Using the Separation Quotient Parameter on the AQUIOS CL Flow Cytometer

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INTRODUCTION

Separation Quotient (SQ) is a mathematically calculated parameter that is specific to the AQUIOS CL flow cytometer. It uses population separation as an indicator of relative sensitivity and resolution. SQ is tracked and is a good representation of the sample and run quality.

The detailed definition of *Separation Quotient* can be found in the AQUIOS Tetra System Guide but can be understood as the difference between the 2 SD points divided by the difference of the mean channel numbers. For readability, the number is multiplied by 10. The values are then expressed on a scale of <0 to 10. The larger the separation quotient, the better the separation.

Simply put, *Separation Quotient* is used as an index that indicates how well two different populations are separated. SQ is used as a form of quality control.

The *Separation Quotient* index is measured continuously for both samples and QC controls to determine whether the Cytometer and the AQUIOS Tetra-1 Panel, and/or the AQUIOS Tetra-2+ Panel reagents are performing optimally for each and every patient sample.

On the AQUIOS CL flow cytometer, the separation quotient is a function of instrument laser alignment and preparation quality. The separation quotient is used as a form of quality control.

CURRENT METHODS FOR INSTRUMENT ALIGNMENT (OPTICAL OPTIMIZATION)

Flow cytometers started out as instruments that were custom built and marginally stable. Eventually, these instruments were manufactured by companies that sold equipment such that one could acquire two instruments that were built the same, ensuring standardization instrument-to-instrument. However, the instruments were still very sensitive, and keeping the instrument in full alignment (optically optimized) was still a challenge. Ultimately, the challenges associated with keeping the instrument optically optimized were minimized with newer technologies. Despite these improvements, keeping an instrument optically optimized remains a challenge that the flow cytometry industry faces.

With these challenges in mind, procedures for running tests on flow cytometers focused on first verifying that the flow cytometer is optically optimized. In traditional flow cytometry, microspheres are typically used to determine optical optimization. These microspheres usually have a uniform fluorescence value. The results obtained from running the fluorospheres show the optical optimization of the flow cytometer by checking the stability of the flow stream and its relationship to the laser beam. These fluorospheres are used as an indirect measurement that correlates to the actual operation of a flow cytometer.

In traditional flow cytometry, the fluorosphere run is then followed by a process control to ensure that the reagents used are performing properly. This determination requires review by a laboratory professional. A knowledgeable user makes the determination based on the process control assay values and experience in interpreting data presentation.

The AQUIOS CL flow cytometer does not require fluorosphere runs to determine optical optimization. Instead, the AQUIOS CL flow cytometer improves upon the inefficiency of having to perform these runs by monitoring various characteristics of the run, one of which is Separation Quotient. This eliminates the need for fluorosphere reagents by analyzing population separation using a software calculation based on light scatter, and/or fluorescence. It also eliminates the need for a subjective determination of optical alignment since Separation Quotient provides objective criteria to confirm that the instrument is optimized for that application. Refer to the *Daily Quality Control on the AQUIOS CL System for the Tetra Application* Product Bulletin for a detailed look at the QC process on the AQUIOS CL flow cytometer.

USING SEPARATION QUOTIENT EFFECTIVELY

As optics optimization in a flow cytometer decreases, two things are affected: Resolution and Sensitivity.

1. Resolution

Resolution is the ability to measure two particles with the same quantity of fluorescence and assign them the same value resulting in the capability to distinguish two populations of similar intensity.

Typically, fluorospheres with a known coefficient of variation (CV) are used to measure resolution. Both Forward Scatter (FS) and fluorescence CV are measured for each fluorosphere to determine resolution. An increase in CV indicates increased variance in the values assigned to each cell population's distinct quantity of fluorescence. This increased variance can be visually detected by the spread of each peak. The greater the spread, the lower the resolution.

The AQUIOS CL flow cytometer monitors resolution daily by tracking the Separation Quotient values of the AQUIOS IMMUNO-TROL controls. The AQUIOS CL flow cytometer also uses the SQ index to monitor the resolution for every patient sample.

