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August 2011 | Detection of Intracellular Cytokines in T Lymphocytes using the BD FastImmune™ Assay on the BD FACSVerse™ System

Detection of Intracellular Cytokines in T Lymphocytes using the BD FastImmune™ Assay on the BD FACSVerse™ System

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BD Biosciences

Application Note

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Summary

Intracellular cytokine staining (ICS) is a flow cytometry–based assay that can detect cytokine production by immune cells in combination with cell surface markers. BD FastImmune™ intracellular cytokine detection kits are designed to detect cytokines such as interferon gamma (IFN-γ), interleukin 2 (IL-2), and tumor necrosis factor alpha (TNF-α), along with the activation marker CD69 expressed by antigen-activated CD4+ and CD8+ T lymphocytes in whole blood. The BD FACSVerse™ system includes the cytometer, BD FACSuite™ software for acquisition and analysis, and BD FACSuite research assays for use with specific reagent kits. Based on the BD FastImmune Intracellular Detection Kits, the BD FastImmune CD4 3-color (3C) assay in BD FACSuite software provides acquisition, analysis, and reporting functions for generating reliable and consistent ICS data. This application note describes a BD FastImmune CD4 3C assay for the detection of CD4+ lymphocytes producing IFN-γ upon antigen-specific stimulation with a cytomegalovius peptide pool (CMV pp65). Further, the BD-defined assay was customized to include three additional markers—CD3, CD8 and intracellular IL-2—to create a 6-color user-defined assay for characterization of polyfunctional CD4+ and CD8+ T lymphocytes.
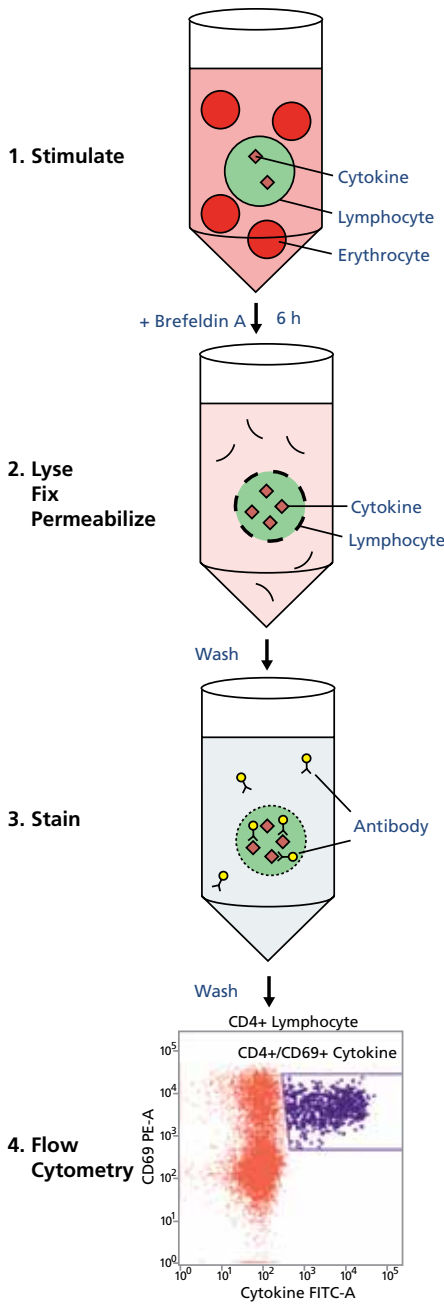
Figure 1. Overview of the BD FastImmune Intracellular Cytokine assay.

Introduction

Cytokines are soluble proteins that play an important role in immunity, inflammation, and hematopoiesis. They are produced by a variety of cell types and secreted in response to specific and non-specific stimuli. Cytokines are known to regulate cell growth and differentiation and mediate normal and pathological immune responses. Monitoring cytokine profiles of T lymphocytes in response to physiological and non-physiological immunomodulators is important for researchers investigating basic immunological function and disease pathogenesis.

T-cell responses play a pivotal role in a wide variety of immune function phenomena such as immunoregulation, modulation of physiological functions, infectious disease, sepsis, vaccination, cancer, organ transplantation, and organ-specific diseases. The detection of T cells that have the capability to produce cytokines is of particular importance to researchers investigating the role of cytokines in these scenarios.

To detect cytokines in appropriate populations of T lymphocytes, they are initially characterized based on the surface and intracellular expression of CD3 and CD4 (helper T cells) or CD8 (cytotoxic T cells). T-helper cells can be further classified by the types of cytokine that they produce upon activation. For example, CD3+CD4+ T cells producing IFN-γ, IL-2, and TNF-α are known as T helper 1 (Th1) cells. Th1 cells orchestrate cell-mediated immune responses and help eliminate intracellular pathogens such as viruses. CD3+CD4+ T cells that produce IL-4, IL-5, IL-6, IL-10, or IL-13 are known as T helper 2 (Th2) cells. Th2 cells regulate humoral immune responses against extracellular infections such as bacteria and multicellular parasitic organisms. In addition, T cells that produce mixed profiles of the Th1 and Th2 cytokines have also been identified, as well as T cells that produce other subsets of cytokines such as Th9 cells, Th17 cells, and follicular helper T cells. For further details, visit **bdbiosciences.com/tcell**.

Various techniques such as ELISPOT, in situ hybridization, immunohistochemistry, limiting dilution analysis, and single-cell PCR allow the enumeration of cytokine-producing cells. However, flow cytometry is a powerful analytical technique for characterization of cytokine signatures of various subsets of T lymphocytes. Using multicolor flow cytometry, individual cells can be simultaneously analyzed for multiple parameters including size and granularity, as well as the expression of surface and intracellular proteins. It provides visual and statistical information by precisely defining cell subsets based on surface and intracellular phenotype.

