A Guide to Absolute Counting Using the BD Accuri™ C6 Flow Cytometer

Technical Bulletin

Contents

Introduction

The BD Accuri C6 personal flow cytometer can simplify cell analysis by counting absolute cell numbers directly (per unit sample volume). Such counts are essential in many research applications, including enumerating leucocytes, B cells, T cells, and platelets in human blood, measuring microorganism concentrations in purified water, and determining the viability of cultured cell lines.

The microprocessor-controlled peristaltic pump system accurately monitors the sample volume pulled per run. The direct counts correlate highly (r^2=0.999) with, and are as precise as, counts performed with counting beads, and are more precise than counts obtained by hemocytometer.

BD Accuri C6 software displays the volume (in µL) as data in the statistics tables, and automatically calculates counts per µL for any gated population. Users can display the counts in a data view on the BD Accuri software Statistics tab.

These guidelines contain recommendations, tips, and techniques to help maximize the accuracy of absolute cell counts using the BD Accuri C6.

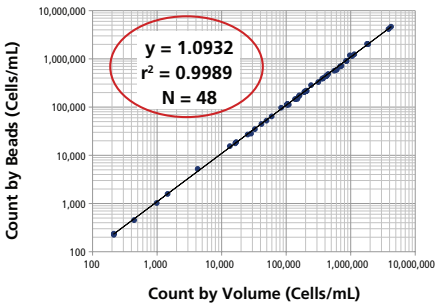


Figure 1. Comparison of absolute cell counts measured by direct volume vs counting beads.

Serial dilutions of Jurkat, 3T3, and U937 cells, and T cells, B cells, and platelet samples from four human peripheral blood donors, were counted on the BD Accuri C6 by two methods. X-axis values represent absolute cell counts measured against volume sampled directly, while y-axis values are relative to the number of counting beads detected.

Preventive maintenance

As with any laboratory instrument, optimal performance requires proper system maintenance. The following table shows the suggested preventive maintenance routine. For detailed procedures, see the BD Accuri *C6 Flow Cytometer Instrument Manual.*

Table 1. Recommended preventive maintenance procedures.

Frequency	Task	Supplies and parts
After every experiment	Run 0.22 µm-filtered deionized (DI) water at the Fast rate for 3 to 5 minutes to inhibit clogging.	0.22 µm-filtered DI water
Daily (if the cytometer is not shut down)	Run the decontamination and cleaning cycles.	0.22 µm-filtered DI water, Cat. Nos. 653154, 653155, and 653157
Between uses	Place a tube of 0.22 µm-filtered DI water on the Sample Introduction Probe (SIP), or if using the BD CSampler™ option, place the SIP in the wash station.	0.22 µm-filtered DI water
Monthly	Clean the flow cell by performing an extended flow cell clean.	Cat. No. 653159
Bi-monthly	Replace the peristaltic pump tubing, in-line sheath filter, and bottle filters.	Cat. Nos. 653146, 653148, and 653147

Performance validation

Prior to running experimental samples, validate accurate counting by using a reference count bead in the experimental buffer, using the same sample volume and tube as in the experiment. If bead counts are within 20% of the expected value (based on information provided by the bead manufacturer), proceed with sample collection. If bead counts are not within 20% of expected values, proceed with following fluidics calibration procedure.

Fluidics calibration

Perform the following in order:

1. Ensure that the fluid levels in the Sheath, Cleaning, and Decontamination bottles are sufficient to cover the inlet tubing and that there are no “kinked” fluidic lines.
2. Run a decontamination cycle from the Instrument Menu or by shutting down and restarting the cytometer.
3. Within 5 minutes of completing the decontamination cycle, place a 12 x 75-mm tube containing 750 µL of 70% ethanol on the SIP. Acquire 400 µL using the Fast fluidics setting.
4. Within 5 minutes of completing the ethanol run, place a 12 x 75-mm tube containing 1,500 µL of 0.22 µm-filtered DI water on the SIP. Acquire 400 µL using the Fast fluidics setting.

5. Within 5 minutes of completing the water run, place a calibration sample on the SIP. Select Instrument > Calibrate Fluidics.

    - Calibration should be performed in the same tube type as the experimental sample.
    - Calibration should be performed using a sample of the same or similar viscosity as the samples to be analyzed. For example, if lysed human peripheral blood samples are to be acquired, lysed human peripheral blood should be used during calibration.
    - The calibration procedure consumes approximately 220 µL. To account for this, the volume in the calibration sample tube should be 110 µL more than the average volume used with subsequent test samples. For example, if using 1,000-µL samples, perform calibration with 1,110 µL in the tube. The values determined by the BD Accuri C6 are based on the average sample height in the tube during the calibration.
    - If sample volumes >50 µL are to be acquired from the sample tube, the calibration volume should take this into account and the average volume in the sample tube during the acquisition should be used. For example, if 100 µL is to be acquired from a 1,000-µL sample, the average volume would be 950 µL.

