CFlow User Guide

Science is hard. Flow cytometry should be easy.®
Figure 3-1 CFlow Workspace after selecting File, New CFlow File from the CFlow file menu .............. 18
Figure 3-2 The Plot Spec Dialog Box. Changing these settings only affects the view of the data. No data is changed in, or lost from, the data file ................................................................. 18
Figure 3-3 Threshold Settings dialog box ......................................................................................... 19
Figure 3-4 Warning message when raising a threshold value will result in data loss ...................... 19
Figure 3-5 CFlow template after collection of Sample A1, human peripheral blood ....................... 20
Figure 3-6 Use of Zoom Tool for a “close-up” view of the lymphocyte population in a human peripheral blood sample ................................................................. 20
Figure 3-7 Polygonal gating tool was used to draw P1 on the 2D plot above. Other available gating tools are Rectilinear and Quadrant ................................................................. 21
Figure 3-8 Opening a Density plot defaults to a linear FSC-A vs. SSC-A plot ................................. 21
Figure 3-9 Changing density plot axes parameters and scaling ...................................................... 22
Figure 3-10 Selecting a gating option, which includes all events from a given gate, i.e. Plot 2 is now gated to include “P1 on all events” ............................................................. 22
Figure 3-11 Plot 2 has been gated to include P1 (all events) ........................................................... 23
Figure 3-12 Gating choice options for plots: Include, Exclude, and Intersection ............................ 23
Figure 3-13 Adding Vertical or Quadrant Markers to the Gating list .............................................. 23
Figure 3-14 Selecting to display the 1st decade of data on a default log data plot. a. Open the Plot Spec dialog box. b. Uncheck “Hide 1st decade” boxes for FL1-A and FL2-A, and set the “Min Value” for both parameters to 0 ................................................................. 24
Figure 3-15 A CFlow Workspace with 5 added 2D fluorescence plots. Plots required for tutorial: FL1-A vs. FL2-A; FL1-A vs. FL3-A; FL2-A vs. FL3-A; FL4-A vs. FL3-A; FL1-A vs. FL4-A ................................. 24
Figure 3-16 Sample number 2 has been added to well A2 in the CFlow Workspace ........................ 25
Figure 3-17 Changing axes labels using the axis label drop-down menu and the Rename Parameters option ................................................................. 25
Figure 3-18 Changing axes labels using the axis label drop-down menu and the Rename Parameters option. Note that once the label CD45 PE-Cy7 has been entered it will now appear in the drop-down menu when the Select option is chosen. ................................................................. 26
Figure 3-19 Saving and naming a CFlow File ....................................................................................... 26
Figure 3-20 New file name, “Human PB”, appears in the upper left corner of the CFlow workspace after saving and naming the CFlow file ................................................................. 27
Figure 3-21 Fluorescence spillover of positive PE-Cy7 signal from FL3 into a. FL1-A, and b. FL2-A, c. The PE-Cy7 signal does not spillover into FL4-A ................................................................. 27
Figure 3-22 Selection and placement of Quadrant Marker ................................................................. 27
Figure 3-23 a. Placement of Quadrant Tool for median calculations on the double negative (lower left) and single PE-Cy7 positive (upper left) populations. b. Median fluorescence values in the Statistics Table for Plot 3, Q1, will all read “NA” until Calculate Median is selected ............................................. 28
Figure 3-24 Quadrant Q2 is selected with mouse click (indicated by bold red) and Calculate Median chosen from the Display drop-down menu ................................................................. 28
Figure 3-25 Statistics Table updated with median values after choosing Calculate Median from the Display drop-down menu ................................................................. 29
Figure 3-26 Opening and using the Compensation Settings dialog box ........................................... 29
Figure 3-27 Results of subtracting spillover of FL3 PE-Cy7 signal from FL1 by 1.35%. Note that the median FL1 channel numbers in Q1-UL (238) and Q1-LL (231) are approximately equal (blue boxes) ................................................................. 29
Figure 3-28 Results of selecting Plot 4, applying Quadrant markers and calculating median FL2 fluorescence channel values ................................................................. 30
Figure 3-29 Results of correcting FL2 by subtracting 4.3% of the signal from FL3 (PE-Cy7). The median FL2 fluorescence channel value for Q2-UL (131) is approximately equal to the median FL2 fluorescence channel value for Q2-LL (134) ................................................................. 31
Figure 3-30 No fluorescence subtraction of FL3 PE-Cy7 signal is needed from FL4, as indicated by comparison of median FL4 values for Q3-UL (290) and Q3-LL (263) ................................................................. 31
Figure 3-31 Correction of spillover from FITC FL1 into FL2. a. Before fluorescence subtraction. b. After calculation and application of appropriate percentage subtraction of FL1 from FL2 ................................................................. 32
Figure 3-32 Gating region R1 has been drawn on Plot 3 to include the CD45 PE-Cy7 positive events ................................................................. 32
Figure 3-33 Gating Plot 2 (CD3-FITC vs. FL2-A) to include events in gates R1 and P1 ................................................................. 33
Figure 3-34 Gate for Plot 2 (CD3-FITC vs. FL2-A) includes events in gates R1 and P1, after following steps 1-3 in Adding and Applying Additional Gates ......................................................... 34
Figure 3-35 Blue boxes show desired 4 log channel to view for Plots 2 and 6. ...........................................34
Figure 3-36 Setting Min and Max channel values for the X and Y axes using the Plot Spec dialog box. ....35
Figure 3-37 Result of axes view adjustment to 4 logs for Plots 2 and 6 .........................................................35
Figure 4-1 The Analyze Tab .........................................................................................................................36
Figure 4-2 Selecting plots to copy from the Collect tab ..................................................................................37
