

APPLICATION NOTE

Detecting Intracellular Cytokines in Activated Lymphocytes

Scope

Cytokines are soluble proteins that have a significant role in the immunoregulation of lymphocyte responses.^{1,2} Specifically, cytokines regulate the growth, differentiation, and function of a wide variety of cells and mediate normal and pathological immune responses. Cytokines are unique proteins that can have both effector and regulatory activities. In fact, recent research has shown that cytokines can have multiple functions, target many cellular subsets and can be expressed by diverse cellular subsets.^{3,4}

Early studies attempting to relate cytokine expression and T-lymphocyte function were based on activities of cloned cell populations.⁵ Although investigations using T-lymphocyte clones demonstrated distinct patterns of cytokine synthesis, ie, T_H1 (IL-2, IFN- γ) and T_H2 (IL-4, IL-5, IL-10), these studies were difficult to interpret because the functional correlation of cloned T lymphocytes to in vivo T-lymphocyte behavior is largely unknown.⁶

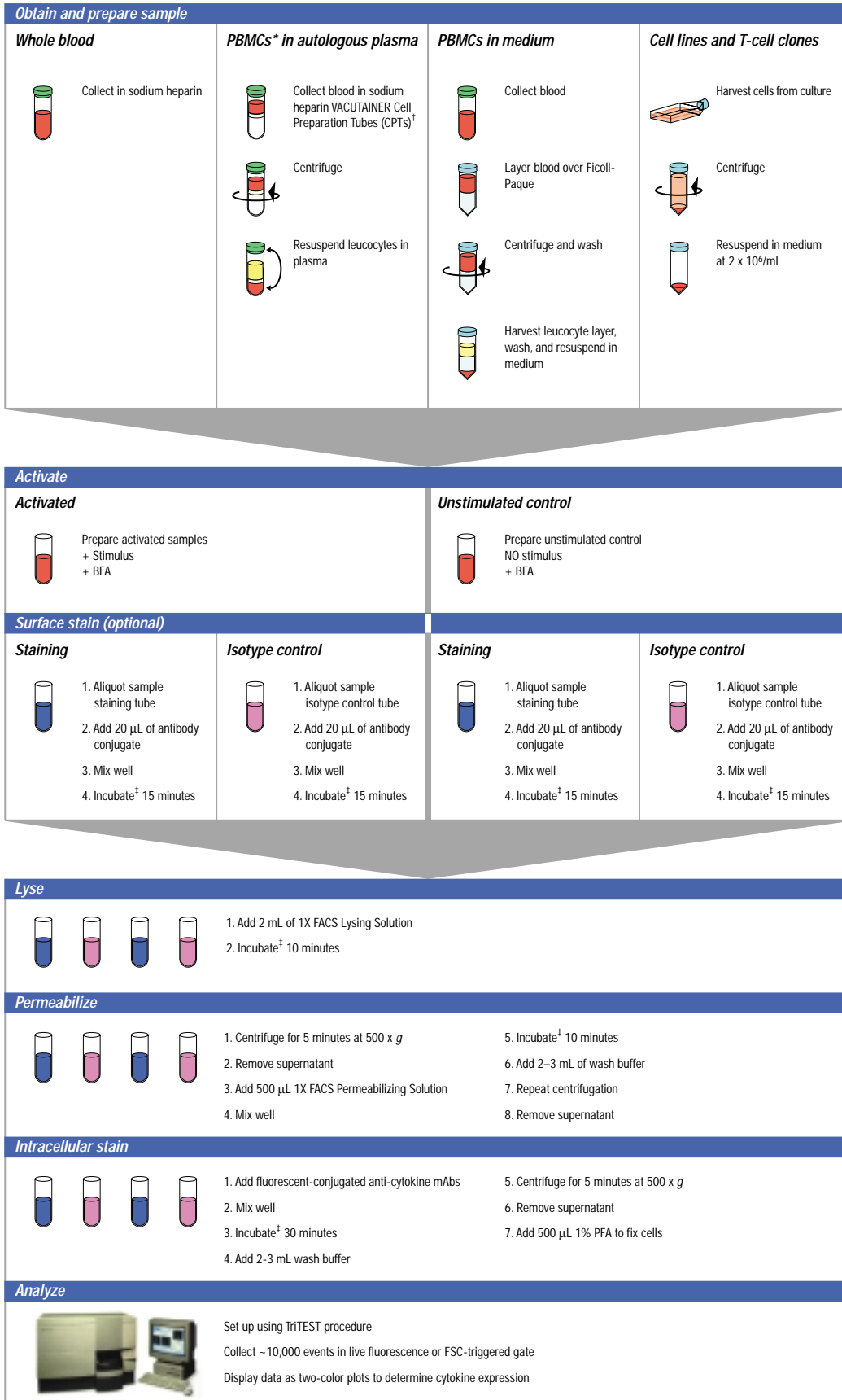
Recently, Jung et al⁷ and Picker et al⁸ have adapted a method to detect the intracellular expression of cytokines after incubation with drugs, such as monensin or Brefeldin A (BFA). This process disrupts intracellular Golgi-mediated transport and allows cytokines to accumulate, yielding an enhanced cytokine signal that can be detected by flow cytometry. This unique method can detect multiple cytokines per cell and discrete cellular populations that express a particular cytokine. These are important advantages when studying cytokine responses to specific stimuli.

