APPLICATION NOTE

FLOW CYTOMETRY

CyAn™ ADP | 9 Color No-Lyse Lymphocyte Subset Enumeration

It is important to determine the relative and absolute numbers of the various lymphocyte sub sets to ascertain if a sample is abnormal. The lymphocyte population consists of three major types: T lymphocytes (CD3+), B lymphocytes (CD19+) and Natural Killer lymphocytes (CD56+/CD3-); each of these groups can be further subdivided, *e.g.,* CD4+ T lymphocytes, CD8+ T lymphocytes, CD5+/- B lymphocytes, CD8+/- NK lymphocytes. A nine-color panel of 10 antibodies was created, which allowed easy identification of each lymphocyte group and some of their sub types.

Currently for lymphocyte sub set enumeration it is typical to use a Lyse No Wash technique, in which cells are stained with antibody for 10–15 minutes, after which red blood cells are removed by adding a lysing reagent. After approximately 10–15 minutes incubation, the sample is acquired on the flow cytometer without prior washing. We have developed a technique in which samples can be acquired using a CyAn ADP LX 9 Color flow cytometer, without lysing the red blood cells, saving 10–15 minutes in preparation time. Sample acquisition time on the instrument can also be reduced significantly by using high sample flow rates of 150–200 µL/min.

A gating strategy has been developed that permits the removal of the majority of non-lymphocyte events, allowing easier identification of the lymphocyte populations and their sub sets. The addition of reference counting beads also allows for the single platform determination of absolute cell counts.

Materials and Methods

Peripheral blood from a normal healthy adult donor was collected by venepuncture into a tube containing EDTA anticoagulant; testing was performed within 24 hours.

Before making up the antibody combinations, it is imperative to pre-titrate out all antibodies used to ensure they are present at optimum concentration.

For all data acquisition a CD45 trigger must be used to eliminate the red blood cells.

Instrument Set Up

Prepare Tubes 1–11 as shown in Table 1. Add 5 µL of CD45 and 5 µL of the CD3 to Tubes 1–10, 5 µL of the 9 color mix to Tube 11, and add 50 µL of well-mixed blood to each tube. If absolute counts are required then it is important that "wet tip reverse pipetting[1]" be used to dispense the 50 µL of sample for Tube 11. Incubate for 10–15 minutes then add 500 µL of Phosphate Buffered Saline (PBS) mix and acquire. For Tubes 1–10, use a slow to moderate flow rate (~100 µL/min). Place Tube 1 (unstained) on the instrument, and using a CD45 CY vs. SSC plot (Figure 1), adjust the Cascade Yellow PMT so the lymphocyte cloud is seen clearly. Please note that the FSC may appear increased and the SSC population may appear very elongated in No Lyse preparations. Set a region enclosing the lower half

Table 1

Tube #	Name	FITC	RPE	RPE-TR	RPE-Cy5	RPE-Cy7	Pacific Blue	Cascade Yellow	APC	APC-Cy7
1	Unstained	–	–	–	–	–	–	CD45	–	–
2	CD3 FITC	CD3	–	–	–	–	–	CD45	–	–
3	CD3 RPE	–	CD3	–	–	–	–	CD45		–
4	CD3 RPE-TR	–	–	CD3	–	–	–	CD45	–	–
5	CD3 RPE-Cy5	–	–	–	CD3	–	–	CD45	–	–
6	CD3 RPE-Cy7	–	–	–	–	CD3	–	CD45	–	–
7	CD3 APC	–	–	–	–	–	–	CD45	CD3	–
8	CD3 APC-Cy7	–	–	–	–	–	–	CD45	–	CD3
9	CD3 Pacific Blue	–	–	–	–	–	CD3	–	CD45	–
10	CD3 Cascade Yellow	–	–	–	–	–	–	CD3	CD45	–
11	9-color	CD14/CD15	CD56	CD19	CD4	CD5	CD8	CD45	CD20	CD3

