BD Pharmingen™

Intracellular Cytokine Staining Starter Kit - Mouse

Instruction Manual

Catalog No. 559311

BD

Kit Contents

Note: Store the following items at 4°C.

- **BD Pharmingen™ Stain Buffer (FBS):** 1 vial, 125 ml
- **BD Cytofix/Cytoperm™ Buffer:** 1 vial, 25 ml
- **BD Perm/Wash Buffer:** 1 vial, 25 ml (10×)
- **PE-conjugated anti-mouse IL-2:** 1 vial (25 tests)
- **PE-conjugated anti-mouse IFN-γ:** 1 vial (25 tests)
- **PE-conjugated anti-mouse 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
- **MiCK-1 Cytokine Positive Control Cells:** 1 vial (5×10^6 cells)

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

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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.[1] 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.[1-5]

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.[1,3-8] 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.[9,10] 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.[2]

Staining of intracellular cytokines depends on the identification of cytokine-specific monoclonal antibodies that are compatible with a fixation-permeabilization procedure.[11-13] 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.[14] 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.

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 mouse spleen cells (splenocytes) in culture and stain these activated cells for intracellular accumulations of IL-2, IFN-γ 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 differentation factor while IFN-γ 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 mouse spleen cell population and the subsequent intracellular staining to detect IL-2-, IFN-γ-, and TNF-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.[5,15] The kit includes two vials of the Leukocyte Activation Cocktail, which provides a sufficient amount of reagent to stimulate approximately 1-1.5 × 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.)

MiCK-1 Cytokine Positive Control Cells: MiCK-1 Control Cells are an activated and paraformaldehyde-fixed mouse splenocyte population shown to express IL-2, IFN-γ and TNF using intracellular staining and flow cytometric analysis. The vial contains approximately 5 x 10^6 cells, which is sufficient for 20 tests (2.5 x 10^5 /test). 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 buffer 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 (FBS)*: 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×) containing both fetal bovine serum* and saponin and the presence of some amount of precipitate is common. Dilute to 1× with deionized H_2O 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× BD Perm/Wash Buffer through a 0.45 micron filter. The BD Perm/Wash Buffer should be stored at 4°C. Dilute to 1× with deionized H_2O before use.

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

Antibodies for Intracellular Staining

PE-conjugated Anti-mouse IL-2: A test-sized dilution of PE (phycoerythrin)-conjugated anti-mouse IL-2 antibody (clone JES6-5H4) suitable for intracellular staining. A vial containing 25 tests (20 µl/test) of PE-anti-mouse IL-2 antibody is included in this kit and should be stored at 4°C.

PE-conjugated Anti-mouse IFN-γ: A test-sized dilution of PE-conjugated anti-mouse IFN-γ antibody (clone XMG1.2) suitable for intracellular staining. A vial containing 25 tests (20 µl/test) of PE-anti-mouse IFN-γ antibody is included in this kit and should be stored at 4°C.

PE-conjugated Anti-mouse TNF: A test-sized dilution of PE-conjugated anti-mouse TNF antibody (clone MP6-XT22) suitable for intracellular staining. A vial containing 25 tests (20 µl/test) of PE-anti-mouse 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 PE-conjugated rat IgG_1 and rat IgG_{2b} 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-mouse IL-2, IFN-γ, and TNF antibodies included in this kit. The cocktail is a mixture of anti-mouse IL-2 (clone JES6-5H4), anti-mouse IFN-γ (clone XMG1.2) and anti-mouse TNF (clone MP6-XT22). 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.

   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. 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.

5. 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 used to induce the 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 mouse splenocytes. This kit also provides the methodology and reagents necessary for the stimulation of mouse splenocytes to produce a variety of cytokines including IL-2, IFN-γ, and TNF. The information given in the next section describes the dissociation of a mouse spleen to produce a single cell suspension. The protocol for activating these cells to induce production and accumulation of IL-2, IFN-γ, and TNF is described in ***Activation of Mouse Splenocytes***. A recipe for medium that is suitable for the culture and *in vitro* activation of mouse cells is presented in the **Appendix A**.

