

# FASTIMMUNE™

# Assay System

A Rapid and Comprehensive System for  
Assessing Lymphocyte Function by Flow Cytometry

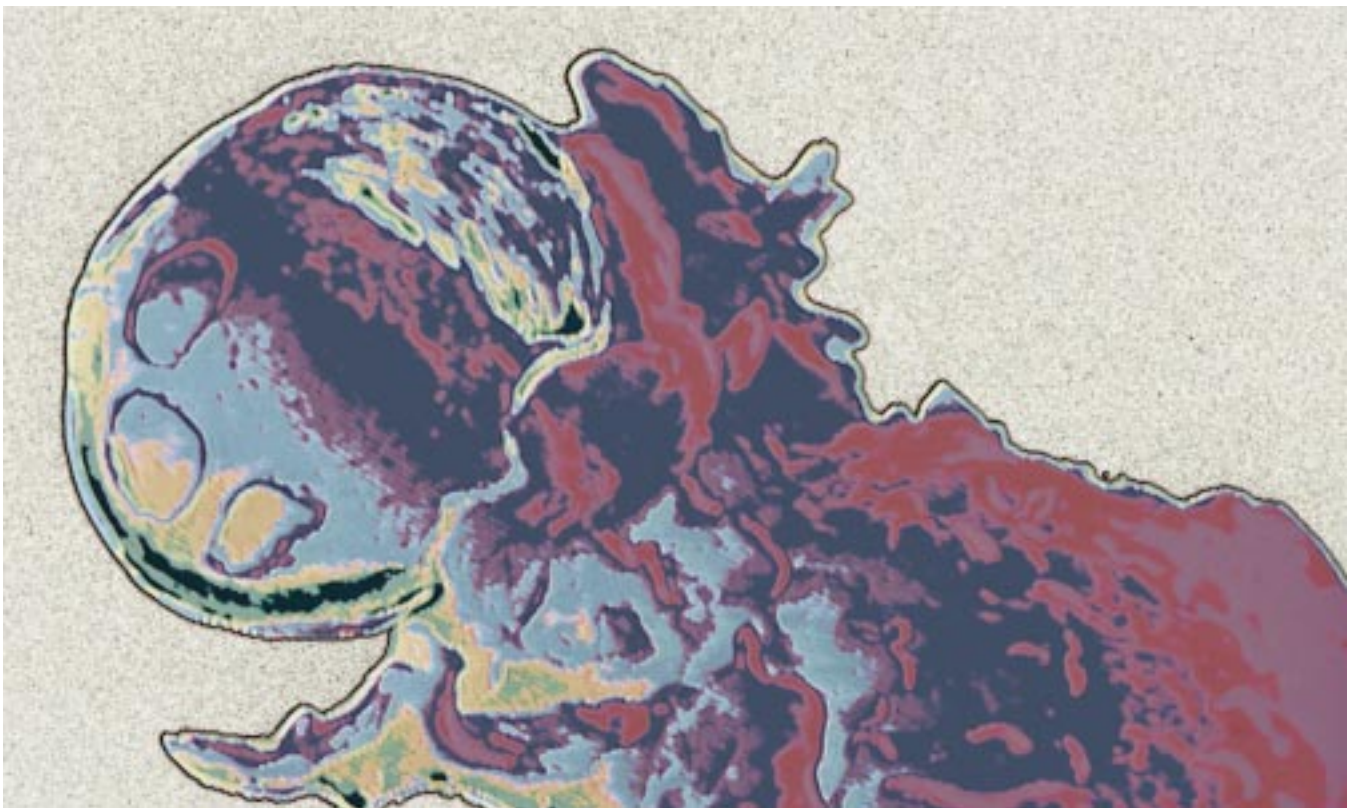
Vernon C. Maino, Ph.D.