At the cellular level, this approach demonstrates the existence of Type 1 and Type 2 polarities in both mouse and human lymphocytes and shows that these polarities can be reversed under appropriate cytokine-enhanced conditions.⁸⁻¹⁰ It is also clear from these studies that only activated cell populations express cytokines; resting normal lymphocytes (T, B, or natural killer [NK]) do not constitutively express cytokines.

The intracellular flow cytometric assay described in this procedure forms the basis of the current BD Biosciences FastImmune™ Cytokine System.* When the anti-cytokine conjugates are used in combination with cell-surface or intracellular phenotyping, the production of cytokines by specific cell types can be assessed.⁸ The assay format takes advantage of the unique chemistry and antibody selection protocols BD Biosciences uses to ensure minimal background fluorescence in resting or cytokine-negative cells. Anti-cytokine monoclonal antibody conjugates from BD Biosciences allow detection at low Ig concentrations because they are specifically selected for high-affinity binding to fixed, nascent, intracellular cytokine epitopes.

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

Figure 1 FastImmune intracellular cytokine assay strategy



* Peripheral blood mononuclear cells.

[†] For more information, contact BD VACUTAINER Division at (800) 631-0174.

[‡] Incubate in the dark at room temperature.

Procedure

This procedure is designed for a broad range of applications and with special consideration to minimizing potential assay problems. Figure 1 outlines a general strategy for using this procedure.

Although designed to be simple and rapid, immune function assays require particular attention to the activation steps and reagents, which must be carefully prepared, stored, and used. We include specific recommendations and guidelines for activation, based upon the experiences of the BD Biosciences Immunology Research group.

Successful activation is critical to achieving meaningful results. Therefore, it is important to collect and prepare cells for activation without introducing agents that will interfere with natural activation processes. Avoid calcium-chelating anticoagulants, such as ACD and EDTA, since they restrict calcium-dependent activation responses. On the other hand, lipopolysaccharide (LPS), a common contaminant of biological reagents, is a potent cell activator and can lead to confusing results.

We recommend you begin with experiments that follow our protocol on normal blood samples before using modified procedures or analyzing abnormal samples.

See Troubleshooting at the end of this procedure if you experience difficulty in achieving the desired results.

Equipment

1. Disposable 12 x 75-mm capped polystyrene Falcon[®] test tubes (BD Labware Catalog No. 2058), or equivalent
2. 37°C incubator with 7% CO₂
3. Vortex mixer
4. Centrifuge
5. Pipetman, or equivalent pipettors
6. FACS[™] brand flow cytometer

Cells

Whole blood

Collect blood for whole blood activation assays into sodium heparin VACUTAINER[®] tubes (BD VACUTAINER Catalog No. 367673). FastImmune

assays are incompatible with lithium heparin, EDTA, and ACD anticoagulants.

For best results, assay blood within 8 hours of collection since a minor loss of activity can be expected beyond 8 hours; typically, the percent of cytokine-positive cells is reduced by approximately 5%. If blood cannot be used within 8 hours, store VACUTAINER tubes horizontally at room temperature.