2. Sensitivity

Sensitivity is the degree to which the cytometer can detect and differentiate between dim fluorescence and a slightly brighter fluorescence. Sensitivity is typically used to compare abilities of different cytometers to resolve dimly stained populations. [1,2]

In flow cytometry terms, sensitivity is a measure of the ability of the system to resolve dim fluorescent signal from background noise and to resolve dim populations. It is possible to measure the exact sensitivity of a flow cytometer by using a mixture of pre-calibrated particles with known equivalent soluble fluorescence units; however, sensitivity is not typically monitored daily.

The AQUIOS CL flow cytometer, monitors sensitivity daily by tracking the Separation Quotient values of the AQUIOS IMMUNO TROL controls. The AQUIOS CL flow cytometer also uses the SQ index to monitor the sensitivity for every patient sample run.

Even though newer flow cytometers give robust performance, linearity and sensitivity can vary between instruments and their manufacturers. However, with the AQUIOS CL flow cytometry system, QC checks associated with instrument performance and validation essentially follow the same principles that are behind the fluorosphere-based QC checks performed by general purpose flow cytometers, but in a simple, fast, automatic, and efficient manner. The AQUIOS CL flow cytometer, in conjunction with quality control reagents, verifies light scatter, electronic volume (EV)[3], fluorescence intensities, and color compensation settings for defined assays. The AQUIOS CL flow cytometer automatically verifies the optical, fluidic, and electronic stability of the instrument.

REAGENT IMPACT ON RESOLUTION AND SENSITIVITY

AQUIOS IMMUNO-TROL Cells and AQUIOS IMMUNO-TROL Low Cells

AQUIOS IMMUNO-TROL Cells and AQUIOS IMMUNO-TROL Low Cells are quality control materials containing stabilized human erythrocytes and leukocytes with a known quantity of surface antigens and are used to monitor system performance for all directly measured and calculated parameters.

The AQUIOS CL flow cytometer uses SQ as a measure of both relative sensitivity and resolution. SQ looks at the difference between the two inner SD points and divides it by the difference between the population means to obtain the SQ value. As the means get closer, sensitivity decreases. Similarly, as the 2 SD points get closer, resolution decreases. When the AQUIOS IMMUNO-TROL Cells and AQUIOS IMMUNO-TROL Low Cells are run, it checks both relative resolution and sensitivity. If the SQ values are out of range, QC fails.

On the AQUIOS CL system, SQ is checked during QC to ensure the system is functioning properly. This initial QC remains critical to check that the results are within the pre-defined limits. The AQUIOS CL system, however, takes it one step farther. SQ is also checked during sample runs as a means to ensure system performance throughout the sample run. The system automatically produces a potential sample notification or gating issue notification for any results that are outside of the SQ range to notify the user of potential system issues with the sample or the run.

AQUIOS Tetra Tests

SQ has been assayed specifically for the fluorescence and scatter of the AQUIOS Tetra application. Every time a patient sample is run, the SQ values of the sample run are compared to acceptable values for the application. SQ provides a quantitative measurement that is used to notify the user when there are issues with the data.

Tests, such as AQUIOS Tetra depend on the ability of a monoclonal antibody to bind to the surface of cells expressing discrete antigenic determinants. Specific cell staining is accomplished by incubating whole blood with the monoclonal antibody reagent.

HOW IT APPLIES TO THE AQUIOS CL FLOW CYTOMETER

Typically, fluorospheres act as a stand-in to predict how well the application will do. Fluorospheres are used as a generic control to test for multiple applications.

SQ is different because it looks at the application itself and provides a direct measurement for instrument performance for the specific application from QC to sample run. The AQUIOS CL system has SQ limits for each population of interest. These limits are defined for separation between the relevant populations for each application. The algorithm calculates the Separation Quotient values, compares them to the pre-defined limits for the specific population, and determines the pass/fail criteria from the tolerances provided for that application.