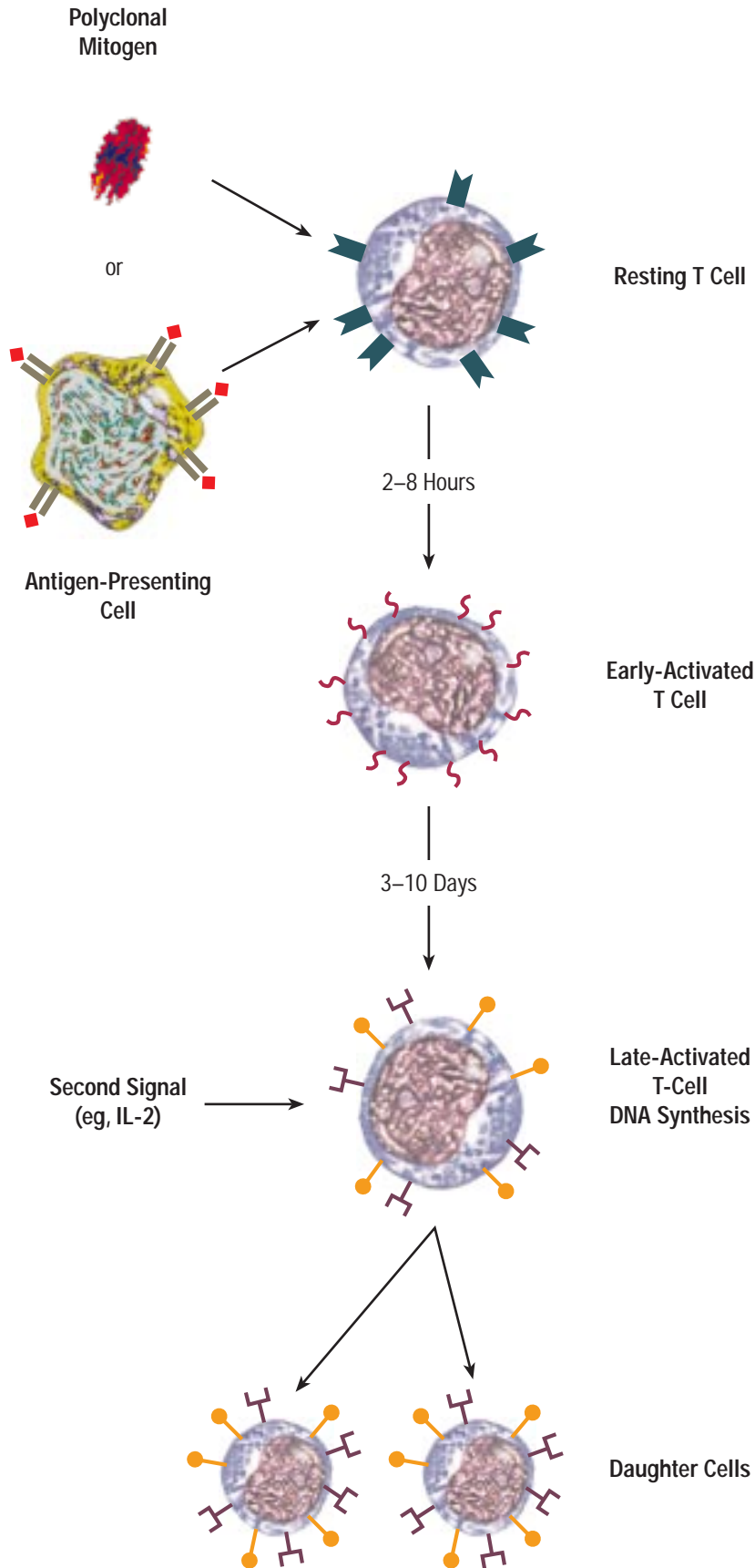
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Lymphocyte phagocytizing cancer cell





## T-Cell Activation

In most cases, activation of T cells requires the presence of accessory cells or antigen-presenting cells (APCs). Under appropriate conditions, in vitro, antigenic, or mitogenic stimuli activate T cells via the T-cell receptor complex. This results in a number of biochemical and morphological changes that culminate in T-cell differentiation and proliferation, and expansion of memory cells. One of the earliest changes noted is the expression of the activation antigen CD69, reaching peak expression within 8 hours of stimulation. Other activation markers, including CD25 (IL-2 receptor), CD71 (transferrin receptor), and HLA-DR are expressed later during this process. Maximum DNA synthesis, as measured by <sup>3</sup>H-thymidine incorporation, is observed 72 to 150 hours following addition of stimulus.

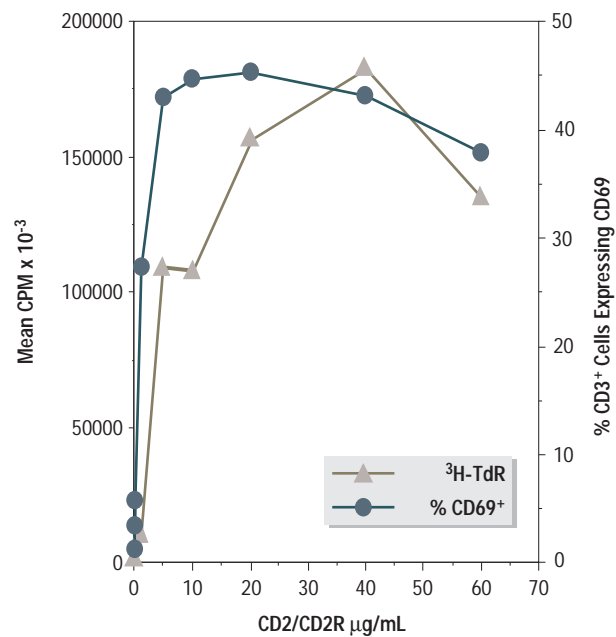
	MHC + Ag
	TcR
	CD69
	HLA-DR
	IL-2R

# Introduction

Multiparameter flow cytometry and fluorescent monoclonal antibody conjugates provide unique tools for identifying functional leucocyte subsets. Isolation of these subsets by cell sorting techniques allows the investigator to assess cellular function of relatively pure populations. While this approach has been very useful for assigning functional properties to leucocyte subsets, new tools are needed to define and quantify functional responses of individual leucocytes to selected stimuli in the whole blood environment.