Figure 4-3 Plots 1C, 3C and 6C opened in the top row of Analyze tab ............................................................37
Figure 4-4 Analyze tab with copied plots in Plot List and selected plot corral ................................................38
Figure 4-5 Analyze Tab: Result of gating Plot 3C on "P2 in all" and Plot 6C on "R2 in (P2 in all)" .......................38
Figure 4-6 Analyze Tab: Row 2 selected to display data from Sample A2 in Plots 1C, 3C and 6C ..............39
Figure 4-7 Analyze Tab view after scrolling down to empty plot corral ..........................................................39
Figure 4-8 Analyze Tab view after adding 2 more rows of Plots 1C, 3C and 6C, and populating with data from Sample A3 and A4 ...........................................................................................................40
Figure 4-9 Analyze Tab: Plots are selected for printing ..................................................................................40
Figure 4-10 Analyze tab: Overlay Tool has been selected .............................................................................41
Figure 4-11 Analyze tab: Overlays of FL1-A data for samples A2, A3 and A4. The Overlay Legend has been opened ........................................................................................................................................41
Figure 5-1 The File Menu .............................................................................................................................42
Figure 5-2 The Edit Menu .............................................................................................................................43
Figure 5-3 Rename Parameters ......................................................................................................................43
Figure 5-4 Rename Parameters, selection options menu ..............................................................................44
Figure 5-5 The Display Menu .......................................................................................................................44
Figure 5-6 Events Display Settings .............................................................................................................44
Figure 5-7 a. Quadrant marker is not selected b. Quadrant Marker selected (bold lines) .........................45
Figure 5-8 a. Statistics table before selecting Calculate Median b. Statistics table after selecting Calculate Median .................................................................................................................................................45
Figure 5-9 a. Statistics table after moving Quadrant markers ......................................................................45
Figure 5-10 The Instrument Menu .............................................................................................................46
Figure 5-11 Threshold Settings ....................................................................................................................46
Figure 5-12 Warning displayed after a change in threshold settings .............................................................47
Figure 5-13 Compensations Settings ...........................................................................................................47
Figure 5-14 The About Menu .......................................................................................................................48
Figure 6-1 When to use VirtualGain ............................................................................................................49
Figure 6-2 Recreate the histogram or copy plots from Collect ......................................................................50
Figure 6-3 Click on the histogram parameter label to reveal the Parameter list ..........................................50
Figure 6-4 Step 7 and Step 8, Aligning plots. ...............................................................................................51
Figure 6-5 Step 9, Pick the sample to align .................................................................................................52
Figure 6-6 Step 11, Move the Peak Definition Marker ..................................................................................52
Figure 6-7 Step 14, To align other samples exactly as the first aligned sample ............................................53
Figure 6-8 Step 15, A black asterisk appears under the Sample to Align plot .............................................53
Figure 6-9 The sample with VirtualGain applied has a black asterisk under the parameter label ...............54
Figure 6-10 Click the asterisk to switch between VirtualGain and native displays. A grey asterisk indicated native data, a black one Virtual Gain applied .................................................................54
Figure 6-11 Overlays automatically display VirtualGain whenever applied ..................................................55
Figure 7-2 The Plate Type drop-down menu ...............................................................................................57
Figure 7-1 CFlowSampler start-up screen ...................................................................................................57
Figure 7-3 The CFlowSampler Manual Collect Tab .....................................................................................58
Figure 7-4 The Run Backflush Cycle window ...............................................................................................59
Figure 7-5 The Run Unclog Cycle window ..................................................................................................59
Figure 7-6 The Calibrate Fluidics window ..................................................................................................60
Figure 7-7 The Auto Collect tab ..................................................................................................................61
Figure 7-8 Selecting an individual sample ....................................................................................................62
Figure 7-9 Applying settings to a set of samples will turn those sample wells the same color ....................63
Figure 7-10 The Auto Collect tab displaying two sets ..................................................................................63
Figure 7-11 The Run Display .......................................................................................................................65
Figure 7-12 The Run Display while collecting data .......................................................................................66
Figure 7-13 The CSampler display after completion of a sample run ...........................................................67
Figure 7-14 Collision detected window ........................................................................................................69
Chapter 1 The Basics - Operating the Accuri® C6 Flow Cytometer®