Average volume = (Starting Volume + Ending Volume)/2
= (1,000 + 900)/2
= 950 µL

6. The cytometer performs a calibration cycle lasting approximately 13 minutes, during which the status traffic light is yellow. Once completed, the traffic light reverts to green with the status message that the BD Accuri C6 is connected and ready.
7. If the status message indicates calibration was successful, repeat the performance validation. If the status message indicates that calibration failed, perform the following troubleshooting. The cytometer will operate normally. However volume measurements for the samples might be incorrect, since the cytometer reverts to the factory-set default fluidics calibration settings.

    - Make sure that the calibration tube did not run dry during calibration.
    - Repeat fluidics calibration from step 1 with a new calibration sample.
    - If calibration fails a second time, replace the peristaltic pump tubing in the cytometer and repeat the calibration routine beginning with step 1.

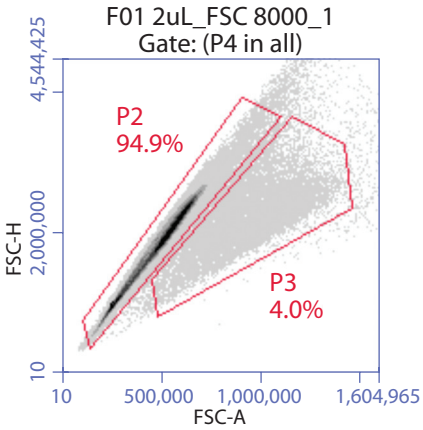
If calibration fails a third time, contact BD Accuri Technical Support.

Sample Preparation

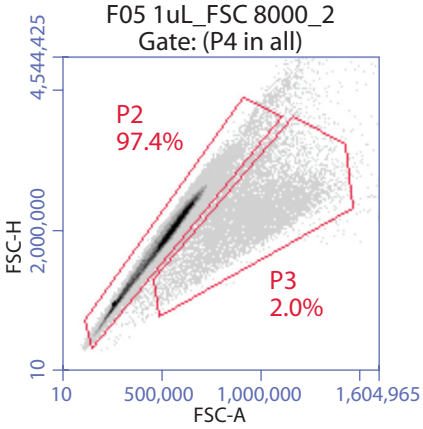
Sample concentration

Volume measurements on the BD Accuri C6 are most accurate with cell concentrations between 1,000 and 5 x 10^6 cells/mL (see Figure 1). Higher concentrations might result in inaccurate counting due to system saturation, so dilute samples if necessary.

A. Primary Sample



B. 1:2 Dilution



C. 1:4 Dilution

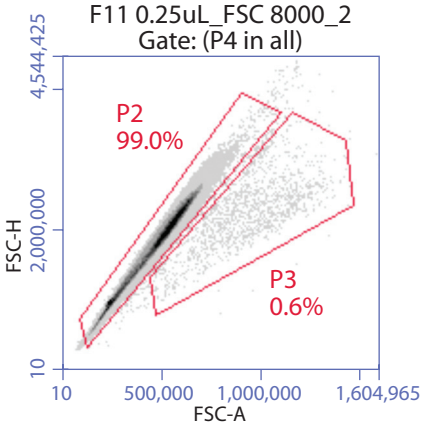


Figure 2. Discriminating cell clumps by plotting FSC-A against FSC-H.

The P2 gate contains single cells, indicated by a linear correlation between FSC-A and FSC-H signals. The P3 gate contains cell clumps, indicated by an increase in FSC-A compared to FSC-H. Results: ***A.*** *At original concentration, 4% of the primary sample is clumped.* ***B, C.*** *The percentage of cell clumps (P3) decreases as the sample is increasingly diluted.*

The ideal cell concentration range for accurate counting varies among cell types, based on considerations such as size, shape, and tendency to clump. The flow rate, relative to sample concentration, should minimize doublets and larger clumps, yet never exceed 10,000 events per second. Verify reported concentration of serial dilutions of the sample result in a linear correlation. We recommend that you test serial dilutions of the sample and compare reported concentrations to verify a linear correlation.

Cell suspension

Assess and minimize cell clumping, either by dilution or a combination of enzymatic and mechanical means. Cells particularly prone to clumping may need to be filtered prior to running on a flow cytometer. Cells must be evenly dispersed throughout the suspension.