Historically, bulk methods, such as tritiated thymidine ( $^3\text{H-TdR}$ ) incorporation, MTT dye uptake, and cytokine secretion, have been the standard approach for evaluating cell-mediated responses to a variety of stimuli.<sup>1-3</sup> Methods for quantifying T-cell activation have typically measured the proliferative responses of activated cells following long-term culture of purified peripheral blood mononuclear cells (PBMCs) in complex synthetic media. These methods are not only lengthy and labor intensive, but also do not provide information about individual lymphocyte subsets responding to particular stimuli. In contrast, multiparameter flow cytometry offers the potential to analyze selected lymphocyte subset responses to a variety of stimuli (eg, pathogens and bioresponse modifiers).

The graph comparing CD69 expression and  $^3\text{H}$ -thymidine incorporation suggests that T-cell activation, as measured by the expression of CD69 on



CD69 Expression vs Tritiated Thymidine Incorporation

Incorporation of  $^3\text{H}$ -thymidine was compared to CD69 expression as a measure of T-cell response to varying concentrations of a co-mitogenic pair of antibodies, CD2 and CD2R. Tritiated thymidine incorporation was measured after 3 days of stimulation of PBMCs in media plus autologous plasma; percent CD69 expression was measured by flow cytometry after 4 hours of stimulation under identical conditions as described in the Method section.

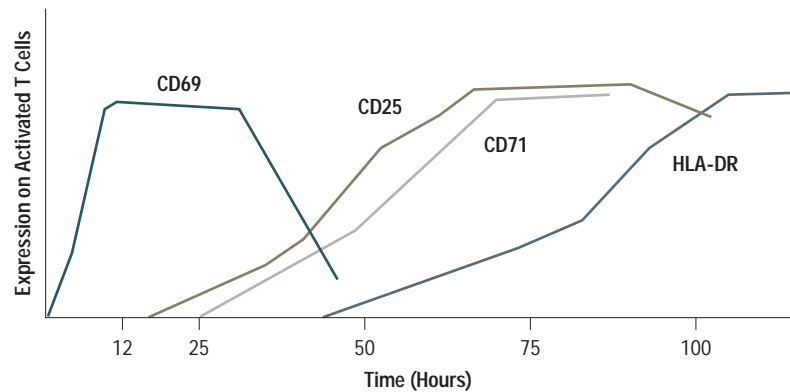
CD3-positive cells, parallels the proliferative response determined by  $^3\text{H}$ -thymidine incorporation. However, it should be noted that the events in T-cell activation measured by the two methods are not equivalent. Expression of CD69 at 4 hours represents an early stage in T-cell activation and consequently reflects the cellular and biochemical events germane to the initial stages of activation. The expression of CD69 does not reflect some of the downstream events involved in signals for proliferation (eg, IL-2R expression). However, a number of recent studies suggest that even within the first 4 hours of activation, a number of committed differentiation pathways become evident (eg, cytokine expression, apoptosis, and anergy) before DNA synthesis and cell division occurs.<sup>4</sup> The availability of new markers to be used in combination with CD69 should provide tools for identifying, at an early stage, committed cells that progress to either effector or anergic pathways of T-cell differentiation.

Becton Dickinson Immunocytometry Systems has developed a unique assay system that directly assesses individual T-lymphocyte subset responses to various physiological and non-physiological stimuli.<sup>5</sup> FASTIMMUNE is a complete reagent system comprised of two- and three-color reagents, isotype and activation controls, and a rapid, no-wash procedure. The assay can be completed within 5 hours in whole blood without tissue culture media or washing steps. The method, based on the expression of CD69, an early activation antigen, utilizes a three-color immunofluorescence staining protocol employing CD3 fluorescence triggering in the FL3 channel. T-cell activation is measured as a function of the percent of each T-cell subset (FL1) that expresses CD69 (FL2).

The FASTIMMUNE Assay System provides rapid analysis of individual lymphocyte subset responses to mitogenic and antigenic ligands in whole blood. The reagent system provides a new window on current lymphocyte phenotyping, enabling basic and clinical researchers to investigate dynamic functional properties of individual T-, B-, or NK cell subsets beyond the limited information provided by bulk population assays.

The CD69 antigen is one of the earliest markers expressed on all activated T, B, and NK lymphocytes, following stimulation by a variety of mitogenic agents.<sup>6-8</sup> The stimulus used can influence the distribution of CD69 expression on lymphocyte subsets. The CD69 molecule, also designated as the activation inducer molecule (AIM),<sup>6</sup> early activation antigen (EA-1),<sup>9</sup> Leu<sup>TM</sup>-23,<sup>7,8</sup> and MLR-3 antigen,<sup>10</sup> is a phosphorylated disulfide-linked homodimeric cell surface protein.<sup>11</sup> CD69 is undetectable on the plasma membrane of resting PBMCs, but is rapidly expressed on antigen- or mitogen-stimulated lymphocytes. Expression of CD69 in response to stimuli requires new RNA transcription and protein synthesis<sup>11</sup> and is believed to be integral to the activation process.<sup>8,12</sup>

## CD69—A Unique Functional Marker for T-Cell Activation



### Kinetics of Expression of Activation Antigens on T Cells

The rapid kinetics and transient expression of CD69 on T cells provide a direct measure of response to experimental stimulus, reduce the potential for artifacts due to *in vivo* activation, and allow the assay to be performed with minimal time and effort. These properties make CD69 the marker of choice for assessing immune function by flow cytometry. In contrast, markers that are expressed later require tissue culture and long incubations (3–10 days) and are vulnerable to distortion of results by *in vivo* artifacts.

