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TECHNOTES

Vol 8 No. 1 January 2002

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BD Biosciences
http://www.bdbiosciences.com

Fluorescence Calibration and Standardization

Introduction

Clinical and research laboratories are facing an increasing number of requirements for the validation and standardization of their procedures. Flow cytometry relies on relative measurements of fluorescence and light scatter, and suffers from a lack of widely accepted calibration or standardization practices. Any flow cytometrist can reasonably ask: What do I want to evaluate on an instrument? Does the instrument require sensitivity and linearity measurements for each PMT? How should sensitivity be measured? Applications might require different strategies to address calibration or standardization. As this area of cytometry continues to evolve, changes in this technology can be expected.

First we will define and discuss calibration, standardization, sensitivity, and resolution of flow cytometry instrumentation; then we will describe the use of standards for monitoring instrument performance; finally we will discuss linearity in the context of instrumentation and assay development.

Definitions

Instrument calibration is a commonly discussed topic in compliance documentation. *Calibration* provides a known relationship between the measurement response and the value of the substance being measured,[1] and a calibrator contains a known value of the substance being measured. However, true fluorescence calibration, as this definition suggests, might not yet be possible. Although relative fluorescence standardization is commonly used with available microbead standards, there are no certified calibrators for fluorescence intensity.

Calibration particles are available with a measured number of specific fluorochromes, and these are used in an application-specific manner, such as quantitative fluorescence cytometry. Calibration particles, such as BD QuantiBRITE PE beads, are available with fluorescence intensity measured in units of molecules of equivalent soluble fluorochrome (MESF). These calibration particles are assigned values by the manufacturers but without reference to established certified fluorescent standards.

A *standard* is a material against which other materials can be compared,[1] and standardization is the process of comparing a result to a standard. Cytometry standards exist in the form of fluorescent microspheres that can be used to check performance within and between instruments. Schwartz et al proposed a classification system for flow cytometry fluorescence standards based on the degree to which a standard particle simulates a stained cell.[2] Since there are multiple processes involved in measuring a property of a particle, the most established and practical approach is to standardize at a system level.

WEB LINKS

http://bpf.med.harvard.edu/Pages/techs/O/IDT/Fluorescence_spectrum.html

The most fundamental performance characteristic that can be measured and standardized is *sensitivity.* Sensitivity can be defined in terms of either detection threshold or resolution—two dissimilar concepts. Detection threshold has to do with the smallest amount of signal that can be detected.[3] Resolution has to do with the ability to resolve dimly stained cells from unstained cells in a mixture.[4] Although instruments can have the same detection threshold, they can differ significantly in the ability to resolve a dimly stained population.[5] Chase and Hoffman, 1998, describe the relationship between signal, noise, and *resolution.* Resolution can be measured as the coefficient of variation (CV) of the signal of one population. Resolution will be impacted by the uniformity of illumination of the particle, the uniformity of the fluorescence label of the particle, background light, and photoelectron noise.

The *linearity* of the instrument electronics, including amplifiers, produce electronic outputs, which are proportional to their inputs.[4,7] Uniform fluorescent particles are commonly used to check the linearity of fluorescence detection. As an example, signal input from aggregated particles have an anticipated doubling or tripling of fluorescence intensity due to the presence of particle doublets or triplets. This should not be confused with the linearity and reportable range of biological assays, which will be affected by many factors, both instrumental and assay-dependent.[6] Linearity is discussed further in this article.

