essential reagents for the activation of a human peripheral blood cell population and their subsequent intracellular staining to detect cytokine-producing cells. Positive and negative controls are included to assist the investigator in utilizing this procedure. The reagents provided in the Starter Kit are described below.

## Cell Activation Reagents and Controls

**Leukocyte Activation Cocktail:** The cocktail is a ready-to-use polyclonal cell activation mixture containing a phorbol ester (Phorbol 12-Myristate 13-Acetate, PMA), a calcium ionophore (Ionomycin), and a protein transport inhibitor (BD GolgiPlug™, containing brefeldin A). This mixture is utilized to elicit a primary cytokine response from T cells. Stimulation of cells using the Leukocyte Activation Cocktail will result in cytokine production that is localized in the rough endoplasmic reticulum of cytokine-producing cells. This localization of cytokines is caused by the protein transport inhibitor, brefeldin A.<sup>5,15</sup> The kit includes two vials of the Leukocyte Activation Cocktail, which provides a sufficient amount of reagent to stimulate approximately  $1 - 1.5 \times 10^8$  cells. These vials are shipped separately on dry ice and should be stored at -80°C. (Each vial should be thawed once and used. Repeated freeze/thaw cycles will inactivate the contents.)

**HiCK-1 Cytokine Positive Control Cells:** HiCK-1 Control Cells are an activated and paraformaldehyde-fixed human peripheral blood derived mononuclear cell population shown to express IL-2, IFN- $\gamma$  and TNF using intracellular staining and flow cytometric analysis. The vial contains approximately  $5 \times 10^6$  cells, which is sufficient for 20 tests. This vial is shipped separately on dry ice and should be stored at -80°C.

## Buffers for Intracellular Staining

**BD Pharmingen™ Stain Buffer (FBS)\*:** This buffer is designed for the suspension, washing and flow cytometric analysis of cells for their expressed levels of cell surface antigens. This buffer contains 2% fetal bovine serum\* and 0.09% sodium azide. A 125 ml vial of BD Pharmingen Stain Buffer is included in this kit and should be stored at 4°C.

**BD Cytofix/Cytoperm™ Buffer:** This buffer is a single-step cell fixation and permeabilization reagent designed for intracellular staining. BD Cytofix/Cytoperm contains a mixture of paraformaldehyde and saponin that serves to preserve morphology, fix cellular proteins, and permeabilize the cell for subsequent immunofluorescent staining of intracellular cytokines. A 25 ml bottle of BD Cytofix/Cytoperm Buffer is included in this kit and should be stored at 4°C.

**BD Perm/Wash™ Buffer:** This is a permeabilization and wash buffer that maintains cellular permeability and facilitates intracellular staining. BD Perm/ Wash Buffer consists of a concentrated stock solution (10 $\times$ ) containing both fetal bovine serum\* and saponin and the presence of some amount of precipitate is common. Dilute to 1 $\times$  with deionized H<sub>2</sub>O before use. The presence of precipitate will not effect the performance of the buffer. If desired the precipitate can be removed before use by passing the diluted 1 $\times$  BD Perm/Wash Buffer through a 0.45 micron filter. The BD Perm/Wash Buffer should be stored at 4°C.

\* Source of all serum proteins is from USDA inspected abattoirs located in the United States.

## Antibodies for Intracellular Staining

**PE-conjugated Anti-Human IL-2:** A test-sized dilution of PE (phycoerythrin)-conjugated anti-human IL-2 antibody (clone MQ1-17H12) suitable for intracellular staining. A vial containing 25 tests (20  $\mu$ l/test) of PE-anti-human IL-2 antibody is included in this kit and should be stored at 4°C.

**PE-conjugated Anti-Human IFN- $\gamma$ :** A test-sized dilution of PE-conjugated anti-human IFN- $\gamma$  antibody (clone B27) suitable for intracellular staining. A vial containing 25 tests (20  $\mu$ l/test) of PE-anti-human IFN- $\gamma$  antibody is included in this kit and should be stored at 4°C.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

**PE-conjugated Anti-Human TNF:** A test-sized dilution of PE-conjugated anti-human TNF antibody (clone MAb11) suitable for intracellular staining. A vial containing 25 tests (20 µl/test) of PE-anti-human TNF antibody is included in this kit and should be stored at 4°C.

**PE-conjugated Isotype Control Cocktail:** A test-sized mixture of PE-conjugated isotype control antibodies of irrelevant specificity suitable for determining background staining due to non-specific antibody binding. This vial contains equal amounts of mouse IgG<sub>1</sub> and rat IgG<sub>2b</sub> isotype control antibodies. A vial containing 25 tests (20 µl/test) of this cocktail is included in this kit and should be stored at 4°C.