Peripheral blood mononuclear cells (PBMCs) in autologous plasma

Prepare PBMCs using BD VACUTAINER Cell Preparation Tubes (CPTs) (BD VACUTAINER Catalog No. 362753) containing sodium heparin. The VACUTAINER CPT is a blood draw tube with a thixotropic matrix that establishes a density-gradient plug upon centrifugation. Refer to the VACUTAINER CPT product insert for detailed information. Red blood cells (RBCs) and granulocytes pass through the plug to the bottom of the tube while the leucocytes and plasma remain above the plug. By gently inverting the tube, the leucocytes can be conveniently resuspended in the plasma and activated like whole blood in this assay.

Before storage, centrifuge CPTs and resuspend PBMCs in the autologous plasma by gently inverting each tube several times. Store each CPT at room temperature on its side. Assay the blood no later than 24 hours after collection.

PBMCs in tissue culture medium

PBMCs can also be separated via Ficoll-Paque density-gradient centrifugation. Use standard techniques and resuspend at 2×10^6 cells/mL in RPMI-1640 with 10% heat-inactivated fetal bovine serum (FBS) for activation.

Cell lines and T-lymphocyte clones

For activation, resuspend cells at 2×10^6 cells/mL in the fresh culture medium typically used to grow the cells.

NOTE: Heat inactivate FBS to denature complement.

Frozen whole blood and PBMCs

Lyse and fix activated whole blood or PBMCs using 1X FACS Lysing Solution; incubate for 10 minutes at room temperature and directly place the tubes in a freezer at -80°C . After thawing, aliquot cells into staining tubes. Wash cells by adding 2 to 3 mL wash buffer and centrifuge for 5 minutes at $500 \times g$; then permeabilize with 1X FACS Permeabilizing Solution and stain.

Selecting, Preparing, and Storing Reagents

The following procedures and reagents have been successfully used by the research laboratories at BD Biosciences.

Reagents used in activation (not provided by BD Biosciences)

1. Phorbol 12-Myristate 13 Acetate (PMA) (Sigma Catalog No. P-8139)
 - a. Reconstitute in DMSO at 0.1 mg/mL.
 - b. Store small aliquots (eg, 20 μ L) at -20°C ; do not refreeze aliquots after thawing.
 - c. Dilute stock 1:100 in sterile PBS (without sodium azide) for each experiment.
 - d. Use PMA at a final concentration of 10 ng/mL of cell suspension. (See Activation section in this procedure for additional information on using this reagent.)
2. Ionomycin (Sigma Catalog No. I-0634)
 - a. Reconstitute in EtOH at 0.5 mg/mL.
 - b. Store at -20°C .
 - c. Dilute stock 1:10 in sterile PBS (without sodium azide) for each experiment.
 - d. Use ionomycin at a final concentration of 1 $\mu\text{g/mL}$ of cell suspension.
3. Staphylococcal enterotoxin B (SEB) (Sigma Catalog No. S-4881)
 - a. Reconstitute in sterile PBS (without sodium azide) at 0.5 mg/mL.
 - b. Store at 4°C .
 - c. Use SEB at a final concentration of 1 $\mu\text{g/mL}$ of cell suspension. (See Activation section in this procedure for additional information on using this reagent.)
4. Brefeldin-A (BFA) (Sigma Catalog No. B-7651)
 - a. Reconstitute in DMSO at 5 mg/mL.
 - b. Store small aliquots (eg, 20 μ L) at -20°C ; do not refreeze aliquots after thawing.
 - c. Dilute stock 1:10 in sterile PBS (without sodium azide) for each assay.

- d. Use BFA at 10 $\mu\text{g/mL}$ of cell suspension for the last 4 to 5 hours of activation.

NOTE: Extensive incubation with BFA will reduce cell viability. (See Activation section in this procedure for additional information on using this reagent.)

5. RPMI-1640 (BioWhittaker Catalog No. 12-167F)
6. PBS without sodium azide (NaN_3), sterile filtered
7. DMSO (Sigma Catalog No. D-8779)
8. EtOH, Gold Shield Ethyl Alcohol, 200 proof
9. Wash buffer, PBS with 0.5% BSA and 0.1% NaN_3 ; store at 4°C .
10. 1% paraformaldehyde (PFA) in PBS; store at 4°C .