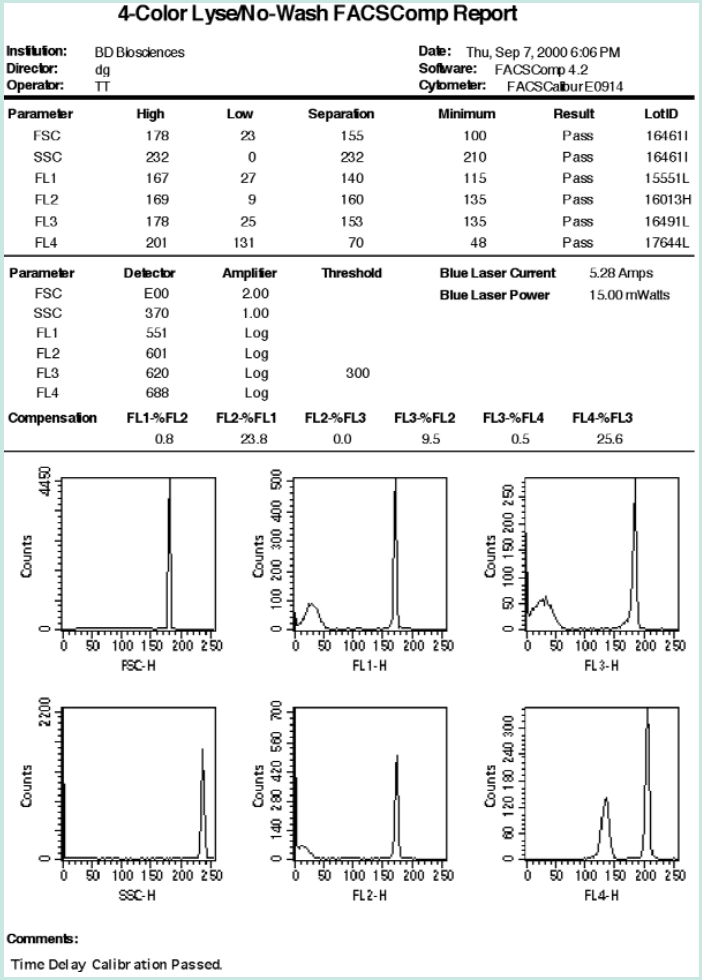
Methods for Measuring Instrument Performance

CaliBRITE™* beads are standards that are used with FACSComp™* software on many BD cytometers. The use of CaliBRITE beads as a standard is prescribed by BD Biosciences, and instructions are found in the *CaliBRITE Beads* package insert and *FACSComp Software User's Guide*. FACSComp software, in addition to other functions, measures the difference in fluorescence intensity of unlabeled beads and moderately bright fluorochrome-labeled beads. See Figure 1.

Bead fluorescence and light scatter in the detection parameters are measured on the cytometer and compared to the expected outcomes for the CaliBRITE beads kit. A *Pass* result is generated and printed by FACSComp software if all parameters demonstrate acceptable separation of labeled and unlabeled beads. A *Fail* result is generated and printed by the software if unacceptable separation is encountered. Any *Fail* result requires some corrective action in order to correct the reason for the failure. CaliBRITE beads are particularly useful for instrument setup in a standard configuration for analyzing lymphocytes from lysed whole blood specimens. CaliBRITE bead products are for in vitro diagnostic use and are manufactured under cGMP in an ISO 9001–certified facility.

WEB LINKS

Table of Fluorochromes
http://pingu.salk.edu/fcm/fluo.html



4-Color Lyse/No-Wash FACSComp Report

Institution: BD Biosciences
Director: dg
Operator: TT
Date: Thu, Sep 7, 2000 6:06 PM
Software: FACSComp 4.2
Cytometer: FACSCaliburE0914

Parameter	High	Low	Separation	Minimum	Result	LotID
FSC	178	23	155	100	Pass	16461I
SSC	232	0	232	210	Pass	16461I
FL1	167	27	140	115	Pass	15551L
FL2	169	9	160	135	Pass	16013H
FL3	178	25	153	135	Pass	16491L
FL4	201	131	70	48	Pass	17644L

Parameter	Detector	Amplifier	Threshold	Blue Laser Current	5.28 Amps
FSC	E00	2.00		Blue Laser Power	15.00 mWatts
SSC	370	1.00			
FL1	551	Log			
FL2	601	Log			
FL3	620	Log	300		
FL4	688	Log			