Separation quotient uses the separation of populations as a means of quantifying the relative resolution and sensitivity of a flow cytometer during QC and during sample runs. It is a statistic that can be used to measure the relative resolution and sensitivity of an instrument for a particular parameter on a particular test and quantify it in terms of sufficiency for running the test.

Separation Quotient provides the ability to define the minimum resolution and sensitivity needs for the cytometer/reagent system using a control to determine that the cytometer/reagent system is performing adequately to run the test on any patient using the statistic defined above.

DEFINING THE STATISTIC

Separation Quotient (SQ) is a mathematically calculated parameter that is specific to the AQUIOS CL flow cytometer and is used as an indicator of relative sensitivity and resolution. SQ is tracked and checks the quality of the sample run.

The Instrument Screen Levey-Jennings charts keep track of the changes over time in the Separation Quotient for AQUIOS IMMUNO-TROL Cells and AQUIOS IMMUNO-TROL Low Cells. See Figure 1 and Figure 2 for an example of Levey Jennings charts of the Separation Quotient for control cells using AQUIOS Tetra-1 and Tetra-2+.

Figure 1 - Levey Jennings - Tetra-1

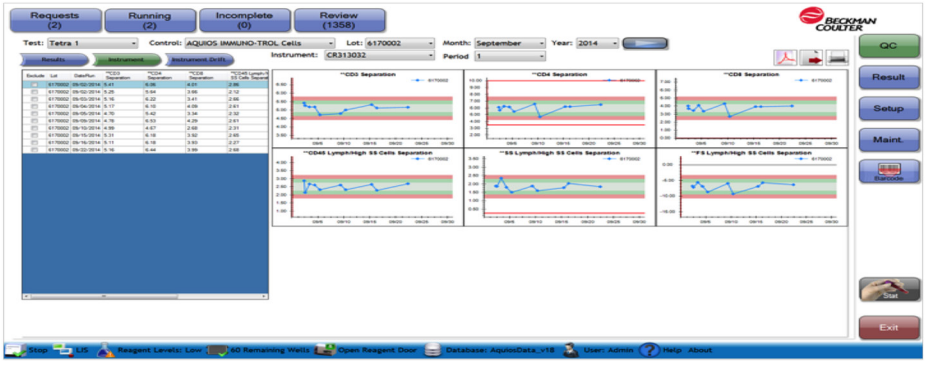


Figure 2 – Levey Jennings – Tetra-2+

The definition of the Separation Quotient statistic is as follows:

Separation Quotient (SQ) is an indicator of relative sensitivity and resolution and can be used as an index that indicates how well separated two different populations are, such as positive versus negative cell populations in fluorescence parameters, sample versus debris, or lymphocytes versus monocytes. The larger the separation quotient, the better the separation.

The separation between two populations for a given parameter is defined as:

1. The difference between the 2 SD points divided by the difference between the mean channel numbers. All calculations being done using the log of the channel numbers to take the variation between channel number and standard deviation (SD) out of the function.

Separation = 10 * 2Std Dev diff/Mean diff

2. The above function returns a number ≤1.
3. For readability, the number is multiplied by 10.

The end result is a numeric means of qualifying performance for a test.

Table 1 Separation Quotient Statistics

Numeric Range	Implication for Results
3 to 10	Excellent separation such that >99% of the data points in the populations are separated from each other
0 to 3	Good separation >2 SD or approximately 95% of the data points in the populations are separated from each other.
0	Point at which populations of 2 SDs touch; approximately 5% of the data points in the populations overlap.
-3-0	Moderate separation at which >5% of the data points in the populations overlap.
<-3	Poor separation which worsens as the numbers decrease.