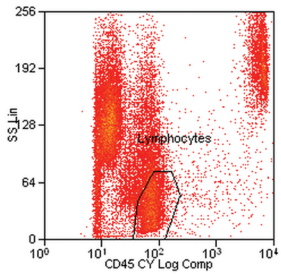
TECHNICAL TIPS

- All antibodies must be pre-titrated to ensure they are present at optimum concentration in the mixture.
- For data acquisition a CD45 Cascade Yellow trigger must be used to eliminate the Red Blood Cells.
- When running the tubes for instrument set up and compensation, it is better to acquire using a low flow rate. This allows for easier identification of the lymphocyte population using a CD45 Cascade Yellow vs. Side Scatter plot.
- Always observe the Time histogram during acquisition to monitor flow rate. If there is an increase or decrease then it is advisable to restart the acquisition.
- For single-platform absolute cell counting it is recommended that "wet-tip reverse pipetting" is used to enure the accuracy of the results.
- Before using the CytoCount beads ensure they have been well suspended by gently rotating the vial. Harsh vortexing can introduce air bubbles into the fluid.

of the lymphocyte cloud and apply this to a series of histogram plots of each individual fluorescence parameter.

Adjust the PMT voltage for each fluorescence parameter in turn to position the negative population, (except Cascade Yellow) with a median channel number of around 3.5. Without adjusting the PMT voltages acquire Tubes 2–10. A plot showing CD45 APC and SSC is required to acquire the CD3 Pacific Blue and Cascade Yellow tubes (Tubes 9 and 10).

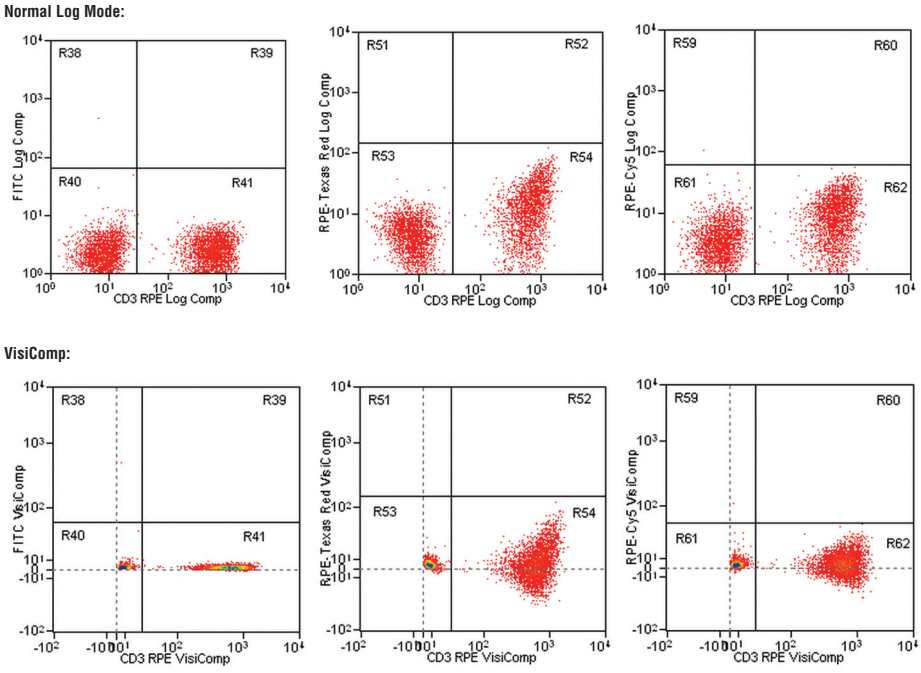
Figure 1



CD45 Cascade Yellow vs. Side Scatter plot showing the positioning of the Lymphocyte gate used for the acquisition of the unstained and CD3/CD45 stained tubes (Tubes 1 – 10).

Set the compensation using the "Auto Compensation" tool in Summit software v4.3., run Tubes 2 – 8 first using the CD45 Cascade Yellow vs. SSC gated lymphocytes, then run Tubes 9–10 using the CD45 APC vs. SSC gated lymphocytes. The compensation settings can be verified using the "VisiComp" function found in Summit software v4.3 (Figure 2).

