Threshold and Analysis of Small Particles on the BD Accuri® C6 Flow Cytometer

Technical Bulletin

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When analyzing small particles, defined as particles smaller than 3.0 µm, on the BD Accuri® C6 flow cytometer, certain items must be considered to ensure proper data acquisition. This note discusses some of the basic concepts needed to perform small particle acquisition and analysis properly.

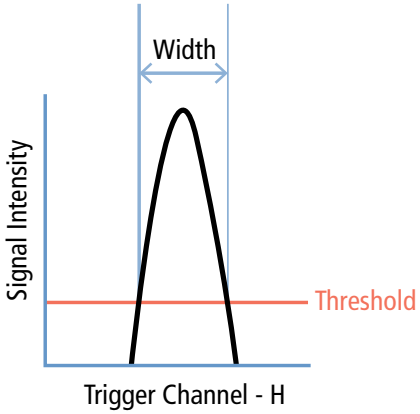

Figure 1. Schematic of a typical signal pulse. To be recorded, the pulse (black line) must have an intensity greater than the threshold value (red line). On the BD Accuri C6, the signal width is defined as the distance between the point at which the signal rises above the threshold and the point at which the signal falls below the threshold (time of flight).

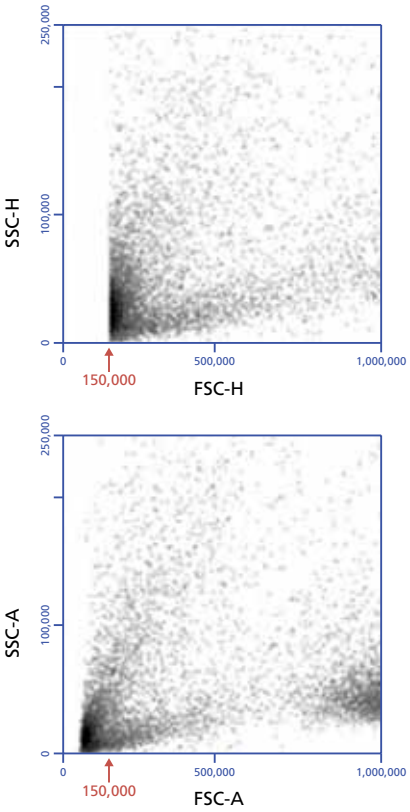

Figure 2. A sample of lysed and fixed human peripheral blood was acquired using an FSC trigger and threshold of 150,000. In the FSC-H vs SSC-H plot, the data begins at 150,000. However, when viewing the Area parameters for the same sample (FSC-A vs SSC-A), the threshold appears to be lower than 150,000.

Thresholds

A threshold is the lowest signal intensity value an event can have for it to be recorded by the cytometer. The trigger channel is the critical parameter used to determine if an event should be recorded. When using the BD Accuri C6, setting the primary threshold also defines the trigger channel. The signal pulse must exceed the level set on the trigger channel to be recorded as an event on any other channel.

On the BD Accuri C6, the default setting of a primary threshold on FSC-H of 80,000 indicates that the system will record an event only if it has an FSC value of 80,000, regardless of any other parameter values. In this example, FSC-H is, therefore, also the trigger channel. Using this setting, the 8-Peak (Cat. No. 653144) and 6-Peak (Cat. No. 653145) Validation Beads can easily be visualized on an FSC vs SSC plot. Typically, thresholds are used to exclude unwanted signals such as those from cellular debris. All thresholds on the BD Accuri C6 are applied using the Height signal measurements. There is no impact on the data if the Area parameter is viewed during data acquisition or analysis. However, this may lead to a discrepancy in which the threshold falls when viewing Area data, as seen in Figure 2. The difference between where the data is thresholded in Area vs Height will vary depending on the particles being analyzed.

The reason for this difference is that the area data is displayed after area scaling factors have been applied. The BD Accuri C6 uses fixed area scaling factors that do not normalize the area data values exactly to the height data values for all sample types.

