Quality Control and Verification of BD FACSCanto™ Clinical CD4 Enumeration Data

Overview

BD FACSCanto™ clinical software is an IVD-cleared product for enumerating lymphocyte subsets of samples stained with BD Multitest™ 4- and 6-color reagents.

BD FACS™ 7-color setup beads are used with BD FACSCanto clinical software to create Lyse/No-Wash and Lyse/Wash instrument settings. Results from the 7-color setup beads can be tracked with Levey-Jennings plots to monitor instrument performance, and can be used for troubleshooting.

Briefly, the software analyzes data in the following manner:

- First, it uses mathematical algorithms to determine where to place an "Expert Gate" around all lymphocytes, based on CD45 PerCP (4-color testing) or CD45 PerCP-Cy™5.5 (6-color testing) vs side scatter (SSC). If BD Trucount™ beads are used in the assay, the non-lymphocyte data is displayed and the beads captured with a gate.

- Next, the software uses interval gates to separate the $CD3^+$ lymphocytes from the $CD3^-$ lymphocytes. Quadrants are also used to classify the appropriate subsets in the CD3 positive ($CD4^+$, $CD8^+$, and $CD4^+8^+$) and CD3 negative ($CD16^+CD56^+$ and $CD19^+$) stained cells.

For a more detailed explanation of the gating algorithm, see the *BD FACSCanto Clinical Software Reference Manual*.

Although BD FACSCanto clinical software is a very sophisticated program, and the algorithms are appropriate for most samples, a technologist must review all dot plots and data before approving the results. The quality control (QC) criteria that the operator uses should verify that an adequate number of lymphocyte events are collected for analysis and that the gate placement around each population or quadrant location is appropriate. If an insufficient number of lymphocytes has been collected (less than the BD- or user-defined criteria), the sample must be reacquired with a new PerCP or PerCP-Cy5.5 threshold to increase the number of cells collected. The new threshold is acceptable as long as it does not cut through the lymphocyte population. Another option is to increase the collection criteria for the lymphocyte population using the Acquisition Targets tab (under the Reagents tab in the Tools menu).

Hallmarks of the lymphocyte subsets

To understand the gating used in BD FACSCanto clinical software, you need to understand the staining characteristics of each population gated and enumerated.

Lymphocytes are bright CD45 staining, brighter than monocytes and neutrophils, and produce low side scatter.
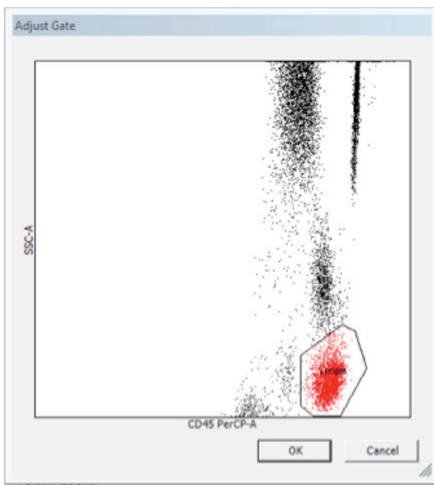

Figure 1: CD45 vs SSC showing lymphocytes

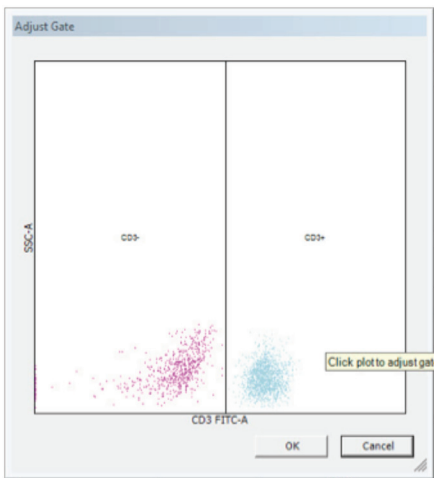CD3+ lymphocytes form a tight cluster as shown in Figure 2.

Figure 2: CD3+ lymphocytes form a tight cluster

CD4+ (3+4+), T-helper cells, also form a tight cluster. See Figure 3

CD8+ (3+8+), T-cytotoxic/suppressor lymphocytes, have a bright cluster and then stream to the negative area. See Figure 3.
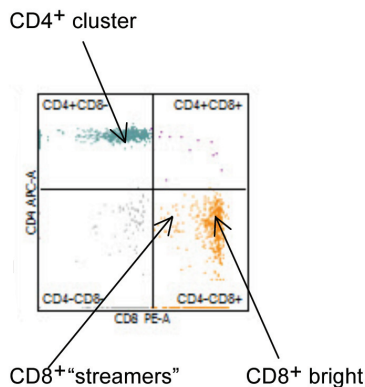

Figure 3. Data file showing typical CD4 and CD8 staining patterns

Visualization of the various populations stained with BD Multitest™ reagent cocktails

