

Practical Protocols the newly accepted CD4 counting procedures

1. Pan-Leucogating

2. BD protocol (abridged version)

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**Protocol No.1
(Version 1.1) -
referred to as PL or
"green protocol"**

**Pan-Leucogating protocol for CD4/CD8 enumeration using
2-colour in 2-tube and haematology WBCC
(double platform)**

Summary: This pan-leucogating protocol has recently been described in Cytometry by Glencross et al. 2001. The protocol has specially been designed to obtain good precision and accuracy, maximum information at low expense. For each sample two double stained tests are set up each containing CD45-FITC and either CD4PE or CD8PE. The protocol operates on double platform in tandem with a haematology analyser that is Quality Controlled and provides WBCC only. If your laboratory can not afford to obtain all three reagent or if your clinicians do not request CD8 counts and CD4/CD8 ratios, then you should only run one single CD45-FITC/CD4-PE tube. The analysis is performed as follows:

A) CD45/CD4 test tube

- 1) Open the CELLQuest program and open a new file
- 2) Set up a dot plot of SSC against FL1 (CD45-FITC)

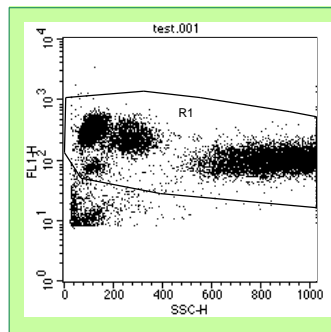


Fig.PL-1

- 3) Region R1 is drawn around all the CD45 positive events, representing the total number of leucocytes in the sample, and enabling their enumeration
- 4) A further dot plot is set up of SSC against FL2 (CD4-PE) gated on region R1 in which only CD45 positive particles are represented

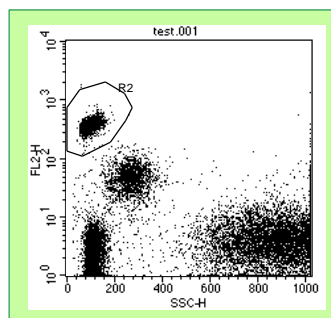


Fig.PL-2

- 5) Region R2 defining CD4 positive lymphocytes is drawn on this plot enabling their enumeration
- 6) A further region R3 may be drawn on the first dot plot to define and enumerate the total number of lymphocytes

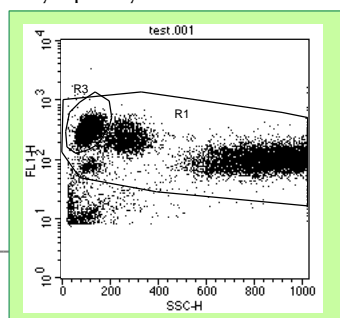


Fig.PL-3

- 7) Select the second dot plot and display the region statistics to give the values for regions R1, R2 and R3 representing the pan leucocyte; CD4 positive T lymphocyte and total lymphocyte numbers, respectively

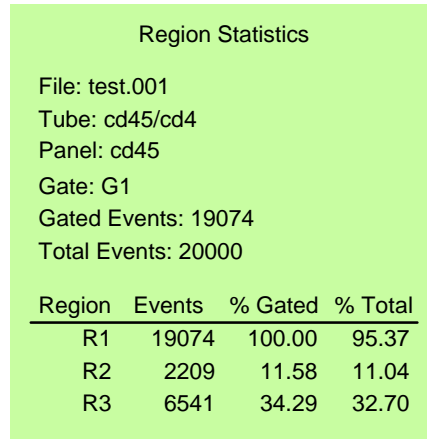


Fig.PL-4

If you can afford it and the clinicians request it continue with the analysis as follows;

B) CD45/CD8 test tube

- 1) Open a new file in the CELLQuest program
- 2) Set up a dot plot of SSC against FL1 (CD45FITC)