Setting the Threshold

When choosing the appropriate threshold value, debris and undesired events should be eliminated without inadvertently eliminating relevant events. If standard or familiar samples are being acquired, the threshold can be set based on experience. However, if this is the first time a particular experiment is performed, it might not be clear which events are the correct ones, so they must be identified. Once the particles have been identified, the threshold should be set low enough to ensure the entire population is captured. One way to do this would be to set the threshold to its lowest value (10). However, setting the threshold very low on any parameter will result in the electronic noise for that channel becoming visible and interfering with acquisition and analysis.

The Noise Floor

On all flow cytometers, every channel has an inherent noise floor. Noise is composed of low-level signals that occur within the electronics and of stray light collected by the optics. This noise appears as events that are collected even when there are no, or very few, “true” events in the sample. For example, as seen in Figure 3, a sample from a tube of 0.2-µm filtered deionized water was acquired at the standard settings, and only a small number of events was detected. These are most likely “true” particulate matter. As the threshold is lowered, more and more events are detected until those events dominate the plot. With the threshold set to 80,000, acquiring sample for 1 minute resulted in only 80 events. However, the same sample re-acquired with a threshold of 1,000 resulted in >200,000 events.

The ideal placement of the threshold is somewhere between the particles of interest and the noise floor. Since the range of samples that can be analyzed on a BD Accuri C6 varies greatly, it is difficult to assign a threshold value that would be appropriate for all samples. Therefore, trial and error are necessary to identify

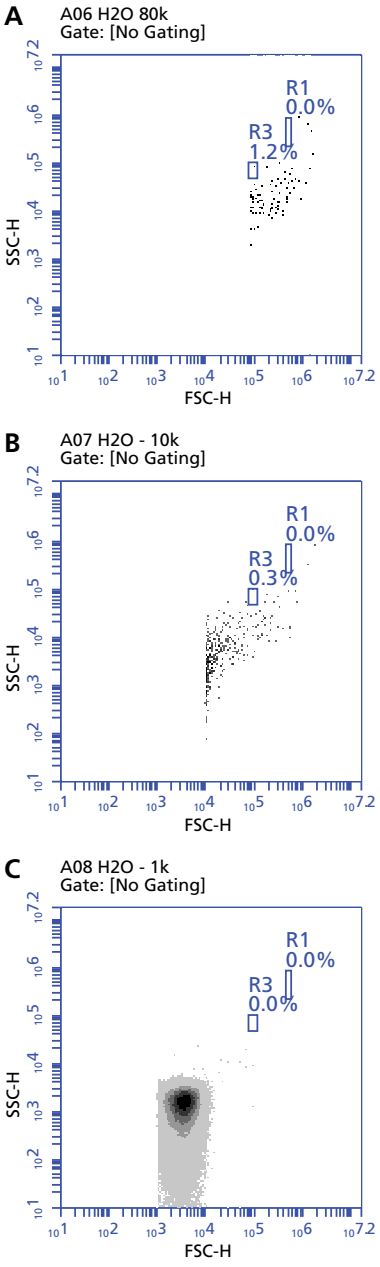

Figure 3. The appearance of the FSC noise floor as the FSC threshold is lowered from: A) 80,000; B) to 10,000; C) to 1,000. Region R1 and region R3 indicate where the Spherotech 8-Peak Validation Beads and a 1-µm bead would fall, respectively. The population in the black region is the noise floor.

the proper threshold value for any given experiment. This is illustrated in Figure 4. Using the default threshold of 80,000, the BD Accuri C6 should be able to visualize a 1-µm bead using an FSC-H vs SSC-H plot. However, since the bead falls very close to the threshold value, this threshold might not be appropriate. Nominally 1-µm beads, or beads of any size, are not created equal and can fall in slightly different locations based on their actual size. Some might be closer to 0.9 µm, while others are 1.1 µm. Some bead samples labeled 1 µm might actually contain a range of particle sizes, for example 0.95 µm to 1.05 µm. Based on the proximity to the bead, a threshold of 80,000 might not be appropriate, since some of the data could be lost.

