

25 T-cell repertoire assays
600 tests (25 x 24 TCR V β determinations)

Contents:

- 8 Vials (A to H) of FITC- and PE-conjugated TCR V β antibodies. Each vial contains a mixture of 3 TCR V β antibodies, corresponding to a total of 24 different specificities.
 - Vial A contains 50 tests (1 mL).
 - Vials B to H contain 25 tests each (0.5 mL).
- Package Insert.
- Quick Reference Card.

1. INTRODUCTION

1.1. Background

T cells play a central role in the immune system as effectors and regulators. In the human, T cells can be defined by serological markers (CD molecules and T-cell receptors, or TCRs). All T cells are CD3 positive, and they can be divided into two subpopulations on the basis of two mutually exclusive types of TCR, $\alpha\beta$ T cells and $\gamma\delta$ T cells (1, 2). Most T cells in peripheral blood and lymphoid organs are of the $\alpha\beta$ type (90 to 99% of all T cells). $\alpha\beta$ T cells can be further dissected into CD4 positive- (about 2/3 of $\alpha\beta$ T cells) and CD8 positive-T cells (about 1/3 of $\alpha\beta$ cells), and very rare CD4-negative/CD8 negative cells.

TCR is a molecular complex which comprises two units: a recognition unit is composed of either $\alpha\beta$ or $\gamma\delta$ heterodimers, which are present on the cell surface in a mutually exclusive manner, and a transducing unit, the CD3 complex, common to $\alpha\beta$ and $\gamma\delta$ heterodimers, which triggers the T cell when the recognition unit is engaged with the antigen (3). The recognition units have to recognize a large variety of antigens and the diversity necessary for this function of recognition is generated by a somatic genomic recombination process which takes place in the thymus (T-cell ontogeny). There are four TCR gene loci (α , β , γ and δ). Each of them is composed of several V segments, very short D segments (for β and δ loci only), and short J segments, and one or two constant C genes. During T cell ontogeny, a given T cell "chooses" at random one V, one D, if any, one J and one C segment. The D and J regions are short but very diverse due to an additional process which adds or deletes nucleotides at random, when the V, D and J segments are linked together. The consequence of this process is that a given T cell displays a single and unique combination on its cell surface. There are in fact 65 V β segments in the β locus, that can be grouped into 25 subfamilies (22 functional families), 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). The table in section 1.4 shows the list of V β segments and corresponding antibodies used in the present kit (covering about 70% of the normal human TCR V β repertoire of CD3+ lymphocytes).

The nomenclature used for the V β segments is the one from Wei et al. (5). The corresponding IMGT gene nomenclature (6, 7) is included in the table 1.4.

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 cells that present the antigen(s) (dendritic cells, macrophages, B cells). This activation results in the clonal expansion of specific T cells that may be followed by anti-TCR V gene usage. Many research studies on T cell repertoire usage in normal and pathological situations, have been performed to look at the human entire repertoire in order to establish potential links. For further information about these research studies please refer to references 8-28.

Many of these investigations were done in the past to distinguish polyclonal from oligoclonal or monoclonal T-cell proliferation using TCR gene probes and primers in Southern blot or polymerase chain reaction (PCR) procedures (13, 29), followed by CDR3 size spectratyping (30, 31), Denaturing Gradient Gel Electrophoresis (DDGE analysis) (18), or, Single-Strand Conformation Polymorphism (SSCP analysis (12, 32). However, these techniques are cumbersome and often not reliable in terms of quantitation and standardization (33). Moreover these molecular techniques do not allow one to perform a simultaneous repertoire analysis on T cell subsets and require cell separation or sorting (8, 21 – 23, 34). More recently, the availability of a large panel of monoclonal antibodies to TCRs, mainly against V β epitopes, permits the study of the TCR repertoire by flow cytometry (20, 28, 35-37). As opposed to the semiquantitative PCR method, antibodies detect the TCR proteins rather than measuring 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, naive/memory markers, etc. (38).

1.2. Description of Product

The IOTest[®] Beta Mark is a multi-analysis tool designed for quantitative analysis of the TCR V β repertoire of human T lymphocytes by flow cytometric analysis. Using an innovative staining principle (see below), this kit simplifies the TCR V β repertoire analysis by reducing the number of tubes to analyze (8 instead of 24) and consequently the time required for obtaining the results. Moreover, this kit makes it possible to study the repertoire on T cell subsets, using antibodies to additional T-cell markers, such as CD3, CD4, and CD8. The kit is composed of 8 vials containing mixtures of conjugated TCR V β antibodies corresponding to 24 different specificities (about 70% coverage of normal human TCR V β repertoire).

The test is intended for use on whole blood samples*. The staining protocol includes a one-step procedure with directly FITC- and PE-coupled antibody mixes (test volume of 20 μ l) and a wash step after staining. A third color T-cell marker (preferentially as PC5-conjugate; not included) is used to gate the specific population. The kit can be used in 3 or in 4 color settings. The test is compatible with different methods of lysis and can be run on Beckman Coulter as well as B-D cytometers.

