AQUIOS Tetra Application Gating Strategy

BECKMAN COULTER Life Sciences

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CHALLENGES TO LYMPHOCYTE GATING

The AQUIOS CL flow cytometer is intended for use as a Single Platform Technology (SPT) for lymphocyte subset analysis and enumeration. SPT is a process in which absolute counts and percentages of lymphocyte subsets are measured from a single tube by a single instrument.(1,2) Recent advances made in SPT enable the AQUIOS CL flow cytometer to address challenges created by lymphocyte gating and data analysis. Many clinical laboratories are turning to SPT for their high daily volume T, B, and NK cell analysis needs since it enables consistent, quick, and simple analysis for routine samples.

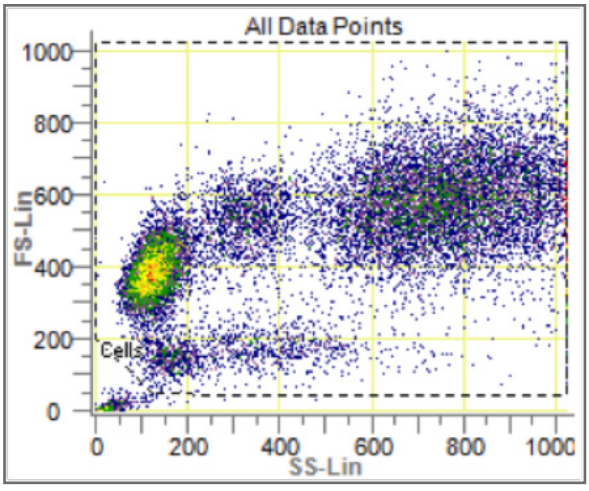
To analyze, quantitate, and measure the different lymphocytes, the flow cytometer uses a data analysis method that can identify different kinds of cells. This process is called “gating”. A flow cytometry data analysis gate is a region drawn around a cell population of interest and is used to "isolate" those cells from a mixture of cells.

Simply put, gates are essentially electronic filters. Gating is a process in which different types of electronic filters with different specificities are applied to flow cytometry data. Thus, a gating process makes it possible to analyze cell subsets of interest from a mixture of cells. This computational process eliminates the need to physically sort cells for further analysis. The operator chooses the specificity of a gate to apply to a mixture of cells, based on the characteristics of the cell morphology, fluorescence of the cell surface, fluorescence of the intracellular markers, or a combination of these traits.

However, biological variations, sample preparation methods, selection of the fluorescent antibody combination, and instrument variability are some of the many factors that pose a challenge to accurately gating lymphocytes.(3, 4) In addition, purity and recovery are important in the calculation of percentages and absolute counts of subsets. Thus, to get absolute counts and calculate the percentage of different cells in lymphocyte subset analysis, the instrument needs to be accurate in establishing the lymphocyte gate.

Several parameters are used to establish gates. The two most common gating parameters are both light scatter parameters Side Scatter (SS) and Forward Scatter (FS). SS is a relative measure of the granularity of the cell. FS is a relative measure of the size of the cell. These two light scatter parameters are frequently used to create gates since they are only dependent on the morphology of the cells and not dependent on any fluorescent staining. Gates using FS and SS are typically created from a single parameter histogram (FS) or from a dual parameter dot plot (FS vs. SS). See Figure 1 for an example of a dual parameter dot plot (FS vs. SS).

Figure 1 - FS vs. SS Dot Plot



The gating parameters are used, either manually or through software methods using complex algorithms, in diagnostic laboratories. In traditional flow cytometry, lymphocyte subset analysis can either be done through automatic gating algorithms or through manual gating by the operator using acquisition software to implement their observations. However, manual gating is subjective, time consuming, and prone to the introduction of errors since a gating scheme must address accuracy, recovery, and purity of the analyzed subset of cells while the operator must assess several parameters on the display screen. In the AQUIOS CL flow cytometer, the automatic gating algorithms aid the operators to meet these challenges while generating accurate and consistent test results.

In addition to manual gating, a variety of factors can affect gating. Some of these factors may do so before the sample is loaded on the flow cytometer. Gating can be influenced by the selection of monoclonal antibody reagents, fluorochrome selection, reagent quality control, compensation, and instrument quality control as well as the parameters selected for analysis and the post data processing gating strategy.