The various plots and colors are reagent specific as shown in the 4-color reagent cocktail in Figures 4 and 5.
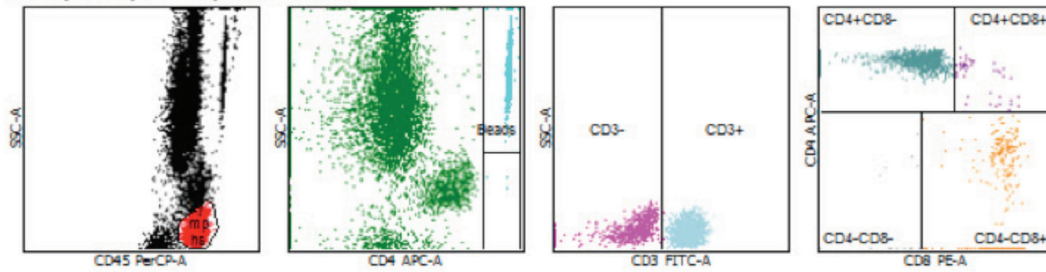

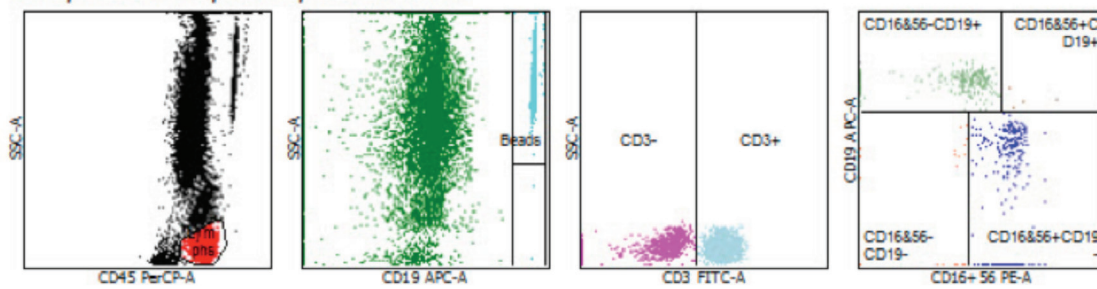**Figure 4.** Visualization of the CD4$^+$ and CD8$^+$ populations

Figure 5. Visualization of the CD19$^+$ and CD16$^+$CD56$^+$ populations

The various plots and colors are reagent specific as shown in the 6-color reagent cocktail in Figure 6.
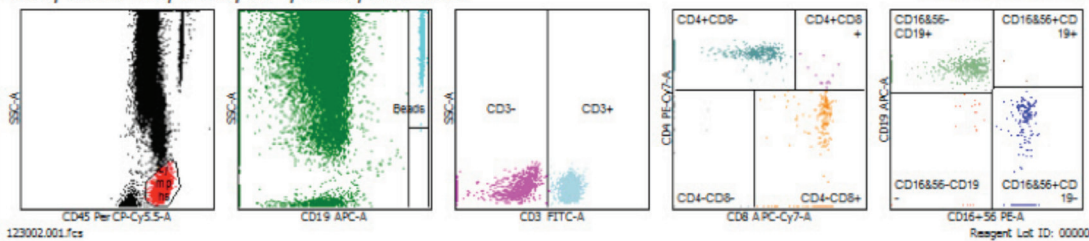

Figure 6. Visualization of the various populations stained with the 6-color BD Multitest™ reagent cocktail

A suggested approach for reviewing BD FACSCanto clinical software lymphocyte subset results

The following is a suggested list of important items to consider when reviewing or interpreting BD FACSCanto clinical data:

Step 1. Determine if a "Sample Quality questionable" error message is on the lab report or "Needs Review" is in the Status column of the worklist.

> If so, can the gate be redrawn to capture the lymphocytes? If the lymphocyte population is not properly captured, then a manual gate must be drawn to properly delineate the lymphocytes in the sample.

> If the gate cannot be redrawn with confidence, then the sample needs to be restained. If restaining does not resolve the issue, sample integrity has been compromised, results cannot be confidently reported, and the specimen must be redrawn.

Common CD45 gating issues

Under certain circumstances or with certain diseases, issues with the CD45 gating may be observed. Examples are as follows:
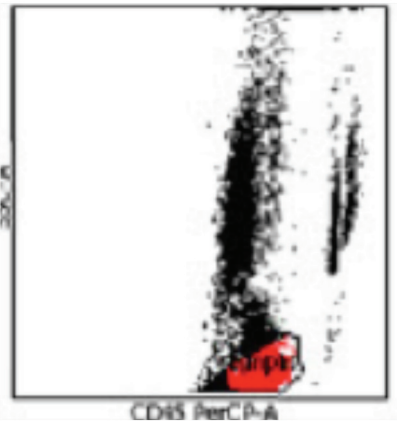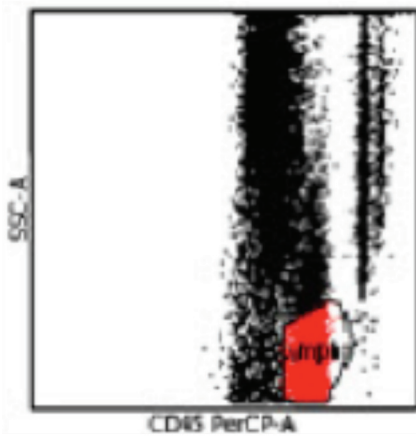