Introduction to CFlow and Running the C6
The C6 Flow Cytometer system includes CFlow or CFlow Plus software for controlling the instrument, acquiring data, generating statistics, and analyzing results. Throughout this User Guide, CFlow refers to both CFlow and CFlow Plus unless indicated otherwise.

CFlow Workspace
The CFlow workspace provides access to all the functions needed to acquire and analyze data. CFlow is divided into three separate tabs: Collect, Analyze and Statistics. This section focuses on data acquisition and the Collect tab.

The Collect tab appears when CFlow is launched and is used to acquire data from samples processed on the C6. The Collect tab enables physical control of the C6, provides access to settings needed to specify the threshold and stop criteria as well as many analytic functions.

Figure 1-1 shows a partial Collect tab. The Sample Grid is laid out in the configuration of a 96-well plate to facilitate both organization of experiments and data acquisition from sample tubes. CFlow acquires each sample into its own well in the Sample Grid. The wells can be filled with data in any order that is convenient. White wells do not contain data and are available to use for data collection. Blue wells contain data. A red outline around a well indicates that well is currently selected for viewing or collecting data.

Instrument Controls
The left-hand column of the CFlow Collect tab provides access to all instrument controls needed to collect data. Before data collection can begin, the Traffic Light below the Sample grid must be green and the words “C6 Cytometer is connected and ready” appear in the message box.

Getting Started
This section provides a step-by-step guide for running validation beads. The validation will demonstrate many of CFlow features and ensure the C6 is working properly before running experimental samples. All the individual features of CFlow are described in detail in Chapter 4.

Complete the initial instrument set-up as outlined in the C6 Flow Cytometer Instrument Manual, Section 4, before continuing.

Starting CFlow
Open CFlow by double clicking on the CFlow icon on the desktop. The message box in CFlow will say Cytometer Not Connected.

C6 Startup Procedure
1. Check sheath bottle (blue ring) fluid level and fill if needed. Accuri recommends the use of 0.2 µm filtered, deionized, H₂O with bacteriostat added (Accuri Part# KR-220). CFlow will alert the user if the bottles need attention when the C6 is turned on and CFlow is running.
2. Empty the waste tank (red ring).
3. Check Cleaner (green ring) and Decontamination Solution (yellow ring) bottles. Fill if necessary.
4. Replace the tube on Sample Introduction Probe (SIP) with a fresh tube containing at least 2 mL filtered, deionized H₂O.

5. Firmly press the On/Off button on the front of the unit to turn on the C6. A blue light will flash and then stay illuminated. After ~20 seconds, the CFlow traffic light will show yellow. After ~60 seconds, the C6 pumps will start to run. When powered on, the C6 will automatically flush the fluidics lines with sheath. This process takes approximately 3 minutes.

6. When the C6 is ready for data collection, the CFlow traffic light will change to green. Begin data
collection when the Traffic Light is green and the C6 Status Message Box reads **C6 and CFlow are connected and ready**.

For optimal performance, the C6 should be on for at least 15 minutes prior to processing samples.

**C6 Improper Shut Down Recovery Message**

If the message shown in Figure 1-2 is seen at startup, the C6 will take approximately 6 minutes to recover and return to the green-light, ready state, as opposed to the usual 3 minute startup procedure. This may occur on initial C6 startup when the unit is first received. It will also occur after an interruption of power to the unit.

![C6 Improper Shut Down Recovery Message](image)

**Running Validation Beads**

Accuri provides an initial set of pre-diluted validation beads. Store the beads at 4°C in the dark. The beads expire within 2 weeks of receipt. Refer to **Reagents Required** for ordering information.

Validate the performance of the C6 with validation beads each day that the cytometer is used. The C6 should be able to distinguish a defined number of peaks within each fluorescence channel. If these peaks are not discernable there may be a problem and any data collected may be flawed.

It is important to collect and save a bead data file every day that data is collected. The user can easily address many C6 issues with assistance from Accuri Technical Support. If a problem is experienced that requires telephone assistance, Technical Support may ask for data from the bead file to assist in troubleshooting.

**Reagents Required**

1. Spherotech 8-Peak Validation Beads (Accuri Part# QA-100) or the prediluted 12x75 mm tube from Accuri labeled 8-Peak Beads
2. Spherotech 6-Peak Validation Beads (Accuri Part# QA-110) or the prediluted 12x75 mm tube from Accuri labeled 6-Peak Beads
3. Sheath fluid: Deionized, filtered water (0.2 µm filter) plus Bacteriostatic Concentration Solution (Accuri Part# KR-220)

**Running the Bead Samples**

1. Be sure that the C6 has been switched on for at least 15 minutes.
2. Verify that the file named **“Bead Template.c6t”** has been copied to the computer attached to the C6. This file is on the CFlow CD shipped with the C6 and also available on the Accuri website.
3. Double-click on the bead template file to open or load the file from the CFlow **File** Menu.
4. Place an empty 12x75 mm tube on the SIP.
5. Click on “BACKFLUSH”.
6. Place a fresh tube with 2 mL filtered, deionized H₂O on the SIP.
7. Click on well A1 to select it. Well A1 should now have a red box around it.
8. Deselect the **Run Unlimited** check box under **Run Limits** in the Instrument Control Panel.
9. Select the time check box (Min Sec) in the Instrument Control Panel and input 2 minutes.

10. In the Control Fluidics section of the Control Panel, select Slow. The flow rate should display 14µL/min and Core Size should display 10 µm.