Reagents for immunophenotypic staining (BD Biosciences reagents)

11. Monoclonal antibody conjugates for surface staining

The choice of specific surface markers will depend on your particular phenotyping strategy. For example, CD45 PerCP (BD Biosciences Catalog No. 347464) is useful for triggering on all lymphocytes. CD3 PerCP (BD Biosciences Catalog No. 347344) is a good choice for fluorescent triggering on CD3⁺ T lymphocytes in a multicolor assay. CD4 PerCP (BD Biosciences Catalog No. 347324) and CD8 PerCP (BD Biosciences Catalog No. 347314) are good choices for triggering on specific T-lymphocyte subsets. CD19 PerCP (BD Biosciences Catalog No. 347544) and CD20 PerCP (BD Biosciences Catalog No. 347674) can be used to identify B lymphocytes, while CD56 and CD14 are the markers of choice for NK lymphocytes and monocytes, respectively.

12. FACS Lysing Solution

FACS Lysing Solution (BD Biosciences Catalog No. 349202) is required when using PBMC preparations, cultured cells, and whole blood. For this method, it serves three purposes:

- a. lyses RBCs in whole blood preparations
- b. fixes the external epitopes
- c. assists in permeabilization

FACS Lysing Solution is supplied as a 10X concentrate. Before use, dilute 1:10 in deionized

water; refer to the product insert for instructions. Do not dilute in PBS or other buffers.

13. FACS Permeabilizing Solution

BD Biosciences has developed FACS Permeabilizing Solution (BD Biosciences Catalog No. 340457) to ensure consistent sensitivity and low background staining. This proprietary reagent is specifically designed to overcome the limitations of saponin-based permeabilizing reagents common to many intracellular staining protocols. Saponin, a compound derived from plants, is a common source of variability in intracellular immunophenotypic staining because of its heterogeneous composition. FACS Permeabilizing Solution also eliminates the necessity of freezing cells overnight, which some protocols recommend to increase permeability.

FACS Permeabilizing Solution is supplied as a 10X concentrate. Before use, dilute 1:10 in deionized water; refer to the product insert for instructions. Do not dilute in PBS or other buffers.

14. Monoclonal antibody conjugates for intracellular staining

The choice of specific intracellular markers will depend on your particular phenotyping strategy. For example, the two-color IFN- γ FITC/IL-4 PE reagent (BD Biosciences Catalog No. 340456) is a popular choice for simultaneously assessing Type 1 and Type 2 immune responses with fluorescent triggering on CD3 PerCP or other markers.

Successful intracellular staining demands superior antibodies against the intracellular targets. Intracellular assays that utilize secretory inhibitors, such as BFA, detect developing protein in the Golgi apparatus. Conformationally, the nascent protein can be different from the secreted form. Therefore, antibodies that work well in detecting secreted cytokine can perform poorly in intracellular assays. BD Biosciences screens many monoclonal antibodies for each cytokine under actual intracellular assay conditions.

Activation

Activation is performed in the presence of BFA, which inhibits intracellular transport of proteins,^{8,9,11} so antigens and cytokines produced during activation will be retained inside the cell. The unstimulated control sample should also contain BFA. See the Assay Control section in this procedure. All activation procedures outlined are performed in 12 x 75-mm capped polystyrene test tubes (Falcon Catalog No. 2058). The reagent concentrations indicated are final concentrations in the activation mixture using reagent preparations described previously.

1. PMA + ionomycin (PMA + I)

- Dilute whole blood or PBMCs in plasma 1:1 with RPMI 1640 without serum. (This dilution procedure is required only for PMA + I activation. Cells that have already been resuspended at 2×10^6 /mL in medium need not be further diluted with RPMI.)
- Stimulate with 10 ng/mL of PMA (10 μ L of working solution described previously per mL of blood) and 1 μ g/mL of ionomycin (20 μ L of working solution per mL of blood) in the presence of 10 μ g/mL of BFA (20 μ L of working solution per mL of blood).
- Incubate for 4 hours at 37°C, 7% CO₂ with tube caps loosened to allow entry of CO₂-containing air. (While a CO₂ incubator is preferred to ensure proper control of pH, the incubation can also be carried out in a water bath with each tube tightly capped.)