Figure 7: Aged blood sample

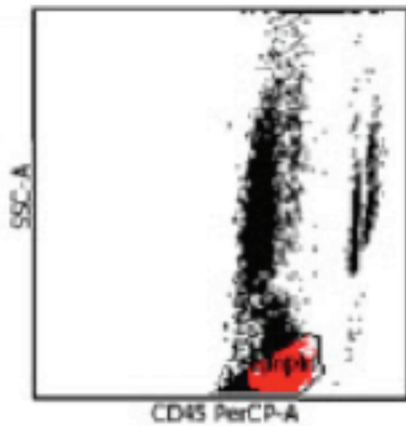**Figure 8:** Inadequately lysed sample
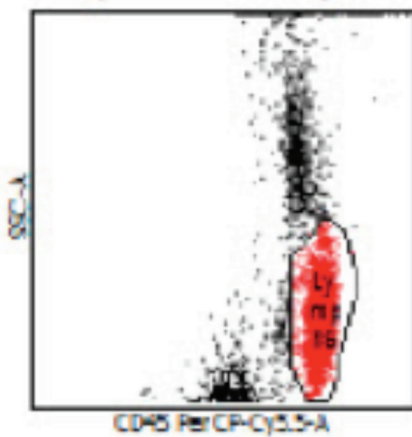
Figure 9: Lipemic sample

Figure 10: Elongated lymphocyte population due to improper optimization of side scatter voltage

Lipemic samples may be washed before staining to remove the lipids, which cause a compression of the side scatter. The laboratory would need to validate the washing protocol to ensure that the washing procedure does not affect absolute counts generated.

The side scatter voltage needs to be altered such that the granulocytes can be observed in the CD45 vs SSC plot. This ensures that the Expert gate can capture the lymphocyte population.

The Troubleshooting section in the *BD FACSCanto Clinical Software Instructions for Use* has specific details and recommendations to resolve these issues and related problems.

Specimens showing relative monocytosis can be inaccurately gated for lymphocytes. The inclusion of monocytes in the lymphocyte gate is determined by the presence of dim CD4 staining cells, since monocytes dimly express CD4 and high SSC in the CD3⁻ population. Data demonstrating that monocytes have been included in the lymphocyte gate is shown in Figure 11.
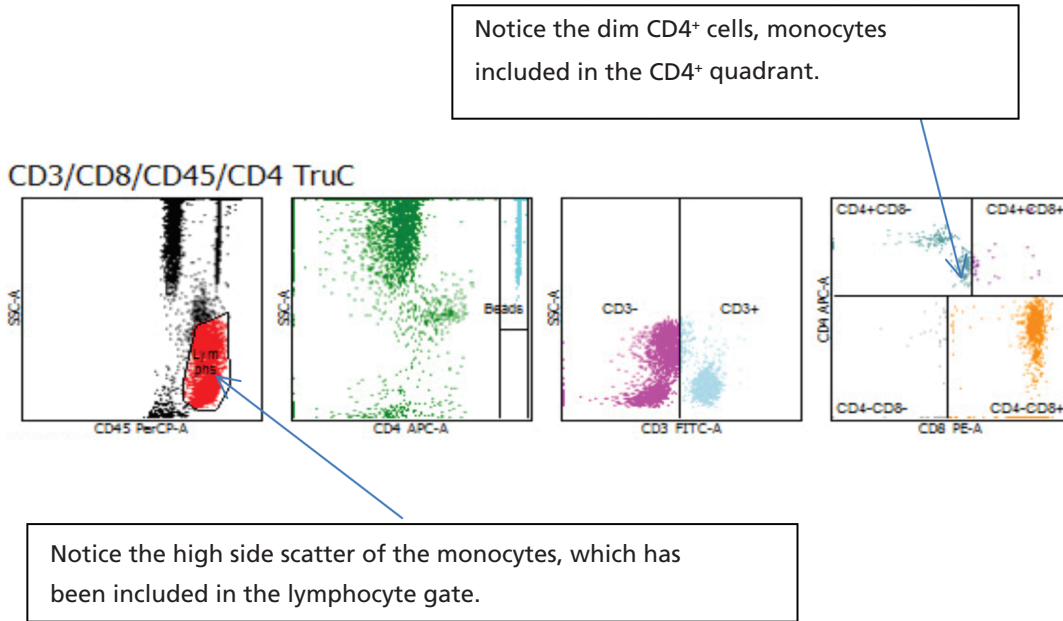
Notice the dim CD4⁺ cells, monocytes included in the CD4⁺ quadrant.

Notice the high side scatter of the monocytes, which has been included in the lymphocyte gate.