11. Click on the Threshold button to set the Threshold to 80,000 on FSC-H (default).

12. Press Run. While data is being acquired, well A1 will flash blue.

13. Click Pause then the Delete Sample Data button in the Control Panel of the CFlow screen to clear data collected during the run. The data well should change from blue to white and the counters will reset.

14. Deselect the time check box and click the Events check box. Verify that it is set to 50,000 events in Ungated Sample. If not, enter 50,000 events and select the Ungated Sample check box.

15. Replace the tube on the SIP with a tube containing suspended 8-peak beads. Vortex the tube prior to placing it on the C6.

16. Click RUN to start acquisition. Acquisition will automatically stop after 50,000 total events are acquired. Do not click on ADD TO. ADD TO is used to add more data to a well that already contains data. The R1 region may not encompass the main population of bead events on the FSC-H vs. SSC-H plot. This will be addressed in Analyzing and Recording Your Validation Bead Data.

17. Name this sample including the date processed in the text box just above the Sample Grid. Samples can be named at any time; before, during, or after collection.

18. Remove the beads tube and wipe off the end of the SIP with a Kimwipe, or comparable material, to minimize bead carryover. To further minimize carryover, click Backflush while holding a reservoir or empty tube under the SIP. Fluid should drip or stream out of the SIP.

19. Place a tube with suspended 6-peak beads on the SIP. Vortex the tube prior to use.

20. Click on well E1 to advance to well E1. Verify that the Events check box is still selected and set to 50,000 events in Ungated Sample.

21. Click RUN. The R2 region on the FSC-H vs. SSC-H plot in the second row of the CFlow bead file may not encompass the main population of beads.

22. Name this sample including the current date in the text box just above the Sample Grid.

23. Remove the beads tube from the SIP and wipe the end of the SIP.

24. Place a tube with 2 mL of filtered, deionized H2O on the SIP and advance to any empty data well.

25. Select the time check box (Min Sec) in the Instrument Control Panel and set it for 2 minutes. Click RUN.

26. Leave the tube on the SIP after the C6 stops running.

Always end each data collection session on the C6 by running filtered, deionized H2O for 2 minutes. Leave this tube on the SIP between sessions and when shutting off the C6.

**Saving the Data**

Saving data as a CFlow file will save the CFlow workspace in its entirety, including collected sample data, plot layouts, gating, color compensation, and threshold settings that have been added. Changes made in the Collect, Analysis or Statistics tabs will be saved in a single comprehensive (and often large) data file. Data collected on the C6 should always be saved as a CFlow file.

There is no need to export after every sample run. Data from individual sample wells can be exported from CFlow as FCS 3.0 files at any time.
Save Your Data as a CFlow File

1. In CFlow, under the File menu select Save CFlow File (Figure 1-3) and save the bead data file as a “Validation Bead File”. Use this file each day to collect Validation Bead data, advancing to a new well in row A or E for 8-peak and 6-peak beads, respectively. Include the date in the Sample Naming Field when naming each day’s bead sample.

**CAUTION:** CFlow does not automatically save CFlow files to the computer hard drive. The data is saved locally in RAM until it is saved as a CFlow file or exported as FCS files. CFlow allows switching between data wells to view data even when data is not stored to the hard drive.

2. End every session of data collection on the C6 by placing a tube containing 2 mL of filtered, deionized H₂O on the SIP. Run for 2 minutes in an empty data well to clear the last sample’s residue from the SIP. Leave the tube on the SIP after the run has finished.

Analyzing and Recording your Validation Bead Data

Analyze the bead data using the Collect tab of CFlow.

1. Click on the well in the Bead File which contains the 8-peak bead data to select it (will be A1 if following previous procedure).

2. On the first FSC-H vs. SSC-H plot (scatter plot) in the bead file, adjust the pre-drawn region R1 to encompass the main population by clicking on the region (it will have thicker lines when selected) and dragging it so it looks like Plot 1 in Figure 1-4. This should contain 75-85% of all events in R1. There is usually a “shadow” population slightly higher in FSC-H than the main cluster of beads; this is normal for these particular beads (bead doublets or clumps). Do not include this group in R1.

3. View the next 3 plots - FL1-H, FL2-H, and FL3-H. They should be gated on the scatter region, R1. The phrase “**R1 in all**” should be visible next to the gray GATE button at the top of each of these 3 fluorescence plots (Figure 1-5). If not, click the GATE button and select the option “**R1 on all events**” from the pop-up gating list.

4. Position the predrawn horizontal Marker Region around the top (brightest, far-right) peak on each of the 3 fluorescence plots.

Use the **Zoom Tool**, located in the lower right corner of each plot (the button with the magnifying glass), to zoom in on the top peak. Then, adjust the marker tightly around the peak. Click on the **Expand Tool** (the button with a square and four arrows) to zoom back out.

5. Compare the bead run to the manufacturing results sent with the C6. If the C6 is performing properly, data should look similar to that in Figure 1-6 for the 8-peak beads. There should be one main
population of beads on FSC-H vs. SSC-H, 8 discernable peaks on FL1-H and FL2-H, and 6 peaks on FL3-H. FL4-H performance will be checked with the 6-peak beads in a later step.