2. SEB

- Activate undiluted blood with 1 μ g/mL of SEB in the presence of 10 μ g/mL of BFA.
- Incubate for 4 to 6 hours at 37°C.

3. CD2/CD2R (BD Biosciences Catalog No. 340366)

- Activate undiluted blood with 20 μ L of CD2/CD2R per mL of blood in the presence of BFA.
- Incubate for 4 to 6 hours at 37°C.

4. CD3

- a. Activate undiluted blood with immobilized CD3¹² in the presence of BFA.
- b. Incubate for 4 to 6 hours at 37°C. CD5 PerCP (BD Biosciences Custom Conjugate) and CD45 PerCP (BD Biosciences Catalog No. 347464) are recommended for FL3 fluorescence triggering because the CD3 antigen is modulated by crosslinking of CD3.

NOTE: High-concentration, low-azide CD3 is available through the BD Biosciences Custom Conjugate Program. Contact your local BD Biosciences representative for more information.

5. CD28

Use CD28 (BD Biosciences Catalog No. 340975) at 10 µg/mL to enhance activation responses to various stimuli, including SEB, CD3, and CD2/CD2R.

Assay Controls

Proper system controls simplify troubleshooting. Before modifying the procedure, it is strongly recommended that you establish proficiency with the assay by using normal human samples described in this procedure. At the start, prepare all of the controls outlined below using normal human donor samples. As you gain confidence in your results, you can eliminate the activation and intracellular staining controls. Figure 4 in the Troubleshooting section illustrates how the activation and intracellular staining controls are useful in troubleshooting. However, you should run unstimulated and isotype controls for each sample in the assay.

1. Unstimulated control

The unstimulated control is used to assess the level of residual cytokine synthesis from *in vivo* activation. Run this control for all samples. As the name implies, the unstimulated control is prepared by incubating the blood during the activation step with 10 µg/mL of BFA, but without a stimulus.

2. Isotype controls

Fluorescent-conjugated isotype control antibodies are used at matching concentrations to detect non-specific binding to cells due to the class of the mouse monoclonal antibody. The FastImmune Cytokine System utilizes standard anti-KLH isotype controls

specially formulated for intracellular detection systems. These intuitive controls save time and resources by eliminating the need for expensive and tedious cross competition with unlabeled recombinant cytokines.

3. Activation control

The activation control utilizes surface expression of CD69 to assess whether activation has been achieved. If the expected level of CD69 is not seen, there is a problem with the activation step of the assay. Specifically, one of the reagents used in the activation step can be inactive, expired, or improperly prepared; or, a solvent can be contaminated. Make fresh preparations of the stimuli and try again.

- a. Activate one aliquot of blood with PMA + I (as described previously) but omit the BFA.

NOTE: Anti-secretory agents like BFA prevent surface expression of CD69 and must be omitted to permit surface expression and detection.

- b. Surface stain only with CD69 PE/CD3 PerCP (BD Biosciences Catalog No. 340368). Omit the permeabilization and intracellular staining steps of the procedure.

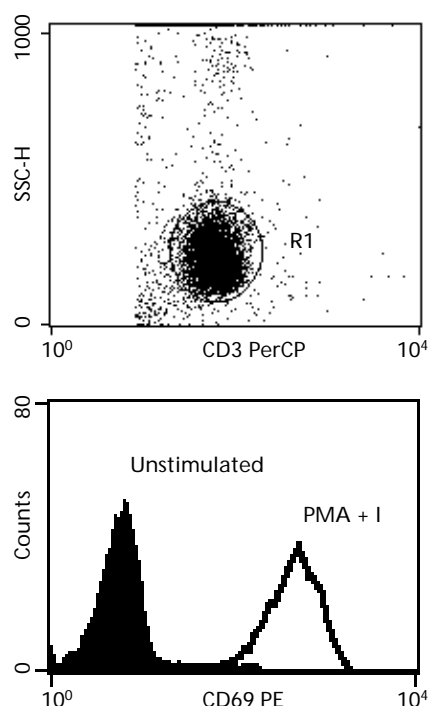


Figure 2 Activation control

- c. Analyze results by fluorescence triggering on FL3 and assessing CD69 staining in the CD3 gated events. Surface staining for CD69 should be greater than 90% positive (Figure 2).