**Purified Blocking Antibody Cocktail:** A test-sized mixture of three purified and unconjugated anti-cytokine antibodies suitable for use as a specificity control for intracellular staining. This mixture will block the intracellular staining of the PE-conjugated anti-human IL-2, IFN-γ, and TNF antibodies included in this kit. The cocktail is a mixture of anti-human IL-2 (clone MQ1-17H12), anti-human IFN-γ (clone B27), and anti-human TNF (clone MAb11). A vial containing 15 tests (20 µl/test) of this cocktail is included in this kit and should be stored at 4°C.

## Warnings and Precautions

### 1. Danger

BD Cytofix/Cytoperm™ Buffer (Fixation and Permeabilization Solution) contains 4.2% formaldehyde.

### 2. Hazard statements

Harmful 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 respiratory irritation.

### 3. Precautionary statements

Wear protective clothing / eye protection.

Wear protective gloves.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

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 skin irritation or rash occurs: Get medical advice/attention.

4. **HiCK-I Cytokine Positive Control Cells:** This product contains human blood, serum, or cells, a potential biohazardous material. Use universal precautions when handling. Handle as if product were capable of transmitting disease. Material used in this product has been tested using FDA approved methods and found 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.
5. The BD Perm/Wash™ Buffer contains less than 0.1% sodium azide and saponin. 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.
6. Fluorescently conjugated antibodies contain less than 0.1% 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.

## Cell Preparation and Activation

The most critical step for detection of intracellular accumulations of cytokines by intracellular staining is the isolation and activation of a cell population to induce production of the cytokine of interest. Without properly activated cytokine-producing cells, cultured in the presence of a protein transport inhibitor, the ability to detect intracellular accumulations of cytokines in most cases is severely compromised. This kit provides a proven method for the isolation of human peripheral blood mononuclear cells (PBMCs). This kit also provides the methodology and reagents necessary for the stimulation of human PBMCs to produce a variety of cytokines including IL-2, IFN- $\gamma$ , and TNF. The information given in the next section describes the isolation of human PBMCs from whole blood.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

The protocol for activating these cells to induce production and accumulation of IL-2, IFN- $\gamma$ , and TNF is described in **Activation of Human PBMCs**. A recipe for media that is suitable for the culture and *in vitro* activation of human cells is presented in **Appendix A**.

## Isolation of human PBMCs

The human PBMCs isolation protocol described below is a reliable method; alternative procedures for isolation of human lymphocytes may also be useful. On average  $1 - 2 \times 10^6$  cells/ml of blood can be isolated using this procedure. Keep in mind that up to  $5 \times 10^7$  cells can be activated using a single vial of the Leukocyte Activation Cocktail. The volume of human blood recommended below will, on average, yield the maximum number of PBMCs for activation by a single vial of the Leukocyte Activation Cocktail.

### Protocol for Isolation of Human PBMCs

1. Draw 9 ml of human blood per BD Vacutainer® tube (Cat. No. 366480), a total of 5 tubes should be drawn. It is recommended that blood be collected using collection tubes containing sodium heparin.
2. Transfer equal volumes of blood to two 50 ml conical tubes (Falcon® Cat. No. 352098). There should be approximately 20 - 25 ml of blood per tube once transfer is complete.
3. Add an equal amount of room temperature  $1\times$  PBS to each 50 ml conical tube.
4. Add 8 ml of Ficoll-Paque™ density gradient solution (Amersham Biosciences AB, Cat No. 17-0840-02) to four fresh 50 ml conical tubes.
5. Layer diluted blood on top of the Ficoll-Paque™ density gradient, with care being given not to allow blood to pass below or mix with the Ficoll-Paque™ layer. Diluted blood should be transferred evenly to all 4 Ficoll-Paque™ gradient tubes so that they contain similar volumes.
6. Balance the tubes in centrifuge carriers with care being given not to disturb the gradient interface.
7. Centrifuge the gradient tubes at  $150 - 200 \times g$  for 20 minutes at room temperature without braking.
8. Discard the plasma/platelet layer from the top of the density gradient in each tube by aspiration.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

9. Harvest the PBMCs layer off the top of the Ficoll-Paque™ density gradient and pool into a single 50 ml conical tube.
10. Add excess 1× PBS to bring the volume in the 50 ml conical tube containing the isolated lymphocytes to 40 ml.
11. Centrifuge the tube at  $150 - 200 \times g$  for 5 minutes at 10°C with centrifuge braking.
12. Aspirate the supernatant to isolate the cell pellet. Resuspend pellet in 25 ml of complete RPMI (see *Appendix A* for a recipe for complete RPMI useful for this procedure).
13. Centrifuge tube at  $150 - 200 \times g$  for 5 minutes at 10°C with centrifuge braking.
14. Aspirate the supernatant to isolate the cell pellet and proceed to the next section on setting up the cell activation culture.