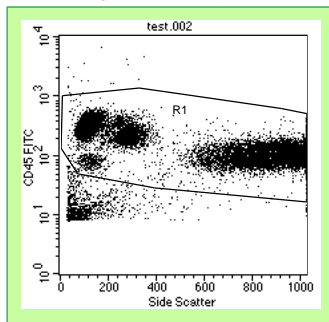


Fig.PL-5

- 3) Region R1 is drawn (or copied and pasted from the previous file) around all the CD45 positive particles representing the total number of leucocytes, and enabling their enumeration in the sample
- 4) A further dot plot is set up of SSC against FL2 (CD8PE) gated on region R1 in which only CD45 positive particles are represented

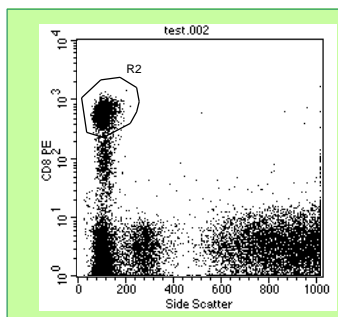


Fig.PL-6

- 5) Region R2, defining CD8 positive lymphocytes, is drawn on this plot enabling their enumeration

- 6) As previously, a further region R3 is drawn (or copied and pasted from the previous file) on the first dot plot to define and enumerate the total number of lymphocytes

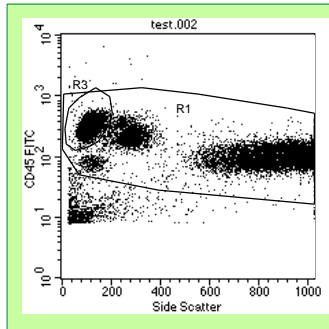


Fig.PL-7

- 7) With the second dot plot selected, the region statistics are displayed to give the values for regions R1, R2 and R3 representing the pan-leucocyte, CD8 positive and total lymphocyte numbers respectively

Region Statistics			
File: test.002			
Tube: cd45/cd8			
Panel: cd45			
Gate: G1			
Gated Events: 18927			
Total Events: 20000			
Region	Events	% Gated	% Total
R1	18927	100.00	94.64
R2	2035	10.75	10.17
R3	6467	34.17	32.34

Fig.PL-8

C) Calculation of results

The results obtained can yield absolute values if an haematological analyser is also used to evaluate the specimen, and if the results are linked by relating to the leucocyte (WBC) counts on each platform.

Results may be expressed as;

Absolute CD4 and CD8 T lymphocyte counts (see above)

CD4 and CD8 positive cells as a percentage of total leucocytes and/or total lymphocytes

The sum of CD4 plus CD8 positive cells representing the number of functional T cells as an absolute count - and/or as percentage of total leucocytes and/or total lymphocytes

The CD4:CD8 ratio

These possibilities are discussed in the "Simple Concepts in Affordable Clinical Flow Cytometry" slide show (see Slide 6).

Protocol No.2 (abridged version 1.1) - referred to as BD or "blue protocol"

Becton Dickinson protocol for CD4/CD8 enumeration using MultiTest IMK kit with TruCOUNT tubes

Summary: This laboratory hand-out is an abridged version of the original protocol provided to MultiTest users. Our intention here is to adhere, strictly, to the BD protocol. We are only simplifying it, unchanged. It is now in the same format as the other techniques shown here. The BD method uses whole blood stained with four antibody reagents coupled to different fluorochromes, and incorporating beads for absolute counting. This is one, expensive, option. The other option is not to use beads but export, from a Haematology analyser, the values obtained for absolute lymphocyte counts in a parallel tube of the same blood sample. The third option, is to use fixed, stained cells as "bead-substitutes" (Protocol No.4 below).

To analyse the bead-containing four colour acquisition of CD3-FITC; CD8-PE; CD45-PerCP and CD4-APC treated blood;

- 1) Open the CELLQuest program
- 2) Open a new file
- 3) Set up a dot plot of SSC against FL3 (CD45-PerCP)
- 4) Region R1 is drawn around the beads to enable their enumeration

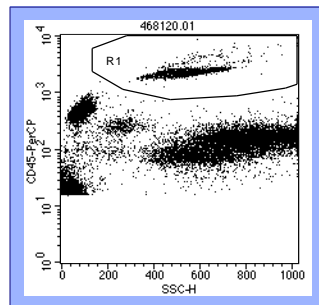


Fig.BD-1

- 5) A further region R2 is drawn around the CD45 positive lymphocytes to enable their enumeration