Figure 2

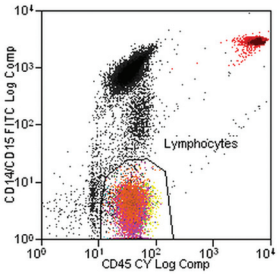


Representative plots showing the Auto Compensation derived compensation for the CD3 RPE tube (Tube 3). The top plots shows the normal log mode and the lower show the VisiComp plots.

Once PMT voltages and compensation settings have been set, the 9-color tube (Tube 11) can be acquired. If absolute counts are required, then, just prior to acquisition, add, by means of wet tip reverse pipetting, 50 µL of well-mixed CytoCount beads and mix. Since we do not require the scatter parameters, the sample flow rate can be increased to 150–200 µL/min. In the example shown a total of 35,000 total events were acquired in just over 30 seconds, yielding approximately 11,500 lymphocyte events.

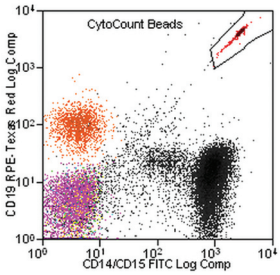
Gating Strategy

Gating strategy is shown in Plots 1–13 below.



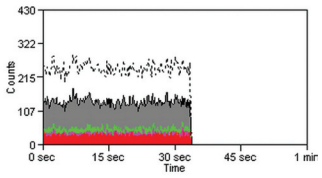
Plot 1 – CD45 Cascade Yellow vs. CD14/CD15 FITC. (Ungated)

This plot is used to ensure the CD45 Cascade Yellow trigger is not set too high so that it is clipping the lymphocyte cloud, or too low to include red blood cells. A gate (Lymphocytes) is created to identify the lymphocytes and eliminate the Neutrophils, Eosinophils, Monocytes and debris.



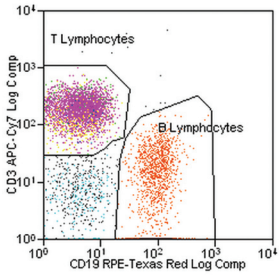
Plot 2 – CD15 vs. CD19 RPE-TR (Ungated)

This plot is used to identify the CytoCount beads.



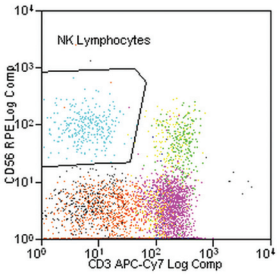
Plot 3 – Time (Ungated)

This plot is a single histogram of Time and is used to monitor the flow rate over the acquisition period.



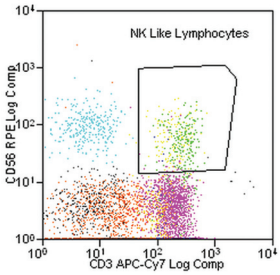
Plot 4 – CD19 RPE-TR vs. CD3 APC-Cy7 (Lymphocyte gate applied)

This plot is to identify the CD3+ T Lymphocytes and the CD19+ B Lymphocytes.



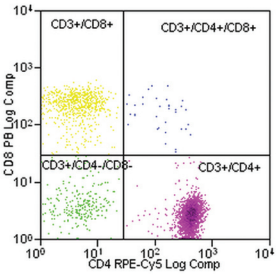
Plot 5 – CD56 RPE-TR vs CD3 APC-Cy7. (Lymphocyte gate applied)

This plot is used to identify the CD56+/CD3- Natural Killer lymphocytes.



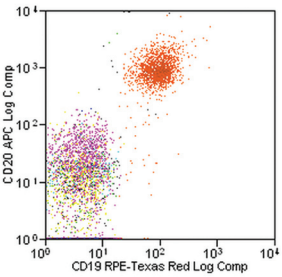
Plot 6 – CD56 RPE-TR vs. CD3 APC-Cy7. (Lymphocyte gate applied)

This plot is used to identify the CD56+/CD3+ Natural Killer Like lymphocytes. Note the NK and NK like lymphocyte gates can be derived from the same CD56 RPE-TR vs. CD3 APC-Cy7 plot they have been separated here for clarity.