**Note:** One alternative to the protocol described above can be found in section 7.1 of the Current Protocols in Immunology. (J.E. Coligan, *et al.*, eds Greene Publishing Associates and Wiley-Interscience, New York.)

## Activation of human PBMCs

Once the human PBMCs have been isolated, they will be used in a primary activation culture in order to induce cytokine production. This activation culture will involve stimulating the human PBMCs for 4 hours using the T-cell Activation Cocktail in the presence of a protein transport inhibitor.

### Protocol for Activation of Human PBMCs

1. To the 50 ml conical tube containing the cell pellet obtained from the protocol on isolation of human PBMCs described above, add 10 ml of complete RPMI and thoroughly resuspend the cell pellet.
2. Count the number of cells isolated using a hemacytometer and a light microscope or by a similar method.
3. Add complete RPMI to the cell suspension to bring the cell concentration to  $1 - 2 \times 10^6$  cells/ml.
4. Transfer the cell suspension to a 6-well culture plate (Falcon Cat. No. 353046) in 6 ml aliquots per well. Use only one 6-well culture plate in this procedure. It is not necessary to use every well on the plate if an insufficient number of cells were isolated during the isolation procedure. Discard excess cells.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

5. Rapidly thaw the Leukocyte Activation Cocktail at 37°C in a water bath and add 10 µl/well into the cell suspension in each well of the 6-well culture plate.
- Note:** After use, discard the unused portion of Leukocyte Activation Cocktail.
6. Place the 6-well culture plate at 37°C in a humidified CO<sub>2</sub> incubator for 4 hours.
  7. Remove the plate from the incubator after a 4 hour incubation. Harvest cells from each well by agitation and aspiration using a 10 ml serological pipet (Falcon Cat. No. 357551), or equivalent. Cells should be transferred to a fresh 50 ml conical tube.
  8. Centrifuge cells at 150 - 200 × g for 5 minutes at 10°C.
  9. Aspirate the supernatant and resuspend cells in 20 ml of complete RPMI. Repeat step (8) above.
  10. Discard the supernatant and resuspend cells in BD Pharmingen™ Stain Buffer (FBS) to a final concentration of approximately 2 × 10<sup>7</sup> cells/ml. Use the starting number of cells activated on the plate as an estimate of the current cell number when resuspending cells at this point.
  11. Proceed to the Staining Protocol described in the next section of this manual.

## Appendix A

### Recipe for Complete RPMI

- |  |         |
|--|---------|
| 1. RPMI-1640 (BioWhittaker Cat. No. 04-558B)                                 | 500 ml  |
| 2. Penicillin/Streptomycin/L-Glutamine (100×)<br>(Gibco® Cat. No. 10378-016) | 5 ml    |
| 3. Low IgG Fetal Bovine Serum<br>(HyClone Cat. No. SH30151.03)               | 37.5 ml |
| 4. 2-Mercaptoethanol (1000×)<br>(Gibco® Cat. No. 21985-023)                  | 0.5 ml  |

Mix thoroughly and store at 4°C.

**Note:** Sources for media ingredients are intended as suggestions. Comparable reagents from other sources may be applicable.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

# Intracellular Staining Procedure

Following the primary activation of the human PBMCs, it is now possible to stain the activated cells and the HiCK-1 Cytokine Positive Control Cells for intracellular cytokines. The intracellular staining procedure requires the fixation, permeabilization and subsequent staining of the activated cell population with fluorochrome-conjugated anti-cytokine antibodies. This kit is designed to detect intracellular accumulations of human IL-2, IFN- $\gamma$ , and TNF.

Information on thawing and resuspending the HiCK-1 Positive Control Cells is described in *Resuspension of the HiCK-1 Positive Control Cells*, page 13. The intracellular staining protocol is given in *Intracellular Staining Protocol*, page 14. Appendix B at the end of this section contains a detailed cell staining template for the first experiment. In addition, technical hints on intracellular staining are also provided.