Dissociation of Mouse Spleen

The mouse spleen dissociation protocol described below is a reliable method; alternative procedures for separation of a mouse spleen sample into a single cell suspension may also be useful. Keep in mind that up to 5×10^7 cells can be activated using a single vial of the Leukocyte Activation Cocktail. A single mouse spleen will, on average, yield sufficient splenocytes for activation by a single vial of the Leukocyte Activation Cocktail.

Protocol for the Preparation of a Mouse Spleen Single Cell Suspension:

1. Harvest or obtain a whole spleen from a "normal" strain of mouse (eg, BALB/c or C57B1/6).
2. Insert a 70 µm cell strainer (Falcon® Cat. No. 352350) in a 50 ml conical tube (Falcon Cat. No. 352098).
3. Place the spleen in the cell strainer and rinse spleen cells with 3 - 5 ml complete RPMI (see ***Appendix A*** for medium recipe).
4. Dissociate the spleen by gently pressing it through the cell strainer using a syringe plunger. Rinse cells through the strainer using complete RPMI.
5. Centrifuge splenocyte suspension at 400 - 500 × g for 5 minutes at 10°C with centrifuge braking.
6. Discard supernatant by aspiration.
7. Lyse red blood cells by resuspending cell pellet in 3 ml ACK lysing buffer (see ***Appendix A*** for recipe) or 1 × BD PharmLyse™ Buffer (Cat. No. 555899). Incubate cells for 3-5 minutes at room temperature.
8. Add 27 ml complete RPMI to the cell suspension.
9. Centrifuge cell suspension tube at 400 - 500 × g for 5 minutes at 10°C with centrifuge braking.
10. Discard supernatant by aspiration and resuspend cell pellet in 30 ml of complete RPMI.
11. Centrifuge the tube at 400 - 500 × g for 5 minutes at 10°C with centrifuge braking.
12. Aspirate the supernatant to isolate cell pellet and proceed to the next section for setting up the cell activation culture.

Note: One alternative to the protocol described above can be found in section 3.1.3 of the Current Protocols in Immunology. (J.E. Coligan, et al., eds Greene Publishing Associates and Wiley- Interscience,New York.)

Activation of Mouse Splenocytes

Once the mouse splenocytes have been isolated as per ***Dissociation of Mouse Spleen***, page 10, they will be used in a primary activation culture in order to induce IL-2, IFN-γ, and TNF production. This activation culture will involve stimulating the mouse splenocytes for 4 hours using the Leukocyte Activation Cocktail.

Protocol for Activation of Mouse Splenocytes

1. To the 50 ml conical tube containing the splenocyte cell pellet, add 10 ml of complete RPMI and thoroughly resuspend the cell pellet.
2. Count the number of cells isolated by using a hemocytometer 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 × 10^6 cells/ml.
4. Transfer the cell suspension to a 6-well culture plate (Falcon Cat. No. 353046) in 6 ml/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.
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: Wear safety glasses and protective lab clothing when thawing the activator. After use, discard the unused portion of Leukocyte Activation Cocktail, do not refreeze.

6. Place the 6-well culture plate at 37°C in a humidified CO_2 incubator for 4 hours.
7. Remove the plate from the incubator after 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 400 - 500 × 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 2×10^7 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 on page 14 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×) (Gibco® Cat. No. 10378-016)	5 ml
3.	Low IgG Fetal Bovine Serum (HyClone Cat. No. SH30151.03)	37.5 ml
4.	2-Mercaptoethanol (1000×) (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.

Recipe for ACK lysing buffer:

1.	NH_4Cl (0.15 M)	8.29 g
2.	$KHCO_3$ (1.0 M)	1.00 g
3.	Na_2EDTA (0.1mM)	37.2 mg
4.	H_2O	800 ml

Add water and adjust pH to 7.2-7.4 with 1 N HCl.