Figure 1 shows an overview of the BD FastImmune ICS assay. The basic workflow of the ICS assay used in this application note is as follows:

1. Whole blood or peripheral blood mononuclear cells (PBMCs) are stimulated to produce cytokines using specific antigens which can be whole viruses or proteins, pools of peptide mixes, or individual peptides. Polyclonal stimuli for T cells are often used as positive controls. These include phorbol 12-myristate 13-acetate (PMA) combined with the calcium ionophore (ionomycin), the superantigen staphylococcus enterotoxin B (SEB), or the mitogen phytohaemagglutinin (PHA).

2. Extracellular secretion of cytokines is blocked using a protein transport inhibitor such as brefeldin A or monensin.

3. Typically IFN-γ, IL-2, and TNF-α can be detected after 6 hours. The length of stimulation might vary depending on the nature of the antigen and the cytokine to be detected (see the Tips and Tricks section).

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4. Whole blood is lysed, fixed, and stained with antibodies to surface markers, and then permeabilized to enable staining of intracellular cytokines.

5. Multiparameteric data is acquired and analyzed using flow cytometry.

BD FastImmune CD4 Intracellular Cytokine Detection Kits are designed for the detection of intracellular cytokines (IFN-γ, IL-2, or TNF-α) and expression of the activation marker CD69 in antigen-activated CD4$^+$ and CD8$^+$ T lymphocytes in whole blood. Research applications include studies of T-cell responses to antigens such as CMV, human immunodeficiency virus (HIV), herpes viruses, and tumor antigens. In addition to cytokine production, the expression (upregulation) of CD69 helps identify activated T cells.

BD FACSVerse System and BD FACSuite Software

The BD FACSVerse system is a high-performance flow cytometer that incorporates easy-to-use, task-based workflows. The system streamlines every stage of operation from automated setup through data analysis and reporting. The system includes unique features such as the BD™ Flow Sensor option for volumetric counting, automated procedures for setting up the instrument and assays, and configurable user interfaces that provide maximum usability for researchers. These functions are integrated to provide simplified routine applications while simultaneously providing powerful acquisition and analysis tools for more complex applications. In addition, the BD FACS™ Universal Loader option (the Loader) provides compatibility for using either tubes or plates for samples, with or without barcoding for sample identification and tracking.

The BD FastImmune CD4 3C assay in BD FACSuite software is a specific module based on the BD Fastimmune Intracellular Cytokine Detection Kit and contains all the acquisition, analysis, and reporting functions for generating data to determine the cytokine expression profile and phenotype of T lymphocytes present in whole blood samples.

Pre-defined assays such as the BD FastImmune CD4 3C assay can be also be used as a starting point for creating custom experiments and assays to suit the needs of researchers. These user-defined assays can then be run in a worklist and deployed to other BD FACSVerse cytometers within the laboratory or to an external site.

Objective

The objective of this application note is to show proof-of-principle data to demonstrate the following:

• The ease of use of the BD FastImmune 3-color CD4 intracellular IFN-γ detection kit and BD FastImmune CD4 3C assay for detection of CD4$^+$ lymphocytes producing IFN-γ after stimulation with CMV pp65.

• Customization of a 6-color user-defined assay using the BD FastImmune CD4 3C assay as a template by adding CD3 and CD8 surface markers and intracellular IL-2 for characterization of polyfunctional CD4$^+$ and CD8$^+$ T lymphocytes.

Methods

Kits

Product Description	Vendor	Catalog Number
BD FastImmune 3-color CD4 Intracellular IFN-γ Detection Kit, Antibody Cocktail (IFN-γ FITC, CD69 PE, CD4 PerCP-Cy™5.5), Isotype Control Cocktail, Co-Stimulatory Antibodies (CD28/CD49d), Brefeldin A (BFA) Solution, EDTA Solution, BD FACS™ Lysing Solution (10x), BD FACS Permeabilizing Solution 2 (10x)	BD Biosciences	340970

Antibodies

Specificity	Clone	Fluorochrome	Isotype	Vendor	Catalog Number
CD3	SK7	APC-H7	Ms IgG$_1$, κ	BD Biosciences	641397
CD8	SK1	APC	Ms IgG$_1$, κ	BD Biosciences	340584
IL-2	MQ1-17H12	PE-Cy™7	Rat IgG$_{2a}$, κ	BD Biosciences	560707

Reagents and Materials

Product Description	Vendor	Catalog Number
BD Falcon™ conical tubes,15-mL Tubes	BD Biosciences	352096
BD FACSuite CS&T Research Beads	BD Biosciences	650621 (50 tests) 650622 (150 tests)
BD FACSuite FC Beads - 4c Research Kit	BD Biosciences	650625
BD FACSuite FC Beads - 4c Plus Research Kit	BD Biosciences	650626
BD Pharmingen™ Stain Buffer (BSA)	BD Biosciences	554657

BD FACSVerse Instrument Configuration

Wavelength (nm)	Detector	Dichroic Mirror (nm)	Bandpass Filter (nm)	Fluorochrome
488	A	752 LP	783/56	PE-Cy7
	B	665 LP	700/54	PerCP-Cy5.5
	D	560 LP	586/42	PE
	E	507 LP	527/32	FITC
640	A	752 LP	783/56	APC-H7
	B	660/10 BP	660/10	APC

Software

Product Description	Catalog Number
BD FACSuite Research Assay Software	651363

Specimens

Blood specimens were collected from normal donors who consented to participate in an Institutional Review Board–approved protocol.

Sample Preparation

Whole blood was collected from CMV-seropositive donors into BD Vacutainer® tubes (heparin), and prepared as outlined in the BD FastImmune 3-Color CD4 Intracellular Cytokine Detection Kit Technical Data Sheet (TDS). Staphylococcal enterotoxin B (1 µg/mL) from Staphylococcus aureas (SEB, Sigma-Aldrich, No. S-4881) was used as a non-specific positive control.[1] Antigen-specific stimulation was performed using 1.7 µg/mL of CMV pp65 – Recombinant Protein Human CMV (JPT Peptide Technologies) for 6 hours in the presence of brefeldin A.