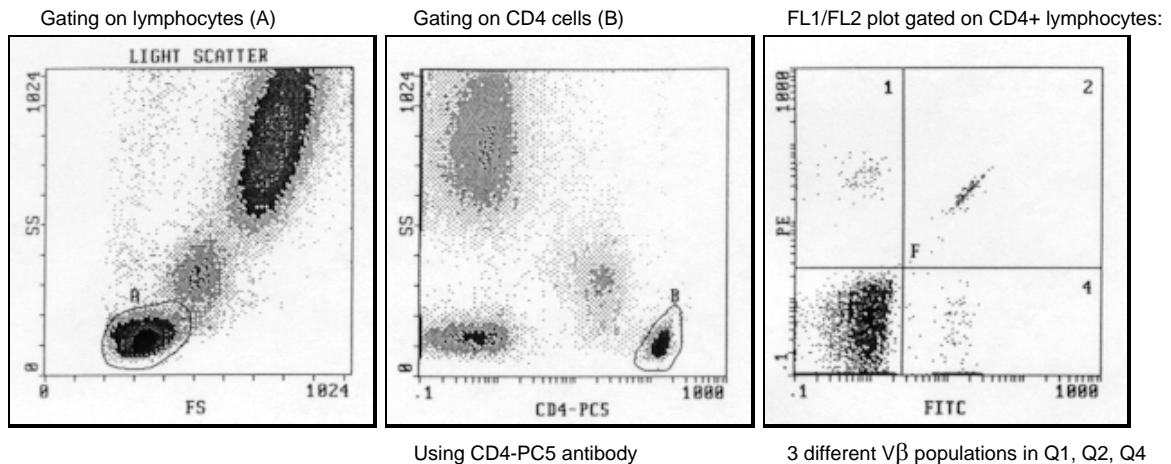
The IOTest Beta Mark includes a control for fluorescence PMT and compensation settings. Reagent A is used for this purpose without addition of a T-cell marker (to include 25 control tests, vial A contains 50 tests).

* Other blood preparations, such as PBMC (ficollated blood samples), and other body fluids may be processed by the IOTest Beta Mark but the procedure has not been standardized.

The IOTest Beta Mark is intended For Research Use Only. Not For Use In Diagnostic Procedures.

1.3. Principle of Assay

The test - simple and fast repertoire analysis, while retaining all information from each individual antibody staining - is achieved by combining 3 TCR V β -specific reagents in a single test but with only two colors. One TCR V β antibody is conjugated to FITC, another one to PE, and the third to both FITC and PE. In this way, the third V β -stained population shows up in the diagonal of the Quadrant 2 in a FL1/FL2 histogram, as shown below:



1.4. Reagent Composition

The 24 antibodies are regrouped into 8 vials, A to H, as shown below, leading to 8 reagent mixtures (tube A contains 50 tests for 25 control tests and 25 sample tests):

Tube	Volume / No. of tests (20 µL / test)	Vβ * (#) / Fluorochrome	Clone (Refs)	Isotype (species)	
A	1.0 mL / 50 tests	Vb 5.3 (TRBV5-5) Vb 7.1 (TRBV4-1, TRBV4-2, TRBV4-3) Vb 3 (TRBV28)	PE PE+FITC FITC	3D11 (35, 39) ZOE (40) CH92 (35, 39, 41)	IgG1 (mouse) IgG2a (mouse) IgM (mouse)
B	0.5 mL / 25 tests	Vb 9 (TRBV3-1) Vb 17 (TRBV19) Vb 16 (TRBV14)	PE PE+FITC FITC	FIN9 (35, 41) E17.5F3 (9, 35, 39, 43) TAMAYA1.2 (9, 35, 39)	IgG2a (mouse) IgG1 (mouse) IgG1 (mouse)
C	0.5 mL / 25 tests	Vb 18 (TRBV18) Vb 5.1 (TRBV5-1) Vb 20 (TRBV30)	PE PE+FITC FITC	BA62.6 (35, 39) IMMU157 (35, 44) ELL1.4 (35, 39)	IgG1 (mouse) IgG2a (mouse) IgG (mouse)
D	0.5 mL / 25 tests	Vb 13.1 (TRBV6-5, TRBV6-6, TRBV6-9) Vb 13.6 (TRBV6-6) Vb 8 (TRBV12-3, TRBV12-4)	PE PE+FITC FITC	IMMU222 (35, 39) JU74.3 (35, 39) 56C5.2 (35, 39, 43)	IgG2b (mouse) IgG1 (mouse) IgG2a (mouse)
E	0.5 mL / 25 tests	Vb 5.2 (TRBV5-6) Vb 2 (TRBV20-1) Vb 12 (TRBV10-3)	PE PE+FITC FITC	36213 (35, 39) MPB2D5 (45, 46) VER2.32 (35, 45)	IgG1 (mouse) IgG1 (mouse) IgG2a (mouse)
F	0.5 mL / 25 tests	Vb 23 (TRBV13) Vb 1 (TRBV9) Vb 21.3 (TRBV11-2)	PE PE+FITC FITC	AF23 (35, 39) BL37.2 (39) IG125 (14, 35, 39)	IgG1 (mouse) IgG1 (rat) IgG2a (mouse)
G	0.5 mL / 25 tests	Vb 11 (TRBV25-1) Vb 22 (TRBV2) Vb 14 (TRBV27)	PE PE+FITC FITC	C21 (35, 39, 42) IMMU546 (35, 39, 42) CAS1.1.3 (35, 42, 45)	IgG2a (mouse) IgG1 (mouse) IgG1 (mouse)
H	0.5 mL / 25 tests	Vb 13.2 (TRBV6-2) Vb 4 (TRBV29-1) Vb 7.2 (TRBV4-3)	PE PE+FITC FITC	H132 (35, 47) WJF24 (not published) ZIZOU4 (not published)	IgG1 (mouse) IgM (rat) IgG2a (mouse)

* Nomenclature from Wei et al. (5)

IMGT nomenclature (6, 7)

1.5. Storage Conditions and Stability

After use, vials should be stored at 2 – 8°C in the box of origin. Each reagent is stable up to the expiration date stated on the vial when stored at 2 – 8°C in the absence of light. Do not use after the expiration date. Do not freeze.