Figure 11: Sample with monocytes included in lymphocyte gate

Samples may contain a large number of basophils which can also be included in the lymph gate. The inclusion of the basophils affects the percentages and absolute counts reported for each population. Figure 12 and Figure 13 show data files exhibiting this gating.
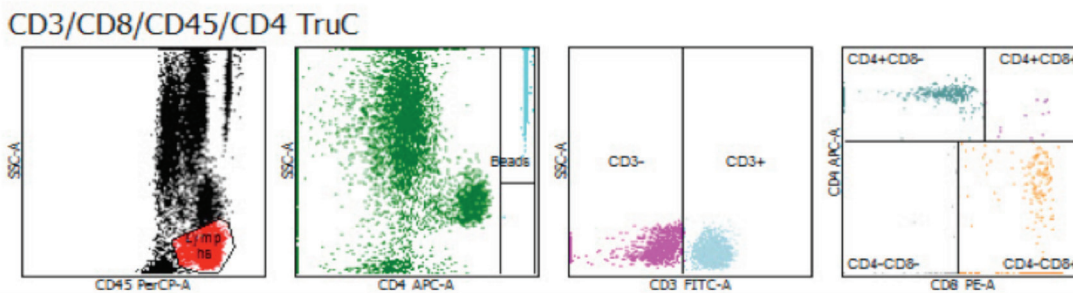

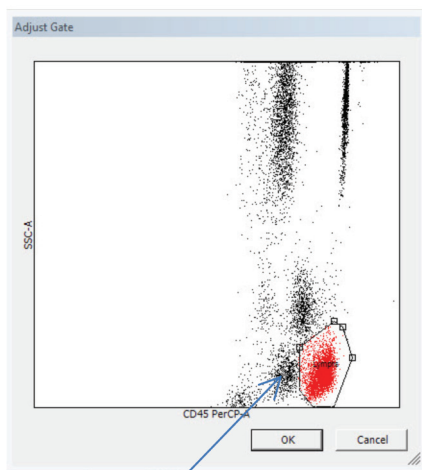**Figure 12:** Basophils included in the lymphocyte gate

Basophils

Figure 13: An enlarged view of a sample with numerous basophils present

Step 2. Verify that the CD3 interval gate is in the appropriate location.

> Some samples might have dimmer staining CD3$^+$ cells than others, requiring adjustment of the interval gate to reflect the correct population position.

> Verify that there are not high SSC CD3$^-$ events. If so, return to step 1.

> NOTE: Commercial controls can have dimmer CD3$^+$ populations than fresh blood. See the Staining patterns seen with BD™ Multi-check and other process controls section for details and examples.

Step 3. Verify the quadrant location to properly capture the CD4$^+$ and CD8$^+$ cells.

> Are the CD4$^+$ and CD8$^+$ quadrants appropriately placed? Populations should not be bisected with a quadrant marker.

> If not, adjust the quadrants to capture the population of interest.

Common CD4/CD8 gating issues

In some patients you may see an increase in the CD4$^+$CD8$^+$ population. Typically this value is less than 5%. This population can be due to interfering substances present in the patient's blood or certain diseases such as hepatitis C. See Figure 14.
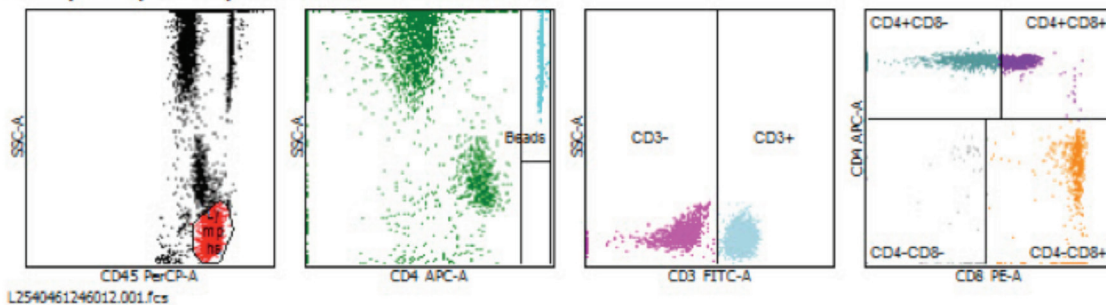

Figure 14: Interfering substances

In some cases, the sample can be washed with PBS before staining, and appropriate subset data is reportable. The laboratory must validate the wash procedure before using a single-platform method to report out the absolute counts.

