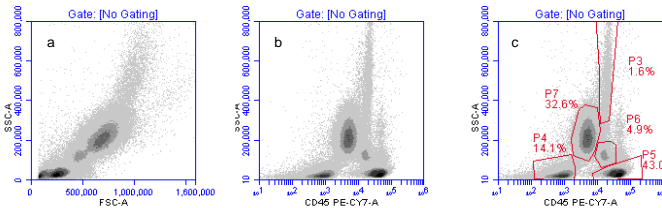


# Protocol

Identification and Enumeration of Human Peripheral Blood Cell Populations with the Accuri C6 Flow Cytometer<sup>®</sup> System

## Introduction

Several unique features of the Accuri C6 Flow Cytometer System make it an ideal platform on which to perform no-wash, differential analysis of human peripheral blood samples. First, the fixed voltage detectors of the C6 simplify data collection and reduce the potential for data loss due to signal over- or under-amplification. Second, the broad dynamic range of the C6 allows peripheral blood populations as varied in size as platelets and eosinophils to be easily analyzed in the same data file. Third, the easy-to-use Zoom Tool feature of the CFlow<sup>®</sup> software gives the user precise control when setting gates. Lastly, the use of volume measurement provided by CFlow Plus software allows calculation of the cell concentration per  $\mu\text{L}$  of original peripheral blood for each of five populations: platelets, lymphocytes, monocytes, granulocytes and eosinophils (Figure 1).



**Figure 1:** Ungated plots of human peripheral blood collected on the C6 after whole blood was stained, and red cells lysed, in a no-wash protocol. (a) FSC-A vs. SSC-A; (b) CD45-PE-Cy7 vs. SSC-A; (c) CD45-PE-Cy7 vs. SSC-A showing pre-set gates for 5 populations: P3, eosinophils; P4, platelets; P5, lymphocytes; P6, monocytes; P7, granulocytes.

## Sample Preparation

A no-wash staining procedure is outlined. Any protocol utilized should minimize sample handling to reduce cell loss and result in more accurate population counts. All pipetting steps should be performed as accurately as possible, changing tips in between each sample.

1. Collect blood in any standard anti-coagulant tube (heparin, sodium citrate or EDTA). Keep blood at room temperature (RT), rocking gently, until ready to stain.
2. See Table 1 for a suggested staining tube layout. Antibodies should be directly conjugated to fluorochromes and be titrated prior to use to determine the optimal staining concentration.
3. Aliquot 50 to 100  $\mu\text{L}$  of whole peripheral blood into standard 12x75 mm or 1.5 mL microfuge tubes.
4. Add appropriately diluted, directly conjugated antibodies following an experimental plan which includes single-stained controls (Table 1).

5. Stain cells for 20 minutes at RT, in the dark, with gentle shaking.
6. Add red cell lysis buffer of your choice at the manufacturers recommended dilution. A red cell lysis buffer containing a fixative is the best choice.
7. Vortex each tube, **gently**, after adding lysis buffer. Place tubes in dark and allow lysis to proceed for 10 to 20 minutes.
8. If red cell lysis buffer does not contain a fixative, analyze samples as soon as possible after lysis step. Fixed samples can be stored in the dark at 4<sup>o</sup> C for up to 3 days.

Tube	FITC	PE	PE-Cy7	APC
1	Isotype	Isotype	Isotype	Isotype
2	<b>CD41</b>	Isotype	<b>CD45</b>	Isotype
3	Isotype	<b>CD11b</b>	<b>CD45</b>	Isotype
4	Isotype	Isotype	<b>CD45</b>	Isotype
5	Isotype	Isotype	<b>CD45</b>	<b>CD8</b>
6	<b>CD3</b>	Isotype	<b>CD45</b>	Isotype
7	<b>CD3</b>	<b>CD4</b>	<b>CD45</b>	<b>CD8</b>
8	<b>CD14</b>	<b>CD11b</b>	<b>CD45</b>	Isotype

**Table 1.** Suggested staining tube layout for four-part differential

## Data Collection on the C6

NOTE: Calculating the numbers of cells per  $\mu\text{L}$  requires the use of CFlow Plus software during data acquisition. Gently resuspend the cells in each sample immediately before placing tube on the C6.

