Bulletin 9336

A Five Color Application for the Cytomics™ FC 500

V Beta Repertoire Analyses of Naïve and Effector T Cell Subsets

INTRODUCTION

T cells play a central role as effectors and regulators of the immune response. In the human, T cells can be defined by characterizing the expression of clustered and non-clustered T cell antigens and by examining the composition of the T Cell Receptor (TCR). All mature T cells are CD3 positive and can be divided into two subpopulations on the basis of two mutually exclusive types of TCRs, αβ T cells and γδ T cells.[1,2] Most T cells in the peripheral blood and lymphoid organs (90-99%) are of the αβ type. αβ T cells can be further sub classified into $CD4^+$ (≈ 2/3) and CD8 positive (≈ 1/3) T cells and very rare CD4 negative/CD8 negative T cells. In contrast, γδ T cells, which are $CD4^-/CD8^-$ or $CD8^-/CD4^+$, comprise 1-10% of T cells in peripheral blood and lymphoid tissues.

The TCR molecular complex is comprised of two subunits. The recognition subunit is composed of either αβ or γδ heterodimers that are present on the cell surface in a mutually exclusive manner. The CD3 complex is the transducing subunit and is common to all αβ and γδ heterodimers. It triggers the T cell when the recognition unit is engaged with the antigen.[3] The TCR must recognize a large variety of antigens specifically and the diversity necessary for this function is generated by a gene rearrangement process which takes place in the thymus during T-cell ontogeny. The consequence of this process is that a given T cell displays a single and unique TCR subunit combination on its cell surface. There are at least 65 Vβ segments in the β locus, that can be grouped into 25 subfamilies, with each member of a given family having more than 75% homology at the nucleotide level with at least one other member of the same subfamily.[4]

During the cellular immune response, T cells react to specific antigen(s). Only those T cells having a TCR specific for a given antigen are triggered by interaction with specialized antigen presenting cells. This activation results in the clonal expansion of specific T cells that may be followed by TCR Vβ gene usage. Many research studies on the T cell repertoire in both normal and pathological situations have been performed to look at repertoire diversity. Immunocompetence is dependent on TCR repertoire diversity.

Many of these investigations have been performed in order to distinguish polyclonal from oligoclonal or monoclonal T-cell proliferations using TCR gene probes and primers in Southern blot or polymerase chain reaction (PCR) procedures.[5, 6] However, these techniques are cumbersome and often not reliable in terms of quantitation and standardization.[7] Moreover these molecular techniques do not allow for Vβ repertoire analysis on T cell subsets unless cell separation or sorting techniques are used.[8-13]

More recently, the availability of a large panel of monoclonal antibodies to TCRs, mainly against Vβ epitopes, allows one to study the TCR repertoire by flow cytometry.[14] As opposed to semi quantitative PCR methods, antibodies detect the unique TCR epitopes rather than measuring DNA/RNA levels. Moreover the use of antibodies in multicolor immunofluorescence allows one to study functional subsets of T cells defined by specific markers such as CD4, CD8, activation markers, naïve/memory markers, etc.[15]

The following protocol describes a five color, nine-parameter application that has been performed on the FC500. It was first presented by Dr. Albert Donnenberg during the ISAC International Congress in May, 2002. This application allows the determination of approximately 70% of known Vbeta specificities, as well as the T cell subset and memory/naïve status of the expressing cells in a total of eight tubes.

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MATERIALS AND METHODS

Instrumentation

A Cytomics™ FC 500 flow cytometer equipped with a single argon laser, five fluorescence channels and RXP software (Version 1.0) was used for this study. The ImmunoPrep/TQ-Prep™ Workstation System was used for red blood cell lysis during specimen processing for flow cytometric analysis.

Reagents

The following reagents were obtained from Beckman Coulter Inc. and used for daily instrument set-up and for preparation of stained samples.