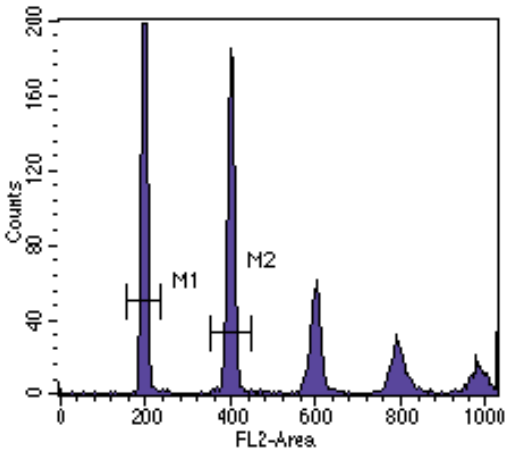
Compensation	FL1-%FL2	FL2-%FL1	FL2-%FL3	FL3-%FL2	FL3-%FL4	FL4-%FL3
	0.8	23.8	0.0	9.5	0.5	25.6

Comments:
Time Delay Calibration Passed.

Figure 1 FACSComp Summary Report with results for a four-color lyse/no-wash instrument setup

If an assay requires fluorescence signal resolution, the operator should use an additional means of measuring instrument performance. Resolution is required for DNA ploidy analysis. Propidium iodide (PI)-labeled chicken erythrocyte nuclei (CEN) are used to check the instrument's ability to resolve PI-stained nuclei and to demonstrate linearity of the FL2 area parameter. See Figure 2. These reagents are available in BD Biosciences' DNA QC Particles Kit† (Cat. No. 349523). Please note that this test is fluorochrome- and application-specific, and does not reflect the ability of the FL2 parameter to test other signal types such as phycoerythrin.

The CV is typically measured on the CEN singlet population, as seen in Figure 2 in marker M1.



Sample ID: CEN
X Parameter: FL2-A FL2-Area (Linear)

Marker	Geo Mean	CV
All	327.42	59.69
M1	200.92	1.95
M2	400.45	1.80

Figure 2 CEN singlets, doublets, and multiplets, useful indicators of linearity

As a practical means of monitoring relative signal intensity and resolution for multiple parameters, the log fluorescence intensity and CV of *hard-dyed* beads can be monitored on an instrument with settings optimal for these particles. The benefit of measuring the CV and mean fluorescence intensity on a day-to-day basis is to provide consistent performance data on the instrument. Factors that will affect performance in terms of CV and intensity include: uniformity of illumination of the particles in the core stream; the effects of *noise,* such as background light, bubbles; and accumulation of protein in the flow cell.

As an example, SPHERO™ Rainbow QC Kit,* (Cat. No. RQC-4K) can be used to provide relative standardization for FL1, FL2, FL3, and FL4 fluorescence intensity and resolution.

The product contains a mixture of 3.0-mm particles having eight different fluorescence intensities. Instrument settings appropriate for the particles or cells that you want to test can be established on the instrument, and these particles can be used as a standard to monitor instrument sensitivity. See Figure 3.