Figure 1-4 Proper position of R1 on Plot 1 and Gate selection for Plot 2

Figure 1-5 Location of Zoom Tool and Expand Tool, and effect of Zoom
6. Select the well containing the 6-peak bead data (should be well E1).

7. Adjust the pre-drawn region R2 in the second FSC-H vs. SSC-H to encompass the main data peak similar to the procedure for the 8-peak beads. This population should look like an exclamation mark. The R2 region should encompass both major populations (Figure 1-7).

8. Confirm that the FL4-H plot is gated on the region R2. If not, click on the “GATE” button and select the option “R2 on all events” from the pop-up menu.

9. Adjust the Marker in the FL4-H plot to be tightly placed around the top (brightest) peak. Refer to Figure 1-7 for an example of good 6-peak bead data on a C6.

10. Record the number of peaks, the Mean channel numbers and CVs for the top peaks, and for the forward scatter of the beads for each parameter in the Accuri Cytometer Log. Additional copies can be found on the CFlow CD and on the Accuri website.

Please send a copy of the bead file, from the first time beads are run on the C6 after installation, to Accuri Technical Support: dmack@accuricytometers.com.
Figure 1-7 Example of 6-peak SPHERO APC Calibration Particles, shown in FL4-H. The peaks in FL1-H, FL2-H and FL3-H are not relevant.

Using the Statistics Tab in CFlow to Generate a Table of Mean Peak Channel and CV values

A convenient way to monitor the Validation bead data, and thus the C6 performance, is to store the 8- and 6-peak validation bead data in a single CFlow file, saving the data from each day in its own well. Then, using the Statistics tab, a table can be created of the Mean channel numbers and CVs for the top peaks, and for the forward scatter of the beads to make it easy to determine if the C6 performance is stable.

In Figure 1-8, the C6 in question had been performing with a FSC-H CV of between 2% and 2.5% for over a month using the SPHERO Rainbow Calibration Particles. However, on the last day shown, the FSC-H CV had increased substantially as had the top peak CVs for FL1-H and FL2-H. This could indicate either bead degradation or a slightly dirty flow cell. Refer to Troubleshooting for possible fixes.

Also refer to Troubleshooting if any of the following conditions are experienced:

1. Very broad or multiple peaks for FSC-H on the 8- or 6-peak beads, excluding the shadow doublet population, or
2. Fewer than 8 peaks visible on FL1-H and/or FL2-H, or fewer than 6 peaks on FL3-H, or
3. Brightest peak CVs are >5.0% in any channel.
Troubleshooting

If there are very broad or multiple peaks for FSC-H, fewer than 8 peaks visible on FL1-H and/or FL2-H, fewer than 6 peaks on FL3-H or the brightest peak CVs are >5.0% in any channel check the following:

1. If bead data was acquired using the Slow rate (Under Fluidics Control in the Control Panel). If not, select Slow, re-suspend the beads by vortexing or flicking the tube, and recollect the data.

2. The age of the beads. If the beads have been diluted for more than 2 weeks, kept at room temperature or warmer, or exposed to light for long periods of time, their performance may be degraded. Make up new bead suspensions and run the bead sample again.

3. If there is a bubble or clog in the flow cell.
   a. Run the bead sample again.
   b. Remove the tube from the SIP, place an empty tube under the SIP and click Unclog. When the Unclog cycle is finished (Traffic Light will be green), run the bead sample again.
   c. Remove the tube from the SIP, place an empty tube under the SIP and click Backflush. When the cycle is finished (Traffic Light will be green), run the bead sample again.

For further troubleshooting, refer to FAQs at www.AccuriCytometers.com or call Accuri Technical Support at 1.734.994.8000.
Chapter 2 Getting the Most from the C6

Materials Needed to Operate and Maintain the C6

- Sheath fluid: Deionized, filtered water (0.2 µm filter) plus Bacteriostatic Concentration Solution (Accuri Part# KR-220)
- Decontamination Solution: 10X Concentrate (Accuri Part # KR-200)
- Cleaning Solution: 10X Concentrate (Accuri Part# KR-210)
- C6 Flow Cytometer Maintenance Kits which include replacement Fluidic Bottle Filters (Accuri Part# CP-130 and CP-135), In-line Sheath Filters (Accuri Part# CP-140) and Peristaltic Pump Tubing (Accuri Part# CP-105)
- Validation beads (refer to page 7)

System Cleaning and Validation
Following a few simple rules ensures C6 optimal performance.

Keep the C6 Clean

- Always follow the startup and shut down procedures outlined in the C6 Flow Cytometer Instrument Manual in order to keep the SIP and flow cell clean, and the fluidics system free of air bubbles. Decontamination fluid is run through the C6 fluidics system at each shut down. This helps to keep the SIP and flow cell free of debris and protein build-up but may not prevent build-up from occurring.
- Always leave a tube containing at least 2 mL of filtered, deionized H₂O on the SIP in between uses, and at shut down.
- Replace the following User Serviceable Parts of the C6 every two months.
  - Sheath bottle filter
  - Decontamination and Cleaning solution bottle filters
  - In-line Sheath filter inside the C6
  - Peristaltic pump tubing
- Refer to the C6 Flow Cytometer Instrument Manual, Section 5, for replacement procedures and Section 6 for order information.