If the threshold is lowered to 10,000, it is a sufficient distance from the beads to prevent accidental loss of any particles of interest. This can be verified by checking the number of beads in region R3. With the threshold set to 10,000, there are ~30,700 events in region R3. At the threshold of 80,000, there are ~31,300 in the region, indicating no loss of bead data at the higher threshold. However, at the lower threshold value, a second population begins to appear near the threshold. Based on the noise floor criteria, this new population is the noise floor. In the example with the lower threshold, >84% of the data is the 1-µm beads, compared to >99% at the higher threshold. To minimize the effects of the noise floor, a threshold somewhere between 10,000 and 80,000 should be chosen in this case. For example, if a threshold of 40,000 is selected, most of the noise is eliminated, and the majority of the events acquired are 1-µm beads, as indicated by the percentage of total events increasing back up to almost 99% (~31,000 events in region R3).

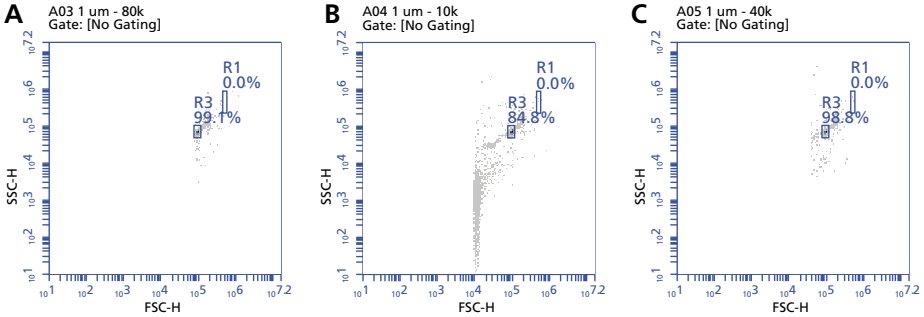

Figure 4. The effect of different FSC thresholds when analyzing a 1-µm bead. A) FSC-H threshold of 80,000; B) FSC-H threshold of 10,000; C) FSC-H threshold of 40,000. Region R3 encompasses the 1-µm bead. Region R1 indicates the position of the Spherotech 8-Peak Validation Beads. The black box indicates the position of the noise floor.

As a general guide, if there is a large difference between the particles of interest and the noise floor, set the threshold no more than 1 log decade lower than the particles of interest. However, if this is not possible, minor overlaps of the noise floor will not impact results as long as the number of "true" events outweighs the number of noise events.

When determining the appropriate threshold, the threshold setting can be changed while acquiring data. This is useful when determining the proper setup. However, the sample acquisition should always be restarted if the threshold value was changed during a previous acquisition. This ensures proper application of the desired threshold value.

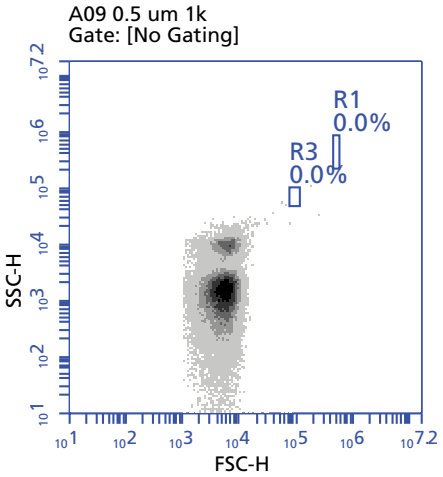

Figure 5. Acquisition of a 0.5-µm particle using an FSC trigger and FSC-H primary threshold of 1,000. Two populations, the 0.5-µm bead and noise, are evident.