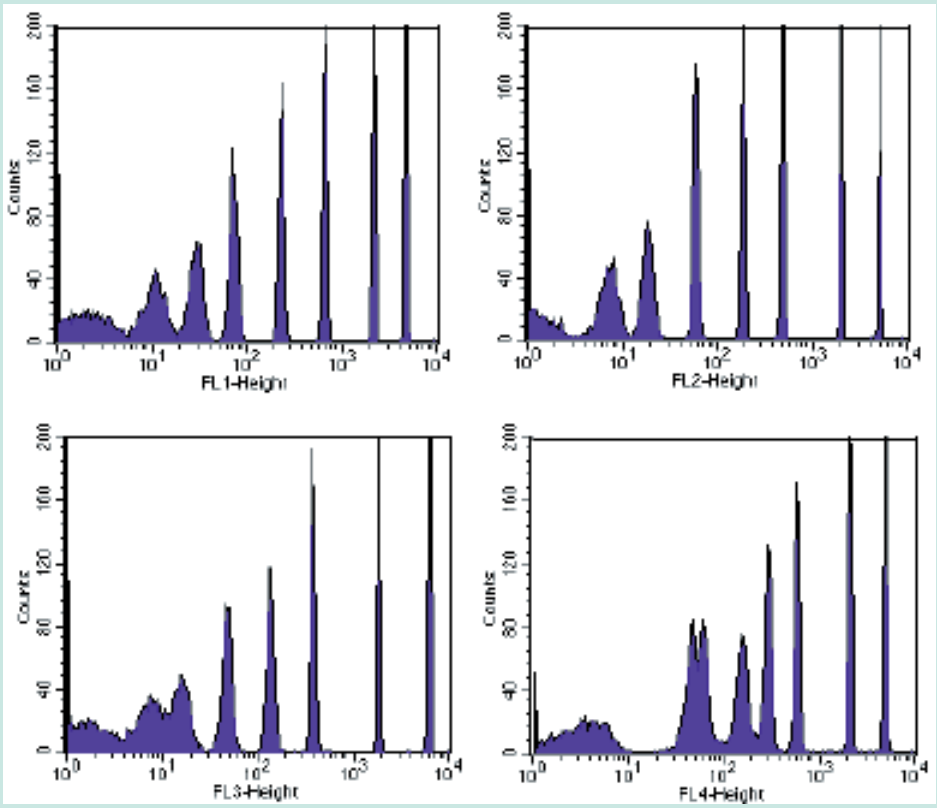


Figure 3 SPHERO Rainbow Calibration Particles analyzed on a four-color FACSCalibur™

Data acquisition was performed with logarithmic amplification.

The instrument response for the FL1, FL2, and FL3 data demonstrates typical resolution for instruments tested at our San Jose facility. On the same instrument, the FL4 data demonstrates the best resolution on all systems tested. Although significant instrument-to-instrument variation in resolution should be expected, these particles are quite useful as a control for periodically monitoring performance on the same instrument.

WEB LINKS

Fluorescence Spectra
http://www.drmr.com/abcon/allspec.html

Hard-dyed beads are embedded with fluorochromes different from those typically used in labeling cells, including FITC, PE, PerCP, and APC. The instrument response will be different because hard-dyed beads do not have the same fluorescence spectra as these standard fluorochromes. Since cells differ from beads in their autofluorescence characteristics, commercially available process control cells can be used to demonstrate fluorochrome-specific emissions when labeled with fluorochrome-conjugated antibody.

Linearity

Fluorescence linearity can be affected by many factors, including optical alignment, laser power, and electronic offsets but is generally impacted by amplifier calibration. This is set at the factory and should not change when the instrument is shipped. As mentioned previously, fluorescence linearity can be measured by using fluorescent particles or nuclei stained for DNA fluorescence, or both. Particle doublets and multiplets give simple multiples of the signal of a single fluorescent particle. This is generally applied as a means of verifying that DNA ploidy results are not impacted by the signal linearity of the instrumentation. It is important to note that for many clinical applications, such as lymphocyte subset enumeration, establishing the linearity of the assay might be more important than the linearity of the instrumentation. The key is to establish the linearity of the reportable range. If, for example, a laboratory is reporting CD4 absolute count values, the reportable range should be contingent upon the linearity of CD4 absolute count values.

Summary

As the technology continues to evolve, several steps can be taken to standardize and monitor the performance of a flow cytometer. CaliBRITE beads provide information on signal separation, perform automatic compensation, and establish instrument settings appropriate for peripheral blood specimens. QuantiBRITE beads are used to calibrate the FL2 parameter in terms of MESF. Resolution, signal intensity, and linearity are established using SPHERO Rainbow Calibration Particles, other microbead standards, and DNA-stained particles.