Stimulated cells were stained according to the BD FastImmune 3-Color CD4 Intracellular Cytokine Detection Kit TDS. For the 6-color user-defined assay, the additional antibodies were added to cells at the same time as the antibodies provided in the kit.

Instrument Setup

The basic workflow for BD FACSVerse instrument setup is shown in Figure 2. Performance quality control (QC) was performed using BD FACSuite CS&T Research beads as outlined in the *BD FACSVerse System User's Guide*.[2] The BD FastImmune CD4 3C assay setup was then performed following the instructions in the *BD FACSuite Software Research Assays Guide*.[3]

BD FastImmune CD4 3-Color Intracellular Cytokine Detection Assay

Data was acquired using a BD FACSVerse system and BD FACSuite software using the pre-defined BD FastImmune CD4 3C assay. As shown in Figure 3, a worklist was created from the assay and the samples were acquired automatically using the Loader with an acquisition criteria of 30,000 CD4+ lymphocyte events for each tube. The reference settings for compensation were automatically applied. The data was automatically analyzed and a report was generated.

The report generated from the BD FastImmune CD4 3C assay includes the following plots and gates for unstimulated (negative control), positive control (SEB-stimulated), and antigen-specific stimulated samples. As shown in Figure 5, each section of the report contains the following plots for each sample:

- SSC-A vs FSC-A with a gate for lymphocytes
- SSC-A vs CD4 PerCP-Cy5.5-A with a gate for the CD4+ lymphocyte population
- CD69 PE-A vs Cytokine FITC-A with a gate for the cytokine expressing CD69+ population

A summary of assay results with statistics for unstimulated (negative control), positive control (SEB-stimulated), and antigen-specific stimulated samples is automatically generated in the lab report. The summary table includes:

- Absolute number of lymphocyte events
- Absolute number of CD4+ lymphocyte events
- Absolute number of CD69+Cytokine+ events
- CD69+Cytokine+ events as a percentage of CD4+ lymphocytes
- FITC (cytokine) median fluorescence intensity (MFI) of the CD4+CD69+ Cytokine+ population.

In addition, the following cross-tube result is calculated to determine cellular response in CD4+ Lymphocytes:

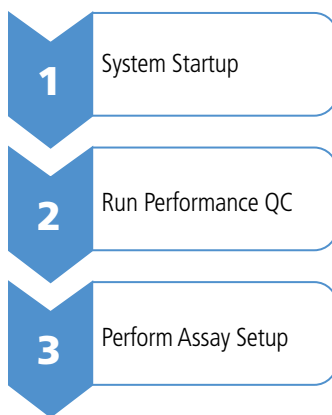Cell Response CD4+ Lymphocytes = % Cytokine-producing CD4+ lymphocytes from stimulated sample – Unstimulated control.

1 System Startup

2 Run Performance QC

3 Perform Assay Setup

Figure 2. Workflow for instrument setup.

1 Create Worklist

2 Select BD FastImmune CD4 3C assay

3 Adjust Gates if Necessary

4 Acquire & Analyze Data

5 Print Report
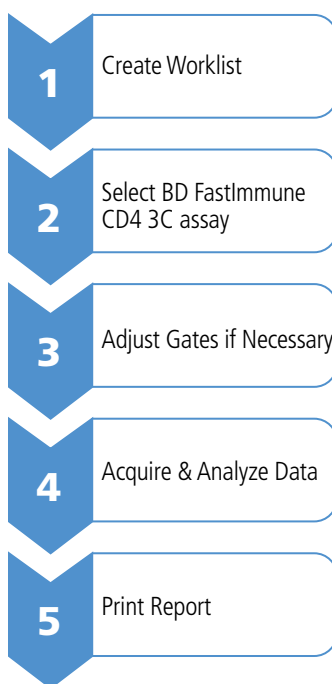
Figure 3. BD FastImmune CD4 3C assay workflow.

1 Create Experiment from the BD FastImmune CD4 3C assay

2 Customize Tube Properties

3 Customize Plots & Gates

4 Create Report

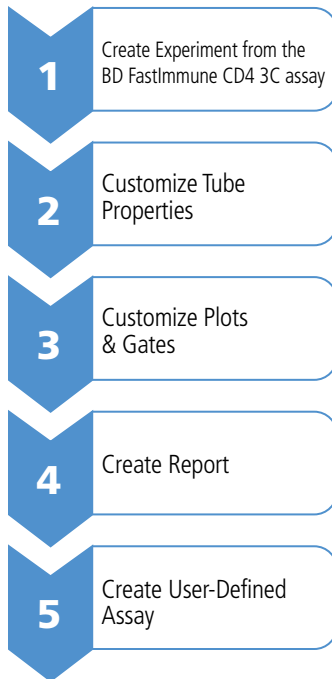**5** Create User-Defined Assay

Figure 4. Workflow for creating a user-defined assay from a BD-defined assay.

User-Defined Assay

The BD FastImmune CD4 3C assay was used as a starting point for creating a 6-color user-defined assay to accommodate three additional markers: CD3 APC-H7, CD8 APC, and IL-2 PE-Cy7 in order to discriminate T-cell (CD3$^+$) subsets (CD4$^+$ and CD8$^+$) and to detect an additional cytokine, IL-2. Figure 4 shows the general workflow for creating a user-defined assay from a BD FastImmune CD4 3C assay. Tube properties for the user-defined assay were customized to include three additional fluorochromes (APC, APC-H7, and PE-Cy7). Photomultiplier tube voltages (PMTVs) were adjusted for the added fluorochromes and lot-specific spillover values for APC-H7 and PE-Cy7 were calculated. For details about adding fluorochromes, see the *BD FACSVerse System Reference*.[4]

The following additional plots and gates were created:

- SSC-A vs CD3 dot plot with a gate for the CD3$^+$ lymphocyte population

- CD8 vs CD4 dot plot with gates for the CD4$^-$CD8$^+$ and CD4$^+$CD8$^-$ populations