1.6. Statment of Warnings

The IOTest Beta Mark is intended For Research Use Only. Not For Use In Diagnostic Procedures.

- 1) All results obtained with the kit are for use in research applications only. They are not for use in diagnostic applications. All products and materials recommended for use with the kit will be considered "For Research Use Only. Not For Use in Diagnostic Procedures."
- 2) Monoclonal antibody preparations contain 0.1% sodium azide. Sodium azide, under acidic conditions, yields hydrazoic acid, an extremely toxic compound. Disposal into sewage systems should be accompanied with large quantities of running water to avoid dangerous buildup in metal piping and potential explosions.
- 3) Specimens, samples and all material coming in contact with them should be handled as if capable of transmitting infection and disposed of with proper precautions.
- 4) Never pipet by mouth and avoid contact of samples or reagents with skin and mucous membranes.
- 5) Do not expose reagents to strong light or to heat during storage or use.
- 6) Avoid microbial contamination of all reagents, or erroneous results may occur.
- 7) Stained and lysed samples must be acquired no later than 24 hours, if stored at 2 - 8°C in the dark, but optimally within 2 hours.

- 8) Ensure that at least 10,000 lymphocyte events are counted in the lymphogate, or erroneous results may occur. For accurate determination on small V β populations, the acquisition of 10,000 gated T lymphocytes is recommended.
- 9) The use of incubation or mixing times, or temperatures other than those specified, may give erroneous results.
- 10) The use of lysis buffers, isotypic control antibody, or additional T-cell antibody for gating, other than those specified, may give erroneous results.
- 11) Use good laboratory practices when handling this reagent.

2. PROCEDURE FOR SAMPLE PROCESSING

2.1. Materials Required but not Supplied

- 1) A typical sample analysis, using a series of 8 test-tubes and 2 control-tubes, requires at least 1 mL of fresh (less than 24-hour old) whole blood.
- 2) Additional PC5-conjugated antibodies for gating of T lymphocyte subsets.
Important: *The reagents of the IOTest Beta Mark have been validated with the use of one of following PC5-conjugated T-cell antibodies from the Beckman Coulter catalog:*
 - PN IM2635U CD3-PC5 (clone UCHT1) 100 tests
 - PN IM2636U CD4-PC5 (clone 13B8.2) 100 tests
 - PN 6607011 CD8-PC5 (clone T8) 50 tests**The use of additional T-cell antibodies for gating other than those specified, may give erroneous results.**
- 3) Isotypic control.
 - PN IM2663U Mouse IgG1-PC5 100 tests**The use of isotypic control antibody other than the one specified, may give erroneous results.**
- 4) Lyse-and-fix buffers.
Important: *The reagents of the IOTest Beta Mark have been validated with the use of ImmunoPrep™ reagent for Beckman Coulter Cytometers, and OptiLyse® B Lysing Solution for Becton Dickinson cytometers.*
After red blood cell lysis and leucocyte fixation steps, a wash step is required.
 - PN 7546999 ImmunoPrep 250-300 tests
 - PN IM1400 OptiLyse B Lysing Solution 250 tests**The use of lyse-and-fix buffers other than those specified, may give erroneous results.**
- 5) Ice water bath.
- 6) Plastic test-tubes (12 x 75 mm).
- 7) Standard pipettes (20 μ L, 100 μ L, 2 mL) and tips.
- 8) Vortex mixer.
- 9) Timer.
- 10) Flow cytometer.

2.2. Reagent Preparations

- 1) The two color reagents of the IOTest Beta Mark are used directly, i.e. as calibrated in the vials, without further dilution.
- 2) In the following 3-color staining method, the CD3-PC5 antibody (clone UCHT1, PN IM2635U, ready-to-use) is employed as additional marker for gating of T lymphocytes.
- 3) The isotypic control Mouse IgG1-PC5 (PN IM2663U) is ready-to-use.

2.3. Specimen Preparation

- 1) Fresh (less than 24 hour old) whole blood samples can be processed with the IOTest® Beta Mark.
- 2) Collect peripheral blood samples, aseptically into a sterile evacuated blood collection tube with anticoagulant (EDTA is strongly recommended). Anticoagulated specimens should be stored at room temperature (18 – 25°C) until staining (within 24 hours).

2.4. Sample Staining Methodology

1. For each blood sample, label 10 tubes and pipet the reagents into the corresponding tubes as described below:

Tube	Settings*	Reagent Mix
Control tube 1	PMT1, PMT2, PMT4 & Compensations FL1-%FL2, FL2-%FL1, FL4-%FL2	20 µL of vial A + 10 µL of IgG1 PC5 vial
Control 2	Compensation FL2-%FL4 & subset gating	10 µL of CD3-PC5 vial
Test tube A	—	20 µL of vial A + 10 µL of CD3-PC5 vial
Test tube B	—	20 µL of vial B + 10 µL of CD3-PC5 vial
Test tube C	—	20 µL of vial C + 10 µL of CD3-PC5 vial
Test tube D	—	20 µL of vial D + 10 µL of CD3-PC5 vial
Test tube E	—	20 µL of vial E + 10 µL of CD3-PC5 vial
Test tube F	—	20 µL of vial F + 10 µL of CD3-PC5 vial
Test tube G	—	20 µL of vial G + 10 µL of CD3-PC5 vial
Test tube H	—	20 µL of vial H + 10 µL of CD3-PC5 vial

* for Beckman Coulter cytometers (note that for BD[®] cytometers, you need to use FL3 instead of FL4, PMT4 becomes PMT3).