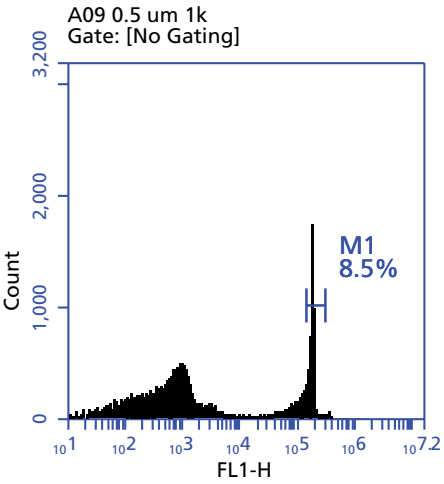

Figure 6. Identification of the fluorescent signal of the 0.5-µm particle using the FL1 parameter. Region M1 is the fluorescent bead.

Identifying Particles of Interest

Before the threshold can be set properly, it might be necessary to determine which events are really the particles of interest in the samples. With larger particles or cells, this is relatively straightforward using the guidelines previously described. However, with smaller particles, especially those under 1.0 µm, this can be problematic. For example, Figure 5 shows a 0.5-µm particle acquired on a BD Accuri C6 using an FSC trigger and threshold of 1,000. There are clearly two populations, and yet only one type of particle was run.

There are two ways to make the identification:

1. Use another characteristic of the particle, such as fluorescence, to positively identify the particles in the FSC vs SSC plot, or
2. Compare against the instrument noise floor.

Of these two options, the first is preferred. This allows for distinguishing a single particle type from other particle types in addition to distinguishing from the noise.

Identification of Fluorescent Particles

In the 0.5-µm beads example, a fluorescence signature from the bead can be used, since this particular 0.5-µm bead is labeled with a fluorochrome that can be detected using the 533/30 filter in the FL1 channel. The FL1 data acquired previously using an FSC trigger and threshold of 1,000 indicates a bright peak just above 100,000, identified with marker M1, and some other dim or nonfluorescent events (Figure 6).

If the M1 region is used as a gate and the FSC vs SSC plot is viewed with the M1 gate applied (backgating), a single population is evident (Figure 7). A region has been drawn around this population so that when the M1 gate is removed from the FSC vs SSC plot, the fluorescent 0.5-µm beads population is clear.

Using this technique, various particle types or populations could be distinguished from each other as long as each one has a unique fluorescent marker.

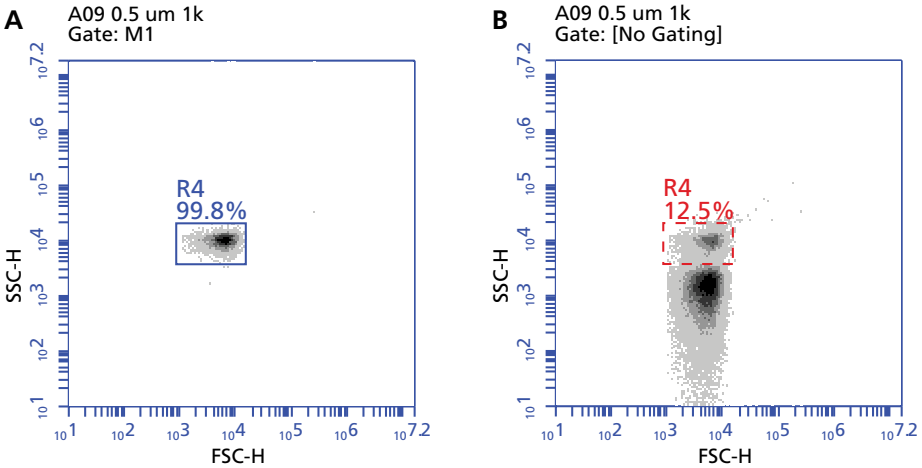

Figure 7. Identification of the 0.5-µm bead from the noise by backgating on the fluorescence of the 0.5-µm bead seen in FL1. A) Identification of the fluorescent beads after using a fluorescent backgate. B) Removal of the gate on the FSC vs SSC plot to distinguish the bead population from the other population (noise).