## Resuspension of the HiCK-1 Positive Control Cells

### Thawing and Resuspending the HiCK-1 Positive Control Cells

1. Remove the HiCK-1 cell vial from the -80°C freezer and quickly thaw at 37°C in a water bath (consult the *Technical Data Sheet for the HiCK-1 Positive Control Cells* for safety information).
2. Transfer 0.5 ml of HiCK-1 cell suspension into a 15 ml conical tube and bring the volume to 5.0 ml using the BD Pharmingen™ Stain Buffer. Return the remaining 0.5 ml of HiCK-1 cells to the -80°C freezer for subsequent use.
3. Centrifuge the 15 ml conical tube at 400 - 500  $\times$  g for 5 minutes at 10°C. Aspirate the supernatant and agitate the tube to disrupt the cell pellet.
4. Resuspend the cell pellet in 2.5 ml of BD Pharmingen Stain Buffer and repeat step (3) above.
5. Finally, resuspend the cell pellet in 0.5 ml of BD Pharmingen Stain Buffer. The cells are now ready to be used for intracellular cytokine staining.
6. Proceed to the staining protocol.

**Note:** For optimal results, thaw the HiCK-1 vial immediately prior to beginning the intracellular staining protocol. The human PBMCs activation culture should already be completed by the time the HiCK-1 Cells are being thawed.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

# Intracellular Staining Protocol

## Protocol for Intracellular Staining

1. The activated PBMCs obtained from the stimulation culture that were resuspended in BD Pharmingen Stain Buffer at  $2 \times 10^7$  cells/ml can now be aliquoted and distributed into the microwells of a 96-well plate (Falcon Cat. No. 353072) [see *Appendix B*, page 16].
2. Transfer the activated human PBMCs or HiCK-1 Positive Control Cells in 50  $\mu$ l/well into the 96-well plate. Use only the number of wells necessary. For the first staining experiment using this kit, the staining template in the Appendix for this section can be used to determine the number of sample wells necessary.
3. Add 100  $\mu$ l of BD Pharmingen Stain Buffer to each well. Centrifuge plate at  $400 - 500 \times g$  for 5 minutes at  $10^\circ\text{C}$  to pellet cells.
4. Aspirate supernatant from each sample well. Agitate plate to disrupt cell pellets.
5. Add 100  $\mu$ l of BD Cytotfix/Cytoperm™ Buffer to each sample well. Incubate the plate for 20 minutes at room temperature. This step will fix the human cell morphology and permeabilize the activated cells for subsequent intracellular staining.

**Note:** The procedure can be stopped at this point. Repeat steps 3 and 4. Resuspend the cells in 100  $\mu$ l/well of BD Pharmingen™ Stain Buffer. Cover the plate and store the cells at  $4^\circ\text{C}$  overnight, or up to 3 days. To proceed, repeat steps 3 and 4. Resuspend the cells in 100  $\mu$ l/well of BD Perm/Wash Buffer™ and proceed to step 6.

6. Add 100  $\mu$ l of  $1 \times$  BD Perm/Wash™ buffer to each sample well and centrifuge the plate at  $150 - 200 \times g$  for 5 minutes at  $10^\circ\text{C}$ .

**Note:** Saponin is a reversible permeabilization agent. It is important to also add BD Perm/Wash™ Buffer to those sample wells receiving antibody to ensure permeabilization of the cells during the staining and washing procedure.

7. Aspirate supernatant from each sample well and agitate plate to disrupt cell pellets.
8. Repeat steps (6) and (7) above.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

9. Add Purified Blocking Antibody Cocktail to the desired sample wells in 20 µl aliquots. Additionally add 30 µl of BD Perm/Wash™ buffer to the same sample wells, each well should now have a total volume of 50 µl.

**Note on Blocking Controls:** To test the specificity and non-specific binding of the PE-conjugated anti-cytokine antibodies, it is useful to pre-incubate the cells with excess unconjugated anti-cytokine antibody. Sample wells used for staining with blocking controls will receive both unconjugated and PE-conjugated antibody and have a total final volume of 100 µl. The increased reagent volume in these wells will not affect the staining results. This kit provides an unconjugated antibody blocking control, recombinant proteins can also be used as specificity blocking controls. Some recombinant protein preparations are not useful as specificity blocking controls (eg, mouse and human IFN-γ and IL-12).

10. Add PE-conjugated Isotype Control Cocktail and PE-conjugated anti-cytokine antibodies to the desired sample wells in 20 µl aliquots. Additionally add 30 µl of BD Perm/Wash buffer to the same sample wells, a total volume of 50 µl is added to each well.
11. Add 50 µl of BD Perm/Wash Buffer to the sample wells designated as autofluorescence controls.
12. Incubate the 96-well plate for 15 minutes at room temperature (also can be done on ice).
13. Add 100 µl of BD Perm/Wash Buffer to each sample well and centrifuge the plate at 400 - 500 × g for 5 minutes at 10°C.
14. Aspirate the supernatant and agitate plate to disrupt the cell pellets.
15. Repeat steps (13) and (14) above.
16. Transfer the contents of each well to a correspondingly numbered sample tube (Falcon Cat. No. 352008) using 200 µl of BD Pharmingen™ Stain Buffer. Bring the final volume in each tube to 400 µl using BD Pharmingen Stain Buffer.
17. The samples are now ready for data acquisition and analysis on a flow cytometer. See Flow Cytometric Acquisition and Analysis of Data on page 17 for more details.