2. Pipette 100 µL of the well-mixed blood sample to the bottom of the test tubes.
Important: Do not allow blood to remain on the inner tube walls. Remove traces with a cotton swab.
3. Incubate at room temperature (18 – 25°C) for 20 minutes in the dark.
4. Proceed to manual or automated fix and lyse step according to the procedure recommended for the reagent used.
5. **Important: for lyse-and-fix methods without wash (such as ImmunoPrep and OptiLyse B), the following wash step is required for optimal results:**
Add 3 mL of PBS, centrifuge for 5 minutes at 300 x g at room temperature and discard supernatant (by aspiration).
6. Resuspend the cell pellet in 1 mL of PBS containing 0.5% formaldehyde (0.5 mL may be needed for faster acquisition times) and store at 2 – 8°C in the dark until data acquisition is performed (within 24 hours).
7. Samples are now ready for flow cytometric analysis.

3. METHOD OF ANALYSIS

Important notes:

- a) The IOTest® Beta Mark-prepared samples must be acquired no later than 24 hours, if stored at 2-8°C in the dark, but optimally within 2 hours.
- b) FL1/FL2 compensations should be done on Control tube 1 (containing reagent A) in order to obtain optimal compensation values for the TCR Vβ antibody staining.
- c) The FL2-%FL4* compensation should be done on Control tube 2 for each antibody used in the third color, for optimal compensation setting.

* for Beckman Coulter cytometers (FL2-%FL3 for B-D cytometers)

- d) *The settings must be checked for each blood sample.*
- e) *Polymorphisms have been described, and some blood samples may be negative for the expression of TCR V β 7.2 (Tube H, see ref. 48) or V β 20 (Tube C, see ref. 49).*

3.1 Instructions For COULTER EPICS[®] XL[™]/XL-MCL[™] Brand Flow Cytometer

Read instructions carefully before use.

3.1.1 Recommended Protocol Set-up for Analysis

The flow cytometer must be equipped to detect Forward Scatter, Side Scatter and three fluorescence channels: FL1, FL2 and FL4.

Creation of Histograms:

Create 6 histograms as follows [X vs Y]:

Create Histogram 1 as Side Scatter vs Forward Scatter.

Create Histogram 2 as FL1 vs. FL2.

Create Histogram 3 as FL1 vs. FL4.

Create Histogram 4 as FL2 vs. FL4.

Create Histogram 5 as FL4 vs. SSC.

Create Histogram 6 as FL1 vs. FL2 (Analysis Histogram).

Creation of Regions

1. On Histogram 1, create amorphous Region A for the lymphocyte gate (with low Side Scatter low Forward Scatter).
2. Create Quadstat (Region B) on Histogram 2 and display events from Region A.
3. Create Quadstat (Region C) on Histogram 3 and display events from Region A.
4. Create Quadstat (Region D) on Histogram 4 and display events from Region A.
5. Create rectangular Region E (in our example, CD3-positive lymphocyte events) on Histogram 5.
6. Create Quadstat (Region F) on Histogram 6 and display events from Region E. A minimum of 5,000 events must be acquired in gate E, 10,000 events are recommended, however.

Flow Cytometer Setting

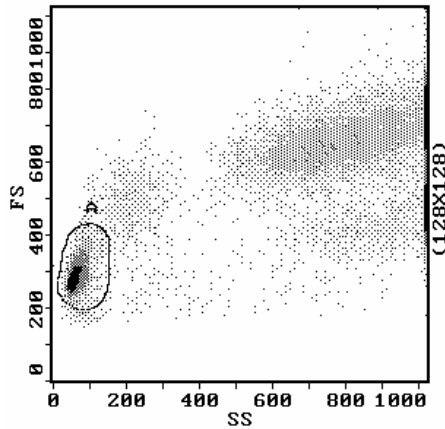
1. Verify that the flow cytometer is properly aligned and optimized for light scatter and fluorescence intensities of the FL1, FL2 detectors, and FL4 detectors. Verify that color compensation is set for standard three-color operation. Refer to the instrument manual for additional instructions.
2. Vortex test tubes for five seconds.
3. Perform data acquisition on flow cytometer. A minimum of 10,000 lymphocyte events (gate A) must be analyzed for settings.
4. Adjust the discriminator [threshold] and regions by analyzing Control tube 1.

3.1.2 Example for Analysis

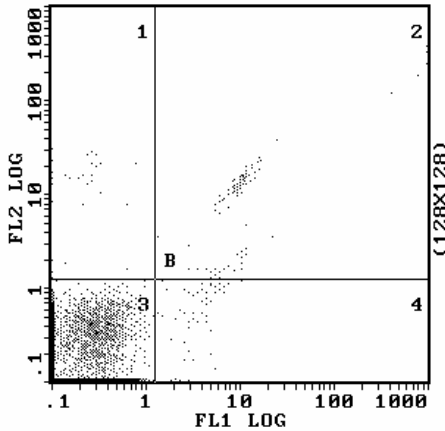
In the following figures, the histograms are not displayed in an ascending number order, as displayed on the protocol. They correspond to a series of steps that are to be followed to analyze a whole blood sample, processed as described above.

Note: Pictures feature histograms obtained on a COULTER EPICS XL-MCL flow cytometer equipped with SYSTEM II[™] Software (version 3.0).

