Technical Note
A Guide to Absolute Counting on the Accuri C6 Flow Cytometer

The Accuri C6 Flow Cytometer® simplifies cell analysis by performing absolute cell counts, eliminating the need for counting beads, or dual-platform hemacytometer counts. The microprocessor-controlled peristaltic pump system allows the sample volume pulled per run to be determined. The volume (in µL) appears as data in the CFlow® Software statistics tables, and the counts per µL for any gated population is automatically calculated and can be selectively displayed in the Statistics Tab data view. These guidelines cover the factors to consider when performing absolute cell counting with the C6.

Preventative Maintenance

As with any lab instrument, optimal performance requires proper system maintenance. Accuri suggests the following preventative maintenance routine:

- To prevent clogging, run filtered deionized water (di H_2O) on fast for 3-5 minutes at the end of each experiment.
- Run decontamination and cleaning cycles each day of use. These cycles are run automatically during machine shut-down.
- Keep the C6 wetted by placing a tube of di H_2O on the SIP between uses. Note: this is done automatically with the CSampler® by placing the SIP in the wash station when idle or at system shut-down.
- Thoroughly clean the flow cell each month by performing an extended flow cell clean (KR-235).
- Replace peristaltic pump tubing (CP-105), in-line sheath filter (CP-138) and bottle filters (CP-132) every 2 months.

Performance Validation

Prior to running samples for absolute counting, validate system fluidics performance by running Accuri Volume Validation Beads (QA-120) according to product instructions.

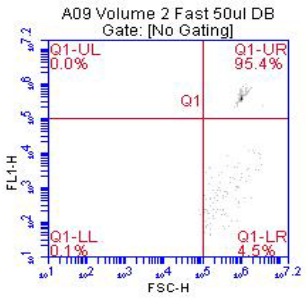

Figure 1. Validation of C6 performance using Accuri Volume Validation Beads. The fluorescent Volume Validation Beads are depicted in the upper right quadrant (Q1-UR).

Counting Capabilities

Concentration Range	1,000 – 5 x 10^6 cells/mL
Sample Medium	Cell growth media (+10% serum)
	PBS (+2-5% BSA or FBS)
	di H_2O (beads)
Sample Type	Cell lines
	Primary cells
	Beads
	Bacteria**
Sample Volume	75µL – 4mL (12x75mm tube)
	75µL - 900µL (1.5 mL microcentrifuge tube)
Fluidics Speed	Medium
	Fast
	Custom: see Flow Rate section

Table 1. Examples of C6 counting capabilities. **For special instructions when counting small particles including bacteria, refer to Analyzing Small Particles Technical Note.

Sample Concentration Range - The C6 will accurately measure volume in samples with concentrations ranging from 1000 - 5 x 10^6 cells/mL (Figure 2). Concentrations in excess of those recommended may result in inaccurate counting due to system saturation. The appropriate cell concentration range that can be analyzed will vary between cell types, based on considerations such as size, shape and tendency to clump. In addition, the flow rate used, relative to sample concentration, should minimize doublets and larger clumps, and must never allow the event rate to exceed 10,000 events per second. It is therefore recommended that users compare reported concentrations of serial dilutions of their sample to verify a linear correlation.

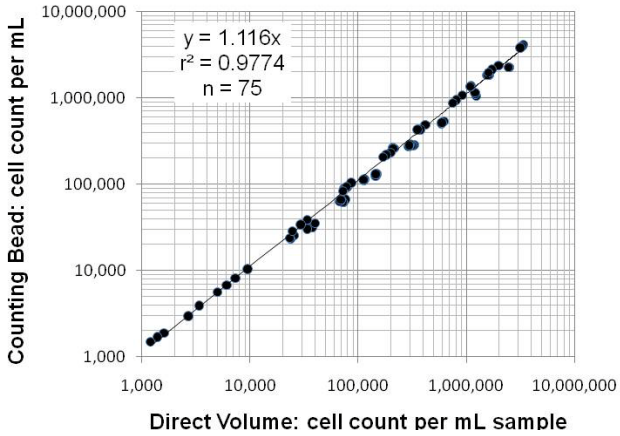

Figure 2. Comparison of a range of cell concentrations measured by C6 direct-volume and counting beads.