Manual gating of CD4$^+$ and CD8$^+$ populations

There may be situations in which the CD8 events are on the axis. This phenomenon can show up in one of two ways. The first is when some of the events are above the axis and can be used to verify the placement of the quadrant marker. See Figure 15.
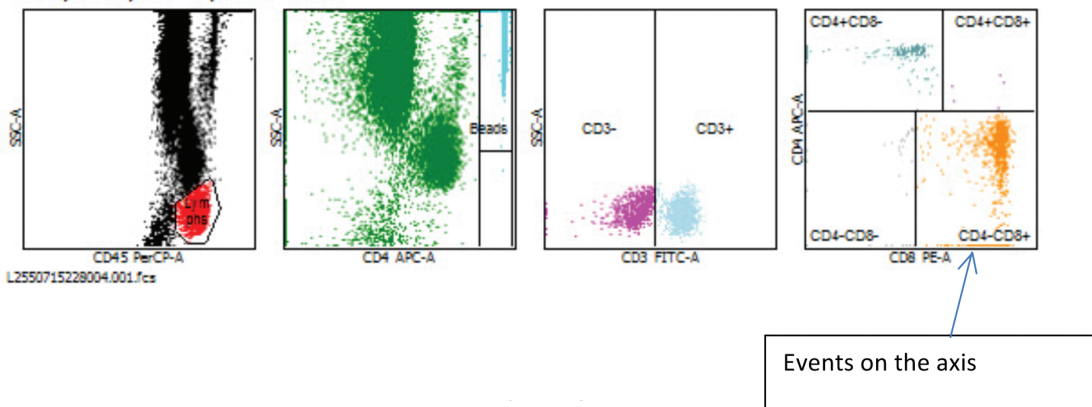

Events on the axis

Figure 15: Quadrant marker verification is possible

As you can see, there are a number of events that have piled up on the x-axis. By looking for valleys, or gaps, in the events, a decision can be made to verify or move the quadrant marker.

In the second example, all of the CD8 events are on the CD8 axis, which makes it extremely difficult to verify or adjust the quadrant marker. See Figure 16.
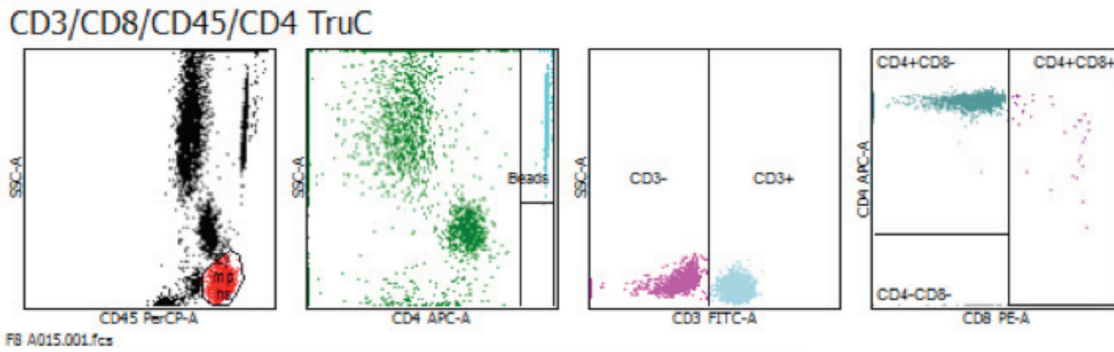
Figure 16: Quadrant marker verification unlikely

The first consideration is whether you can see any CD8 events above the baseline. If this is the case, as in Figure 15, there are some valleys between the various populations and locations to place the quadrant marker. The valleys in this plot are very close to the right-hand boundary of the CD4+ cells. This is a good, consistent, visual location to place the quadrant marker.

The data in Figure 16 is extremely difficult to gate in BD FACSCanto clinical software. One option is to verify the gating in this file in BD FACSDiva™ software. Figure 17 shows similar data analyzed in BD FACSDiva software. Contact Clinical Application Support or your Technical Application Specialist for help in creating this analysis template.
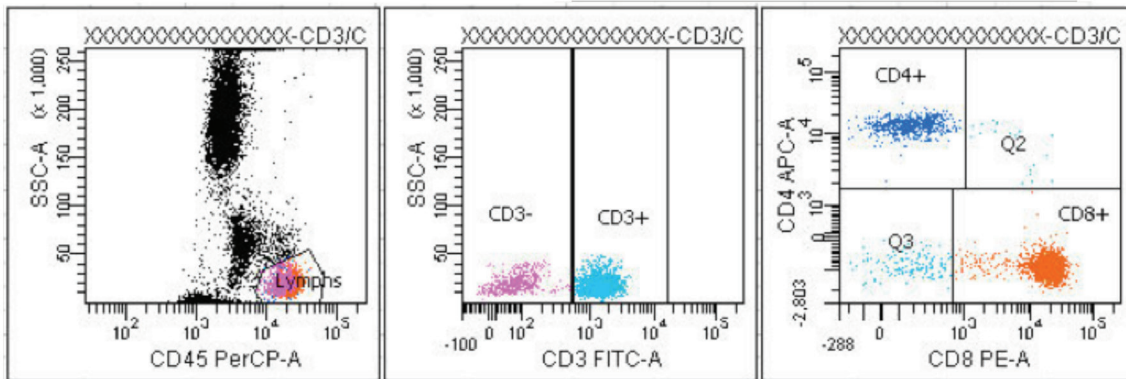

Figure 17: Data similar to Figure 16 analyzed in BD FACSDiva software

Another approach to take when seeing such data as in Figure 16 is to perform an optimization procedure. This optimization procedure used to restore the CD3+CD8+ events above the baseline is different from that detailed in the *BD FACSCanto Clinical Software Reference Manual* or the BD FACSCanto Operator's Manual from Customer Education. This alternative optimization is described in the protocol *Optimizing for Cells on the Axis with BD FACSCanto™ Clinical Software*. Adjusting the APC-% PerCP compensation for the 4-color BD Multitest reagent or the PE-Cy™7 - %APC-Cy7 compensation for the 6-color BD Multitest reagent will bring the CD8 events off the axis and make it easier to verify the placement of the quadrant marker.