**Note:** This intracellular staining protocol can be performed in a 96-well plate (as described above) or in individual tubes. The volume and amount of antibody used for staining should not change. A general description for intracellular staining using tubes can be found at <http://www.bdbiosciences.com/research/multicolor/tools/resources.jsp>

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

## Appendix B

### Staining Template for Staining HiCK-1 and Activated Human PBMCs

Well #	Cell Type	1st Step	Fixation and Permeabilization	Washing Step	IC Staining Step	Final Wash Step
A1	HiCK-1 Cells	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	BD Perm/Wash Buffer	BD Perm/Wash Buffer Washes
A2	HiCK-1 Cells	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Isotype Control Cocktail	BD Perm/Wash Buffer Washes
A3	HiCK-1 Cells	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	PE Anti-human IL-2	BD Perm/Wash Buffer Washes
A4	HiCK-1 Cells	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Purified Blocking Ab cocktail/PE Anti-human IL-2	BD Perm/Wash Buffer Washes
A5	HiCK-1 Cells	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	PE Anti-human IFN- $\gamma$	BD Perm/Wash Buffer Washes
A6	HiCK-1 Cells	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Purified Blocking Ab cocktail/PE Anti-human IFN- $\gamma$	BD Perm/Wash Buffer Washes
A7	HiCK-1 Cells	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	PE Anti-human TNF	BD Perm/Wash Buffer Washes
A8	HiCK-1 Cells	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Purified Blocking Ab cocktail/PE Anti-human TNF	BD Perm/Wash Buffer Washes
C1	Activated PBMCs	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Perm/Wash <sup>TM</sup> Buffer	BD Perm/Wash Buffer Washes
C2	Activated PBMCs	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Isotype Control Cocktail	BD Perm/Wash Buffer Washes
C3	Activated PBMCs	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	PE Anti-human IL-2	BD Perm/Wash Buffer Washes
C4	Activated PBMCs	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Purified Blocking Ab cocktail/PE Anti-human IL-2	BD Perm/Wash Buffer Washes
C5	Activated PBMCs	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	PE Anti-human IFN- $\gamma$	BD Perm/Wash Buffer Washes
C6	Activated PBMCs	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Purified Blocking Ab cocktail/PE Anti-human IFN- $\gamma$	BD Perm/Wash Buffer Washes
C7	Activated PBMCs	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	PE Anti-human TNF	BD Perm/Wash Buffer Washes
C8	Activated PBMCs	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Purified Blocking Ab cocktail/PE Anti-human TNF	BD Perm/Wash Buffer Washes

**Note:** We recommend leaving column B empty.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

## Helpful Tips on Intracellular Staining

1. Fixation/Permeabilization of cells: It is critical for intracellular staining that the cells be fixed prior to or concurrently with permeabilization. If cells are unfixed at the time of permeabilization, the integrity of the cell is compromised usually leading to cell lysis.
2. Continued permeabilization of cells: Saponin, the permeabilization agent in BD Cytofix/Cytoperm™ and BD Perm/Wash™ Buffers, is a reversible permeabilizing detergent and is necessary for antibody to effectively permeate the cell. Therefore it is necessary to have saponin present in all steps requiring permeabilized cells.
3. Overcoming background staining: When performing surface staining, if background staining with the PE-conjugated Isotype Control Cocktail is unusually high, it may be necessary to block the Fc receptors on the activated human PBMCs prior to fixation and permeabilization. This can be done effectively with normal sera prior to addition of cells to the staining plate.

## Flow Cytometric Acquisition and Analysis of Data

The primary method of analyzing intracellular staining of activated cells stained with direct fluorochrome-conjugated anti-cytokine antibodies is by flow cytometry. This rapid high-throughput technique of acquiring and analyzing staining data from large numbers of cells allows quantification of cytokine-producing cells in a mixed population of cells. This section provides advice for the acquisition and analysis of stained cell samples by flow cytometry. A basic familiarity with flow cytometric operating principles is required for using this kit. This manual is not intended to provide instructions on the operation of a flow cytometer or to describe a flow cytometer in technical terms. For additional information on flow cytometry and flow cytometers it is recommended that Howard M. Shapiro's textbook, *Practical Flow Cytometry (3rd Edition, Wiley-Liss, New York)* be used as a reference.<sup>16</sup> A brief description of flow cytometry can also be found in the Technical Protocols section of our website <http://www.bdbiosciences.com/research/multicolor/tools/resources.jsp>.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