As a rule of thumb, an operator should choose standardization methods that are appropriate for the type of analysis performed. Instructions and published protocols are available from suppliers and other sources. Evaluation of performance variables such as signal intensity, separation, and CV can be monitored on the instrument over a period of time by using Levy-Jennings charts established by the laboratory. This approach provides an objective means of identifying trends, shifts, and anomalies in the collected data.

WEB LINKS

Center for Fluorescence Spectroscopy
http://cfs.umbi.umd.edu/

References

1. Henderson LO, Marti GE, Gaigalas A, Hannon WH, Vogt RF, Jr. Terminology and nomenclature for standardization in quantitative fluorescence cytometry. *Cytometry.* 1998;33:97–105.
2. Schwartz A, Marti GE, Poon R, Gratama JW, Fernandez-Repollet E. Standardizing flow cytometry: a classification system of fluorescence standards used for flow cytometry. *Cytometry.* 1998;33:106–14.
3. Schwartz A, Fernandez-Repollet E, Vogt R, Gratama JW. Standardizing flow cytometry: construction of a standardized fluorescence calibration plot using matching spectral calibrators. *Cytometry.* 1996.26:22–31.
4. Shapiro HM. *Practical Flow Cytometry*. New York, NY: Wiley-Liss; 1995.
5. Chase ES, Hoffman RA. Resolution of dimly fluorescent particles: a practical measure of fluorescence sensitivity. Cytometry. 1998;33:267–279.
6. Hoffman RA. *Methods in Cell Biology Vol. 63: Standardization and Quantitation in Flow Cytometry.* Academic Press; 2001.
7. Bauer KD, Duque RE, Shankey VT. *Clinical Flow Cytometry Principles and Application, Instrumentation*. Baltimore, MD: Williams and Wilkens; 1998.

* For Research Use Only. Not for use in diagnostic or therapeutic procedures.

† For In Vitro Diagnostic Use.

CD15 and Ammonium Chloride

CD15, clone MMA, FITC (BD Cat. No. 347423 [RUO] or Cat. No. 340703 [ASR]) has been tested and is routinely used with FACS™ Lysing Solution as an RBC lysing reagent. We have observed that this clone can cause granulocyte lysis when whole blood specimens are treated with an ammonium chloride–based lysing solution. This has been demonstrated when cells are stained before lysing, or stained after lysing. Please exercise caution when inspecting data, validating reagents, or changing lysing solutions.

Compensation Effects Observed with FITC-Bright Populations

A compensation issue has come to our attention that we would like to illustrate and explain. As you know, FITC-labeled reagents emit fluorescence in FL1, FL2, and FL3.