Instrument Set-up Reagents

1. Flow-Check™ Fluorospheres – 6605359
2. Flow-Set™ Fluorospheres – 6607007
3. PC7 (770/488) Setup Kit – 6607121
4. CYTO-COMP™ Cell Kit – 6607023
5. Compensation Reagents:

   IOTest CD45-FITC – IM0782
   IOTest CD45-PE – IM2078
   IOTest CD45-ECD – IM2710
   IOTest CD45-PC5 – IM2653
   IOTest CD45-PC7 – IM3548

Sample Preparation Reagents

1. IOTest CD45RA-ECD – IM2711
2. IOTest CD27-PC5 – 6607107
3. IOTest CD4-PC7 – 6607101
4. IOTest CD8-PC7 – 6607102
5. IOTest Beta Mark Kit – IM3497

The composition of the eight reagent tubes in the Beta Mark Kit is shown in the following table.

Tube	Volume/No. of tests (20 μL/test)	Vβ*/Fluorochrome		Clone	Isotype (species)
A	1.0 mL / 50 tests	Vb 5.3 Vb 3 Vb 7.1	PE FITC PE+FITC	3D11 CH92 ZOE	IgG1 (mouse) IgM (mouse) IgG2a (mouse)
B	0.5 mL / 25 tests	Vb 9 Vb 16 Vb 17	PE FITC PE+FITC	FIN9 TAMAYA1.2 E17.5F3	IgG2a (mouse) IgG1 (mouse) IgG1 (mouse)
C	0.5 mL / 25 tests	Vb 18 Vb 20 Vb 5.1	PE FITC PE+FITC	BA62.6 ELL1.4 IMMU157	IgG1 (mouse) IgG (mouse) IgG2a (mouse)
D	0.5 mL / 25 tests	Vb 13.1 Vb 8 Vb 13.6	PE FITC PE+FITC	IMMU222 56C5.2 JU74.3	IgG2b (mouse) IgG2a (mouse) IgG1 (mouse)
E	0.5 mL / 25 tests	Vb 5.2 Vb 12 Vb 2	PE FITC PE+FITC	36213 VER2.32 MPB2D5	IgG1 (mouse) IgG2a (mouse) IgG1 (mouse)
F	0.5 mL / 25 tests	Vb 23 Vb 21.3 Vb 1	PE FITC PE+FITC	AF23 IG125 BL37.2	IgG1 (mouse) IgG2a (mouse) IgG1 (rat)
G	0.5 mL / 25 tests	Vb 11 Vb 14 Vb 22	PE FITC PE+FITC	C21 CAS1.1.3 IMMU546	IgG2a (mouse) IgG1 (mouse) IgG1 (mouse)
H	0.5 mL / 25 tests	Vb 13.2 Vb 7.2 Vb 4	PE FITC PE+FITC	H132 ZIZOU4 WJF24	IgG1 (mouse) IgG2a (mouse) IgM (rat)

Instrument Set-up

Daily Instrument set-up was accomplished using the automated features of RXP Software as described in sections 2 and 3 of the Cytomics™ FC 500 Operator's Guide (PN 4277273A).

1. Alignment and fluidic verification was performed with Flowcheck beads.
2. Target voltages were determined and standardized with a mixture of Flowset and PC7 Set-up beads (10 drops of Flowset and 5 drops of 770 beads). The target channels for each parameter are shown in the table below and can be used only as a guideline due to lot to lot variation in Flowset values. Two target channels for the forward scatter channel are shown with Flowset detected as a lower peak and PC7 Set-up beads as a higher peak.
3. Full Matrix compensation was performed using CD45-FITC, PE, ECD, PC5, and PC7.
4. A panel was created for test analysis and all cyto-settings were passed into the panel setup.

Peak Targets: 5 Color	
Parameter	**Targets**
FS	102.0
FS770	163.0
SS	478.0
LFL1	15.5
LFL2	24.5
LFL3	26.6
LFL4	17.4
LFL5	83.3