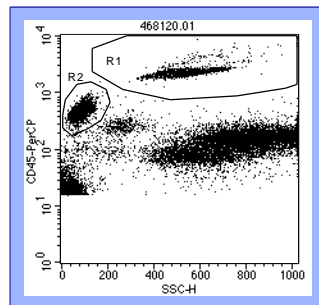


Fig.BD-2

- 6) A further dot plot is set up of SSC against FL1 (CD3-FITC) which is gated on region R2, and in which only CD45 positive lymphocytes are represented

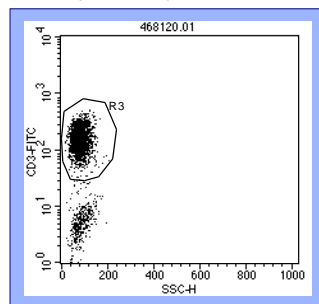


Fig.BD-3

- 7) Region R3 defining CD3 positive T cells is drawn on this plot enabling their enumeration
- 8) A third dot plot displaying FL4 (CD4-APC) against FL2 (CD8-PE) is set up, gated on region R3, in which only CD3 positive T lymphocytes are represented
- 9) By setting a quadrant on this dot plot, the CD4 positive (CD3+,CD4+,CD8-) and CD8 positive T cells (CD3+CD8+,CD4-) are enumerated, together with minority populations such as CD3+, CD4-, CD8- T cells and CD3+, CD4+, CD8+ double positives.

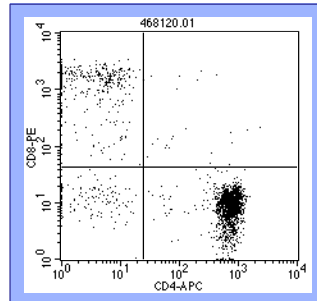


Fig.BD-4

- 10) Display the region statistics of the first dot plot (SSC against FL3) to give the values in regions R1, R2 and R3, representing beads, CD45 positive lymphocytes and CD3 positive T cells respectively

Region Statistics			
File: 468120.01			
Tube: CD3/CD8/CD45/CD4 Tru			
Panel: 4 Color TBNK + TruC			
Gate: No Gate			
Gated Events: 22000			
Total Events: 22000			
Region	Events	% Gated	% Total
R1	1770	8.05	8.05
R2	2337	10.62	10.62
R3	1963	8.92	8.92

Fig.BD-5

- 11) Display the quadrant statistics of the third dot plot to show the values of CD4 positive and CD8 positive T cells within the CD3 positive T cell population

Quadrant Statistics			
File: 468120.01			
Tube: CD3/CD8/CD45/CD4 TruC			
Panel: 4 Color TBNK + TruC			
Gate: G3			
Gated Events: 1963			
Total Events: 22000			
Quad	Events	% Gated	% Total
UL	401	20.43	1.82
UR	24	1.22	0.11
LL	130	6.62	0.59
LR	1408	71.73	6.40

Fig.BD-6

- 12) To obtain the absolute counts for these values using the TruCOUNT beads, the procedure indicated in the *BD Training Manual* is followed (Page 5– 89). Using the TruCOUNT beads the following formula can be used to determine the absolute CD4 positive T cell count.

$$\begin{array}{l}
 \text{Events enumerated in the} \\
 \text{quadrant representing} \\
 \text{the CD4 positive T cells} \\
 \text{Number of bead events} \\
 \text{in region R1}
 \end{array}
 \times
 \begin{array}{l}
 \text{Total number of TruCOUNT beads} \\
 \text{in the tube pellet} \\
 \text{(value provided by BD on package)} \\
 \text{Blood volume (50?l)}
 \end{array}$$

e.g. $\frac{1408}{1770} \times \frac{50100}{50?1} = 797 \text{ CD4 positive T cells per ?l}$

Similarly the absolute CD8 positive T cell count can be determined by substituting the appropriate quadrant value to this equation.

- 13) If TruCOUNT beads are not used, absolute counts can be obtained using haematology analyser values for lymphocytes and total white blood cells.