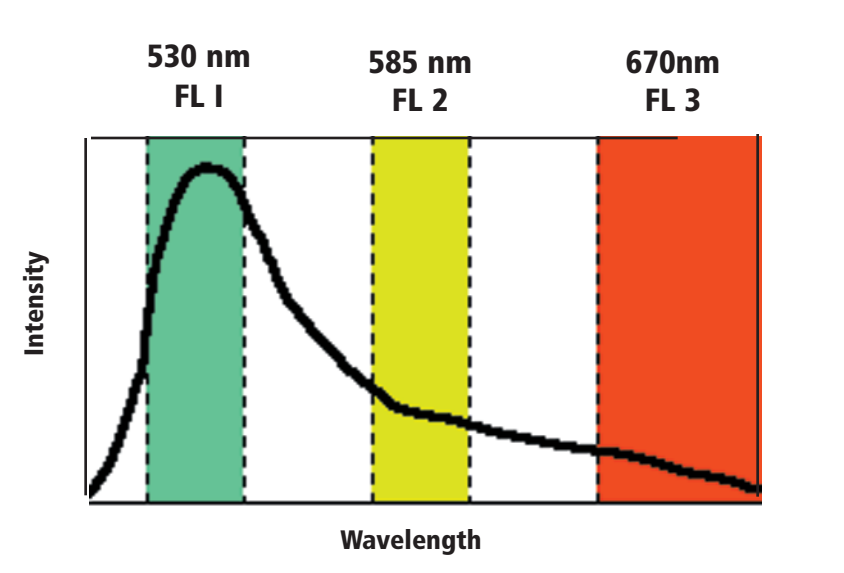


Figure 1 Relative FITC fluorescence emissions captured by the FL1-, FL2-, and FL3-emission filters of the FACSCalibur

The FL3–%FL2 compensation setting is usually set to a value appropriate for removing PE emission from FL3. Although it is possible to use the FL3–%FL2 compensation network to remove a portion of the FITC signal from the FL3 detector, it will not provide an appropriate degree of correction for FITC when set up for PE. Since the FACScan™, FACSort™, and FACSCalibur do not have an FL3–%FL1 compensation network to correct this overlap, the compensation settings will be suboptimal.

Compensation Effects Observed with FITC-Bright Populations

Under these conditions, bright FITC-labeled cells demonstrate a decreased FL3 fluorescence. Consequently, for cells stained with an appropriate FL3 fluorophore, the FL3-positive population might exhibit a pattern of fluorescence smeared toward the origin. See Figure 2. Because the FL3 staining pattern is affected, you might need to alter regions and markers to properly capture the FL3-positive, but dim events.