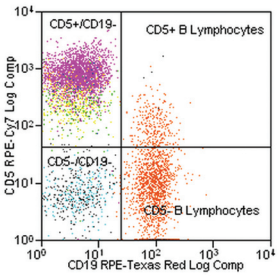
Plot 7 – CD4 RPE-Cy5 vs. CD8 Pacific Blue. (T Lymphocyte gate applied)

This plot is used to define the CD8+ T lymphocytes, CD4+ T lymphocytes, CD4 and CD8+ T lymphocytes and T lymphocytes that are CD4 and CD8 negative.



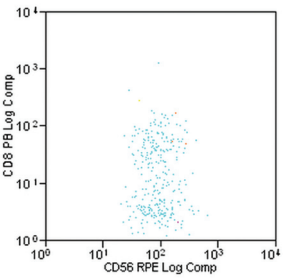
Plot 8 – CD19 RPE-TR vs. CD20 APC. (Lymphocyte gate applied)

This plot is used to check the staining intensity of CD20 on the B lymphocytes since the levels can vary in some disease states (*i.e.,* decreased levels can be observed in B Cell Chronic Lymphocytic Leukemia B-CLL).



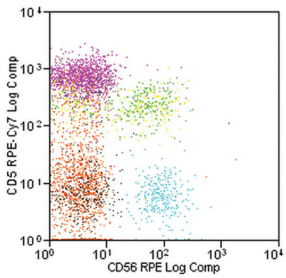
Plot 9 - CD19 RPE-TR vs. CD5 RPE-Cy7 (Lymphocyte gate applied)

This plot is used to define the CD5+ B lymphocytes, whose numbers can increase in some disease states (*i.e.,* increased levels can be observed in some auto immune disorders and B Cell Chronic Lymphocytic Leukemia B-CLL).



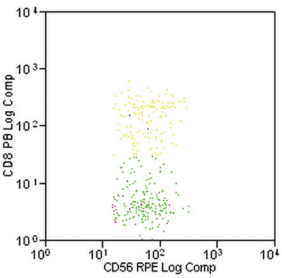
Plot 10 – CD56 RPE vs. CD8 Pacific Blue (NK Lymphocyte gate applied)

With this plot you can identify NK lymphocyte that are CD8dim and CD8-.



Plot 11 – CD56 RPE vs. CD5 RPE-Cy7 (Lymphocyte gate applied)

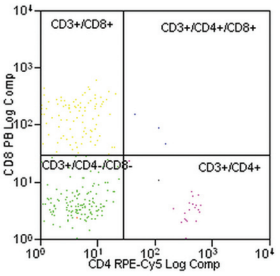
This plot shows the NK lymphocytes are CD5- whereas the NK Like lymphocytes are CD5+.



Plot 12 - CD56 RPE vs. CD8 Pacific Blue (NK Like Lymphocyte gate applied)

With this plot you can identify NK Like lymphocytes that are CD8+ and CD8 -.

Note: Plots 10–13 were created to demonstrate the ability of being able to further refine the set sub analysis.



Plot 13 - CD4 RPE-Cy5 vs CD8 Pacific Blue (NK Like Lymphocyte gate applied)

With this plot you can identify NK Like lymphocytes that are either CD8+ only, CD4+ only, CD4+/CD8+ or CD4-/CD8-.

Deriving the populations as percentage of total lymphocytes [2]

Total lymphocyte is made up from T, B and NK lymphocytes.