Beckman Coulter recognizes these issues and, in order to enhance and simplify analysis of heterogeneous cell populations, these instruments have been standardized and calibrated, reagents have been optimized, and reagent quality control provides proof that the instrument is properly setup for producing accurate results. The AQUIOS CL flow cytometer makes gating decisions simpler by minimizing human error through a combination of defined monoclonal reagent antibody panels with standardized analysis protocols and intelligent gating algorithms. The AQUIOS CL flow cytometer data and algorithms, therefore, contribute to producing accurate results for the immunologic assessment of patients having or suspected of having immune deficiency.

AQUIOS TETRA TESTS

The AQUIOS CL flow cytometer is designed to run pre defined and well-characterized tests. Beckman Coulter's AQUIOS Tetra system tests for simultaneous identification and enumeration of T, B, and NK lymphocytes in whole blood are proven for reliability and precision and complement the AQUIOS CL system.

AQUIOS Tetra tests are based on the ability of monoclonal antibodies to bind to the surface of cells expressing discrete, or a combination of discrete, antigenic determinants.

The AQUIOS Tetra-1 Panel CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5 and AQUIOS Tetra-2+ Panel CD45-FITC/(CD56+CD16)-RD1/CD19-ECD/CD3-PC5 monoclonal antibody reagents are each a combination of four or five murine monoclonal antibodies respectively, conjugated to the specified fluorochrome and specific to the cell surface antigen. The AQUIOS Tetra-1 Panel and Tetra-2+ Panel can be run individually or together in sequence by using the AQUIOS Tetra Combo test definition.

Specific staining of leukocytes is accomplished by incubating whole blood with the monoclonal antibody reagent. The red blood cells (RBCs) are then removed by lysis and the remaining leukocytes are analyzed by flow cytometry.

ACCURATE LYMPHOCYTE COUNTS – PURITY AND RECOVERY

Purity and recovery in flow cytometry always present as a tradeoff in gating. It is necessary for the operator to choose the appropriate balance between purity and recovery.

In the clinical management of immune deficiency diseases, accurately counting the absolute cell numbers of leukocyte subsets and measuring the percentage of individual subtypes in blood is critical. The purity of lymphocyte gates influence the accuracy of absolute counts. Lymphocytes can be identified and separated from other leukocytes by gating on a Forward Scatter (FS) /Side Scatter (SS) dot plot. However, the traditional light scatter morphology gate is unreliable for samples with a high number of non-lymphocyte leukocytes.(5)

Lymphocyte gate purity and recovery can be greatly enhanced by gating on bright CD45 (pan leukocyte marker) fluorescence and the relative low SS of lymphocytes. This gating method, when used in combination with FS/SS, is now widely accepted and used ensures >95% lymphocyte gate purity and recovery.(6)

In some instances, the CD45/SS gating method is not enough to capture the lymphocytes that escape the traditional lymphocyte light scatter gate due to reduced light scatter. Biological variations, sample preparation methods, reagents, and fluorochromes are some of the factors contributing to the diminished light scatter characteristics of lymphocytes. To increase the accuracy of the lymphocyte gate, Beckman Coulter employs a parameter called Electronic Volume (EV) as a measure of relative cell volume. The AQUIOS CL flow cytometer incorporates this parameter to gate lymphocytes using Electronic Volume versus Side Scatter in a dual parameter gate. This additional gating element, added to the repertoire of lymphocyte subset analysis gating strategy, improves the purity and recovery of total lymphocytes in a way that balances purity and accuracy with speed.

How it's Done on the AQUIOS CL Flow Cytometer

The AQUIOS CL flow cytometer is designed to balance both purity and recovery through cross-checks of the T, B, and NK cells. Some cells' properties are altered during the sample preparation process resulting in different FS or SS profiles than usual.(5) This can cause certain sub-populations to end up in a different location of the dot plots than would be expected. Therefore, these cells, often referred to as "escapees,"(7, 8) are missed by conventional gating and can lead to skewed results.

On a typical flow cytometer, all of the lymphocytes are first identified, then broken down into four main groups: T-cells, B-cells, NK-cells, and other cells. The problem with this approach is if the initial definition of lymphocytes is incorrect, the breakdown is also incorrect. Therefore, properly gating the lymph region in the CD45/SS graph is critical to the accuracy of results. Minor errors in placement can result in a significant impact on results.

The AQUIOS CL flow cytometer approaches gating from the bottom up. The cytometer builds the lymphocytes from the four main groups to minimize the impact of inaccurate gating of the lymph region in the CD45/SS graph. It is critical that the boundary between the lymphocytes and monocytes is placed correctly. If gated too tightly, good lymphocyte purity is achieved, but recovery will be impacted. If gated too loosely, good recovery is achieved, but it will result in poor lymphocyte purity. To strike an optimum balance between purity and recovery, the AQUIOS CL system allows for loose lymphocyte definition to maximize recovery and creates a tight lymphocyte definition to help maximize purity. The system uses additional lymphocyte specific markers (CD3 and CD19) in combination with a loose gate definition to identify sub-sets. Conversely, the system tightens the gate definition for NK cells and other cells that have a higher risk of being contaminated with the introduction of monocytes. To correct for the effect of "escapees," the AQUIOS CL system uses seven parameters.