5. Add H_2O to 1 Liter.

6. Filter sterilize through a 0.2-µm filter and store at room temperature.

Note: The recipe for ACK lysing buffer was obtained from section 3.1.5 of the Current Protocols in Immunology. (J.E. Coligan, *et al.*, eds Greene Publishing Associates and Wiley-Interscience, New York.)

Intracellular Staining Procedure

Following the primary activation of the mouse splenocytes, it is now possible to stain the activated cells and the MiCK-l 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 mouse IL-2, IFN-γ, and TNF.

Information on thawing and resuspending the MiCK-1 Positive Control Cells is described in ***Resuspension of the MiCK-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 MiCK-1 Positive Control Cells

Thawing and Resuspending the MiCK-1 Positive Control Cells

1. Remove the MiCK-1 cell vial from the -80°C freezer and quickly thaw at 37°C in a water bath (consult the Material Safety Data Sheet included with this kit for safety information).
2. Transfer 0.5 ml of 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 MiCK-1 cells to the -80°C freezer for subsequent use.
3. Centrifuge the 15 ml conical tube at 400 - 500 × 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 MiCK-1 vial immediately prior to beginning the intracellular staining protocol. The mouse splenocyte activation culture should already be completed by the time the MiCK-1 Cells are being thawed.

Intracellular Staining Protocol

Protocol for Intracellular Staining

1. The activated splenocytes obtained from the stimulation culture that were resuspended in BD Pharmingen Stain Buffer at 2×10^7 cells/ml can now be aliquoted and distributed into the microwells of a 96-well plate (Falcon Cat. No. 353072) (see ***Appendix B***).

2. Transfer the activated mouse splenocytes or MiCK-1 Positive Control Cells in 50 µl/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 µl of BD Pharmingen Stain Buffer to each well. Centrifuge plate at 400 - 500 × g for 5 minutes at 10°C to pellet cells.

4. Aspirate supernatant from each sample well. Agitate plate to disrupt cell pellets.

5. Add 100 µl of BD Cytofix/Cytoperm™ Buffer to each sample well. Incubate the plate for 20 minutes at room temperature. This step will fix the mouse 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 µl/well of BD Pharmingen™ Stain Buffer. Cover the plate and store the cells at 4°C overnight, or up to 3 days. To proceed, repeat steps 3 and 4. Resuspend the cells in 100 µl/well of BD Perm/Wash™ Buffer and proceed to step 6.

6. Add 100 µl of 1 × BD Perm/Wash™ buffer to each sample well and centrifuge the plate at 400 - 500 × g for 5 minutes at 10°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.

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

Appendix B

Staining Template for Staining MiCK-1 and Activated Mouse Cells:

Well #	Cell Type	1st Step	Fixation and Permeabilization	Washing Step	IC Staining Step	Final Wash Step
A1	MiCK-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	MiCK-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	MiCK-1 Cells	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	PE Anti-mouse IL-2	BD Perm/Wash Buffer Washes
A4	MiCK-1 Cells	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Purified Blocking Ab cocktail/PE Anti-mouse IL-2	BD Perm/Wash Buffer Washes
A5	MiCK-1 Cells	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	PE Anti-mouse IFN-γ	BD Perm/Wash Buffer Washes
A6	MiCK-1 Cells	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Purified Blocking Ab cocktail/PE Anti-mouse IFN-γ	BD Perm/Wash Buffer Washes
A7	MiCK-1 Cells	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	PE Anti-mouse TNF	BD Perm/Wash Buffer Washes
A8	MiCK-1 Cells	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Purified Blocking Ab cocktail/PE Anti-mouse TNF	BD Perm/Wash Buffer Washes
C1	Activated splenocytes	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Perm/Wash Buffer	BD Perm/Wash Buffer Washes
C2	Activated splenocytes	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 splenocytes	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	PE Anti-mouse IL-2	BD Perm/Wash Buffer Washes
C4	Activated splenocytes	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Purified Blocking Ab cocktail/PE Anti-mouse IL-2	BD Perm/Wash Buffer Washes
C5	Activated splenocytes	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	PE Anti-mouse IFN-γ	BD Perm/Wash Buffer Washes
C6	Activated splenocytes	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Purified Blocking Ab cocktail/PE Anti-mouse IFN-γ	BD Perm/Wash Buffer Washes
C7	Activated splenocytes	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	PE Anti-mouse TNF	BD Perm/Wash Buffer Washes
C8	Activated splenocytes	BD Pharmingen Stain Buffer Wash	BD Cytofix/Cytoperm Incubation 20°C	BD Perm/Wash Buffer Washes	Purified Blocking Ab cocktail/PE Anti-mouse TNF	BD Perm/Wash Buffer Washes