4. Intracellular staining control

The intracellular staining control assesses intracellular staining of CD69 in conjunction with the results of the activation control to pinpoint whether permeabilization and intracellular staining are executed properly. If the activation control is greater than 90% positive but a comparable level of CD69 is not detected by intracellular staining, there is a problem with the permeabilization or intracellular staining step of the assay. Make sure you have followed the procedure exactly as written, and try again.

- Activate one aliquot of blood with PMA and ionomycin in the presence of BFA.
- Omit the surface staining step. Stain intracellularly only with CD69 PE/CD3 PerCP (BD Biosciences Catalog No. 340368).
- Analyze results by fluorescence triggering on FL3 and assessing CD69 staining in the CD3 gated events. Intracellular staining for CD69 should be greater than 90% positive (Figure 3).

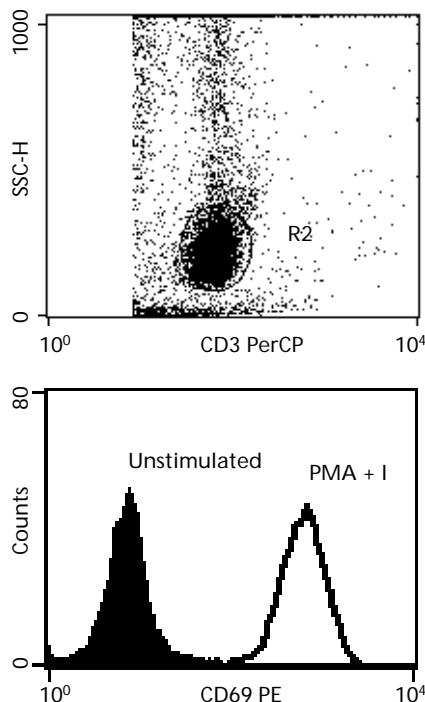


Figure 3 Intracellular staining control

Staining

Anti-cytokine antibodies can be used in conjunction with antibodies against cell surface antigens for the purpose of studying specific lymphocyte populations. CD3 PerCP, CD4 FITC, and CD8 FITC are most commonly used by the BD Biosciences research laboratories for T-lymphocyte subsetting. The CD4, CD14, and CD56 antigens are modulated by PMA activation to varying degrees in most donors; therefore, they are not useful for phenotypic subsetting in PMA-activated samples.

To simultaneously stain all epitopes after permeabilization, add all antibody conjugates at the same time. Surface staining reagents should be titrated if used in the intracellular staining step to ensure optimal results. Intracellular staining of all surface markers should be compared to surface staining to ensure that they are comparable. Staining of CD3, CD4, and CD8 antigens can be performed either on the cell surface prior to permeabilization, or during the intracellular staining step after permeabilization. This does not apply to all antigens because epitopes vary in their sensitivity to paraformaldehyde fixation.

Surface staining

- Add BD Biosciences surface staining reagent to 12 x 75-mm tubes.
- Add 100 μ L of diluted PMA + I activated blood or 50 μ L of undiluted whole blood (activated by other stimuli) to the surface staining reagents. (Dilution of whole blood or PBMCs in plasma in RPMI is only required for PMA + I activation. Cells that have already been resuspended at 2×10^6 cells/mL in medium need not be diluted further with RPMI.)
- Mix well and incubate for 15 minutes at room temperature in the dark.

Permeabilization and intracellular staining

- Add 2 mL of 1X FACS Lysing Solution prepared according to the package insert. Incubate for 10 minutes at room temperature. When staining PBMCs or cultured cells, add FACS Lysing Solution to fix the surface epitopes and optimize the permeabilization process.

NOTE: PMA-activated whole blood does not always lyse completely.

2. Centrifuge for 5 minutes at 500 x *g* and remove the supernatant. Avoid disturbing the pellet. Add 500 μ L of 1X FACS Permeabilizing Solution prepared according to the package insert and mix well. Incubate for 10 minutes at room temperature in the dark.
3. Add 2 to 3 mL of wash buffer and centrifuge for 5 minutes at 500 x *g*. Remove the supernatant.
4. Add fluorescent-conjugated anti-cytokine mAbs. Mix well and incubate for 30 minutes at room temperature in the dark.
5. Add 2 to 3 mL wash buffer and centrifuge for 5 minutes at 500 x *g*. Remove the supernatant and add 500 μ L 1% PFA.