- CD69 histograms of CD4$^-$CD8$^+$ and CD4$^+$CD8$^-$ events with markers for the CD69$^+$ populations

- IL-2 vs IFN-γ dot plots for both the CD4$^-$CD8$^+$ and CD4$^+$CD8$^-$ populations

Once the user-defined assay was created, it was used in a worklist and samples were acquired automatically using the Loader. For details about creating a user-defined assay and creating experiments from assays, see the *BD FACSVerse System User's Guide*.[2]

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Results and Discussion

Detection of IFN-γ and CD69 in CMV pp65–Activated T Cells

Using the BD FastImmune Intracellular Cytokine Detection Kit and the BD FastImmune CD4 3C assay, activated T cells were identified based on the expression of CD69 and production of IFN-γ. The data in the lab report (Figure 5) from a CMV seropositive donor shows the differences in CD4+ T-cell activation among unstimulated, SEB (positive control), and antigen-specific responses to stimulation with a CMV pp65 peptide pool. Activation with SEB stimulates IFN-γ production through general (non-specific) stimulation of the T-cell receptor (TCR), while activation with a CMV pp65 peptide pool specifically activates antigen-experienced CD4+ memory T cells. The inclusion of the CD69 marker facilitates the detection of cytokine-producing cells since only the CD69+ T cells produced IFN-γ. In addition, it is important to include CD4+(dim) cells since some of the activated CD69+IFN-γ+ T cells down-modulate the expression of their surface receptors upon activation (note the distribution of purple events in the CD4+ lymphocyte population). Overall, a difference of 0.14 in the percentage of cytokine-producing CD4+ lymphocytes was observed in response to CMV pp65 when compared to the unstimulated control.

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✺BD FastImmune CD4 3C v1.0: Lab Report

|		
Cytometer Name: BD FACSVerse	Software Name & Version: FACSuite Version 1.0.0.1477	Operator Name: BDAdministrator
Cytometer Serial #:123456789		Report Date/Time: 07-Jul-2011 01:50:47

Tube Name: Unstimulated

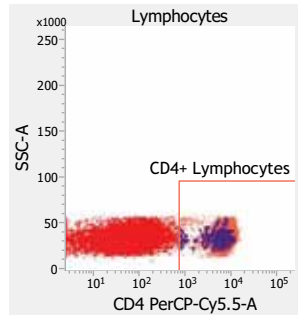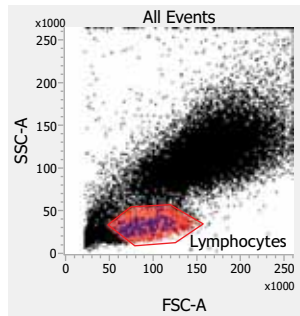| Sample ID	PBMC	Acquisition Date	07-Jul-2011
Antigen	None	Acquisition Time	01:48:29
Cytokine	IFN-γ	Sample Type	PBMCs
Incubation Time	6 h	Concentration	0.0
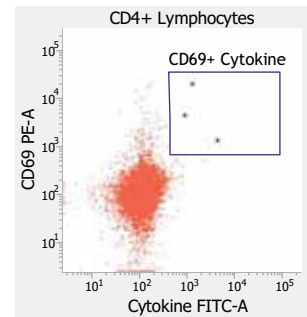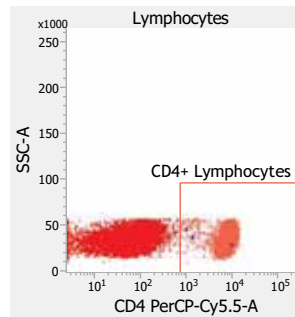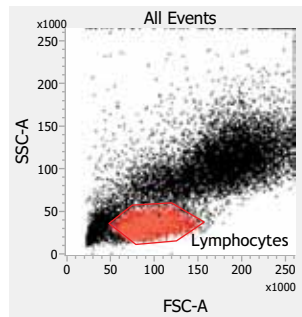
Cytometer Name: BD FACSVerse

Cytometer Serial #:123456789

Software Name & Version:
FACSuite Version 1.0.0.1477

Operator Name: BDAdministrator

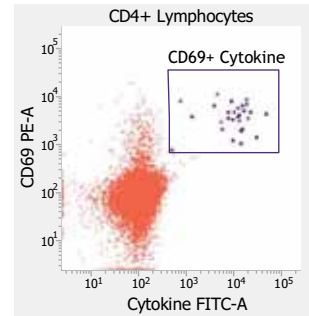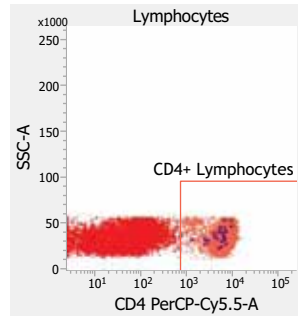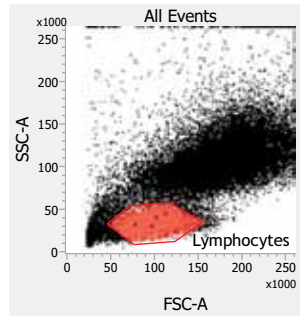Report Date/Time: 07-Jul-2011 01:50:47

Tube Name: Positive Control (SEB)

Sample ID	PBMC	Acquisition Date	07-Jul-2011
Antigen	SEB	Acquisition Time	01:51:27
Cytokine	IFN-γ	Sample Type	PBMCs
Incubation Time	6 h	Concentration	1 µg/mL
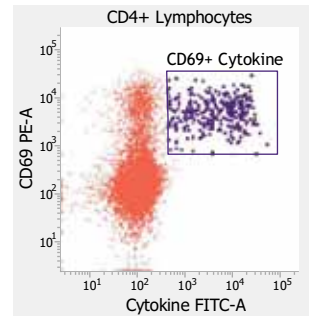
Tube Name: Stimulated (CMV pp65)