T-cell receptor gamma/delta cells (TCR γ/δ)

Increased TCR γ/δ cells, >5%, can be seen in pediatric patients, some HIV+ patients, patients with an infection or inflammatory process, patients who have undergone a splenectomy, and in autoimmune and other diseases. The following figure shows an increase in CD3⁺CD4⁻CD8⁻ cells. When reviewing data from TCR γ/δ patients with a higher than normal T sum, the difference may be as great as 10%–15%. See Figure 18.
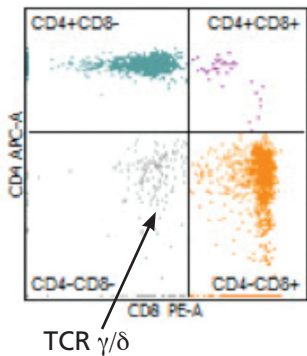

Figure 18: Plot showing TCR γ/δ cells

Step 4. Verify the quadrant location to capture the CD19⁺ and CD16⁺CD56⁺ cells. See Figure 19.

Are the CD19⁺ and CD16⁺CD56⁺ quadrants appropriately placed? Populations should not be bisected with a quadrant marker.

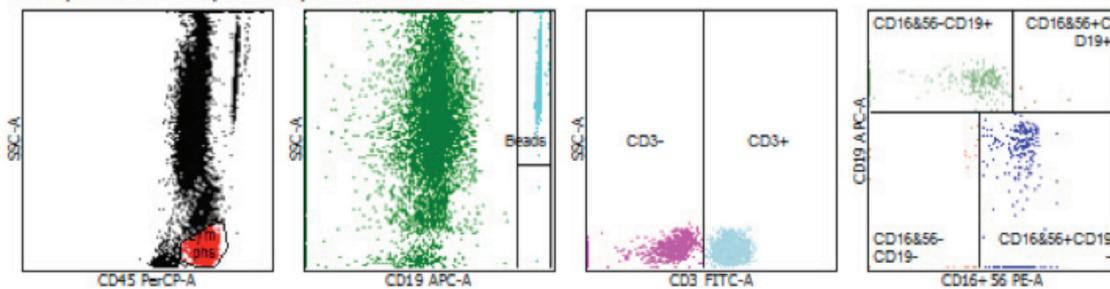If not, adjust the quadrants to capture the population of interest.

Figure 19: Normal B-cell (CD19⁺) and NK cell (CD16⁺CD56⁺) patterns

Step 5. Verify the TruCount gate. This gate should include all beads. All events to the right of the main population should also be included. See Figure 20.
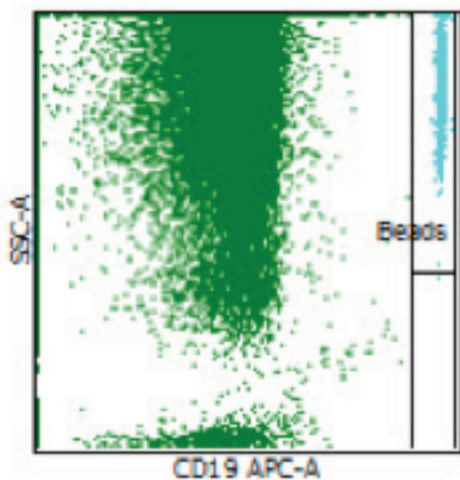

**Figure 20:** Adequate capture of BD Trucount beads

Step 6. Verify other QC messages.

Double check that the collection criteria are met: 2,500 lymphocytes and 500 beads.

- If lymphs are low, recollect the sample with higher threshold or lymph criteria.

- If beads are low, dilute the sample with PBS and restain. The laboratory should perform a validation of this deviation. See the following example.

A sample with a high WBC is submitted for testing. Again, the bead count may be very low and the less than 500 beads collected error message may be displayed. If the sample is re-gated, the bead error will not be displayed. It is imperative that the absolute values be checked in addition to other QC criteria to ensure that the results are appropriate to be reported. See Figure 21.
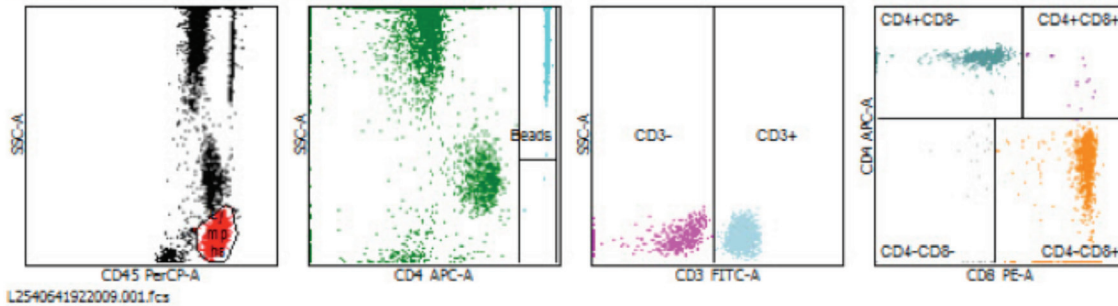
Parameter	Percent	Value/AbsCnt
Lymph Events		3359
Bead Events		970
CD3+	88.42	3361.92
CD3+CD8+	67.55	2568.42
CD3+CD4+	20.54	781.05
CD3+CD4+CD8+	0.51	19.24
CD45+		3802.25
4/8 Ratio		0.30