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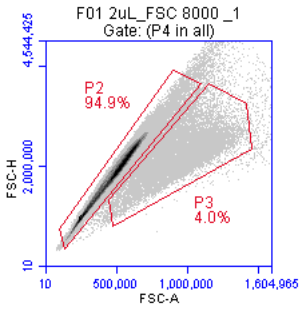
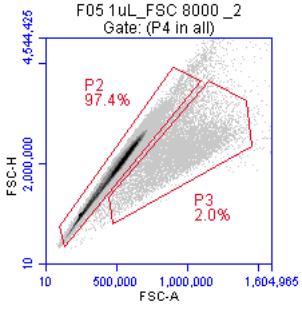
Assessing Cell Clumping in Samples - Minimize clumping, either by dilution or a combination of enzymatic and mechanical means. Cells must be evenly dispersed throughout the suspension, otherwise accurate counting, by any method, cannot be achieved.

Figure 3 illustrates a flow cytometric analysis method for assessing the amount of clumping in samples. Plots of FSC-A versus FSC-H are displayed. Single cells passing the laser beam will have FSC-A and FSC-H signals which correlate linearly and when plotted against each other will fall along a relatively straight line (P2, Figure 3a). Clumps of cells will have larger FSC-A signals relative to their FSC-H signals (P3, Figure 3a) and will fall off the diagonal formed by single cells. Figures 3b and 3c show the FSC-A versus FSC-H profile of the sample in Figure 3a after two 1:2 serial dilutions have been made. The amount of clumping, as reflected by the percentage of cells falling in gate P3, decreases in proportion to the increasing dilution of the sample.

a. Primary Sample



b. 1:2 Dilution



c. 1:4 Dilution

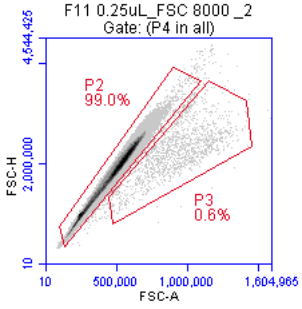


Figure 3. Discriminating cell clumps using a plot of FSC-A versus FSC-H. a) P2 is the single cell gate, where increasing FSC-A channel correlates linearly with increasing FSC-H signal. The P3 gate is cell clumps. b and c) Serial dilutions of the primary sample shown in 3a. The percentage of cell clumps (P3) decreases with increasing sample dilution.

In the example in Figure 3, if the accuracy of cell counts required for the experiment would be impacted by a loss of 4% due to clumping, it is recommended that a dilution of the original cell suspension be made.

Sample Medium - Sample viscosity will affect direct-volume measurements. Accuri recommends resuspending cell samples in phosphor-buffered saline (with or without BSA up to 5%, or fetal bovine serum (FBS) up to 2%). When running samples in viscous buffers such as blood lysis/fixative solutions, Accuri highly recommends verifying cell counts with an alternative method.

Sample Type - Accuri has validated the accuracy of absolute cell counts using a variety of cell types including primary cells (human peripheral lymphocytes, human platelets, and mouse splenocytes), cell lines (Jurkat, Chinese Hamster Ovary (CHO) and 3T3 mouse fibroblasts), and bacteria (see Analyzing Small Particles Technical Note). Care should be taken to maintain mono-dispersements of cells, particularly with cell types prone to clumping. Careful consideration of cell type-specific characteristics, such as size, shape and tendency to clump should be taken into account when determining the appropriate operational concentration range (see Sample Concentration).

Minimum Sample Volume - The minimum sample volume required for accurate counts is dependent on sample fluid height in relation to the SIP position (which will vary with different tube types). Because accurate counting with the C6 is calculated based on a pressure-differential between the SIP and sample liquid, a certain amount of residual volume is required. In addition, there is approximately 25µL of “dead volume” that is pulled into the flow cell (but not analyzed) during fluidics stabilization. Taking these factors into account, accurate counts can be obtained on the C6 with sample volumes as low as 75µL (assuming a run limit of 10µL) in 12x75mm or 1.5mL microcentrifuge tubes. Assuming complete homogeneity of sample, accurate counts can be obtained by analyzing as little as 5µL of sample. Samples with low concentrations may require larger sample volumes to achieve statistical significance for accurate cell counts. Accuri recommends at least 500 cells in the population of interest be counted per run, and that replicate runs be averaged to obtain the most accurate counts.

Flow Rate - The Accuri C6 has 3 standard fluidics settings with pre-optimized flow rate/core size combinations: slow, medium and fast. Due to the nature of the method used to calculate direct-volume measurements, SLOW SPEED SHOULD NOT BE USED TO DETERMINE ABSOLUTE COUNTS. In addition to the standard fluidics settings, CFlow allows the user to set custom flow rates and core sizes. When working with custom settings, the minimum values for flow rate and core size that will give accurate volume measurements are 15µL/min and 16µm respectively.

Tube Types - The C6 is a non-pressurized system; therefore the user is not limited to specific sample tubes. Accurate counting has been validated with 12x75mm tubes and 1.5mL microcentrifuge tubes. Accuracy of counts obtained using other types of tubes should be verified by the user.