Sample ID	PBMC	Acquisition Date	07-Jul-2011
Antigen	CMV pp65	Acquisition Time	01:53:51
Cytokine	IFN-γ	Sample Type	PBMCs
Incubation Time	6 h	Concentration	1.7 µg/mL

Cytometer Name: BD Liberty

Software Name & Version: FACSuite Version 1.0.0.1477

Operator Name: BDAdministrator

Cytometer Serial #:123456789

Report Date/Time: 07-Jul-2011 01:50:47

Result Summary

	Unstimulated	Positive Control	Stimulated
Lymphocyte # Events	72879	67771	71881
CD4+ Lymphocyte # Events	28910	27026	29073
CD69+ Cytokine # Events	8	666	48
FITC MFI of CD4+ CD69+ Cytokine+	2441.0	8351.0	13422
Cytokine+ % of CD4+ Lymphocytes	0.03	2.46	0.17

Cross Tube Result Summary

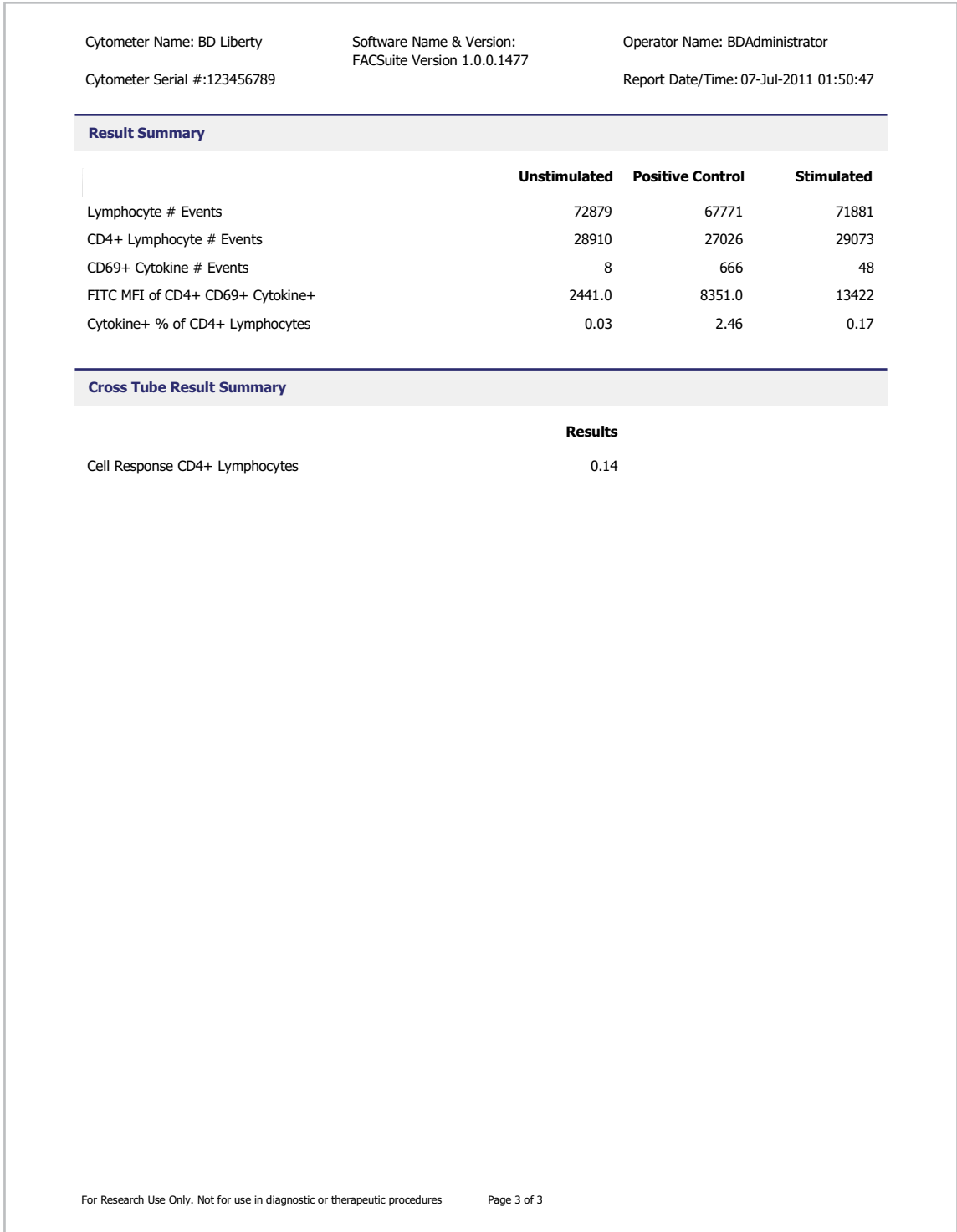|	Results
Cell Response CD4+ Lymphocytes	0.14

Figure 5. FastImmune CD4 3C assay report showing unstimulated, SEB (positive control), and CMV pp65–stimulated samples.
Plots and gates to analyze cytokine response from CD4+/CD69+ lymphocytes are shown for each sample along with a summary of statistics.