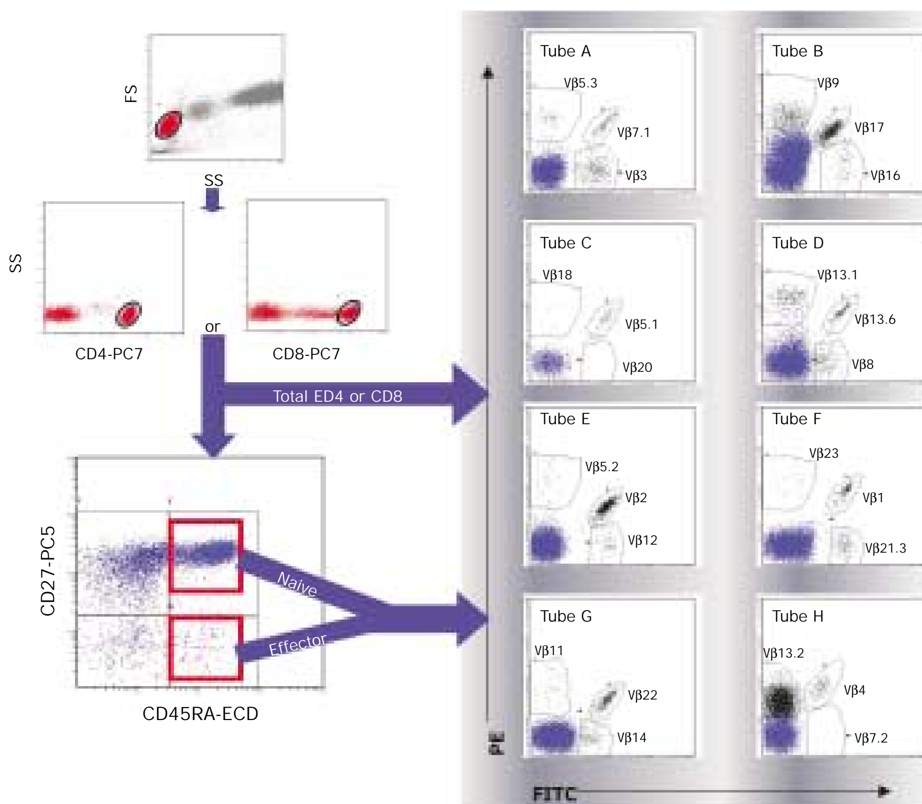
Sample Preparation

1. 100 µL of fresh (< 24 hrs.) EDTA anticoagulated whole blood from each donor was added to the following reagent cocktails containing one of the reagents (A thru H) from the Beta Mark kit and either CD4 or CD8 PC7. Samples with low CD4 counts (< 500/cu mm) were prepared in duplicate and pooled after washing in order to provide adequate cell numbers for analysis.

10 µL CD45RA-ECD
10 µL CD27-PC5
10 µL CD4-PC7 or 10 µL CD8-PC7
20 µL Beta Mark Vial A through H

2. Incubate the reaction tubes for 20 minutes at room temperature and protected from ambient light.
3. Following the incubation period, the RBC component of the reaction mixture is lysed using the ImmunoPrep/TQ-Prep Workstation.
4. The lysed samples are washed with 3mLs of PBS, centrifuged for 10 minutes at 600xg at 25°C, and resuspended in 0.5 mL PBS solution. Samples prepared in duplicate due to low CD4 counts can be pooled following this wash step.

Figure 1



Data Acquisition and Analysis

A sequential gating strategy was employed in order to examine the Vβ repertoire within the total CD4 and CD8 populations as well as within the naïve and memory effector subsets of CD4 and CD8 populations. This gating strategy is depicted in Figure 1. Briefly, the Vβ repertoire of total CD4 or CD8 cells is determined by initially gating lymphocytes in a forward vs. side scatter histogram and then passing these gated events into a side scatter vs CD4 or CD8 histogram. CD4 or CD8 positive events can then be further gated to display the expression of the three Vβ specificities that are provided by each Beta Mark reagent. In order to examine the Vβ repertoire of naïve and memory effector subsets, the gated CD4 or CD8 populations are analyzed for their expression of CD45RA and CD27. The naïve subset is CD45RA+/CD27+ and the effector subset is CD45RA+/CD27-. These additional regions are established and then sequentially gated to display the Vβ expression within these subsets. A minimum of 20,000 CD4 or CD8 events should be collected for each tube. This is important to insure that a statistically relevant number of events are collected within each subset.

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