NOTE: Samples can be stored for up to 24 hours at 4°C in the dark prior to analysis.

Analysis

1. Analyze on a FACS brand flow cytometer.
2. Use CaliBRITE™ beads and appropriate software (FACSCOMP™, version 1.1 or later, or AutoCOMP™, version 3.0.2) for setting photomultiplier tube (PMT) voltages and fluorescence compensation and for checking instrument sensitivity prior to use. Refer to the appropriate TriTEST™ three-color application note for flow cytometric setup, acquisition, and analysis.

NOTE: Proper instrument setup with the correct version of FACSCOMP or AutoCOMP is important for obtaining accurate results with the FastImmune assay. Contact your BD Biosciences representative if you have an older version of either FACSCOMP or AutoCOMP.

3. Acquire data with CellQuest™ or LYSYS™ II software, using a fluorescence or forward scatter (FSC) threshold. Typically, 10,000 gated events is sufficient.
4. Gate on FL3⁺ cells. Display data as two-color dot plots to determine cytokine expression. Data can be analyzed using CellQuest, LYSYS II, PAINT-A-GATE™, or Attractors™ software. With PMA activation, platelets can move into the FL3⁺ gate. In this case, gate on FSC/SSC. In assays with a CD4 trigger, gate on FSC/SSC to exclude monocytes.

Calculate the specific response

As illustrated by the following formula, the specific response of cells to any stimulus is obtained by subtracting % positive events in the isotype control sample from % positive events in the anti-cytokine antibody-stained sample. Then subtract the isotype-corrected response of the unstimulated sample from that of the stimulated sample.

Formula: (AS – AIC) – (US – UIC)

where AS = activated sample
 AIC = activated isotype control
 US = unstimulated sample
 UIC = unstimulated isotype control

Troubleshooting

The following troubleshooting matrix should help you pinpoint potential sources of problems in the assay. The most common sources are the sample preparation and activation steps. The troubleshooting strategy in Figure 4 illustrates how the activation and intracellular staining controls can be used to identify activation problems.

Problem	Possible Cause	Solution	Comments
No intracellular staining of cytokines or CD69	Cells not activated	Activation reagents not prepared correctly. See the Procedure section for stimulus preparation and storage.	>90% CD3 ⁺ T lymphocytes should be CD69 ⁺ after 4 hour-PMA + I activation.
	Wrong anticoagulant used for blood collection	Use only sodium heparin anticoagulant in blood collection. Do not use lithium heparin. Do not use ACD, EDTA, or other calcium-chelating anticoagulants.	Calcium is required for lymphocyte activation; calcium-chelating anticoagulants prevent activation.
	Cells not permeabilized	Treat cells with FACS Lysing Solution prior to treatment with FACS Permeabilizing Solution.	FACS Lysing Solution conditions cells for permeabilization.
	BFA inactive or prepared incorrectly	Prepare BFA as directed. Aliquot BFA stocks and store at -20°C.	See the Procedure section for preparation of non-BD Biosciences reagents.
Intracellular staining positive but dim	Wrong concentration of anti-cytokine monoclonal antibodies	Use only BD directly conjugated monoclonal antibodies at recommended concentrations.	IL-4 is often expressed at frequencies below 2% in normal activated T lymphocytes.
	Permeabilized cells not washed prior to intracellular staining	Wash permeabilized cells as per protocol prior to staining.	
Background staining too high	Poor conjugate purification or antibody conjugate breakdown, resulting in free fluorochrome which binds nonspecifically	Use BD FastImmune reagents.	BD Biosciences reagents are carefully designed to minimize background staining. BD Biosciences PE-conjugated isotype controls for intracellular staining are specially formulated for this application. The surface staining isotype controls are formulated differently and may yield high background in intracellular staining applications.
	Antibodies with low affinity for fixed, nascent intracellular antigens requiring high antibody concentrations	Use BD FastImmune reagents.	
	Wrong isotype control: Control Ig used at too high concentration	Use BD Biosciences-matched isotype controls for intracellular staining at recommended concentration (matching the concentration of the test antibody conjugate).	
Unacceptable cell loss during procedure	Cells not recovered during centrifuge washing steps	Centrifuge fixed and permeabilized cells at 500 x <i>g</i> .	Fixed cells have lower density than live cells; therefore, they require higher centrifugal force to pellet.
	Cells not recovered during aspiration steps	Decant supernatant instead of aspirating with vacuum.	
Incomplete RBC lysis	PMA + I activation	Follow procedures for FL3 triggering to eliminate debris and unlysed cells from analysis.	PMA + I-activated whole blood samples can be difficult to lyse. PMA tends to stabilize RBC plasma membranes.
	FACS Permeabilization Solution or FACS Lysing Solution not diluted 1:10 in deionized (DI) water	Follow protocol for dilution of FACS Permeabilization Solution or FACS Lysing Solution.	
	Lysis not carried out at room temperature	Lyse at room temperature.	