Identification of Nonfluorescent Particles

If a nonfluorescent 0.5-µm particle was used, then a similar process could be performed using the noise floor as the reference point. A sample of water could be run at the same threshold settings as the beads sample. In this case, noise would be the only population present. A region could be drawn around the noise to identify and use it as a comparison to the bead sample.

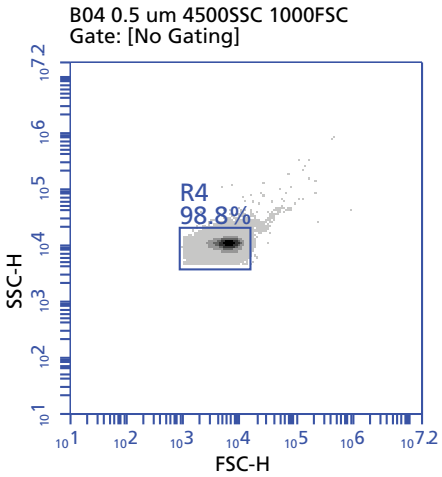

Figure 8. Addition of a secondary SSC threshold to the primary FSC threshold to remove noise.

Using Multiple Thresholds

To ensure proper analysis and to prevent exceeding the maximum data acquisition rate of 10,000 events per second, the majority of the noise should be thresholded out during sample acquisition. In the 0.5-µm bead example, a single FSC threshold would be insufficient. Other options need to be considered for running this sample. To determine the options, review the data for obvious differences in the populations that can be exploited. In Figure 5, the most obvious difference in this example is that the noise has significantly lower SSC than the 0.5-µm bead. The addition of a secondary threshold on SSC could be applied. Adding a secondary threshold imposes an additional minimum value in the data before it is recorded. It does not directly impact the trigger channel and primary threshold. Secondary thresholds can be applied with any primary threshold/trigger channel.

By applying a secondary SSC threshold of 4,000 to the data, the majority of the noise can be removed while retaining the entire bead population.

The sample can therefore be effectively acquired using the FSC trigger, FSC threshold of 1,000, and secondary SSC threshold of 4,000 setup. Similar to primary thresholds, secondary threshold values can be changed at any time. However, the parameter cannot be changed during data acquisition. Secondary thresholds can also be added after the data acquisition. This would be similar to gating out the unwanted data as opposed to never acquiring it in the first place. Increasing the threshold permanently removes events from the data set.

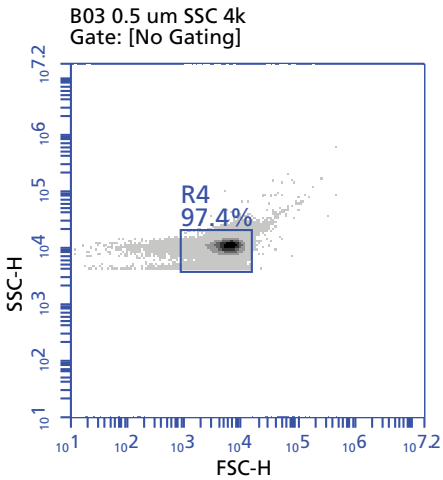

Figure 9. FSC vs SSC plot of a 0.5-µm bead using an SSC trigger and SSC-H primary threshold of 4,000. Region R4 designates the position of the bead using the previous fluorescence backgating (Figures 6 and 7).

Alternative Trigger Channels (Side Scatter and Fluorescence)

The addition of a secondary threshold is only one way to remove noise or debris. Since the noise and 0.5-µm bead can clearly be distinguished in SSC, an SSC trigger could be used to acquire the data. In Figure 9, the same sample was acquired using an SSC primary threshold and trigger of 4,000.