Keep the C6 Validated

- Run the Accuri-recommended Validation Beads each day the C6 is used. Refer to Chapter 1 for detailed instructions on Validation Beads.
- If CFlow Plus is installed on the C6 and volumetric or absolute cell counts are required, it is important that the system is accurately measuring the volume aspirated from each sample.
  - Replace the peristaltic pump tubing every 2 months.
  - Ensure that the SIP and flow cell are clean, and the fluidics lines are free of air bubbles. Refer to C6 Flow Cytometer Instrument Manual, Section 5.4b, Running the Cleaning Cycle and 5.5, Inspection of the Fluidics Lines, for directions.
  - Validate volume measurement using Accuri’s Volumetric Validation Beads or other counting bead product. Refer to C6 Flow Cytometer Instrument Manual.
**Standard Operating Procedures for the C6**

The following are recommended standard operating procedures.

**Instrument Startup**

1. Check fluid levels in all tanks and attend to them if needed.
2. Press the Power Button on the front of the C6 and open CFlow Software.
3. Wait until the Traffic Light in CFlow is green and message reads: **C6 and CFlow are connected and ready.**
4. Place an empty 12x75 mm tube on the SIP.
5. Click **BACKFLUSH** button in CFlow. A small amount of sheath fluid should appear in the tube.
6. Place a fresh tube containing 2 mL of filtered, deionized H₂O on the SIP.
7. In CFlow, Deselect the **Run Unlimited** check box and select the **Time** check box. Enter 2 minutes.
8. Click **RUN**. Wait for run to finish.
9. Click **DELETE EVENTS FROM SAMPLE**.
10. Keep the tube with water on the SIP until experimental samples are ready to be analyzed.

**Care After Running Samples**

Always perform this process after the last sample to ensure cells or other particles are not left in the SIP.

1. Place a tube with 2 mL of Cleaning Solution on the SIP.
2. Select an empty data well.
3. Select the **Time** check box. Enter 2 minutes.
4. Click **RUN**. Wait for run to finish.
5. Remove the tube of Cleaning Solution, and place a tube of 2 mL filtered, deionized H₂O on the SIP.
6. Select the **Time** check box and enter 2 minutes.
7. Click **RUN**.
8. When run is finished, leave the tube of water on the SIP.

**Instrument Shut Down**

1. Perform Care After Running Samples procedure detailed above.
3. When the Power Off process is completed the CFlow Message Box will read **C6 Cytometer not connected.**

**Validation of Instrument Performance**

Follow the directions in Chapter 1 for running the 8- and 6- peak SPHERO Calibration Particles. Use the Accuri C6 Cytometer Log, (spreadsheet located on the CFlow Installation CD) to record top peak CVs and Mean channel numbers for each fluorescence detector.

**Calibrating the Fluidics System for Precise Volume Measurements**

The C6 fluidics system is calibrated for a sample volume of 400 µL in a standard 12x75 mm tube during manufacture. Accuri suggests that you recalibrate your system for your most common tube type and volume to increase the accuracy of volume measurements. If the C6 is being used routinely for volume analysis,
perform volume calibration once a week. For intermittent volume analysis, perform volume calibration prior to each set of experiments requiring it. Volume measurements can only be performed in CFlow Plus software.

To ensure the most accurate volume measurements:
- The peristaltic pump tubing must be less than two months old.
- Run samples at Medium or Fast speeds.
- Calibrate the C6 to an equivalent sample volume/liquid height in tube as the sample to be measured.
- Never perform calibration with less than 150 µL in any type of sample tube.

To Calibrate the C6
1. Place a tube of the same kind to be used in the experiment and containing a volume of sheath fluid close to the sample volume in the experiment + 65 µL on the SIP. For example, if cell samples are diluted to 400 µL in 12x75 mm tubes, then the Fluidics Calibration should be performed with a 12x75 mm tube containing 465 µL of sheath. The minimum sample volume to calibrate a 12x75 mm tube is 150 µL (run calibrate with 215 µL in the sample tube).
2. Under the Instrument Menu, choose Calibrate fluidics. The fluidics calibration will take ~ 5 minutes.

If calibration fails, the C6 will revert to its previous fluidics automatically and will operate normally. However, the volumes recorded will not be optimized for the new sample volume. If more precise measurement is essential, try calibrating again.

Routine Instrument Maintenance

<table>
<thead>
<tr>
<th>Part Number</th>
<th>Description</th>
<th>Replacement Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-102</td>
<td>Replacement SIP Guard</td>
<td></td>
</tr>
<tr>
<td>CP-105</td>
<td>Peristaltic Pump Tubing (Set of 2)</td>
<td>Every 2 Months</td>
</tr>
<tr>
<td>CP-130</td>
<td>Sheath Bottle Filters (Large) (Pak of 5)</td>
<td>Every 2 Months</td>
</tr>
<tr>
<td>CP-135</td>
<td>Cleaner / Decon Bottle Filters (Small) (Pak of 5)</td>
<td>Every 2 Months</td>
</tr>
<tr>
<td>CP-140</td>
<td>In-line Sheath Filters (2 ea.)</td>
<td>Every 2 Months</td>
</tr>
<tr>
<td>CP-158</td>
<td>C6 Flow Cytometer Maintenance Kit (1 year supply of Peristaltic Tubing (CP-105), Bottle Filters (CP-130 and CP-135) and In-line Sheath Filters (CP-140)</td>
<td>Every 2 months</td>
</tr>
<tr>
<td>KR-200</td>
<td>Decontamination Concentrate Solution (10x)</td>
<td>As Needed</td>
</tr>
<tr>
<td>KR-210</td>
<td>Cleaning Concentrate Solution (10x)</td>
<td>As Needed</td>
</tr>
<tr>
<td>KR-220</td>
<td>Bacteriostatic Concentration Solution (For Sheath Fluid) (10 vials)</td>
<td>As Needed</td>
</tr>
</tbody>
</table>