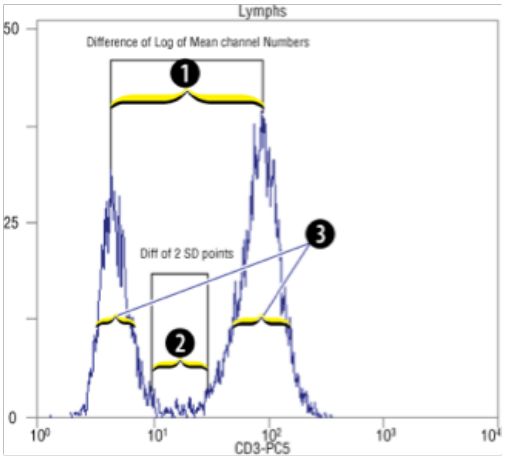
Separation Quotient is used on the AQUIOS CL flow cytometer:

- When running controls, SQ is used along with other measures to decide if QC passes or fails.
- When running samples, SQ is used along with other measures to notify the user of a potential issue with the sample or run.
- SQ is tracked in the Levey-Jennings Plots as an on-going QC check of the system.

EXAMPLES OF SEPARATION QUOTIENT (SQ)

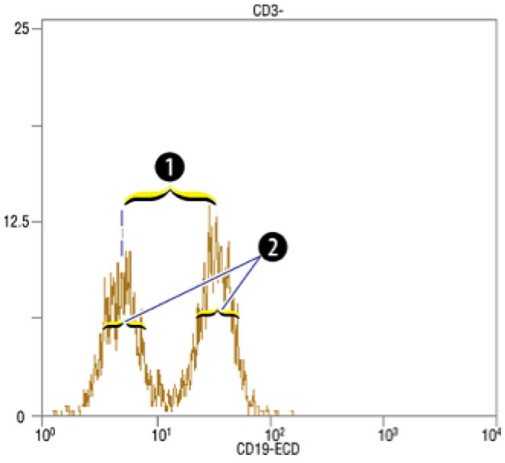
Below are some examples of excellent, good, moderate, and poor separation. Notice in Figure 3 that there is a difference of 2 SD between populations allowing for excellent separation. Compare this to Figure 6 where there is a very small distinction between the two populations of interest. This small distinction between populations is considered to be poor separation.

Figure 3 - Separation Quotient = 4.7 (excellent separation)



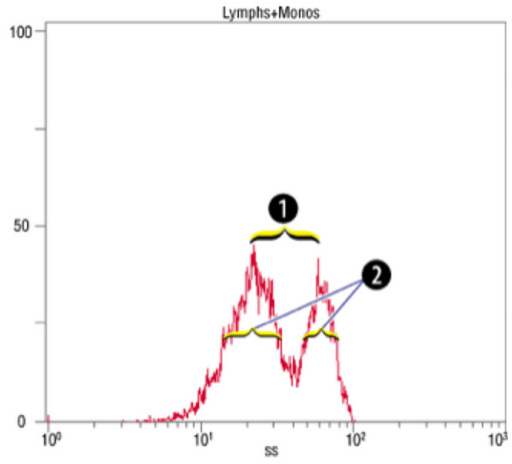
Note: Figure 3 is representative of excellent separation because it displays both good sensitivity and good resolution. The logarithmic difference of the mean channel numbers (1) is a difference greater than two standard deviation points (2), indicating good sensitivity. In addition, the two cell populations have little variance in the values assigned to each cell population's distinct quantity of fluorescence (3), indicating good resolution.

Figure 4 - Separation Quotient = 2.3 (good separation)



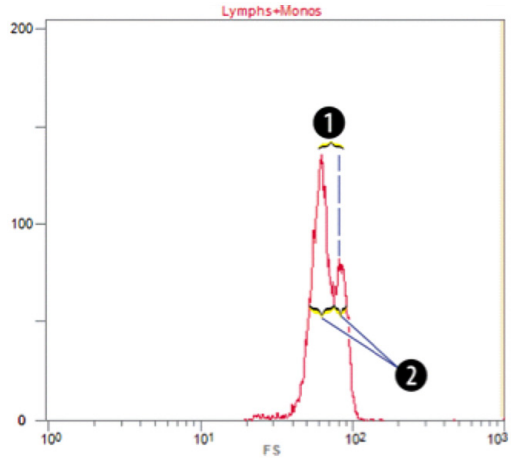
Note: Figure 4 is representative of good separation. It displays good sensitivity, but moderate resolution. The logarithmic difference of the mean channel numbers (1) is greater than two standard deviation points, indicating good sensitivity. However, the two cell populations have more variance in the values assigned to each cell population's distinct quantity of fluorescence (2), indicating poor resolution.