Like other genes expressed early in activation, the CD69 gene exhibits a rapid and transient expression, followed by a rapid decay and degradation at the RNA level.<sup>5,7,11</sup> The pattern of CD69 surface expression is similar for all lymphocyte subsets investigated.<sup>5,7,11</sup> Providing the appropriate stimulus is added, all T cells express CD69 with similar kinetics, which are linear for the first 6 hours.<sup>5</sup> The transient nature of CD69 gene expression is consistent with observed constitutive antigen expression in PBMCs. Therefore, very few circulating T, B, or NK lymphocytes expressing CD69 are present in peripheral blood. In contrast, activation markers like HLA-DR and CD38 are expressed late during T-cell activation and are stably expressed. Consequently, these markers are found on significant numbers of circulating T cells. These properties suggest that CD69 represents a generic marker for lymphocyte activation and is well suited to rapid analysis of discrete subsets of responding cells.

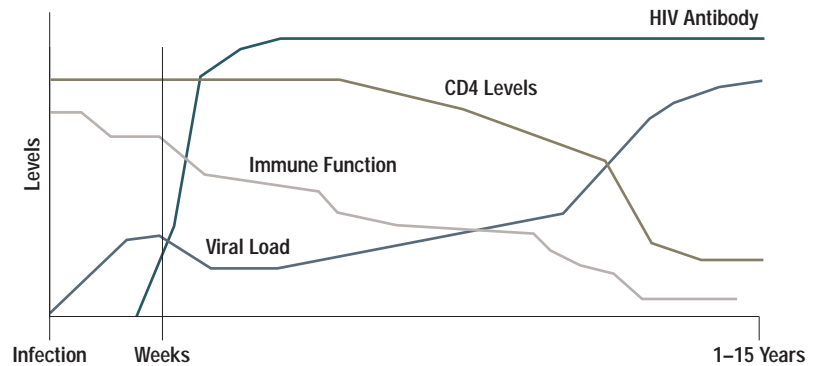
## Broad Range of Applications

Among the many advantages of the FASTIMMUNE Assay System, the following features illustrate the value of the assay in a wide variety of applications:

- Comprehensive, multiparametric analyses:* Most current bulk cellular assays are limited to measuring total population responses. In contrast, multiparameter flow cytometric analysis of early-activation antigen expression allows monitoring of the responses of individual subsets to antigens and mitogens. The rapid format of the FASTIMMUNE Assay System yields incisive answers that can accelerate the rate of discovery and save valuable time and resources.

- Biologically autologous experimental environment:* In addition to ease of use, the measurement of early events of T-lymphocyte activation in whole blood may represent a more physiological response to nominal stimuli. Because whole blood contains cellular and soluble factors that may influence the qualitative and quantitative outcome of an individual T-lymphocyte activation response, the ability to monitor the response directly in whole blood would also reflect these influences. The significance of these factors in clinical situations as it relates to this in vitro flow cytometry-based assay is yet to be determined. However, there is already considerable evidence that soluble factors or cytokines play a major role in regulating immune function.<sup>13,14</sup>

Please see the table in the Summary section for a complete analysis of the benefits of the FASTIMMUNE Assay System.



#### HIV Disease Progression

This diagram is a schematic representation of surrogate marker changes during progression of HIV disease. In addition to significant changes in viral load, CD4 count, and HIV antibody titers during disease progression, a number of investigators have reported diminished immune response potential as a function of a number of cellular and humoral processes.<sup>15</sup> Since both cellular and humoral immune responses are involved in controlling viral replication, understanding the parameters that govern these responses will be paramount to developing effective therapies and reliable monitoring indices of disease progression.

The FASTIMMUNE Assay System promises to be a powerful research tool for answering basic biological questions in a broad range of applications:

- Immune regulation

  - Cytokine/receptor interactions*
  - Co-receptor/ligand interactions*
- Pathogenesis

  - AIDS*
  - Cancer*
  - Autoimmune disorders*
- T-cell subset responses to viral and bacterial antigens
- Cell-mediated responses to opportunistic infections

- Drug/vaccine efficacy assessment
- Chemotherapy—immune status assessment
- Immune status parameters in a variety of conditions

*Transplantation*

*Parasitology*

*Toxicology*

*Nutrition*

*Trauma—surgery, burn, etc*

## Method

### FASTIMMUNE Assay System Overview

The FASTIMMUNE Assay System is comprised of a variety of reagents to ensure ease of use and assay flexibility:

- Three-color reagents for assessing the function of CD4 and CD8 T-lymphocyte populations
- Three-color isotype control reagent
- Activation control
- Two-color, open-system reagent for customized assays

The rapid, no-wash method is carried out in four simple steps as shown in the diagram below. The entire assay may be completed in less than 5 hours with only 9 minutes of hands-on time for a single sample of four tubes (not including instrument setup time). The table below shows typical hands-on and incubation times for each step. Hands-on and elapsed times for large numbers of samples will be longer. Data analysis times will vary depending on the method used. Large numbers of samples can be efficiently analyzed using the automated batch analysis feature of Attractors™ software.