Note: We recommend leaving column B empty.

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™ Buffer and BD Perm/Wash™ Buffer, 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 cell 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 mouse splenocytes prior to fixation and permeabilization. This can be done effectively with BD FcBlock™ antibody (Cat. No. 553141/553142) or 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 and 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. 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

Technical Tips

Technical Tips and Information

1. **Data Acquisition:** When determining instrument settings, it is recommended that an unstained (autofluorescence control) sample for each different cell suspension be used (eg, Samples A1and C1 from the staining template). The light scatter profiles of cells treated with BD Cytofix/Cytoperm Buffer vary little from those of unfixed cells.[14] Normal lymphocyte and monocyte cell populations should be easily discernible by their light scatter characteristics.

2. **Compensation:** 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-mouse TNF in this activation culture).

3. **Data 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 Mouse Splenocytes:

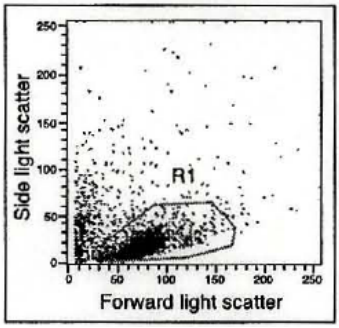

Figure 1. Light scatter profile for activated mouse splenocytes. This figure depicts the forward light- (FSC) and side light-scatter (SSC) characteristics of BALB/c mouse splenocytes stimulated for 4 hours with PMA and ionomycin in the presence of BD GolgiPlug™ (brefeldin A). An electronic region gate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

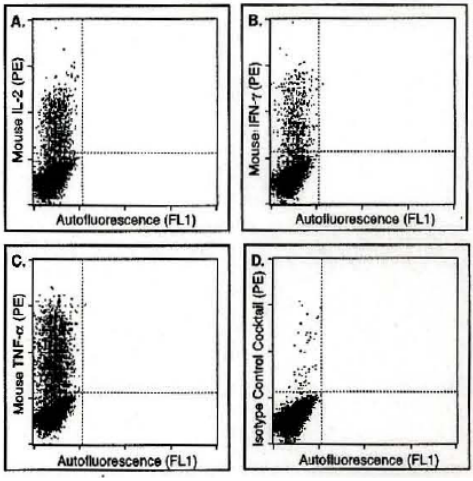

Figure 2. Detection of cytokine-producing cells by intracellular staining of mouse IL-2, IFN-γ, and TNF. Activated BALB/c splenocytes (see ***Figure 1*** for light scattering profile) were stained with either PE-anti-mouse IL-2 (panel A), PE-anti-mouse IFN-γ (panel B), PE-anti-mouse TNF (panel C) or 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-γ 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.