Figure 2 shows how to assess cell clumping using flow cytometry by plotting FSC-A against FSC-H. When single cells in the flow cell pass through the laser beam, their FSC-A and FSC-H signals correlate linearly and plot along a relatively straight line (P2). Clumps of cells will have larger FSC-A signals relative to FSC-H, and the signals will fall off the diagonal formed by single cells (P3). Figures 2B and 2C show the FSC profiles of the primary sample in Figure 2A after one and two 1:2 serial dilutions, respectively. Clumping, shown by the percentage of cells in gate P3, decreases as the sample is increasingly diluted.

In the example in Figure 2, if a loss of 4% of cells due to clumping would affect the experimental accuracy of cell counts, the original cell suspension should be diluted.

Sample medium

Sample viscosity can affect direct-volume measurements. When running samples in viscous buffers such as blood lysis/fixative solutions, validate volume accuracy using reference beads in the sample buffer, and calibrate the fluidics, if necessary.

Sample type

BD Biosciences 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.

Take care to maintain single-cell dispersion, particularly with cell types prone to clumping. Consider cell-type characteristics, such as size, shape, and tendency to clump, when determining the appropriate operational concentration range. See the Sample concentration and Cell suspension sections for more details.

Sample volume

The minimum sample volume required for accurate counts depends on sample fluid height in relation to the SIP position, which varies by tube type. Because accurate counting is calculated based on a pressure differential between the SIP and the sample liquid, some residual volume is required. In addition, approximately 25 µL of “dead volume” is pulled into the flow cell (but not analyzed) during fluidics stabilization.

Taking these factors into account, you can obtain accurate counts on the BD Accuri C6 with sample volumes as low as 300 µL (assuming a volume run limit of 10 µL) in BD Falcon™ 12 x 75-mm tubes. The maximum recommended sample volume in a 12 x 75-mm tube is 2 mL. However, no more than 750 µL on Medium fluidics setting (or 1.5 mL on Fast) should be acquired during a single run.

Acquire only once from any sample tube. Sample height within the tube is critical. We recommend aliquoting sample into separate tubes to obtain and average triplicate measurements for greatest accuracy.

Samples with low concentrations or more clumping may require larger volumes to achieve statistical significance for accurate cell counts. Count at least 500 cells of interest per run. To obtain the highest accuracy, average counts from replicate runs.

Fluidics speed

The BD Accuri C6 offers three standard fluidics settings—Slow, Medium, and Fast—with pre-optimized flow rate and core size combinations. Due to the method used to measure direct volume, *do not use the Slow speed to determine absolute counts*.

In addition to the standard fluidics settings, BD Accuri C6 software allows you to set custom flow rates and core sizes. When working with custom settings, the minimum flow rate and core size values for accurate volume measurements are 15 µL/min and 16 µm, respectively. Volume accuracy using custom flow rate and core size combinations should be verified by the user.

Tube types

Since the BD Accuri C6 is a non-pressurized system, you are not limited to specific sample tubes. BD Biosciences has validated accurate counting with BD Falcon 12 x 75-mm tubes. For other types of tubes, we recommend verifying accuracy of counts using an independent counting method.

Counting with the BD CSampler™ option (BD CSampler)

Note the additional considerations for counting cells when sampling is automated using the optional BD CSampler.

Agitation

One key to obtaining accurate counts when using the BD CSampler is to maintain a homogeneous suspension of samples. The agitate function is designed to help keep cells in suspension by physically agitating the plate or tube rack. Since agitation occurs only between aspirations of each well in a plate, it does not interrupt sample collection.

You can specify the frequency and number (1 to 3 cycles, 15 seconds each) of agitation cycles per well or tube when setting up automated runs. The appropriate agitation frequency is dependent on several experimental factors including cell type, sample medium, and sample volume, and should be determined by the user. Start with one agitation cycle per well or tube and adjust if needed.

Tube and plate type

When using the BD CSampler for absolute counting, use round- or U-bottom 96-well plates, or 12 x 75-mm tubes in the tube rack provided. Agitation is not effective on samples in V-bottom, flat-bottom, or deep-well 96-well plates.

Sample volume

Table 2 shows the range of sample volumes recommended for specific plate and tube types to obtain accurate counts using the BD CSampler. Counting accuracy using alternative plate types should be verified by the user. Plate sample volumes exceeding 50% well capacity might result in sample spillover during agitation.

Table 2. Recommended sample volume ranges for tube and plate types compatible with the BD CSampler.