### Time Requirements for the FASTIMMUNE Assay System

<i>Step</i>	<i>Hands-on Time</i>	<i>Incubation Time</i>	<i>Total Elapsed Time</i>
Activate	1 min	4 hr	4 hr 1 min
Stain*	1 min	15 min	16 min
Lyse/Fix†	1 min	15 min	16 min
Data Collection	6 min	—	6 min
Total	9 min	4 hr 30 min	4 hr 39 min

\* Add approximately 10–30 seconds for each additional sample.

† Fixed samples may be stored up to 24 hours at 4°C for analysis at a more convenient time.

The assay utilizes fluorescence triggering on CD3-positive lymphocytes in the FL3 channel and subsequent two-color analysis of the activation marker (CD69 PE) versus the subpopulation marker (such as CD4 FITC or CD8 FITC). T-cell activation is measured as a function of the percentage of T-cell subsets (FL1) that express CD69 (FL2).

### **FASTIMMUNE Assay System Three-Color Reagents**

Complete three-color reagent systems are available for assessing the function of CD4-positive and CD8-positive T-lymphocyte populations.

- CD4 FITC/CD69 PE/CD3 PerCP
- CD8 FITC/CD69 PE/CD3 PerCP

### **FASTIMMUNE Assay System Controls**

FASTIMMUNE Controls include an isotype control and a positive control for activation. The isotype control,  $\gamma_1$  FITC/ $\gamma_1$  PE/CD3 PerCP, is used to set the boundaries between positive and negative results for the FL1 and FL2 channels. The activation control, CD2/CD2R, is a combination of co-mitogenic monoclonal antibodies that assure that the activation system is working properly.

### **FASTIMMUNE Open System Reagent**

The FASTIMMUNE System includes a two-color reagent, CD69 PE/CD3 PerCP, that allows customized subset analyses. The functions of specific T-lymphocyte subpopulations may be studied by adding FITC-labeled T-cell subset markers to the two-color reagent, as demonstrated in the superantigen application in the Examples section. The open system utilizes the same isotype and activation controls.

## **Basic Procedure**

### **Sample Preparation and Mitogenic Stimuli**

- Whole blood is collected using sodium heparin anticoagulant.
- Mitogenic or antigenic ligands and the FASTIMMUNE activation control are added to separate 200 to 500- $\mu$ L aliquots of heparinized whole blood.
- Stimulated samples and unstimulated control samples are incubated for 4 hours at 37°C in a water bath or an incubator.

### **Three-Color Immunofluorescent Staining**

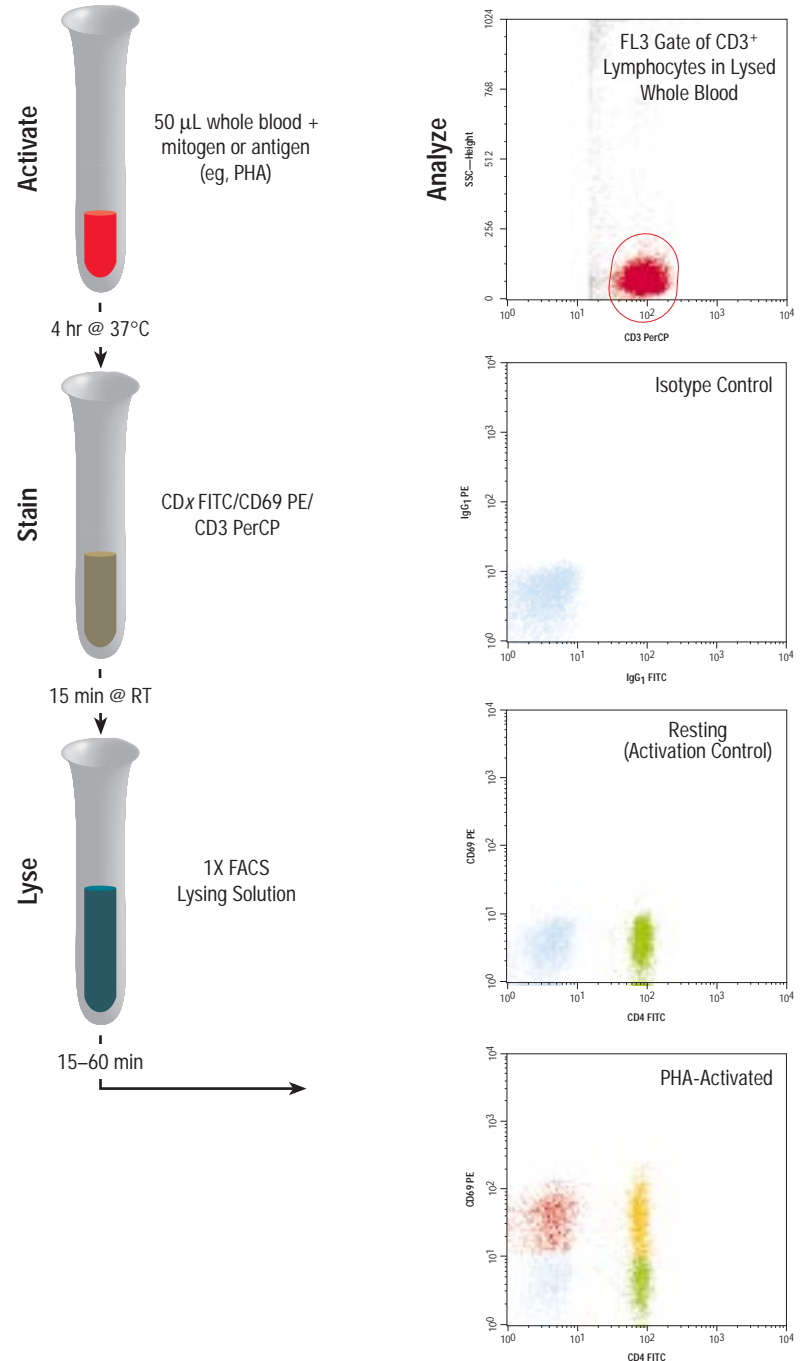
- FASTIMMUNE three-color antibody conjugate combinations, including fluorochrome-labeled isotype-matched staining controls, are added to 50- $\mu$ L aliquots of stimulated and control samples.
- Cells are stained for 15 minutes at room temperature.