The inclusion of a negative control (unstimulated sample) for each donor tested using this assay is mandatory since the baseline level of expression of CD69 and cytokines varies between individuals. An example comparing two different donors is presented in Figure 6 which shows the two-fold difference in the baseline levels of CD69 expression (unstimulated CD69 PE-A vs IFN-γ plots). Moreover, the background response from each individual needs to be taken into consideration in order to establish what constitutes a positive response upon stimulation with a given antigen. In the example shown in Figure 6, the background CD4+CD69+IFN-γ+ lymphocyte response for donor 2 was 0.02% compared to 0.14% in the sample stimulated with CMV pp65. This indicates a 7-fold difference between unstimulated and stimulated sample, thus constituting a positive response. In contrast, more than a 28-fold difference was observed in donor 1.
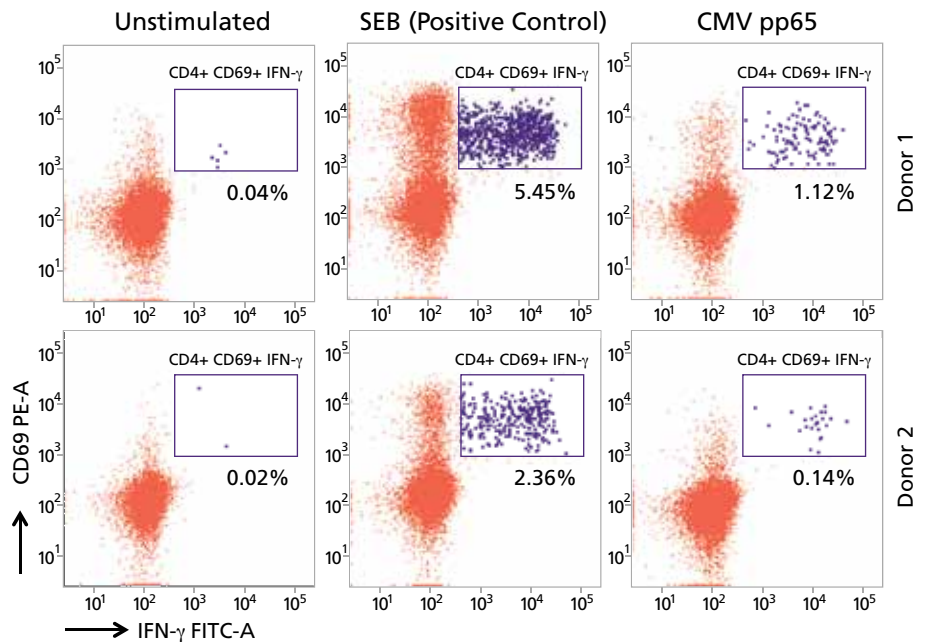

Figure 6. Antigen-specific IFN-γ response in CD4+ and CD69+ lymphocytes. IFN-γ-FITC-A vs CD69-PE-A plots from CD4+ events showing IFN-γ responses from unstimulated (left), SEB-stimulated positive control (center), and CMV pp65–stimulated (right) samples from two donors in a representative experiment.
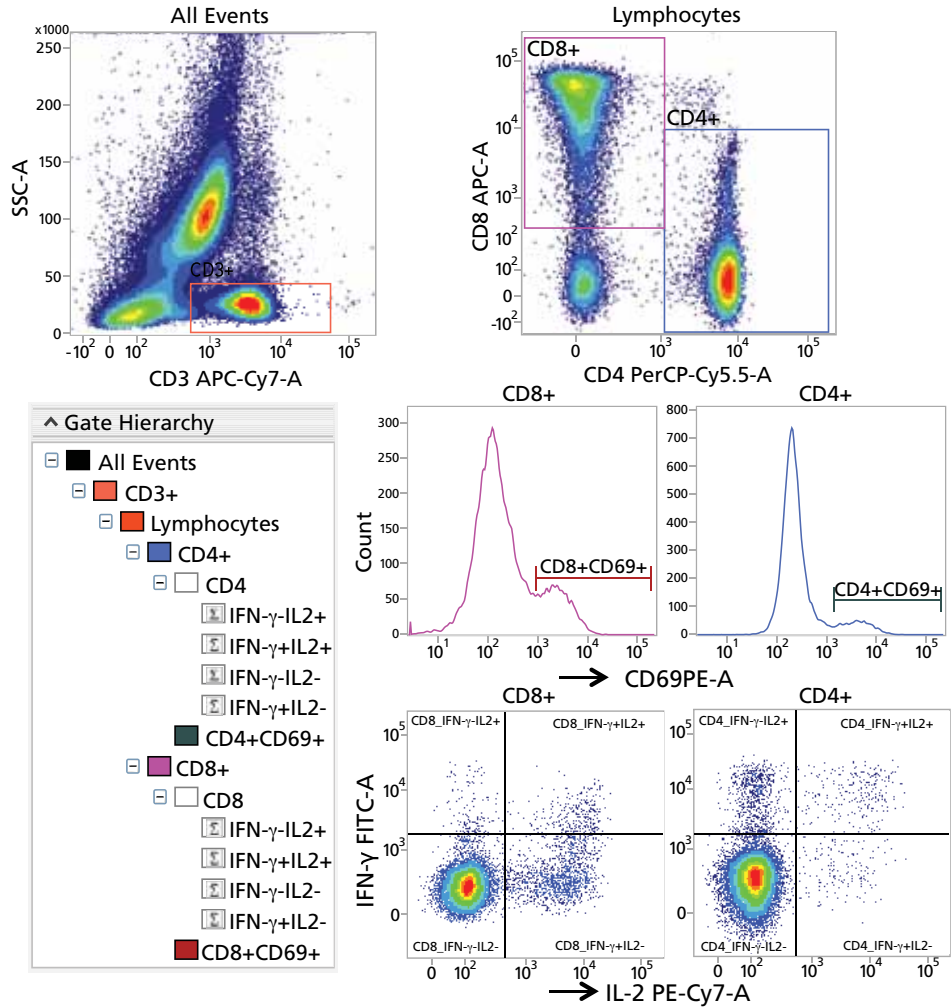
Overall, the data generated from two donors using the BD FastImmune CD4 3C shows differences in baseline levels of CD69 expression and also a variation in the CD4+CD69+IFN-γ+ response after simulation with CMV pp65.

Intracellular IFN-γ and IL-2 Expression in CD3⁺CD4⁺ or CD3⁺CD8⁺ T Lymphocytes Using a User-Defined Assay

Polyfunctional T cells have been reported to secrete more than one cytokine and are implicated in mediating protection against a variety of pathogens.[5-7] Multicolor flow cytometry is an excellent tool for characterizing polyfunctional T cells. Various options for designing multicolor panels are available from BD Biosciences based on a portfolio of anti-cytokine monoclonal antibodies labeled with a variety of fluorochromes. Moreover, these reagents can simply be added to the existing 3- or 4-color BD FastImmune Intracellular Cytokine Detection Kits.