QC Messages
% T-Sum is: 0.33
4/8 ratio is: 0.30

Figure 21: A sample with a WBC nearing the maximum linearity of the BD Multitest assay

Notice that the bead count is nearing the error message flag at 500 beads.

- Occasionally, samples will be received that are not of an immunosuppressed nature. Chronic lymphocytic leukemia (CLL) is one example. A high WBC, few monocytes or neutrophils displayed, a low bead count with a possible error message of less than 500 beads collected (error message not shown) may be a tip-off for this sample. Notice that 83% of the lymphocytes collected are CD3−. Although absolute counts are displayed, the results are suspect due to the few beads collected. The package inserts for both the 4- and 6-color BD Multitest reagents list the upper linearity for a WBC in submitted samples. See Figure 22.
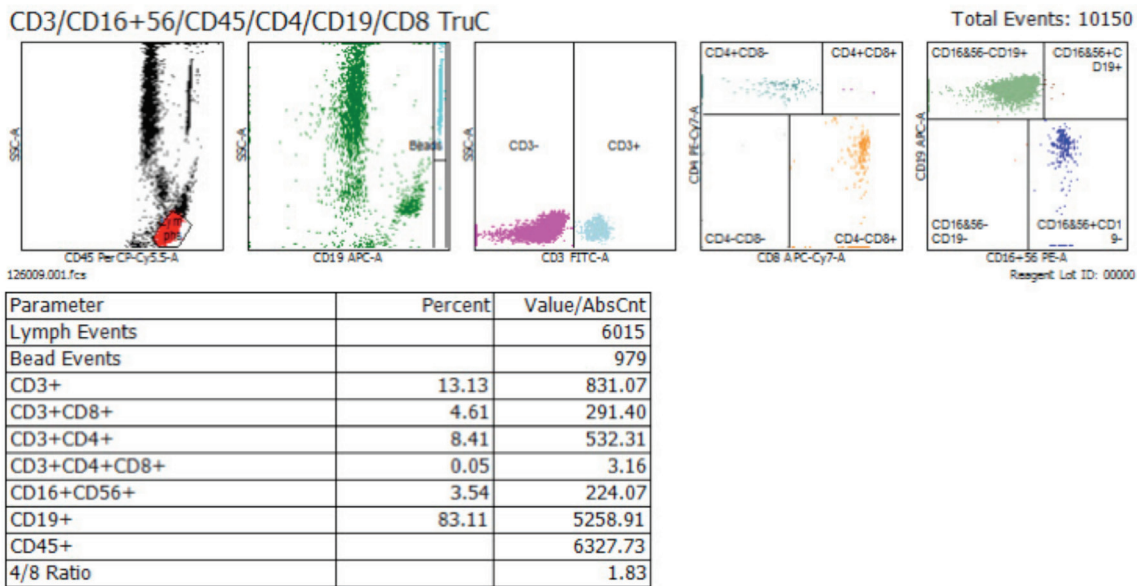
Parameter	Percent	Value/AbsCnt
Lymph Events		6015
Bead Events		979
CD3+	13.13	831.07
CD3+CD8+	4.61	291.40
CD3+CD4+	8.41	532.31
CD3+CD4+CD8+	0.05	3.16
CD16+CD56+	3.54	224.07
CD19+	83.11	5258.91
CD45+		6327.73
4/8 Ratio		1.83

Figure 22: Data from a suspected CLL sample

- Are there other error messages such as T-sum failure, default CD4 gating, or Lymphosum failure? Is the Lymphosum within the acceptable ranges, 95–105 as recommended by the package inserts or software? Are gate or region or quadrant adjustments necessary to bring the Lymphosum within range? The presence of TCR γ/δ cells may affect the Lymphosum as previously discussed.

  What is the CD3% difference between the two tubes if a two-tube panel was run?

  NOTE: Typically the limit is a 5% difference, but this value should be determined by each facility.

  Does the CD3$^{+/-}$ interval gate need to be adjusted? If so, this adjustment usually is necessary on both tubes in the panel.

- Verify the subset percentages and absolute counts.

  Are the results outside the reference range in use?

  Is this expected in light of the suspected condition of the patient?