### **Lyse/Fix**

- Samples are lysed and fixed with 450  $\mu$ L of FACS® Lysing Solution for 15 to 60 minutes at room temperature or 24 hours at 4°C prior to analysis.

### Flow Cytometric Analysis

- Whole blood samples are analyzed by three-color analysis using a FACScan™ or FACSort™ flow cytometer.
- Data are acquired with LYSYS™ II or CELLQuest™ software using fluorescence triggering in the FL3 channel (CD3 PerCP) to gate on the CD3-positive lymphocyte population.
- Data are displayed as two-color dot plots (FL1 vs FL2) to determine the proportion of activated lymphocyte subsets expressing CD69. Data may be analyzed using LYSYS II, CELLQuest, or Attractors software.

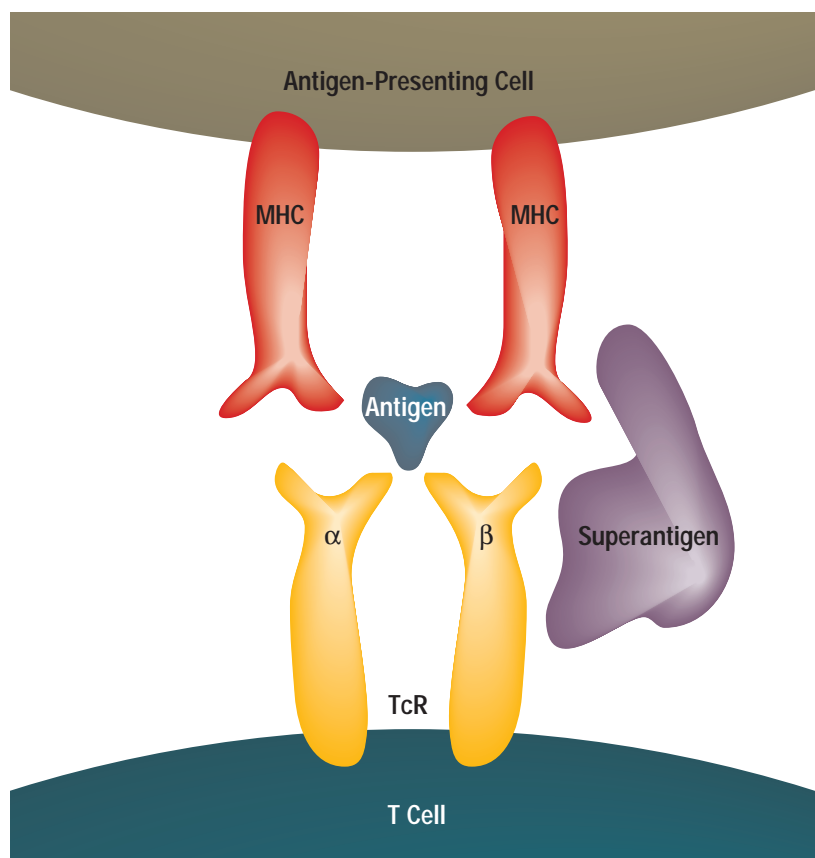


## Examples

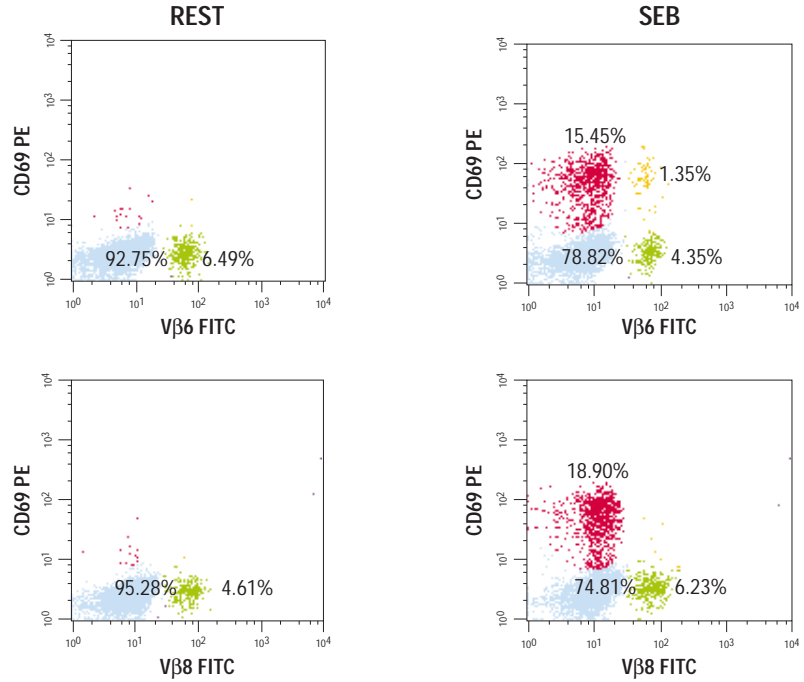
One of the significant advantages of measuring cell activation using multiparameter flow cytometry is that individual subset responses to specific stimuli can be monitored even in the presence of other populations of activated cells. The examples shown below illustrate two applications in which subset-specific analysis can be utilized in important areas of basic and applied research.

### Mechanism of Superantigen Activation of T Cells

Superantigens are proteins derived from various viral and bacterial pathogens that stimulate T cells via the polymorphic determinants on the V $\beta$  chain of the T-cell receptor (TcR) and interact with HLA class II molecules on the antigen-presenting cell. Unlike processed antigen, which is recognized by combinatorial sequences of both  $\alpha$  and  $\beta$  chains of the TcR, superantigens activate