In addition, the system uses dual discriminators: FS and CD45. It is only necessary to satisfy one of the two discriminators for cells to be counted in the analysis. This helps to exclude cells that are both non-fluorescent and too small.

CAPTURE OF "ESCAPEES" AND THE NEW ELECTRONIC VOLUME PARAMETER

Since biology is variable, the traditional FS/SS lymphocyte gate or even the CD45/SS parameter gate are not always sufficient to account for all the lymphocytes that are still present in the data set. These unaccounted for lymphocyte cells are called "escapees".

Escapees are lymphocyte cells with characteristics that can cause them to be missed by traditional lymphocyte gates. These unaccounted for "escapees" affect the accuracy and purity of the lymphocyte gate.(7, 8) The "escapee" phenomenon is caused by aggregates of human lymphocytes with activated platelets and monocytes. Bigger size and granularity of these aggregates result in an altered FS and SS value, which in turn leads the aggregates to be outside the conventional lymphocyte gate. Consequently the aggregates are miscounted, resulting in altered absolute counts.

Multiple factors contribute to aggregate formation. Some of the major factors include monoclonal antibody specificity and IgG subclass, fluorochrome type, monoclonal antibody conjugation, lysing agents, sample processing, and significant donor-to-donor variation. Many of these adverse factors can be mitigated or eliminated by the innovative technology features made available in the AQUIOS CL flow cytometer. These innovative features include preformatted AQUIOS Tetra monoclonal antibody reagents, highly automated sample processing directly from the blood collection tubes without manual sample preparation variation, and the addition of the electronic volume (EV) parameter. EV is unaffected by the cell granularity and so gives an accurate measure of cell volume.(5)

The Electronic Volume measurements are based on the Coulter Principle.(9) The electronic volume (EV) detector measures the cell volume by measuring the electrical impedance of the cell. It works by passing the cell through a flow cell that has two electrodes in a conductive sheath fluid. The impedance of the fluid is defined and as the cell is drawn into the flow cell channel, the volume of the cell displaces an equivalent amount of electrolyte solution. This changes the impedance between the electrodes. This "cell" pulse is a direct correlate of the cell volume. (5)

A gate set using the EV parameter and SS parameter is positioned to recover the lymphocytes that would otherwise be lost in the conventional FS/SS gate. Currently, EV is used for relative sizing in gating and is specific to the AQUIOS CL flow cytometer and not available on any other commercially available clinical flow cytometer.

BALANCED AND AUTOMATED

The intelligent gating algorithm of the AQUIOS CL system software produces high quality data, which takes into account specificity, purity, and recovery of the lymphocytes in a balanced way. This unique ability of the software system to know where to look for cells of interest keeps the need for manual gate adjustments to a minimum. Operators with reviewer/editor rights and/or administrator rights can make adjustments if necessary. For detailed instructions on verifying gating accuracy, see Analytic Reliability Checks in Chapter 5 of the AQUIOS Tetra System Guide.

THE ROLE OF AUTOMATION AND REVIEWER/ADJUSTER

The AQUIOS CL flow cytometer enables the operator to run samples quickly and simply. However, behind the simple workflow of the AQUIOS CL system runs an advanced algorithm to repeatedly trace and gate the cell subsets of interest. Results are automatically generated for specific tests based on gated regions. If the AQUIOS CL software detects anything unusual, it automatically generates system and application-specific flags or notifications. Several features make such automation possible:

SEPARATION QUOTIENT (SQ): A novel, calculated index called Separation Quotient (SQ) plays a major role in the generation of the flags and notifications associated with test results. SQ is a relative measure of sensitivity and specificity and is used as an index that indicates separation of two different populations; such as positive versus negative cell populations in fluorescence parameters, sample versus debris, or lymphocytes versus monocytes. As the AQUIOS CL flow cytometer runs the sample, it measures the SQ in real time, since SQ is used as a quick method of estimating separation and is parameter specific. SQ for a specific parameter such as SS is predefined and can thus enable real time checks and balances.