A- Insert Control tube 1 into cytometer and start event acquisition



2: A



3: A

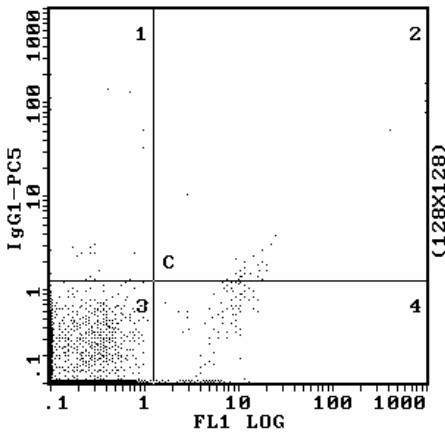


Figure 1

➤ **Step 1: [Control tube 1]**

Observe Histogram 1.

Check whether the Forward and Side Scatter gain parameters of the flow cytometer are optimally set for the processed sample. Adjust the Forward Scatter discriminator to ensure that even the smallest lymphocytes scatter above it and exclude debris. Position Region A to include all lymphocyte events.

Figure 2

➤ **Step 2: [Control tube 1]**

Observe Histogram 2.

Set the cursors of the Quadstat Region B approximately on the value "1" on each of the Log scales (FL1 & FL2). Adjust the PMT1 and PMT2 voltages/gains in order to have the negative population (unstained lymphocytes) entirely in quadrant 3.

Warning: At this step do not take into account the single- and double-stained events (diagonals).

Figure 3

➤ **Step 3: [Control tube 1]**

Observe Histogram 3.

Set the cursors of the Quadstat Region B approximately on the value "1" on each of the Log scales (FL1 & FL4). Adjust the PMT4 voltages/gains in order to have the negative population (negative lymphocytes) in quadrant 3.

Warning: At this step do not take into account the single- and double-stained (diagonals).

4 : A

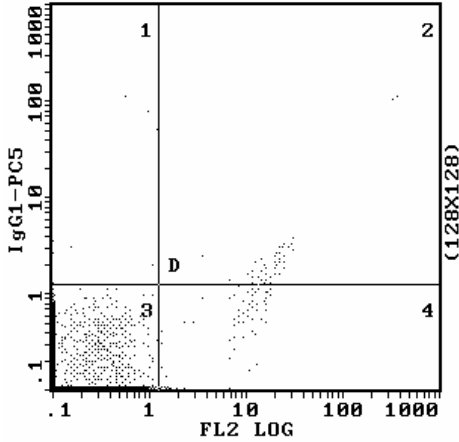


Figure 4

➤ **Step 4: [Control tube 1]**
Observe Histogram 4.

Set the cursors of the Quadstat Region B on the values of the Log scales (FL2 & FL4) as determined in steps 3 and 4.

Verify that the PMT2 and PMT4 voltages/gains are well adjusted (negative population in quadrant 3).

Warning: Again, do not take into account the single- and double-stained events (diagonals).

➤ **Step 5: [Control tube 1] - Observe Histogram 2.**

Adjust the following color compensations:

- FL1-%FL2 (equivalence of Mnl X of regions B3 and B4)
- FL2-%FL1 (equivalence of Mnl Y of regions B1 and B3)

Figure 5: Without setting

2 : A

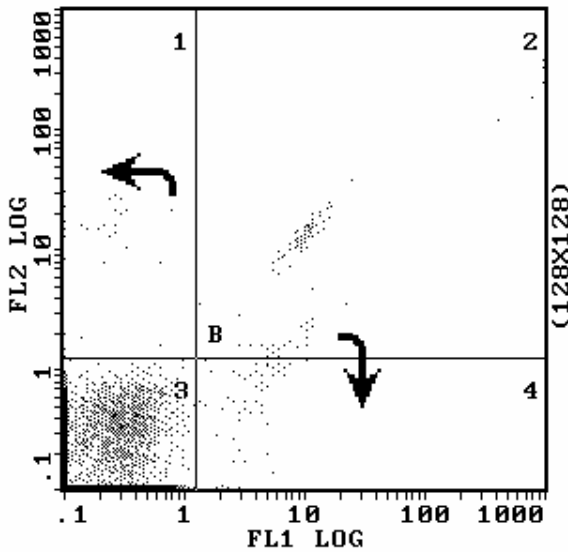
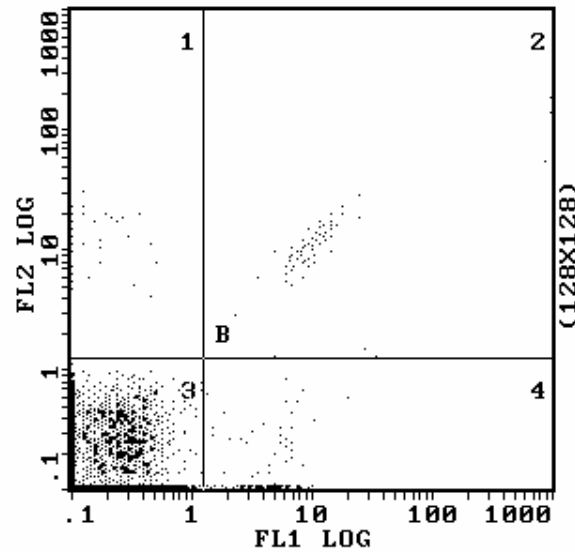


Figure 6: Correct setting

2 : A



➤ **Step 6: [Control tube 1] - Observe Histogram 4.**

Adjust the following color compensation:

- FL4-%FL2 (equivalence of Mnl Y of regions D3 and D4)

Figure 7: Without setting

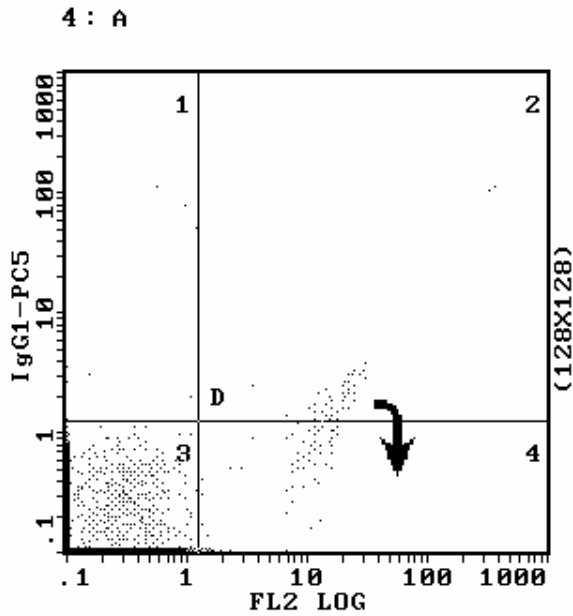
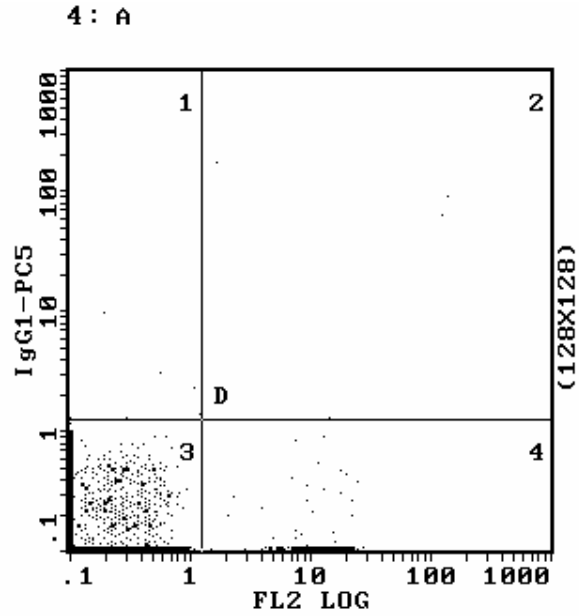


Figure 8: Correct setting



B- Insert Control tube 2 into cytometer and start event acquisition

➤ **Step 7: [Control tube 2] - Observe Histogram 4.**

Adjust the following color compensation:

- FL2-%FL4 (equivalence of Mnl X of regions D1 and D3)

Figure 9: Without setting

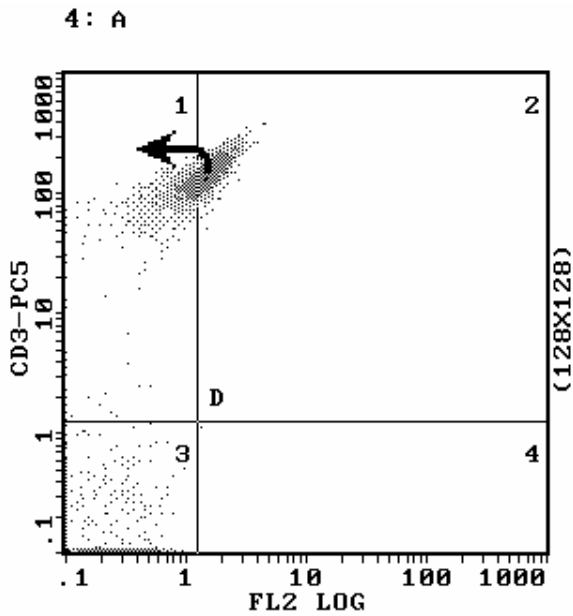
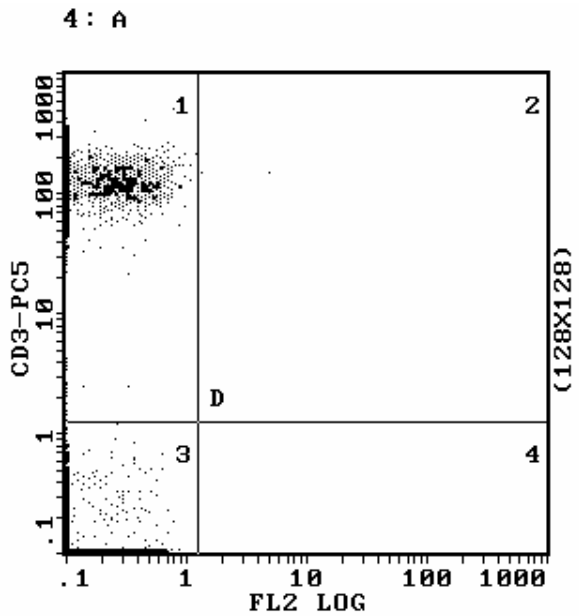


Figure 10: Correct setting



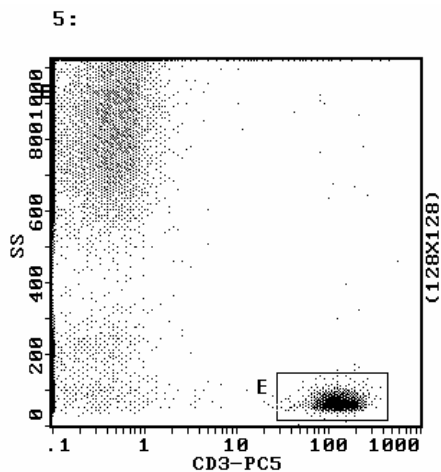


Figure 11

➤ **Step 8: [Control tube 2]**
Observe Histogram 5.