whole subsets of T cells that express the appropriate V $\beta$  chain. Since there are only about 20 families of V $\beta$  chain sequences expressed on human T cells, questions of specificity at the level of the T-cell receptor can be addressed with significant numbers of activated T cells. This example demonstrates the flexibility of the assay to utilize other phenotypic markers for fluorescence triggering and the power of multiparametric analysis to identify functional responses in minor populations.



Model of T-Cell Activation by Superantigen



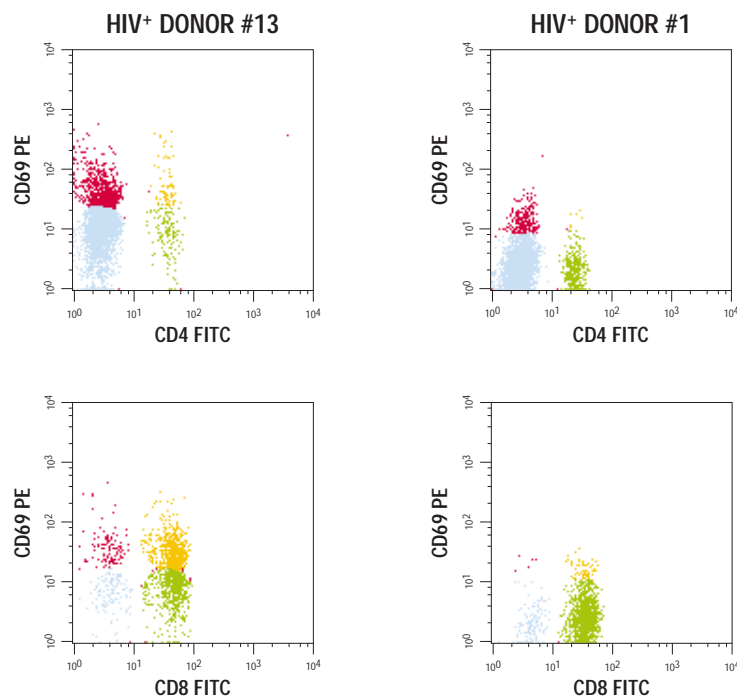
### Activation of Vβ6 but Not Vβ8-Positive T Cells with SEB

Staphylococcal enterotoxin B (SEB) is a bacterial superantigen which reacts with a defined repertoire of Vβ specificities expressed on human T cells.<sup>16,17</sup> For example, SEB has been demonstrated to react with Vβ6 but not with Vβ8.<sup>18</sup> To determine whether the FASTIMMUNE Assay System can be employed to examine specific antigen responses in human whole blood, 4-hour SEB-activated whole blood preparations were analyzed with three-color reagents containing the FASTIMMUNE open system reagents and the appropriate subset markers: anti-Vβ6 FITC/CD69 PE/CD4 PerCP or anti-Vβ8 FITC/CD69 PE/CD4 PerCP. This figure demonstrates that SEB activates Vβ6 T-cell subsets but does not activate Vβ8 subsets, as determined by CD69 expression. These results are consistent with reported observations and demonstrate the utility of multiparameter flow cytometric analysis for the detection of unique T-cell subsets following addition of specific inductive stimuli.