In case of a problem with a Control run, a flag or notification is generated when the SQ is computed to be outside the standard set range. Refer to the AQUIOS IMMUNO-TROL and AQUIOS IMMUNO-TROL Low assay sheets for specific ranges for Controls. For sample runs, a flag/notification is generated if there is an anomaly that has the potential to affect results. This enables the operator to take corrective action quickly. In addition, out of range SQ will result in cell populations falling outside of the pre-set regions in dot plots and histograms in the review screen.

LABORATORY INFORMATION SYSTEM (LIS): LIS have become a major component of many clinical laboratories. Beckman Coulter recognizes the need and has designed the AQUIOS CL flow cytometer to be automation-friendly. The AQUIOS CL system offers the ability to automatically transmit results to the LIS. The AQUIOS CL system can be set up to either auto transmit all results with no flags and no notifications, or hold all results.

Note: QC runs are not exported to the LIS.

While the operator is able to auto transmit test results to the LIS, operators/administrators are still expected to review all samples prior to release of results. This is a critical review role of the operator to ensure that important clinical analysis is performed correctly.

LOAD & GO SYSTEM: The system applies algorithms to check for inconsistencies, and, if one is identified, the system either flags or notifies the operator. Since the AQUIOS CL flow cytometer is a Load & Go system, the operator may elect to receive a text message or an email from the system if a sample is flagged. Only operators with reviewer/editor, or administrator rights can edit these algorithm-defined regions. These warning flags and notifications play an important part in making the AQUIOS CL flow cytometer a true walk-away, and reliable analysis instrument in a clinical laboratory setting.

ABILITY TO ADJUST GATING

The AQUIOS CL flow cytometer automatically gates each sample using a proprietary algorithm that helps to minimize the need to manually adjust gates. The data of a sample that is flagged can be reviewed on the review screen. However, it is possible that operators with reviewer/editor and/or administrator rights may require the ability to minimally adjust gating according to their needs. If the changes are saved to the file, the system will tag these changes with the User Modified notification.

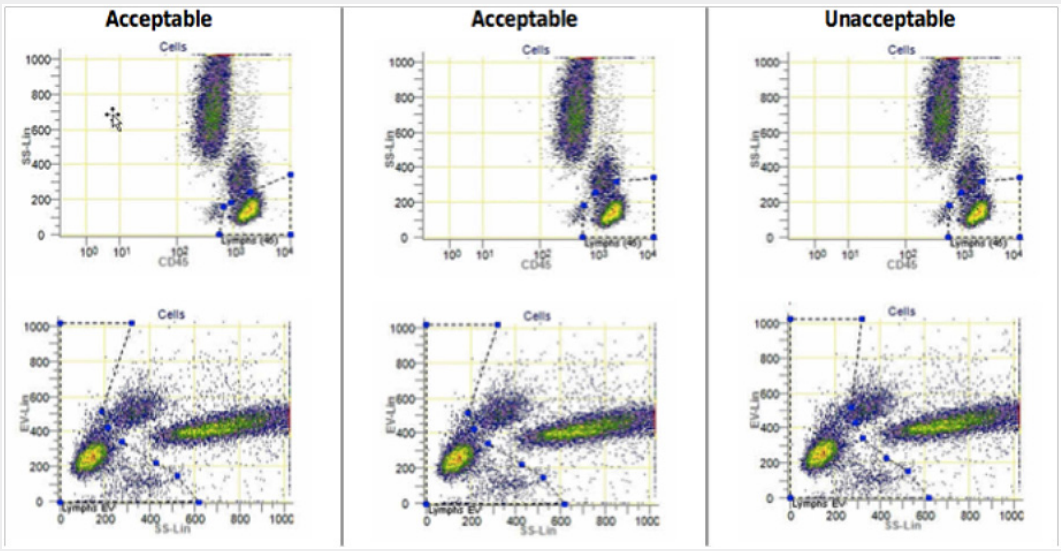
Refer to the AQUIOS Tetra System Guide for a detailed outline of these steps. An example of unacceptable gates and regions is shown in Table 1 and Table 2.

Table 1 – Editing Regions on Detail Plots for AQUIOS Tetra-1 and AQUIOS Tetra-2+

- Lymph gate is defined from the following two plots: SS Lin versus CD45 and SS Lin versus EV.
- Drag or reshape the region using the mouse to edit gate Lymphs (45) to include the lymphocytes which have bright CD45+FITC fluorescence and low SS (see Acceptable example).

  In this analysis, Lymphocytes are included while monocytes (lower CD45 expression and intermediate SS) and basophils (lower CD45 and low SS) are excluded as much as possible.
- Drag the pointer using the mouse to edit Lymphs (EV) gate to include the lymphocytes that have low and medium SS (see Acceptable examples).