Visit www.AccuriCytometers.com for a complete list of available parts and reagents.
The Accuri C6 is a fixed voltage system.
There are no voltage or amplifier gain settings to adjust. The detector settings for light scatter and fluorescence are optimized and balanced during assembly. The Zoom Tool can be used to magnify an area of interest. In Figure 2-1, the Zoom Tool has been used to focus on the lymphocyte light scatter (Figure 2-1a) and surface marker fluorescence (Figure 2-1b).

Fluorescence compensation can be set during or after data collection.
Data collection on the C6 is digital. This means that fluorescence compensation, used to adjust the spectral overlap from one fluorochrome to another, can be applied or adjusted, even after data has been collected and saved in a CFlow file.

Each data well in holds a maximum of 1 million events.
Data can be added to a well, even one already containing events, at any time, up to a total of 1 million events. When a data well already contains data, the well will be colored blue and the RUN button will read ADD TO.
Chapter 3 Using the Collect Tab

Introduction
The Collect tab appears when CFlow is launched and is the only tab which allows control of the C6. The Collect tab is used to set data collection thresholds and gates, stop criteria, and to control the fluidics, including sample collection using the RUN and PAUSE buttons. Collect also allows access to most of the analytic functions in CFlow including the creation of complex gates, setting fluorescence compensation, and generating statistics.

In this chapter, a four-color analysis of human peripheral blood will be used to illustrate many of the Collect tab functions, including:

- Acquiring data on the C6
- Creating plots (histogram, dot or density) in which to view data
- Setting stop criteria and thresholds
- Using regions and markers to create gates and obtain statistics
- Saving and printing plots and data

A CFlow file containing the data used in the following example, “HPB 4 Color Tutorial.c6”, is available from the Accuri website, www.AccuriCytometers.com, and can be used as a tutorial tool. For the example shown here, the C6 has already been started-up and instrument performance validated.

The steps outlined here follow a workflow process where the C6 user is setting and applying gates, opening plots, and applying fluorescence compensation values in real time as data is being collected.

An alternative approach is to collect all data using minimal C6 set-up, with only the appropriate Thresholds, Fluidics rate, and Run Limits set initially. The data for all samples is then collected in quick succession, and analysis performed at a later time, including setting of gates and applying fluorescence compensation.

Experimental Design of Tutorial File
Four tubes are used to assess the CD3⁺CD4⁺ and CD3⁺CD8⁺ cell populations in the example cell analysis. These samples were prepared by staining peripheral blood with directly conjugated antibodies, followed by red cell lysis, according to standard methods.

<table>
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<tr>
<th></th>
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<th>2</th>
<th>3</th>
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<td>Isotype</td>
<td>Isotype</td>
<td>Isotype</td>
<td>CD8</td>
</tr>
</tbody>
</table>

Table 3-1 Experimental design for analysis of CD3⁺CD4⁺ and CD3⁺CD8⁺ cell populations in human peripheral blood. Samples 1 and 2 are the background control and the white blood cell gating control, respectively. Sample 3 is the control required for determining the percent of CD4⁺ and CD8⁺ cells within the CD45⁺CD3⁺ subset.

This experimental design does not contain single-stained fluorescence controls. Based on previous experiments, it is known that the only channels where fluorescence bleedover introduces ambiguity for setting gates are FITC bleedover into PE, and PE-Cy7 bleedover into the FITC and PE channels. Therefore, these samples are sufficient for this tutorial.

Refer to Accuri's online Fluorescence Compensation Tutorial for a more complete discussion of
fluorescence bleedover issues in flow cytometry and the impact it has on experimental design.

**Opening a New CFlow Workspace and Collecting a Data File**

1. Double-click the CFlow software icon on the desktop or, if CFlow is already open, select **File, New CFlow File** from the CFlow file drop-down menu.

2. The Default template is shown in Figure 3-1. Note that only a density plot of linear FSC-A vs. linear SSC-A is displayed. The plot is already "zoomed" to show channels 0 to 1,600,000 on FSC-A and 0 to 800,000 on SSC-A. Click on the **Plot Spec Tool** (lower right hand corner of Plot) to change the axes channel range, or to view axes on a logarithmic scale (Figure 3-2).

3. The Traffic Light below the 96-well grid in the upper left hand corner should be green with the message **C6 and CFlow are connected and ready**.