Figure 5 - Separation Quotient = -0.79 (moderate separation)



Note: Figure 5 is representative of moderate separation. It displays moderate sensitivity and poor resolution. The logarithmic difference of the mean channel numbers (1) is less than two standard deviation points, indicating moderate sensitivity. In addition, the two cell populations have moderate variance in the values assigned to each cell population's distinct quantity of fluorescence (2), indicating moderate resolution.

Figure 6 - Separation Quotient = -4.7 (poor separation)



Note: Figure 6 is representative of poor separation. It displays good resolution, but poor sensitivity. The logarithmic difference of the mean channel numbers (1) is less than two standard deviation points, making it difficult to distinguish between the two cell populations, indicating poor sensitivity. However, the two cell populations have little variance in the values assigned to each cell population's distinct quantity of fluorescence (2), indicating good resolution.

CONCLUSIONS

Separation Quotient (SQ) is a mathematically calculated parameter that is specific to the AQUIOS CL flow cytometer. The AQUIOS CL flow cytometer uses population separation as an indicator of relative sensitivity and resolution. SQ is tracked and is a good quantitative representation of the quality of the sample run specific to the application.

On the AQUIOS CL flow cytometer, the separation quotient is a function of instrument laser alignment and preparation quality. The Separation Quotient is used as a form of quality control.

Separation Quotient helps to make the AQUIOS CL flow cytometer cost effective by eliminating the need for fluorospheres. In addition, SQ makes the AQUIOS CL flow cytometer easy-to-use with SQ quantitative values that facilitate decision making by laboratory professionals. SQ revolutionizes the idea of quality system performance because unlike fluorospheres in traditional flow cytometers, it looks beyond just the QC run and continues to monitor system performance during sample runs.

REFERENCES

1. Hoffman RA, Wood JCS. (2007). Current protocols in cytometry. New York: John Wiley & Sons, Inc. Section 1.8.
2. Shapiro HM. (2003) Practical flow cytometry fourth edition. New York: Wiley-Liss.
3. Coulter, Wallace H., (1953). Means for counting particles suspended in a fluid, Patent number: 2656508

GLOSSARY

CV (Coefficient of Variation): An expression, in percent, of the data spread as related to the mean.
CV% = (SD/Mean) x 100

EV (electronic volume): A relative measurement which uses the Coulter Principle of impedance to measure relative cell volume.

Flow cytometry: A technology for measuring the characteristics of cells or other biological particles as they pass through a measuring apparatus in a fluid stream.

FS (forward scatter): The laser light scattered at narrow angles to the axis of the laser beam. The amount of forward scatter is proportional to the size of the cell that scattered the laser light.

Index: A property or ratio expressed by a number or formula.

Levey-Jennings: Quality Control graph that plots results for controls (y-axis) vs. time (x-axis) in order to illustrate whether the instrument is working well. Distance from the mean is measured in standard deviations.

Monoclonal antibodies: Antibodies produced by a single cell or its identical progeny, specific for a given antigen.

QC (quality control): A comprehensive set of procedures a laboratory sets up to ensure that an instrument is working accurately and precisely.

Resolution: The ability to measure two particles with the same quantity of fluorescence and assign them the same value resulting in the capability to distinguish two populations of similar intensity.

SD (standard deviation): A measure of deviation from the mean.

Sensitivity: The degree to which the cytometer can detect and differentiate between dim fluorescence and a slightly brighter fluorescence.

SQ (Separation Quotient): The difference between the 2 SD points divided by the difference of the mean channel numbers.

PATENTS AND TRADEMARKS

Class I Laser product

FLOW-737TCH12.14-B