1. Power on the C6, check performance with Validation beads, and perform a Fluidics Calibration cycle as outlined in the Accuri C6 Instruction Manual.
2. Open the CFlow Plus Template called "HPB 4 Part Differential".
3. Verify the following settings in the Collect Tab Control Panel:
  - a. Threshold: FSC-H = 80,000
  - b. Run Limit: 100,000 events in R1
  - c. Fluidics: Fast
4. Using Tube 1 collect at least 200,000 total events, and PAUSE data collection manually.
5. Adjust R1 on the FITC vs. PE-Cy7 plot to contain between 0% and 5% background events (Figure 2a).
6. Using Tube 4 (CD45-PE-Cy7 positive) collect data into a new well, allowing the C6 to stop automatically when it reaches the run limit of 100,000 events in R1.
7. Re-adjust R1, if needed, to encompass the CD45-PE-Cy7<sup>+</sup> population (Figure 2b).

### phone

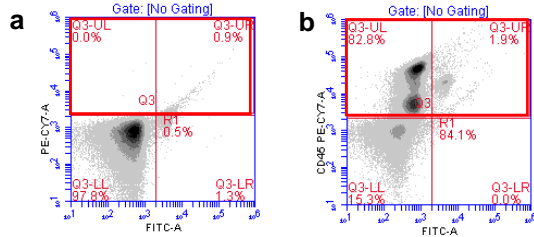
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# Identification and Enumeration of Human Peripheral Blood Cell Populations with the Accuri C6 Flow Cytometer® System

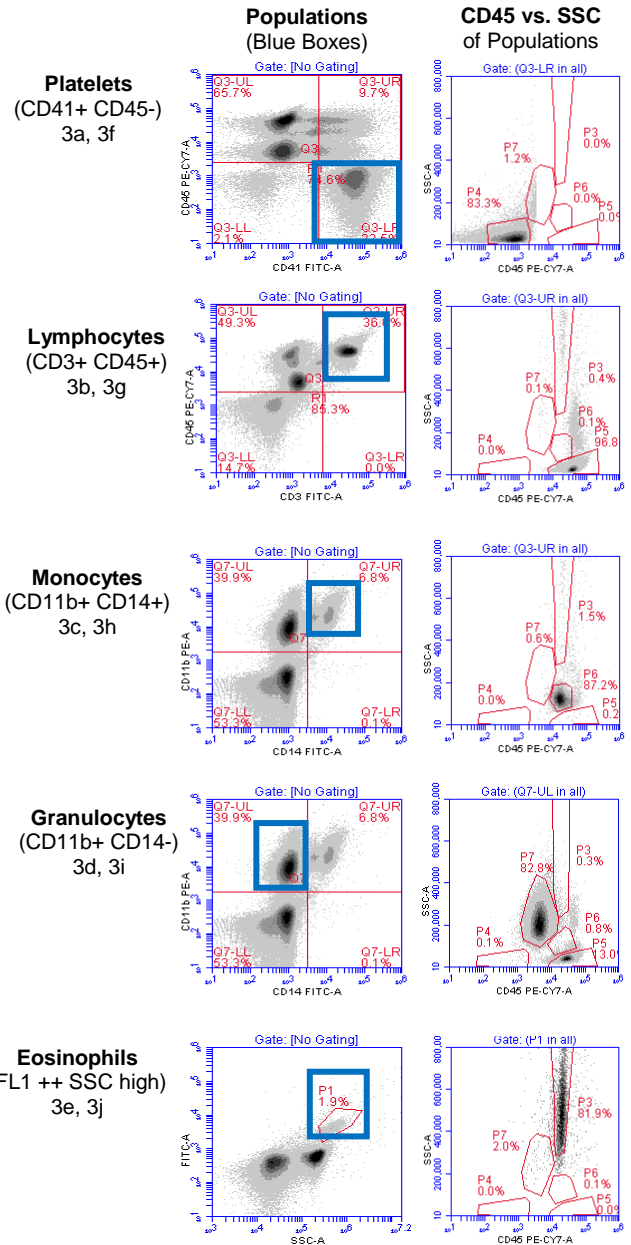
8. Collect data for all remaining tubes, allowing the C6 to stop automatically on a run limit of 100,000 events in R1.



**Figure 2.** Example of R1 gate placement (red box, includes UL and UR quadrants of FITC vs. PE-Cy7 plot) for (a) PE-Cy7 background, Tube 1 and for (b) CD45-PE-Cy7, Tube 4. Refer to Table 1 for tube description.