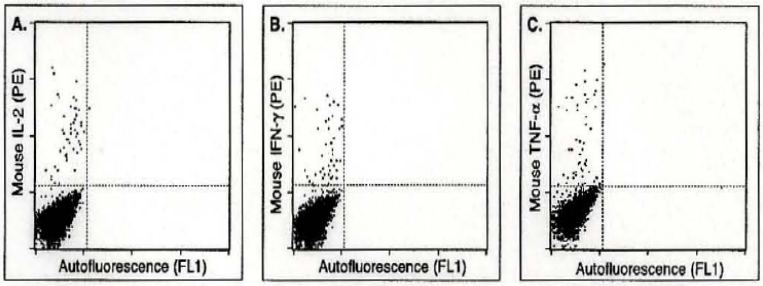

Figure 3. Purified antibody blocking of PE-conjugated anti-cytokine antibody staining. Activated BALB/c splenocytes were simultaneously stained with either PE-anti-mouse IL-2 (Panel A), PE-anti-mouse IFN-γ (Panel B), or PE-anti-mouse TNF (Panel C) and with the Purified Blocking Antibody Cocktail. ***Figure 2*** depicts the unblocked staining profiles for PE-anti-mouse IL-2, IFN-γ, 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 protocol for the primary stimulation of mouse splenocytes. 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 investigator. This kit helps to demonstrate single-color flow cytometric detection of intracellular cytokine detection in an activated cell population. Single-color immunofluorescent staining for a particular cytokine will only allow the researcher to investigate cytokine-producing cells based on their light scattering characteristics (eg, resting versus activated lymphocytes). Using dual or multi-color flow cytometric analysis will allow further investigation of the nature of cytokine-producing cells. Through the use of fluorescent antibodies directed against cell surface markers and various cytokines, the researcher is then able to isolate responsive cell subsets (eg, $CD4^+$ vs. $CD8^+$ T cells; Th1- vs. Th2-like cells) in various activation systems.

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). 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.) In addition, intracellular staining can include detection of cell surface antigens, BrdU uptake and cytokine production. This allows 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.

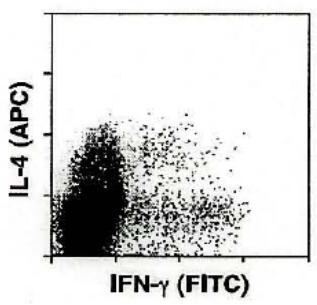

Figure 4. Detection and Discrimination of Th0-, Th1- and Th2-like Cells. *In vitro*-differentiated BALB/c splenocytes were restimulated for 4 hours with PMA and ionomycin in the presence of BD GolgiPlug™. The activated cells were harvested, fixed and stained with APC-anti-mouse IL-4 (clone 11B11, Cat. No. 554436) and FITC-anti-mouse IFN-γ (clone XMG1.2, Cat. No. 554411). 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.

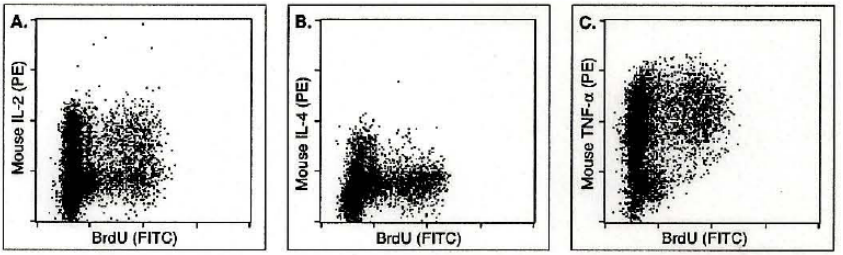

Figure 5. Simultaneous Detection of BrdU incorporation and Cytokine Production by $CD4^+$ mouse splenocytes. Purified CD4+ mouse splenocytes were activated and *in vitro*-differentiated prior to restimulation for 4 hours with PMA and ionomycin in the presence of BD GolgiPlug. During the last 45 minutes of culture, cells were pulsed with 10 µM BrdU. The cells were subsequently stained with FITC-anti-BrdU and either PE-anti-mouse IL-2 (clone JES6-5H4, Cat. No. 554428, Panel A), PE-anti-mouse IL-4 (clone 11B11, Cat. No. 554435, Panel B) or PE-anti-mouse TNF (clone MP6-XT22, Cat. No. 554419, Panel C) by following the BD Pharmingen™ BrdU Flow Kit (Cat. No. 559619) 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.

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Notes

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