This results in a single population and only a small amount of noise. The noise is minimal as evidenced by >97% of the results falling in the original M1 fluorescent backgate in the FSC vs SSC plot. The noise and scatter patterns will be different with the use of different trigger channels, but if the thresholds are set properly, the data should look fairly similar. In many cases, when acquiring small particles, the SSC trigger is more useful than an FSC trigger, as seen in the 0.5-µm bead example.

In the preceding examples, the particles can easily be separated from the noise in either FSC (1.0 µm) or SSC (0.5 µm). Other samples might not be so cooperative. The particles of interest might significantly overlap the noise, complicating acquisition and analysis. In cases such as this, the final option for detecting the particles is to take advantage of fluorescence. As demonstrated earlier, the 0.5-µm particle produces a single bright peak in the FL1 channel. If all the desired particles are the same, ie, have a useful fluorescence signature as is the case here, a fluorescence trigger could be used to acquire the data. A fluorescence trigger would only acquire events that have a fluorescence level above the threshold set. Similar to an FSC threshold, which only records events above

a certain approximate size, a fluorescence trigger only records events brighter than the set threshold.

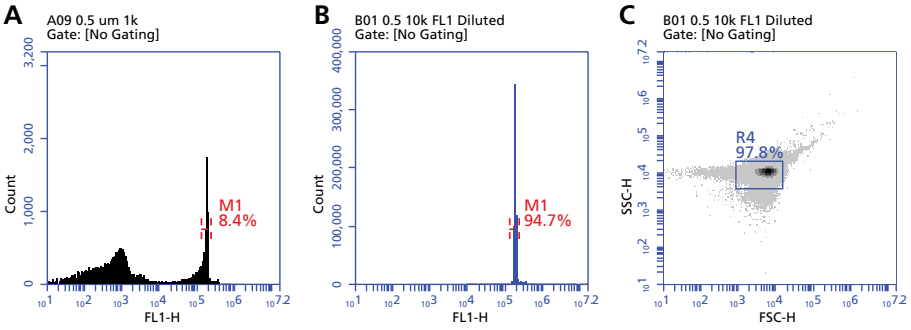

Figure 10. Use of a fluorescence (FL1) trigger to acquire the 0.5-µm bead sample. A) Bead sample acquired using an FSC trigger to identify if and where the fluorescence signature of the bead is in the FL1 parameter. B) Acquisition of the bead sample using an FL1 trigger and primary threshold value of 25,000. C) FSC/SSC results of the bead acquired using an FL1 trigger setting.

If the FSC vs SSC plot is viewed based only on the fluorescent events being acquired (Figure 10C), the beads fall in exactly the same place as before using either the FSC or SSC triggers (the R4 region has not been moved). Using the fluorescence trigger produces data that has much less associated noise.

Data Acquisition Rate

As stated previously, small amounts of overlapping noise will have minimal impact on data acquisition and results. However, large amounts of noise can interfere with proper operation of the cytometer.

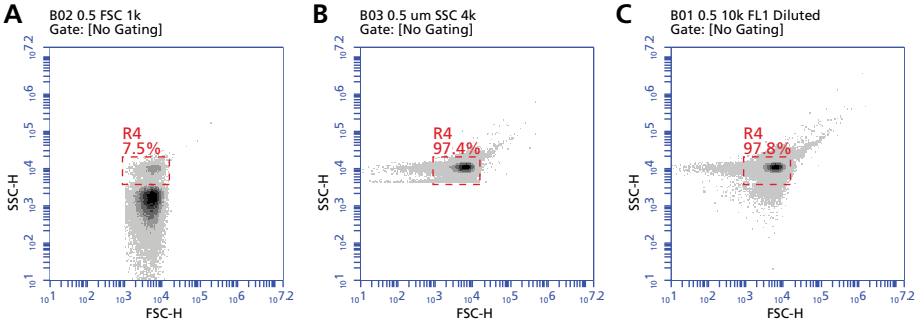

Table 1.