The data in Figure 7 provides an example of a 6-color user-defined assay which was created in BD FACSuite software using the existing BD FastImmune CD4 3C assay as a starting point. The existing 3-color assay was multiplexed with two surface markers (CD8 APC and CD3 APC-H7) and an additional cytokine (IL-2 PE-Cy7). Figure 7, created from the 6-color user-defined assay shows gating scheme, population hierarchy, and statistics for CD4⁺ and CD8⁺ T-cell populations. By incorporating these additional reagents, CD4⁺ and CD8⁺ T lymphocytes producing both IFN-γ and IL-2 simultaneously were clearly distinguishable from cells synthesizing only one of these two cytokines upon stimulation with CMV pp65.

Figure 8 shows the data from two CMV seropositive donors and a summary of all detected responses is presented in Table 1. Similar to the BD FastImmune CD4 3C assay, the inclusion of CD69 in the panel allows identification of activated cells upon stimulation. The inclusion of CD3⁺(dim), CD4⁺(dim), and CD8⁺(dim) populations was also necessary to take into account all cytokine-producing cells since some of these cells down-modulate these receptors upon activation (Figure 7). Moreover, CD3⁺CD4⁺CD8⁺(dim) T lymphocytes are a subset of CD4⁺ T lymphocytes which should also be included in the CD4 gate since some cytokine-producing cells express this phenotype.

Figure 7. Detection of intracellular IFN-γ and IL-2 expression in activated CD4+ and CD8+ lymphocytes using a 6-color user-defined assay. Cells were initially gated on CD3+ (CD3 APC-H7-A vs SSC-A plot), then on lymphocytes based on FSC vs SSC properties. CD4+ and CD8+ T cells were identified by gating the lymphocytes population in the CD4 PerCP-Cy5.5-A vs CD8 APC-A plot. Further, for each T-cell subset, CD69+ populations were gated from CD69 PE-A histograms for CD4+ and CD8+ populations. Expression of IFN-γ and IL-2 was then determined from the CD4+ and CD8+ populations. The population statistics are shown as table and the gating hierarchy is also displayed.
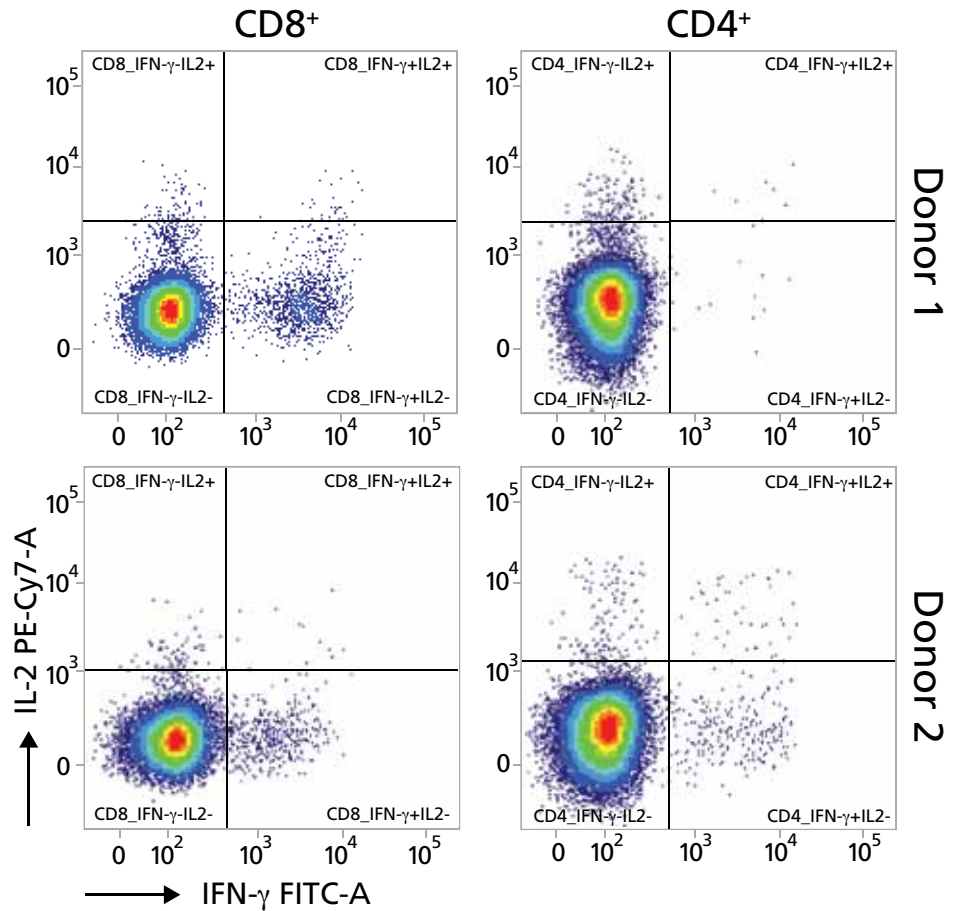
Statistics

Name	Events	% Parent	% Grandparent	% Total
Donor 1 SEB (Positive Control): CD4+CD69+	1,984	5.04	1.07	10.28
Donor 1 SEB (Positive Control): CD8+CD69+	2,750	6.98	1.48	17.67
Donor 1 SEB (Positive Control): CD8_IFN-γ-IL2+	108	0.27	0.06	0.69
Donor 1 SEB (Positive Control): CD8_IFN-γ+IL2+	244	0.62	0.13	1.57
Donor 1 SEB (Positive Control): CD8_IFN-γ-IL2-	13,841	35.15	7.43	88.92
Donor 1 SEB (Positive Control): CD8_IFN-γ+IL2-	1,372	3.48	0.74	8.81
Donor 1 SEB (Positive Control): CD4_IFN-γ-IL2+	652	1.66	0.35	3.38
Donor 1 SEB (Positive Control): CD4_IFN-γ+IL2+	228	0.58	0.12	1.18
Donor 1 SEB (Positive Control): CD4_IFN-γ-IL2-	18,240	46.32	9.80	94.54
Donor 1 SEB (Positive Control): CD4_IFN-γ+IL2-	173	0.44	0.09	0.90

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Figure 8. Multivariate cytokine expression in CD8+ and CD4+ T lymphocytes from two different CMV seropositive donors after CMV pp65 stimulation.