Collect blood			
			
Aliquot blood and activate			
Activation control		Intracellular staining control	
 + PMA and Ionomycin NO BFA		 + PMA and Ionomycin + BFA	
Surface stain			
 CD69 PE/CD3 PerCP	 Isotype control	NO	
Lyse			
YES	YES	YES	
Permeabilize			
NO	NO	YES	
Intracellular stain			
NO	NO	 CD69 PE/CD3 PerCP	 Isotype control
Wash and fix			
YES	YES	YES	YES
Analyze			
 <p>>90% of CD3⁺ cells should be CD69⁺ If activation control is not >90% positive, problem lies in activation method or reagents. If activation control is >90% positive, but staining control is not, problem lies in permeabilization/staining.</p>			

Figure 4 FastImmune cytokine troubleshooting strategy

BD Biosciences publishes this method
as a service to investigators.

Detailed support for non-flow
cytometric aspects of this

procedure may not be

available from BD Biosciences.

References

1. Aggarwal BB, Puri RK. Common and uncommon features of cytokines and cytokine receptors: An overview. In: *Human Cytokines: Their Role in Disease and Therapy*. Cambridge, MA: Blackwell Science; 1995:3-24.
2. Kroemer G, Alboran M, Gonzalo JA, Martinez C. Immunoregulation by cytokines. *Crit Rev Immunol*. 1993;13:163-191.
3. Street NE, Mosmann TR. Functional diversity of T lymphocytes due to secretion of different cytokine patterns. *FASEB J*. 1991;5:171.
4. Paul WE, Seder RA. Lymphocyte responses and cytokines. *Cell*. 1994;76:241.
5. Mosmann RT, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*. 1986;196:2348.
6. Sad S, Mosmann T. Single IL-2 secreting precursor CD4 T cell can develop into either Th1 or Th2 cytokine secretion phenotype. *J Immunol*. 1994;153:3514.
7. Jung T, Schauer U, Heusser C, Neumann C, Rieger C. Detection of intracellular cytokines by flow cytometry. *Immunol Methods*. 1993;159:197.
8. Picker LJ, Singh MK, Zdraveski Z, et al. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood*. 1995;86:1408-1419.
9. Openshaw P, Murphy EE, Hosken NA, et al. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J Exp Med*. 1995;182:1357-1367.
10. Somasse T, Larenas PV, Davis KA, de Vries JE, Yssel H. Differentiation and stability of Th1 and Th2 cells derived from naive human neonatal CD4⁺ T cells, analyzed at the single-cell level. *J Exp Med*. 1996;84:473-483.
11. Ferrick DA, Schrenzel MD, Mulvania T, Hsieh B, Ferlin WG, Lepper H. Differential production of interferon- γ and interleukin-4 in response to T_H1- and T_H2-stimulating pathogens by $\gamma\delta$ T cells in vivo. *Nature*. 1995;373:255-257.
12. Cherwinski HM, Semenuk GT, Ransom JT. Stimulation of a T helper cell class 2 clone with immobilized anti-T cell receptor antibody activates a Ca²⁺ and protein kinase C-independent lethal signal pathway. *J Immunol*. 1992;148:2996-3003.

Patents

FACS Lysing Solution: US Patent Nos. 4,654,312; 4,902,613; and 5,098,849
Phycoerythrin (PE) Conjugates: US Patent No. 4,520,110; European Patent No. 76,695;
Canadian Patent No. 1,179,942
Peridinin Chlorophyll Protein (PerCP): US Patent No. 4,876,190

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