Dot Plots	Trigger	Threshold	Total Events	Events in R4	Events/Second
A	FSC	1,000	53,826	4,052	3,585
B	SSC	4,000	150,477	146,567	10,025
C	FL1	10,000	154,695	151,356	10,309

Figure 11 and Table 1. Impact of large amounts of noise on the event rate. The same sample of 0.5-µm beads was run using similar acquisition parameters (flow rate, time of acquisition) with different threshold settings: A) FSC threshold of 1,000; B) SSC threshold of 4,000; C) FL1 threshold of 25,000. Table 1 shows the differences in event rates resulting from the different threshold settings and resulting impact of noise on the data.

Figure 11 demonstrates the impact of noise on the data. An acquisition with large amounts of noise, similar to when using the low FSC trigger and threshold, can result in faulty data acquisition rates (events per second). This is due to exceeding the maximum acquisition rate, 10,000 events per second. When this occurs, data is lost with no warning and no obvious indicators. In fact most flow cytometers will experience the same issue, without warning or indicators. For example, the FSC threshold run resulted in 4,052 events in region R4 with a total of ~54,000 events. This acquisition was reported at 3,585 events per second, well under the maximum rate of 10,000 events per second. However, the same sample acquired with an FL1 fluorescence trigger resulted in 151,356 events in region R4 out of a total of 154,695 events. This acquisition was reported at 10,309 events per second. Therefore, running the same sample for the same duration of time at the same flow rate yielded different results. When the system is operated above the maximum acquisition rate, events are lost randomly. So, both noise and events of interest are lost.

A similar result can occur if the sample is highly concentrated and the rate exceeds 10,000 events per second, even when using appropriate thresholds that eliminate the noise.

If exact event count or events/µL is needed and/or the sample cannot be cleanly separated from the noise floor, a dilution of the sample should be performed. If BD CFlow® software indicates a similar decrease in events as the dilution, the BD Accuri C6 is acquiring normally and the sample concentration and setup are usable. If the event rate does not decrease with the dilution, the data acquisition rate is more than 10,000 events per second and outside the normal operating range of the system. If this is the case, an effort should be made to remove noise by altering the trigger channel and thresholds and/or diluting the sample. The flow rate setting could also be decreased. However, volume measurements taken on the BD Accuri C6 while acquiring sample at a Slow flow rate are not guaranteed to have <10% error. If absolute volume measurements are not required, decreasing to Slow might be sufficient to lower the event rate to less than 10,000 events per second. Otherwise, dilution of the sample is necessary to obtain an accurate volume measurement.

Conclusion

The BD Accuri C6 personal flow cytometer can analyze platelets, microbes, and many other types of particles smaller than 3.0 µm. Successful acquisition and analysis of small particles depend on accurately differentiating them from debris and noise.

This Technical Bulletin provides guidelines for setting triggers and thresholds, identifying particles of interest, and managing data acquisition rates when acquiring small particles on the BD Accuri C6. It includes data examples that use beads of known sizes to represent various kinds of small particles.

Summary of Recommendations

General Guideline	Specific Recommendations
Adjust the primary threshold for FSC-H to eliminate noise while recording all the particles of interest.	By default, the trigger channel is FSC-H and the primary threshold is 80,000.
	If necessary, adjust the FSC threshold to fall between the particles of interest (ideally no more than one log decade lower) and the noise floor.
	You may try out different threshold settings while acquiring data. However, always restart data acquisition once the final threshold has been set.
If necessary, identify particles of interest.	If possible, identify and backgate particles of interest using fluorescence.
	Otherwise, use a sample of water to identify the noise floor.
If FSC cannot discriminate between particles of interest and noise …	Either add a secondary threshold on SSC or fluorescence, or …
	Use SSC or fluorescence as an alternative trigger channel.
Make sure the data acquisition rate does not exceed 10,000 events per second.	Either adjust the trigger channel and thresholds to remove noise, or …
	Dilute the sample, or …
	Decrease the flow rate setting (unless absolute volume measurements are required).

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