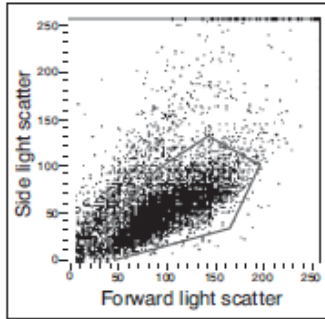
## Technical Tips

### Technical Tips and Information

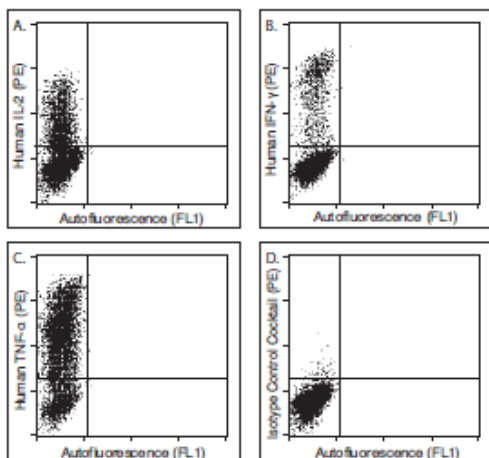
1. **Sample Acquisition:** When determining instrument settings, it is recommended that an unstained (autofluorescence control) sample for each different cell suspension be used. The light scatter profiles of cells treated with BD Cytotfix/Cytoperm™ Buffer vary little from those of unfixed cells.<sup>14</sup> Normal lymphocyte and monocyte cell populations should be easily discernible by their light scatter characteristics.
2. **Compensation Settings:** This kit provides only single color reagents. If compensation setting changes are needed, then it is recommended that the operator use the brightest staining fluorescent anti-cytokine antibody sample (usually anti-human IFN- $\gamma$  in this activation culture).
3. **Sample Analysis:** It is recommended that the staining profile for the PE-conjugated Isotype Control Cocktail be used as a negative control for placing quadrant markers (eg, for bivariate dot or contour plots) or histogram markers. It is also recommended that the region gate be placed around the lymphocyte population (defined by the light scatter profile) and that this gate be used for analysis of samples stained with fluorescent anti-cytokine antibodies. This region gate will isolate the cell population activated by the T-cell Activation Cocktail and exclude cellular debris and instrument noise.

## Appendix C

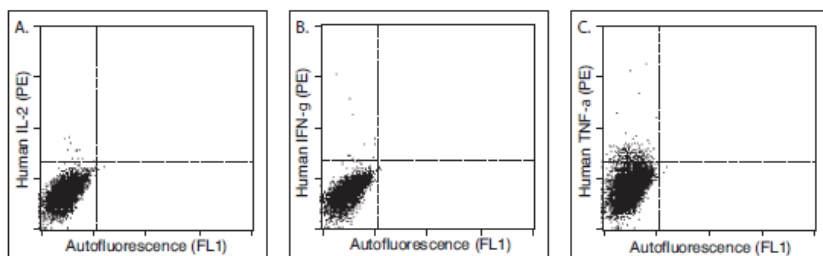
### Representative Staining Data for Activated Human PBMCs:



**Figure 1. Light scatter profile for activated human PBMCs.** This figure depicts the forward light- (FSC) and side light-scatter (SSC) characteristics of human PBMCs that had been stimulated for 4 hours with PMA and ionomycin in the presence of BD GolgiPlug™ (brefeldin A). An electronic region gate has been drawn around the lymphocyte population. This region gate will be used to identify lymphocytes for the expressed levels for several intracellular cytokine proteins (ie, IL-2, IFN- $\gamma$ , and TNF) shown in *Figure 2*. This gate should be used for the analysis of cells stained using the Intracellular Staining Starter Kit.



**Figure 2. Detection of cytokine-producing cells by intracellular staining of human IL-2, IFN- $\gamma$ , and TNF.** Activated human PBMCs (see *Figure 1* for light scattering profile) were stained with either PE-anti-human IL-2 (panel A), PE-anti-human IFN- $\gamma$  (panel B), PE-anti-human TNF (panel C) or the PE-conjugated Isotype Control Antibody Cocktail (panel D). Each panel plots the FL-1 (autofluorescence) and FL-2 (PE emission) event data for each sample. The region gate described in *Figure 1* has been applied to each sample shown in this figure. Due to the region gating used in the analysis of these samples, only data for the lymphocyte cell population (the cell population capable of IL-2 and IFN- $\gamma$  production) is displayed in the panels above. The quadrant markers were set based on the staining profile of cells stained with the Isotype Control Cocktail.