Position Region E to include all CD3-positive lymphocyte events.

Warning: Do not cut off positive events on the CD3 left side of the gate.

C- ANALYSIS STEP: Insert successively tubes A to H into cytometer and start event acquisition.

➤ **Step 9-16 [Tubes A to H]**

Observe Histogram 6 (displaying events from region E).

A minimum of 5,000 T lymphocyte events (gate E) must be analyzed, 10,000 events are recommended, however.

3.1.3 Example data with acquisition on a COULTER EPICS XL/XL-MCL Flow Cytometer

➡ See figure 12 for a complete analysis of a representative 24h-old blood sample (analysis performed with Beckman Coulter System II v3.0 Software).

3.2 Example data with acquisition on a B-D FACSCalibur™ Flow Cytometer

➡ See figure 13 for a complete analysis report of the same blood sample (analysis performed with Beckman Coulter EXPO v2 Analysis Software).

Figure 12: Example data with acquisition on a COULTER EPICS® XL/XL-MCL Flow Cytometer (analysis performed with Beckman Coulter System II v3.0 Software).

The percentage of each Vβ subset in brackets has been added. Total CD3+ lymphocyte Vβ coverage for this sample: 66.93%.

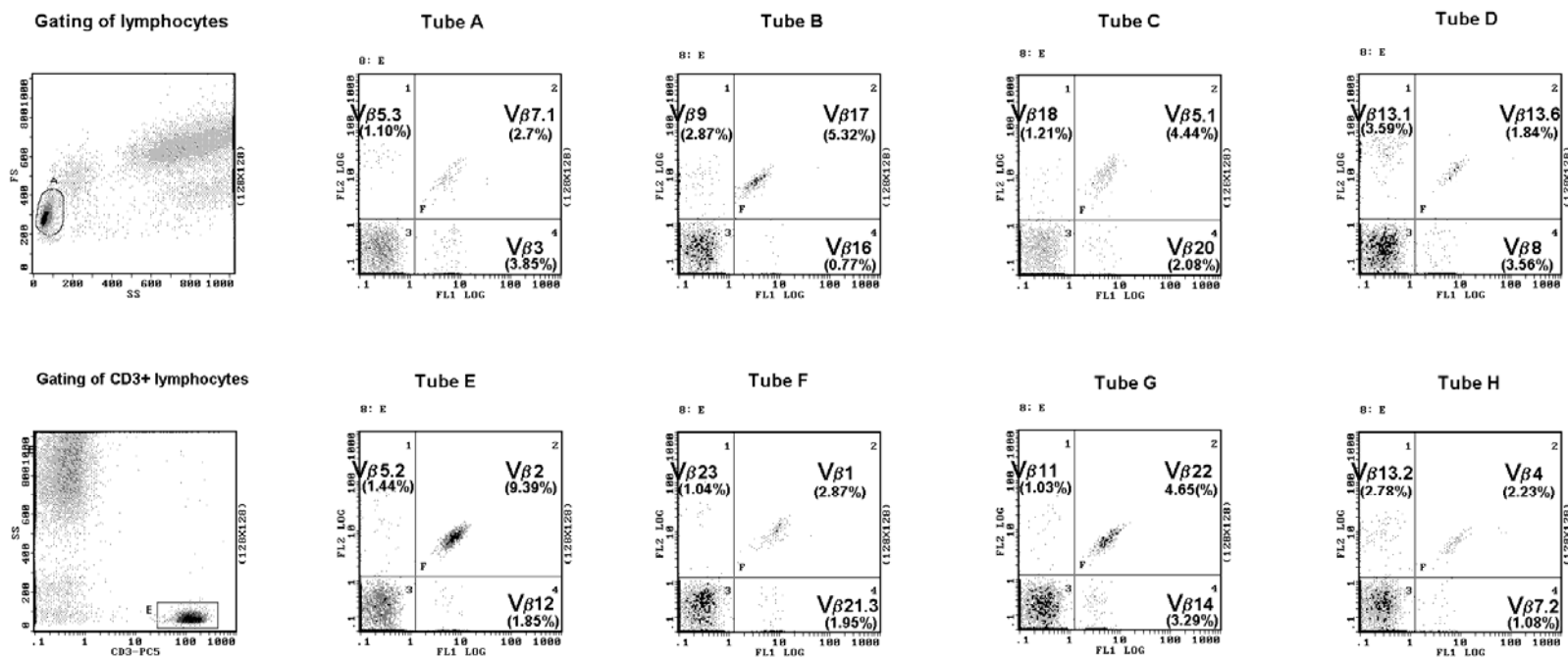
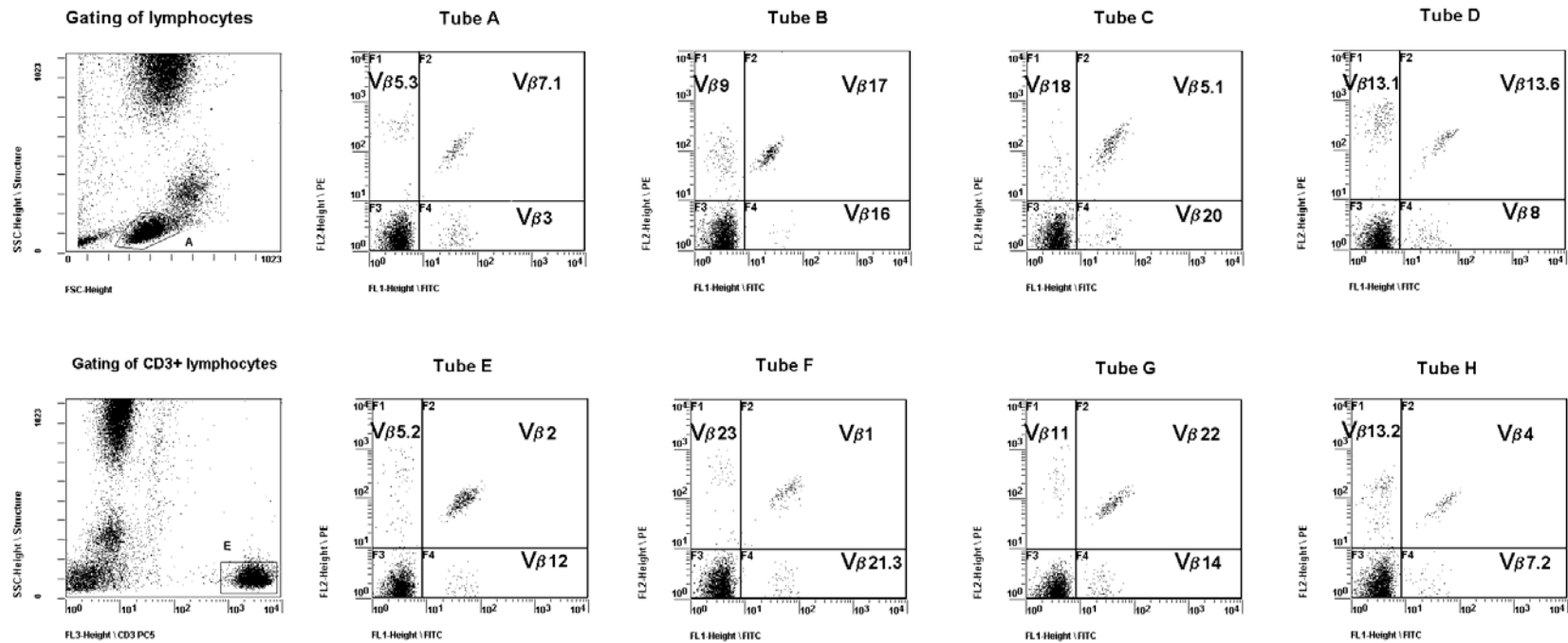