Total number of lymphocyte events =
N° T lymphocyte events (CD3+) +
N° B lymphocyte events (CD19+) +
N° NK lymphocyte events (CD56+/CD3-)

% CD3 =

$$\frac{\text{N° T lymphocyte events (CD3+)}}{\text{Total N° lymphocyte events}}$$

Deriving the absolute cell count (Cells/µL)

Absolute CD3 T lymphocyte count =

$$\frac{\text{N° T lymphocyte events (CD3+)}}{\text{N° CytoCount bead events}} \times \text{CytoCount bead concentration*}$$

** The CytoCount bead concentration is stated on the bottle.*

Table 2

CELL EVENTS								
CD3+ 7311	CD19+ 3081	CD56+ 499	CD3+/8+ 1481	CD3+/CD4+/CD8+ 54	CD3+/CD4+ 5271	CD5+/CD19+ 475	CD3+/CD56+ 517	Beads 4367
ABSOLUTE COUNT								
CD3+ 1744	CD19+ 735	CD56+ 119	CD3+/8+ 353	CD3+/CD4+/CD8+ 13	CD3+/CD4+ 1258	CD5+/CD19+ 113	CD3+/CD56+ 123	Cells/µL
PERCENTAGE OF LYMPHOCYTES								
CD3+ 67.1	CD19+ 28.3	CD56+ 4.6	CD3+/8+ 13.6	CD3+/CD4+/CD8+ 0.5	CD3+/CD4+ 48.4	CD5+/CD19+ 4.4	CD3+/CD56+ 4.7	

Results

Using the method described on the previous pages, it is possible to derive, for any cell population that can be clearly identified, both the percentage that population comprises of the total lymphocyte population and the absolute count of that population. The calculated absolute cell count (Cells/µL) and the populations as a percentage of total lymphocytes are shown in Table 2.

To aid in the visual identification of the various cell types, gates were created defining the population of interest, and color gating was applied. Once the various plots have been created and positioned, and the PMT voltages and compensation settings derived, the protocol can be saved. When opened, the protocol will automatically recall these settings so only minor changes may be required to optimize the settings. For optimal instrument performance it is advisable to regularly clean the instrument and implement a daily quality control procedure to monitor the instrument performance that will alert the user to potential problems.

Discussion

Use of this nine-color No Lyse application can dramatically reduce the total processing time by avoiding the time consuming lysing step, saving at least 10–15 minutes per sample. Nine-color phenotyping means a huge amount of data can be generated from just one tube, and because we only need one tube, only 50 µL of blood is required. Since we are now no longer dependent on scatter parameters, we can acquire samples at sample flow rates of 150–200 µL/min. With this technique we can rapidly screen the lymphocyte population for its major constituents and ascertain if there are any abnormalities. The addition of CytoCount control beads to the sample also allows the direct determination of absolute cell counts.

References

1. Brando. B, Barnett D, Janossy G, Mandy F, et al. "Cytofluorometric Methods for Assessing Absolute Numbers of Cell subsets in Blood." *Cytometry (Communication in Clinical Cytometry) 42:327 – 346 (2000).*
2. Mercolino T.J, Connelly M.C, Meyer E.J, Knight M.D, Parker J.W, Stelzer G.T, DeChirico G. "Immunologic Differentiation of Absolute Lymphocyte Count with an Integrated Flow Cytometric System: A New Concept for Absolute T Cell Subset Determinations."*Cytometry (Communication in Clinical Cytometry) 22:48 – 59 (1995).*

PRODUCT	CODE
CyAn ADP LX 9 Color	CY201
CD15 FITC	F0830
CD14 FITC	F0844
CD56 RPE	R7251
CD4 RPE-Cy5	C7069
CD8 Pacific Blue	PB984
CD45 Cascade Yellow	CA697
CD20 APC	LS386

PRODUCT	CODE
CD3 FITC	F0818
CD3 RPE	R0810
CD3 RPE Cy-5	C7067
CD3 Pacific Blue	PB982
CD3 Cascade Yellow	CA696
CD3 APC	C7225
PBS	S3024
CytoCount Bead	S2366

For research use only – not to be used in diagnostic procedures. Other vendor products used in this application: BD Bioscience and Beckman Coulter.

The protocols in this application note might deviate from the normal recommended protocol/specification guidelines that are included with the Dako product or any other non-Dako product.

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