Tube / plate type	Recommended sample volume
12 x 75-mm tubes	300 µL–2 mL
96-well round- or U-bottom 96-well plates	40–50% well capacity*

*typically 150 µL–200 µL

Summary of recommendations

Table 3. Summary of recommendations for absolute counting on the BD Accuri C6.

Area	Recommendations
Preventive maintenance	Follow recommended preventive maintenance routines.
Sample concentration	1,000–5 x 10^6 cells/mL
Cell suspension	Assess and minimize cell clumping.
Sample medium	Calibrate fluidics when necessary to account for liquid viscosity.
Sample type	Cell lines Primary cells Beads Bacteria*
Sample volume	12 x 75-mm tube: 300 µL–2 mL Users should verify other tube/plate types, calibrate fluidics when necessary.
Fluidics speed	Standard settings: Medium or Fast only Custom settings: Minimum settings are listed below. Appropriate flow rate and core size combinations are experiment specific and should be validated by the user. - Flow rate: ≥15 µL/min - Core size: ≥16 µm
Using the BD CSampler	Use the agitate function if necessary to maintain a homogeneous suspension. Avoid V-bottom, flat-bottom, and deep-well plates. Sample volume: - 96-well round- or U-bottom plates: 40–50% well capacity (150 µL–200 µL) - 12 x 75-mm tubes: 300 µL–2 mL
Troubleshooting	See the Troubleshooting section.

*For special considerations when counting bacteria and other small particles, see the BD Accuri Technical Bulletin *Threshold and Analysis of Small Particles on the BD Accuri C6 Flow Cytometer.*

Troubleshooting

If you suspect problems with absolute counting on the BD Accuri C6, we recommend validating counting accuracy using an alternative method such as adding reference counting beads to the sample. If the cell concentrations differ more than 20% between the two methods, use Table 4 to learn possible explanations and courses of action. It is often helpful to use a time histogram plot to observe consistency of event counts. Spikes or dips in event counts may be indicative of a blockage in the SIP or flow cell.

Table 4. Troubleshooting recommendations for absolute counting with the BD Accuri C6.

Symptom	Cause	Course of action
Reference bead counts too high or low	System not calibrated to sample buffer viscosity	Calibrate fluidics using sample buffer.
	System maintenance required	Replace the pump tubing, bottle filters, in-line sheath filter, and sheath fluid.
	Air bubbles	Perform the BD Accuri C6 Wetting Procedure.
Event counts sporadic	Clogged SIP	Perform the backflush procedure and verify that fluid exits the SIP. If necessary, remove and rinse the SIP.
Event counts sporadic	Clogged or dirty flow cell	Perform the unclog procedure and verify that fluid exits the SIP. If necessary, run a Full System BD Accuri C6 Fluidic Cleaning Routine.

Full System BD Accuri C6 Fluidic Cleaning Routine

1. Place a tube containing 3 mL of BD Accuri Decontamination Solution, working concentration (Cat. No. 653145 or Cat. No. 653155) 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 BD Accuri software, either select the Custom fluidics option and set the Flow Rate to 100 µL/min, or select the Fast option. Set the Run Limit to stop at 5 minutes and click RUN.
4. Remove the tube of Decontamination Fluid from the SIP and replace it with a tube containing 3 mL of BD Accuri Cleaning Solution, working concentration (Cat. No. 653157).
5. Disconnect the blue fluidic line from the Decontamination Bottle and attach it to the Cleaning Fluid Bottle (green ring bottle).
6. Set the Run Limit to stop at 5 minutes and click RUN.
7. Remove the tube of Cleaning Solution from the SIP and replace it with a tube containing 3 mL of 0.22 µm-filtered DI water.
8. Attach the Sheath (blue), Decontamination (yellow), and Cleaning Fluid (green) fluidic lines to their respective bottles.
9. Set the Run Limit to stop at 5 minutes and click RUN.

BD Accuri C6 Wetting Procedure

1. Perform a normal automated fluidics shutdown.
2. Restart the BD Accuri C6, allowing the fluidics to start normally.
3. Place a 12 x 75-mm tube containing 750 µL of 70% reagent grade ethanol on the SIP.
4. Run 400 µL on Fast.
5. Remove the tube and wipe off the SIP.
6. Place a 12 x 75-mm tube containing 1,500 µL of 0.22 µm-filtered DI water on the SIP.
7. Run 400 µL on Fast.
8. Remove the tube and wipe off the SIP.

Reference

1. Rogers C, Dinkelmann M, Bair N, Rich C, Howes G, Eckert B. Comparison of three methods for the assessment of cell phenotype, viability, and concentration in cultures and peripheral blood. American Society for Cell Biology poster, 2009.

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