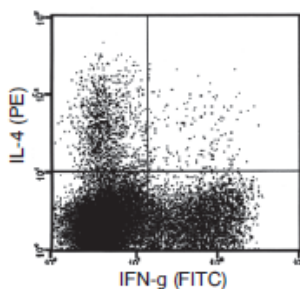


**Figure 3. Purified antibody blocking of PE-conjugated anti-cytokine antibody staining.** Activated human PBMCs were simultaneously stained with either PE-anti-human IL-2 (Panel A), PE-anti-human IFN- $\gamma$  (Panel B), or PE-anti-human TNF (Panel C) and with the Purified Blocking Antibody Cocktail. *Figure 2* depicts the unblocked staining profiles for PE-anti-human IL-2, IFN- $\gamma$ , and TNF. The staining pattern obtained using the PE-conjugated anti-cytokine antibodies were completely abrogated through the addition of the Purified Blocking Antibody Cocktail (*Figure 3, A-C*). The region gate defined in *Figure 1* was used in the analysis of these samples, only data for the lymphocyte cell population is displayed in the panels above. The quadrant markers were set based on the staining profile of cells stained with the Isotype Control Cocktail.

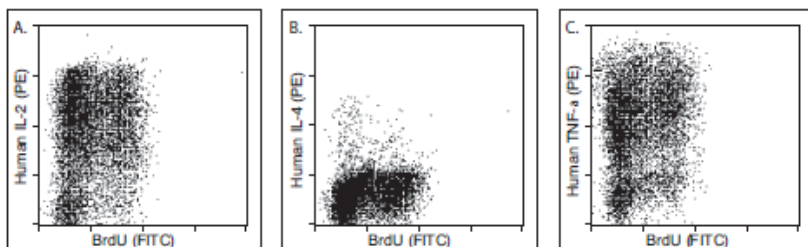
## Intracellular Staining Possibilities (Going beyond this Kit)

This kit has outlined a simple, primary stimulation of human peripheral blood mononuclear cells. As such, it provides a good introduction into cellular activation for inducing cytokine production and subsequent intracellular cytokine staining. This represents just a small example of the possibilities that intracellular staining offers the researcher. This kit helps to demonstrate single parameter flow cytometric detection of intracellular cytokine detection in an activated cell population. Single parameter staining will only allow the researcher to isolate cell population subsets based on their light scattering profiles. Using dual or multi-parameter flow cytometric analysis will allow further investigation of cytokine-producing cells. Through use of an antibody specific for a cell surface marker or multiple anti-cytokine antibodies the researcher is then able to isolate responsive cell subsets (eg, CD4<sup>+</sup> vs. CD8<sup>+</sup> T cells; Th1- vs Th2-like cells) in systems using antigen specific stimulation.

A wide variety of anti-cytokine antibodies, anti-chemokine antibodies, buffers and accessory reagents for intracellular staining are offered by BD Biosciences (see [www.bdbiosciences.com](http://www.bdbiosciences.com)). These reagents, coupled with fluorescent antibodies specific for cell surface antigens will not only allow the detection of cytokine-producing cells but also allow their further characterization (eg, cell lineage and phenotype.) Future developments in intracellular staining will include detection of cell surface antigens, BrdU uptake, and cytokine production. This will allow the researcher to simultaneously analyze cell subsets and evaluate cell proliferation and cytokine production in response to stimulation. The possibilities with the intracellular staining technique are limited only by the experimental design of the researcher and the reagents available for this application.



**Figure 4. Detection and Discrimination of Th0-, Th1- and Th2-like Cells.** *In vitro*-differentiated human PBMCs were restimulated for 4 hours with PMA and ionomycin in the presence of BD GolgiPlug™. The activated cells were harvested, fixed and stained with FITC anti-human IFN- $\gamma$  (Cat. No. 554551) and PE anti-human IL-4 (Cat. No. 554516) and analyzed by flow cytometry. The region gate described in **Figure 1** has been applied to the sample shown in this figure. Due to the region gating used in the analysis of this sample, only data for the lymphocyte cell population is displayed in the panel above.