4. When CFlow is opened, sample well A1 will automatically be selected, as indicated by a red border.

5. Name the current sample by typing into the text box above the 96-well grid. Naming samples is optional, and can be done at any time. If no text is added, the sample will be named according to the
well location, e.g. “Sample A1”.

6. **Set a Run Limit.** For the first sample, a good starting point is to collect 100,000 - 200,000 ungated, total events. The Run Limit can be changed once a gate has been set on the population of interest. Additionally, data acquisition can be stopped at any time or volume limit. Multiple run limits can be chosen; the C6 will stop on the first limit reached.

7. **Set the Fluidics Rate to Slow.** The maximum data rate that the system can accommodate is 10,000 events per second. To ensure the best data resolution, it is recommended that samples be acquired at a rate of 5,000 events per second or less, when possible. Once the data rate has been determined the setting can be adjusted to Medium or Fast if necessary.

8. The default value for thresholds is 80,000. If it is not already set at this value, set the **Primary Threshold** to channel 80,000 on FSC-H (Figure 3-3). Click on the **Set Threshold** button within the Fluidics control area, and a dialog box will open. Set the Primary Threshold on FSC-H to 80,000, and **Apply to All Samples**. This threshold should work well to gate out debris and noise from cell samples freshly isolated from human or mouse tissues.

A lower FSC-H threshold may be needed to effectively analyze small cells such as platelets or bacteria. A higher FSC-H threshold may be needed when working with large cells such as cell lines. The threshold settings can be raised or lowered at any time during analysis. If raising a threshold setting will result in data loss, CFlow will give a warning (Figure 3-4).

9. **Before collecting the first sample,** place an empty sample tube on the SIP and click the **Backflush** button (lower left side of CFlow template). This will clear any residue from the SIP.

10. Gently re-suspend the cells in the sample tube and place on the SIP.

11. Click the **RUN** button to start sample collection. The green Traffic Light will turn yellow and the message will read **Preparing to analyze sample**.

12. Once the fluidics initialization is complete, the Traffic Light will turn back to green and the message...
will read **C6 is collecting data**. Well A1 will flash blue during data collection.

13. After the run limit is reached, well A1 will stop flashing and remain blue, indicating that the well contains data. The **RUN** button will now read **ADD TO** (Figure 3-5). If desired, more data can be collected into well A1 by clicking on the **ADD TO** button. Even after collecting samples in other wells, any well containing data can be returned to, and more events collected, at a later time.

**Creating Plots and Applying Gates**

2. **Use the Zoom Tool to expand the view of the lymphocyte population.** Click on the **Zoom Tool** button (the symbol will turn blue) and use it to encompass the area on which to zoom (Figure 3-6).

3. Choose the **Polygonal** gating tool and draw a region, P1 (Figure 3-7). Left-click on the mouse to anchor each vertice; double-click to close the polygon. The percentage of cells within P1 for that plot appears on the screen automatically. There are two other options available for drawing gating regions, the **Rectilinear and Quadrant** gating tools. These tools will be used in other steps of this tutorial.
3. Open a new plot to display the data collected for FL1 and FL2. This data is currently stored in RAM. Select the Density plot icon from one of the empty plot corrals and a default FSC-A vs. SSC-A plot appears (Figure 3-8). There are two other plot options available, allowing the creation of a Histogram or a Dot plot.

4. Change the newly opened linear FSC-A vs. SSC-A plot to a logarithmic FL1-A vs. FL2-A plot. There are 2 ways to accomplish this:
   a. Click on the Axis label of the plot. A menu will appear, allowing the selection of the desired parameter to display. By default, fluorescence parameters are displayed logarithmically while light scatter parameters are displayed linearly (Figure 3-9 a).
   b. Click on the Plot Spec Tool, select the appropriate parameter from the X-axis and Y-axis menus, and change the radio button selection from linear to log display (Figure 3-9 b).
5. Apply a gate to the FL1-A vs. FL2-A plot. Click on the gray GATE button at the top of the plot to open the gating dialog box (Figure 3-10).

6. Select the gating option Include P1 on all events in the Gate dialog box and click Apply at the bottom of the box. The resulting gated Plot 2 is shown in Figure 3-11. Note that the text next to the gray Gate button will now read (P1 in all) instead of (No Gating).
The events within any gate or series of nested gates can be selected to be included or excluded from a plot. In addition, gating combinations that do not appear as options in the Gating dialog box can be created by selecting the intersection choice (Figure 3-12).

Only Polygon (P), Rectilinear (R), and Marker (M) gating regions that have been drawn will appear automatically in the Gating dialog box list of options. To use Vertical or Quadrant markers for gating, select the appropriate check box in the Gating dialog box and those choices will appear (Figure 3-13).

The initial display of any parameter on a logarithmic scale will be automatically zoomed to a scale that runs from channel 10 to $16.7 \times 10^6$. For the majority of analyses, there are very few events which will fall into channels 0 to 10. Pre-zooming of the plots will save time by reducing the number of zoom steps. However, care must be taken when setting Markers (M), Regions (R) or Polygons (P) that require inclusion of channels lower than 10 on a pre-zoomed plot. It is recommended that users unhide the first decade of data, especially when setting fluorescence compensation, as this will prevent events from being excluded from gating regions. Refer to Figure 3-14a and b, for directions.