Table 1. Summary of cytokine responses from two donors analyzed using a 6-color user-defined assay.

T-lymphocyte subset	Phenotype	% of CD4−CD8+ or CD4+CD8− T lymphocytes with respective phenotype							
		Donor 1				Donor 2			
		Unstimulated	SEB	CMV pp65	Normalized CMV pp65*	Unstimulated	SEB	CMV pp65	Normalized CMV pp65*
CD4−CD8+	CD69+	1.59	17.67	6.95	5.36	2.23	30.12	3.41	1.18
	IFN-γ+IL-2−	0.09	8.81	6.21	6.12	0.11	17.47	2.29	2.18
	IFN-γ+IL-2+	0	1.57	0.17	0.17	0.01	0.88	0.06	0.05
	IFN-γ−IL-2+	0.05	0.69	0.44	0.39	0.08	0.91	0.31	0.23
CD4+CD8−	CD69+	0.26	10.28	0.38	0.12	0.9	30.06	5.12	4.22
	IFN-γ+IL-2−	0.02	0.9	0.07	0.05	0.04	3.35	0.93	0.89
	IFN-γ+IL-2+	0	1.18	0.02	0.02	0	1.08	0.17	0.17
	IFN-γ−IL-2+	0.11	3.38	0.5	0.39	0.07	7.32	0.49	0.42

*The normalized CMV pp65 response was calculated by subtracting the unstimulated response from the CMV pp65 response.

Conclusions

The pre-defined BD FastImmune 3-color CD4 Intracellular detection kits combined with the BD FastImmune CD4 3C assay in BD FACSuite software provide an easy and reliable way to detect cytokine-producing T lymphocytes upon antigen stimulation. The reagents, instrument setup, and analysis strategy have been optimized to obtain the best possible discrimination of these cytokine producing cells which can constitute a very small subset of the total CD4+ or CD8+ T lymphocytes. Using the BD FastImmune CD4 3C assay, we have shown a proof-of-principle experiment for the detection of IFN-γ–producing CD4+/CD69+ T lymphocytes from two donors after activation with CMV pp65. Further, the reagent kit and the tools available in BD FACSuite software allow users to easily multiplex and modify these pre-defined assays for the identification of additional T-cell subsets. An example of a 6-color user-defined assay multiplexed with an additional cytokine IL-2 for characterization of polyfunctional T lymphocytes has also been demonstrated. The BD-defined assays provide a rapid, easy, and customizable way to evaluate specific T-cell responses which have been proven useful in diverse research settings.

References

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3. *BD FACSuite Software Research Assays Guide*. 23-11470-00, Rev 01.

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5. Duvall MG, Precopio ML, Ambrozak DA, et al. Polyfunctional T cell responses are a hallmark of HIV-2 infection. *Eur J Immunol*. 2008;38:350-363.

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Tips for Developing a Multicolor Research Panel

- Use only sodium heparin anticoagulant. Do not use ACD, EDTA, or other calcium-chelating anti-coagulants since they can inhibit lymphocyte activation.

- Always use a positive control such as SEB or PMA/ionomycin to assess the immune competence of the donors tested.

- When using whole protein antigens, optimal CD4 responses can be achieved by adding two costimulatory antibodies: CD28 and CD49d ($\alpha4\beta7$ integrin, or very late antigen number 4 (VLA-4)). These antibodies lower the threshold for activation of antigen-specific cells.

- Always use a negative control for each donor tested to assess the background level of cytokine expression.

- When using whole proteins for stimulation, brefeldin A or monensin should be added after 2 hours of stimulation.

- The length of antigen stimulation and use of brefeldin A and/or monensin varies depending on the cytokine being detected. For example, detection of IFN-γ, IL-2, and TNF-α is optimal after a 6-hour stimulation using brefeldin A as described in this application note. However, detection of IL-10, IL-17, and CD107a requires the presence of monensin. In addition, for optimal detection of IL-10, 12 hours of stimulation is required. Additional information can be found in the antibody TDS.

- When analyzing data generated from activated T-lymphocyte populations, ensure that the CD4^{+} and CD8^{+} gates also include the CD4[(dim)] and CD8[(dim)] populations. If CD4 and CD8 antibodies are used in the same panel, use a CD4 vs CD8 plot. This will allow the gating of CD4^{+}CD8[+(dim)] cells, which often produce cytokines, without inadvertently including the double-positive CD4^{+}CD8^{+} cells as shown in Figure 7.

- Optimal signal-to-noise for intracellular makers is obtained by staining for 60 minutes at room temperature.

- If additional antibodies are added to pre-existing 3- or 4-color BD FastImmune Intracellular Cytokine Detection Kits, antibody titration might be required for optimal staining. The binding of some antibodies can be affected by fixation and permeablization procedures. Staining for these antibodies should be performed before the fixation and permeabilization steps.

- The acquisition of a sufficient number of events of the relevant populations (CD4 or CD8) is mandatory for obtaining accurate results. This number depends on the background levels and the cutoff used to define a positive response. For example, if the CD4 background for antigen-specific cytokine response is, on average, 0.01% and a response is considered to be positive if it is equal or higher to 0.05%, then at least 32,000 CD4^{+} events need to be collected in order for the differences to be statistically significant.