- Are the CD3 absolute counts within the reference range established by the facility? Does the interval gate need adjusting or is a replicate sample processed to rule out pipetting or BD Trucount tube errors?

- After analysis, does the data meet the laboratory's established criteria for reporting a result? If not, restain or have the specimen redrawn. Proceed to the next sample and repeat the process.

Staining patterns seen with BD™ Multi-check and other process controls

Process controls are samples that have been chemically treated to enhance their shelf life. This manufacturing process reduces the fluorescence intensity of populations.

- Commercial process controls frequently will have dimmer than normal CD3 staining. Since the interval gates are defined based on staining patterns observed in fresh whole human blood, the CD3$^+$ interval gate might need to be adjusted on both tubes of a two-tube panel. Verify that each subset is properly classified. Figures 23a shows a fresh whole blood sample stained with the BD Mulitest™ CD3/CD16+CD56/CD45/CD4/CD19/CD8 cocktail for comparative intensities and separations. Figure 23b shows a BD Multi-check control stained with the BD Multitest CD3/CD16+CD56/CD45/CD4/CD19/CD8 cocktail. Figure 23c shows another vendor's control stained with the BD Multitest™ CD3/CD8/CD45/CD4 cocktail.
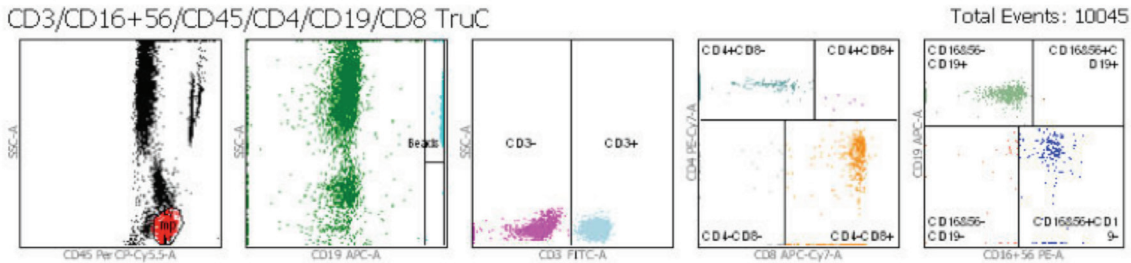

Figure 23a: Fresh whole blood sample showing adequate resolution between the CD3$^+$ and CD3$^-$ lymphocytes
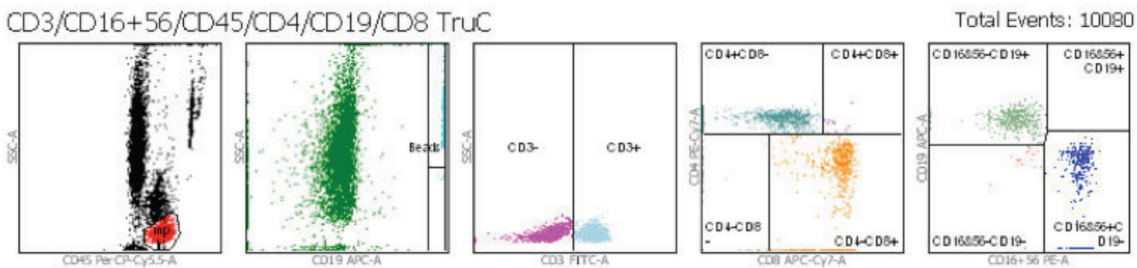

Figure23b: BD Multi-check control showing decreased CD3$^{+/-}$ separation

The lymphocyte gate looks very clean and the decreased fluorescence intensity of the CD3$^+$ population is evident. The CD4$^+$ and CD8$^+$ populations are well resolved and captured.

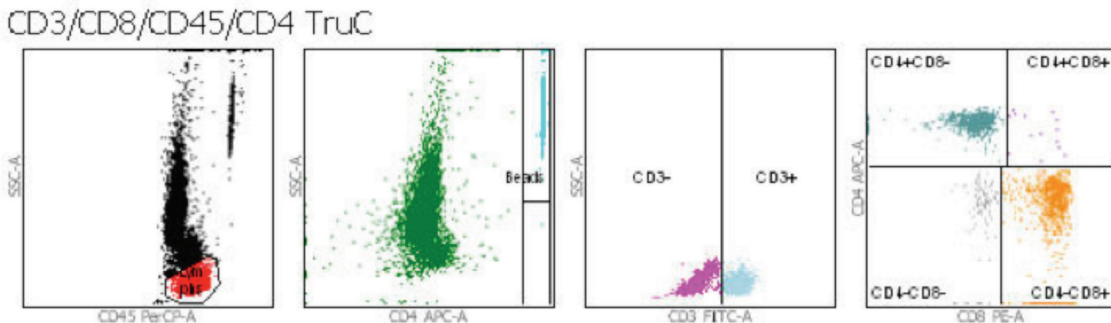CD4 process controls from other suppliers may exhibit the staining pattern seen in the Figure 23c.

Figure 23c: Controls from another manufacturer with decreased CD3$^{+/-}$ separation

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