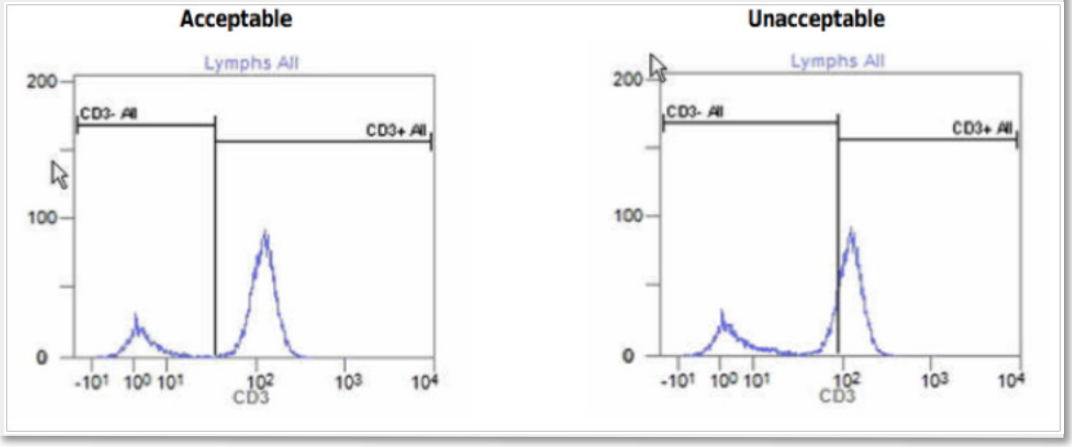
  In this analysis, Lymphocytes are included while monos and basos are excluded as much as possible.



Note: Occasionally, the CD45 gate is loosely set, but this can be acceptable as long as the EV versus SS region is drawn correctly.

Table 2 - Lymphs All CD3 Histogram

- Set a region boundary on the Total CD3+ histogram to encompass the CD3+ population as illustrated in the Acceptable example.



ANALYSIS ALGORITHM OVERVIEW

For more on this, refer to the Analysis Algorithm Overview section in Chapter 2 of the AQUIOS Tetra System Guide.

OVERVIEW OF AQUIOS TETRA GATING STRATEGY

Monocytes and granulocytes in a sample can be excluded by proper gating of lymphocytes on the flow cytometer.(11) Several methods are used in the AQUIOS CL system to ensure specificity and accuracy:

1. The AQUIOS Tetra System software is designed to automatically identify and optimize the lymphocyte gate based on CD45+, EV, light scatter characteristics, and fluorescence parameters (FL1 FL4).

2. The use of EV as an additional differentiating parameter aids in the separation of lymphocytes from other leukocytes and/or debris/noise.

3. The AQUIOS Tetra software monitors nonspecific antibody binding to lymphocytes by automatically placing cursors based on the separation of positive and negative peaks and measuring this difference. This process eliminates the need for an isotypic control.

The AQUIOS gating strategy captures lymphocyte populations and enumerates lymphocyte subsets. The AQUIOS system uses FS (relative size), SS (granularity), and fluorescence parameters (FL1 FL4), as well as the EV parameter (relative cell volume) to separate populations.

For more on gating, refer to Chapter 6 of the AQUIOS CL Flow Cytometer Instructions for Use manual.

CONCLUSIONS

The AQUIOS CL flow cytometer, with its integration of automatic sample preparation, automatic QC, and patient sample analysis offers a significant improvement over conventional flow cytometers and a great tool to meet the challenges of sample preparation, analysis, and gating.

The AQUIOS CL system is a system that uses intelligent software that simplifies gating and data analysis.

The AQUIOS CL system's innovative gating strategy:

- Results in correct lymphocyte subset enumerations providing reproducible results.
- Incorporates the EV parameter and accounts for the various lymphocyte populations in a specimen.
- Offers a balanced approach to purity and recovery of lymphocytes.
- Helps standardize clinical immunophenotyping.
- Presents all around gating solutions at every step of the immunophenotype testing process.

The number of manual and repetitive tasks required in traditional flow cytometry is minimized with the AQUIOS CL flow cytometer and the AQUIOS operator can learn the system quickly, use it efficiently, and produce accurate results consistently with minimal experience required.

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ACRONYMS

SPT:	single platform technology
FS:	forward scatter
SS:	side scatter
EV:	electronic volume
SQ:	separation quotient
LIS:	laboratory information system
QC:	quality control

TRADEMARKS

Class I Laser product

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