Counting With CSampler

When performing absolute counting using CSampler, additional considerations include:

Sample Preparation - When using CSampler, temperature sensitivity should be taken into account, as small sample volumes such as those used in 96-well plates will stabilize to room temperature relatively quickly (within 15 minutes) during the course of a plate run (minimum 45 minutes). For this reason, it is recommended cells be fixed, if possible.

Agitation - The most important CSampler-specific consideration when performing absolute count analysis is maintaining homogeneous suspension of samples. The agitate function is designed to keep cells in suspension through physical agitation of the plate or tube rack. The frequency and number of agitate cycles (1-3 cycles, 15 seconds each) can be selected by the user when setting up an auto-run. Fixed blood cells including lymphocytes and granulocytes will remain in suspension with a single agitate cycle per well/tube over the course of a 96-well plate/24-tube rack run.

Tube/Plate Type - When performing absolute counts from a 96-well plate, Accuri strongly recommends using round/U-bottom plates. Agitate is not effective on V-bottom, flat-bottom and deep-well 96-well plates. CSampler also supports absolute counting using 48-well plates, or 12x75mm tubes in the provided tube rack.

Sample Volume - The following table provides the range of sample volumes recommended for specific plate/tube types in order to obtain accurate counts, without risking sample spill-over during agitation.

Tube/plate type	Recommended Sample Volume
12x75mm tubes	150µL – 4mL
Round or U-bottom 96-well plates	100µL - 250µL**
48-well plates	200µL – 1.3mL

** not to exceed 50% of well capacity

Table 2. Recommended sample volume range for various tube/plate types compatible with CSampler.

Troubleshooting

Validate system performance using volume validation beads. In the case that the volume validation beads are producing counts within 10% of expected, but cell counts are suspected to be inaccurate, possible explanations and recommended course of action include:

Cause	Course of Action
Cell concentration exceeds 5 x 10^6 cells/mL	Prepare serial dilutions, verify linear correlation. Dilute sample.
Sample buffer viscosity too high	Wash and resuspend cells in PBS, or determine absolute counts by alternative method.

In the event bead counts deviate more than 10% of expected with volume validation beads, the following steps are recommended:

Cause	Course of Action
System maintenance required	Replace pump tubing, bottle filters and in-line sheath filter and sheath fluid.
Clogged SIP	Perform backflush, and verify fluid exits SIP. If necessary, remove and rinse the SIP.
Clogged/dirty flow cell	Perform Unclog, and verify fluid exits SIP. If necessary, run a Full System C6 Fluidic Cleaning Routine.
Air bubbles	Perform C6 Wetting Procedure.

If the above recommendations do not resolve absolute count inaccuracies, contact Accuri Cytometers Technical Support:

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Full System C6 Fluidic Cleaning Routine

1. Place a tube containing 3mL of Accuri Decontamination Solution (KR-200 or KR-205) on the SIP.
2. Disconnect the fluidic line from the Sheath Bottle (blue line) and attach it to the Decontamination Bottle (yellow ring bottle).
3. In CFlow Plus or CFlow Sampler, set the fluidics to Custom and set the Flow Rate to 100 µL/min. (If CFlow is being used, set the fluidics to Fast). Set the Run Limit to stop at 5 minutes and press Run.
4. Remove the tube of Decontamination Fluid from the SIP and replace it with a tube containing 3mL of Accuri Cleaning Solution (KR-225).
5. Disconnect the blue fluidic line from the Decontamination Bottle and attach it to the Cleaning Fluid Bottle (green ring bottle).
6. Set Run Limit to stop at 5 minutes and press run.
7. Remove the tube of Cleaning Solution from the SIP and replace it with a tube containing 3mL of 0.2µm filtered di H_2O.
8. Attach Sheath (blue), Decontamination (yellow), and Cleaning Fluid (green) fluidic lines to their respective bottles.
9. Set Run Limit to stop at 5 minutes and press run.

C6 Wetting Procedure

Use this procedure when a C6 is failing to produce accurate volumetric counting results using the Accuri Volumetric Validation Beads (QA-120) and after steps are taken to ensure the C6 is clean.

1. Perform a normal auto-fluidics Shutdown.
2. Restart the C6, allowing normal fluidics Start Up.
3. Place a 12x75mm tube containing 750µL of 70% Reagent Grade Ethanol on the SIP.
4. Run 400µL on FAST.
5. Remove the tube and wipe off the SIP.
6. Place a 12x75mm tube containing 1500µL of 0.2µm filtered di H2O on the SIP.
7. Run 400µL on FAST.
8. Remove the tube and wipe off the SIP.

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