## Disease Model

The functional roles of individual leucocyte subsets in the pathogenesis of a number of diseases (eg, AIDS and autoimmune disease) is an area of active investigation. In addition to enumerating the relative numbers of lymphocyte populations by cell surface immunophenotyping, the ability to monitor functional responses to relevant stimuli provides complementary information about immunological processes involved in disease. The example shown below illustrates differences in the response of the CD4 T-cell subset to a stimulus in two HIV-positive patients with equivalent absolute CD4 cell counts but strikingly different clinical outcomes.

### PWM ACTIVATION CD4 COUNT <15



### CD69 Expression of CD4-Positive T Cells Stimulated by Pokeweed Mitogen: A Comparison of Two HIV-Positive Patients with Similar CD4 Counts

Whole blood preparations obtained from two HIV-positive patients with similar CD4 counts (<15/mm<sup>3</sup>) were compared for their CD4 and CD8 T-lymphocyte subset responses to pokeweed mitogen (PWM). The figure shows that patient #13 exhibits a proportion of CD4-positive T cells responding to PWM. In contrast, the CD4-positive T cells from patient #1 did not respond to PWM. However, a response by the CD8-positive T cells is evident in both patients, although to a lesser degree in patient #1. Although more comprehensive studies comparing this characteristic group of patients have not yet been performed, it is interesting to note that in this example patient #13 remained stable for the duration of the 5-month study, whereas patient #1 progressed rapidly and succumbed to disease. Further studies assessing T-cell subset responses in vitro need to be performed with larger groups of patients to determine whether functional responses can be used synergistically with other cellular and clinical parameters of AIDS to predict disease outcome in individual patients.

# Summary

## Benefits

The FASTIMMUNE Assay System is a rapid and comprehensive research tool for assessing lymphocyte function that has several important benefits over conventional bulk methods, such as  $^3\text{H}$ -thymidine incorporation, MTT dye assays, cytokine secretion, etc:

Benefit	FASTIMMUNE Assay System	Bulk Methods
<i>Fast</i>	<ul style="list-style-type: none"> <li>• &lt;5 hours start to finish</li> </ul>	<ul style="list-style-type: none"> <li>• 3–10 days</li> </ul>
<i>Easy</i>	<ul style="list-style-type: none"> <li>• Whole blood assay</li> <li>• No PBMC fractionation</li> <li>• No tissue culture required</li> </ul>	<ul style="list-style-type: none"> <li>• PBMC fractionation and tissue culture typically required</li> </ul>
<i>Safe</i>	<ul style="list-style-type: none"> <li>• Highly sensitive fluorescent labels</li> <li>• No radioactivity</li> <li>• Sample manipulation and biohazard risk reduced</li> </ul>	<ul style="list-style-type: none"> <li>• Some methods use radioactive components</li> <li>• PBMC fractionation and tissue culture required</li> </ul>
<i>Comprehensive</i>	<ul style="list-style-type: none"> <li>• Multiparameter results accelerate discovery</li> <li>• Functional activity determined simultaneously with phenotype of each individual cell</li> </ul>	<ul style="list-style-type: none"> <li>• Yields bulk result for entire population</li> <li>• Individual or subpopulation responses cannot be discerned</li> </ul>
<i>Expandable</i>	<ul style="list-style-type: none"> <li>• T-cell assay can be customized to address subpopulations</li> <li>• Principle expandable to B cells, NK cells, and monocytes</li> </ul>	<ul style="list-style-type: none"> <li>• Limited expandability</li> </ul>
<i>Biologically Autologous</i>	<ul style="list-style-type: none"> <li>• Whole blood conditions retain cellular and biochemical environment</li> <li>• More accurately represents in vivo conditions</li> </ul>	<ul style="list-style-type: none"> <li>• PBMC fractionation and resuspension in synthetic tissue culture environment required</li> <li>• Cell population and biochemical environment lost</li> </ul>
<i>Time and Cost Effective</i>	<ul style="list-style-type: none"> <li>• Results obtained in hours, not days</li> <li>• No radioactive waste disposal or safety management costs</li> <li>• Multiparameter results accelerate discovery</li> </ul>	<ul style="list-style-type: none"> <li>• Days to weeks required for results</li> <li>• Radioactive waste disposal costs have skyrocketed in recent years</li> <li>• Bulk results yield limited information</li> </ul>
<i>Automation-capable</i>	<ul style="list-style-type: none"> <li>• Compatible with BDIS no-wash reagent technology, sample preparation automation, and data management solutions</li> <li>• Far fewer steps required</li> <li>• Easier to automate</li> </ul>	<ul style="list-style-type: none"> <li>• Many more steps required</li> <li>• Some steps cannot be automated (eg, tissue culture)</li> </ul>

## Potential Future Directions

- New three-color subset reagent combinations
- Reagent and software system for standardization and quantification of activation
- T-cell pathway commitment
- Other cell function measurements
  - Cytokine production*
  - Enzyme activity*
  - Apoptosis*

FASTIMMUNE, Leu, FACScan, FACSort, Attractors, FACStation, LYSYS and CELLQuest are trademarks and FACS is a registered trademark of Becton Dickinson and Company.

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