**Figure 5. Simultaneous Detection of BrdU incorporation and Cytokine Production by CD4<sup>+</sup> human PBMCs.** Purified CD4<sup>+</sup> human PBMCs were activated and *in vitro*-differentiated prior to restimulation for 4 hours with PMA and ionomycin in the presence of BD GolgiPlug™. During the last 45 minutes of culture, cells were pulsed with 10  $\mu$ M BrdU. The cells were subsequently stained with FITC-anti-BrdU and either PE-anti-human IL-2 (clone MQ1-17H12, Panel A), PE-anti-human IL-4 (clone 8D4-8, Panel B) or PE-anti-human TNF (clone mAb11, Panel C) by following the BD Pharmingen™ BrdU Flow Kit Staining procedure. The region gate described in **Figure 1** has been applied to each sample shown in this figure. Due to the region gating used in the analysis of these samples, only data for the lymphocyte cell population is displayed in the panels above.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

## References

1. Carter, L. L., and S. L. Swain. 1997. Single cell analyses of cytokine production. *Curr. Opin. Immunol.* 9:177-182.
2. Parks, D. R., L. A. Herzenberg, and L. A. Herzenberg. 1989. Flow cytometry and fluorescence-activated cell sorting. In *Fundamental Immunology*, 2nd Edition. W. E. Paul, ed. Raven Press Ltd., New York, p. 781-802.
3. Jung, T., U. Schauer, C. Heusser, C. Neumann and C. Rieger. 1993. Detection of intracellular cytokines by flow cytometry. *J. Immunol. Meth.* 159:197-207.
4. Vikingsson, A., K. Pederson and D. Muller. 1994. Enumeration of IFN- $\gamma$  producing lymphocytes by flow cytometry and correlation with quantitative measurement of IFN- $\gamma$ . *J. Immunol. Meth.* 173:219-228
5. Prussin, C. and D. Metcalfe. 1995. Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J. Immunol. Meth.* 188:117-128.
6. Elson, L. H., T. B. Nutman, D. D. Metcalfe and C. Prussin. 1995. Flow cytometric analysis for cytokine production identifies Th1, Th2, and Th0 cells within the human CD4+CD27- lymphocyte subpopulation. *J. Immunol.* 154:4294-4301.
7. Assenmacher, M., J. Schmitz and A. Radbruch. 1994. Flow cytometric determination of cytokines in activated murine T helper lymphocytes: Expression of interleukin-10 in interferon- $\gamma$  and in interleukin-4-expressing cells. *Eur. J. Immunol.* 24:1097-1101.
8. Picker, L. J., M. K. Singh, Z. Zdraveski, J. R. Treer, S. L. Waldrop, P. R. Bergstresser, and V. C. Maino. 1995. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T-cells by flow cytometry. *Blood.* 86:1408-1419.
9. Sallusto, F., C. R. Mackay, and A. Lanzavecchia. 1997. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science.* 277:2005-2007.
10. Austrup, F., D. Vestweber, E. Borges, M. Lohning, T. Brauer, U. Herz, H. Renz, R. Hallmann, A. Scheffold, A. Radbruch, and A. Hamann. 1997. P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflamed tissues. *Nature.* 385:81-83.
11. Sander, B., J. Andersson and U. Andersson. 1991. Assessment of cytokines by 'immunofluorescence and the paraformaldehyde-saponin procedure. *Immunol. Rev.* 119:65-93.
12. Sander, B., I. Hoiden, U. Andersson, E. Moller, and J. Abrams. 1993. Similar frequencies and kinetics of cytokine producing cells in murine peripheral blood and spleen. *J. Immunol. Meth.* 166:201-214.
13. Andersson, U. and J. Andersson. 1994. Immunolabelling of cytokine producing cells in tissues and suspension. In *Cytokine Producing Cells*, eds. D. Fradelizie and D. Emelie. INSERM, Paris. p. 32-49.
14. Lecoeur, H., E. Ledru, M.-L. Gougeon. 1998. A cytofluorimetric method for the simultaneous detection of both intracellular and surface antigens of apoptotic peripheral lymphocytes. *J. Immunol. Meth.* 217:11-26.
15. Fujiwara, T., K. Oda, S. Yokota, S. Takatsuki and Y. Ikehara. 1988. Brefeldina A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J. Biol. Chem.* 263:18545.
16. Shapiro, H. M., *Practical Flow Cytometry*, 3rd Edition, Wiley-Liss, New York.

**For Research Use Only. Not for use in diagnostic or therapeutic procedures.**

## Notes

[illegible]

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

## Notes

This image shows a single sheet of white paper with horizontal ruling lines. The lines are evenly spaced and run across the width of the page. There is no text or other markings on the paper.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

## Notes

[illegible]

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

**United States**

877.232.8995

**Canada**

866.979.9408

**Europe**

32.2.400.98.95

**Japan**

0120.8555.90

**Asia/Pacific**

65.6861.0633

**Latin America/Caribbean**

55.11.5185.9995



**Becton, Dickinson and Company  
BD Biosciences**

2350 Qume Drive

San Jose, CA 95131 USA

(US) Ordering 855.236.2772

Technical Service 877.232.8995

Fax 800.325.9637

**[answers@bd.com](mailto:answers@bd.com)**

**[bdbiosciences.com](http://bdbiosciences.com)**