### Data Analysis

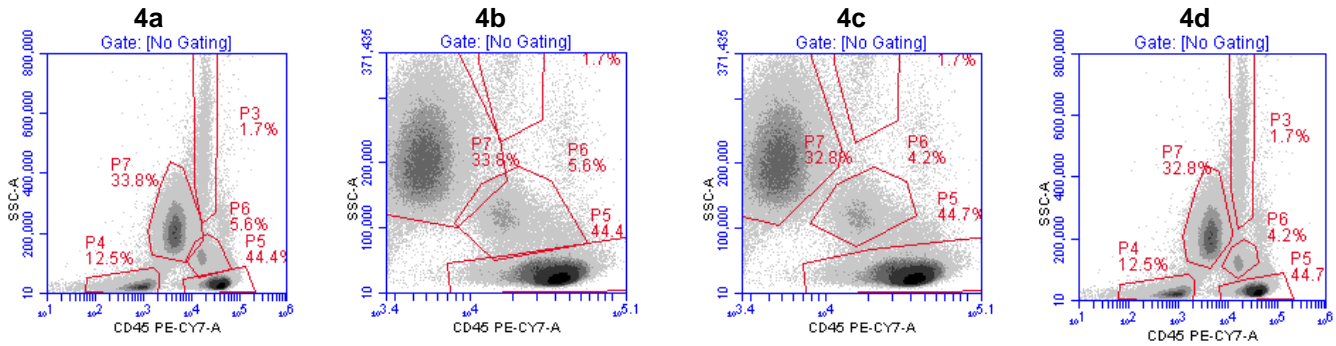
- Verify Fluorescence Compensation Values.
  - Open the compensation matrix in the CFlow Plus software and the C-Comp spreadsheet file provided by Accuri.
  - Ensure that compensation values in the matrix are set to zero.
  - Calculate median values for data in wells A1 through A5, then copy and paste the values into the appropriate cells of the C-Comp Worksheet.
  - Adjust compensation values in the CFlow Plus matrix as required.
- Verify the location of the five populations by backgating on markers specific for each population to the CD45-PE-Cy7 vs. SSC plot. (See Figure 3). Use the Zoom Tool if needed to precisely adjust the population gates (P3 through P7).
  - Platelets:** Use Tube 2 data to gate CD41-FITC<sup>+</sup>, CD45-PE-Cy7<sup>-</sup> cells to Plot 3 (CD45 vs. SSC) and adjust P4 (Figure 3a and 3f).
  - Lymphocytes:** Use Tube 6 data to gate CD3-FITC<sup>+</sup>, CD45-PE-Cy7<sup>+</sup> cells to Plot 3 and adjust gate P5 (Figure 3b and 3g).
  - Monocytes:** Use Tube 8 data to gate CD11b-PE<sup>+</sup>, CD14-FITC<sup>+</sup> cells to Plot 3, and adjust gate P6 (Figure 3c and 3h).
  - Granulocytes:** Use Tube 8 data to gate CD11b-PE<sup>+</sup>, CD14-FITC<sup>-</sup> cells to Plot 3 and adjust gate P7 (Figure 3d and 3i).
  - Eosinophils:** Use Tube 4 data to gate high green autofluorescence, high SSC population in gate P1 on Plot 3 (Figure 3e) and adjust gate P3 (Figure 3j).
  - Use the Zoom Tool in CFlow to aid in drawing precise non overlapping gates on CD45 vs. SSC plot (Figure 4b and 4c).



**Figure 3.** Human peripheral blood populations are first identified by surface markers to aid in adjusting polygons for CD45 vs. SSC population identification. Figure 3a-3e: “Backgating” on surface marker or autofluorescence properties to identify specific populations. Figure 3 f-3j: The CD45 vs. SSC profile of each population identified in Column 1.

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3. Calculate the number of each population per sample tube and per 100µL of original blood sample (Table 2).
  - a. Copy and paste the data columns for gate label, cell number and volume from the CFlow Plus statistics table into a spreadsheet program.
  - b. Calculate the number of cells in each population per tube, and then per volume of original blood sample. (Table 2).



**Figure 4.** Using the Zoom Tool to adjust gates on 5 populations for human peripheral blood analysis. (a) original polygon placement, (b) zoomed plot shows overlap of polygons, (c) adjusted polygons and (d) plot zoomed back to original scale.

Population	CD45 vs. SSC Gate	Cell Number	Volume Pulled (µL)	Sample Volume (µL)	Cells/100µL Blood	K/mm <sup>3</sup>	Normal Range
Lymphocytes	P5	60,827	308.1	2,100	414,642	4.15	0.8 - 5.0
Monocytes	P6	1,154	308.1	2,100	7,869	0.08	0.1 - 1.0
Granulocytes	P7	26,618	308.1	2,100	181,447	1.81	1.4 - 7.5
Eosinophils	P3	2,478	308.1	2,100	16,894	0.17	0.0 - 0.4

**Table 2.** Calculation of cell number per mL of original blood sample for four of five identified populations.