Figure 13: Example data with acquisition on a BD[®] FACSCalibur[™] Flow Cytometer (analysis performed with Beckman Coulter EXPO v2 Analysis Software).



3.4 Presentation of data: Clonogram representation

1. Record the % values corresponding to the 24 V β specificities from the Analysis histogram Nr 6 of tubes A to H (analysis of quadrants 1, 2 and 4 ; see example in figure 12).
2. Arrange % values by V β ascending order and report them on a computer worksheet.
3. Use the worksheet data to create a bar chart termed Clonogram. See below on figure 14, the Clonogram obtained on the analyzed whole blood sample.

CLONOGRAM

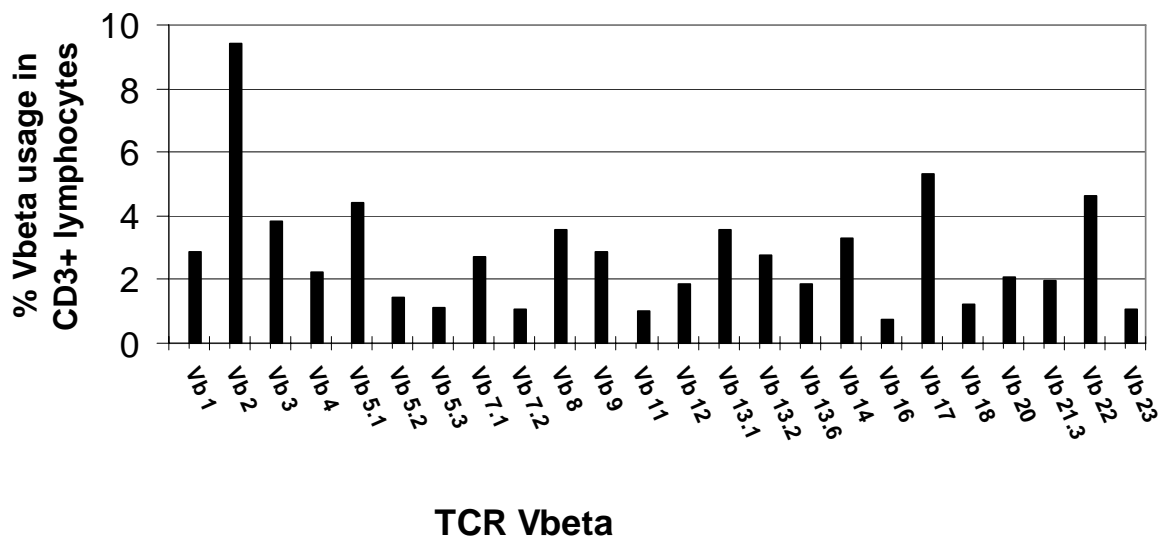


Figure 14: Clonogram representation

TCR V β repertoire in CD3+ T-cells : mean percentages of expression of 24 TCR V β from a normal specimen.

4. RESEARCH REFERENCES

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