WEB LINKS

Science Magazine
http://www.sciencemag.org/

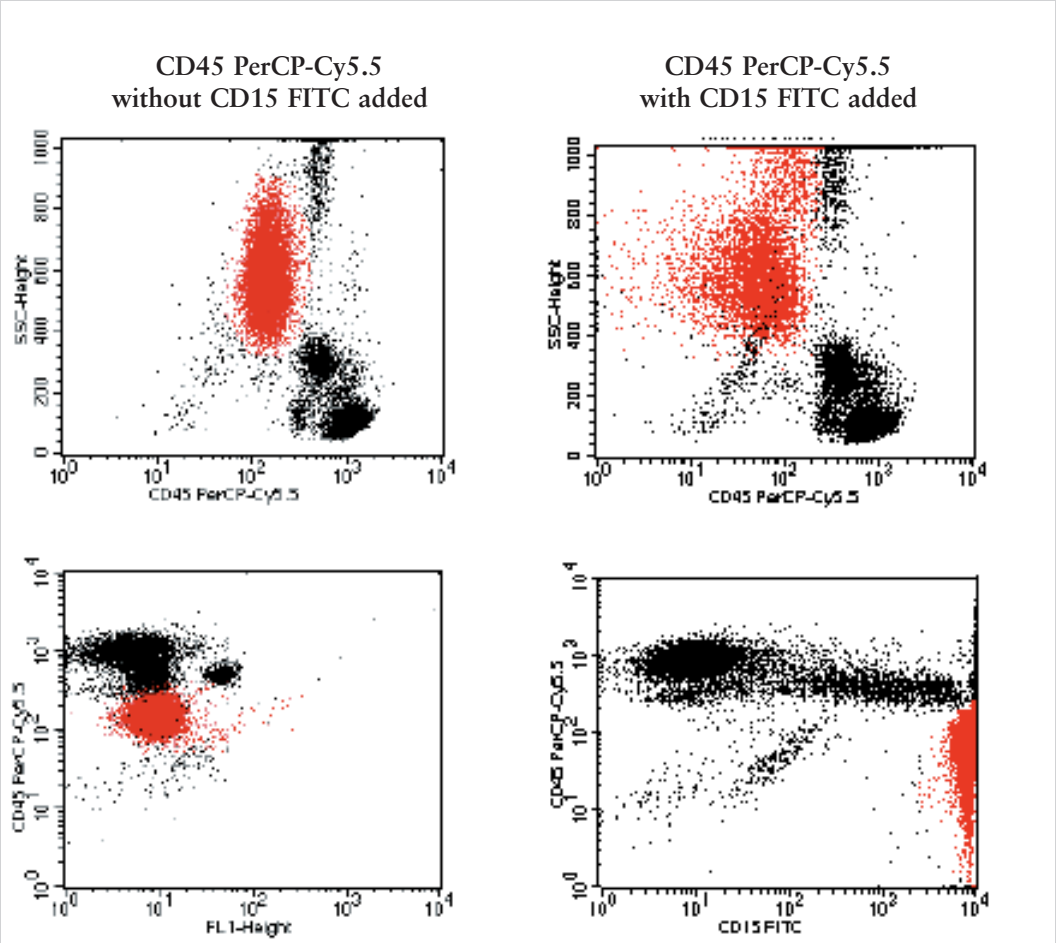


Figure 2 A comparison of fluorescence from two samples

The granulocyte population in Figure 2 is highlighted for comparison purposes. Note the $CD15^+$ granulocyte population exhibits a smeared pattern on the FL3 axis. Since smearing is not observed in the sample without CD15, it illustrates that FITC emission can affect the FL3 staining pattern.

Compensation Effects Observed with FITC-Bright Populations

WEB LINKS

American Journal of Clinical Pathology
http://www.ascp.org/press/periodicals/eajcp/

Quality Control and Verification of MultiSET Data

MultiSET™* software provides automated analysis of samples stained with TriTEST™* three-color and MultiTEST* four-color reagents.

Briefly, the software analyzes data in three steps. First, it sets an Expert Gate around all lymphocytes based on CD45 PerCP versus side scatter (SSC) or around T lymphocytes based on CD3 PerCP versus SSC. Next, it uses Attractors™ software technology to analyze the gated events to compute subset percentages and absolute counts. Finally, the operator reviews the results and is given the option to readjust the gate or attractors regions, or both, to recompute the results. If results are recomputed, the operator chooses which results to report.

For a more complete explanation on the gating algorithm or Attractors analysis refer to the *MultiSET Software User's Guide*.

Although MultiSET is a very sophisticated software, a technologist must review all data plots before signing the results. The quality control (QC) criteria that the operator uses should verify that adequate lymphocyte events are collected for analysis and that the attractors placement around each population is appropriate. If an insufficient number of lymphocytes has been collected (ie, less than the BD or user-defined criteria), the sample must be reacquired with a new FL3 threshold to increase the number of cells collected. The new threshold is acceptable as long as it does not cut through the lymphocyte population.

The following is a suggested list of important items to consider when reviewing or interpreting MultiSET data.

- For tubes containing CD45 PerCP, verify that the CD45 PerCP versus SSC gate is appropriately drawn. Was a Code 3 Sample Integrity error message obtained?
  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.

- Are the CD3 attractors, both $CD3^+$ and $CD3^-$, in the correct location?
  Some samples might have dimmer staining $CD3^+$ cells than others, requiring adjustment of the attractors to reflect the correct population position.
  NOTE: Commercial controls can have dimmer CD3+ populations than fresh blood has.

- Are the subset attractors properly capturing the populations of interest?
  Are the $CD4^+$ and $CD8^+$ attractors appropriately placed?
  If not, adjust the attractors to enclose the population of interest. Repeat for the CD16+CD56 and CD19 attractors.

WEB LINKS

Laser Institute of America
http://www.laserinstitute.org/safety_bulletin/

- Are there other error messages?
- Are the percentages and absolute counts outside the reference range in use? Is this expected in light of the suspected condition of the patient?
- Is the Lymphosum within acceptable ranges, ie, 95–105 as recommended by the package inserts or software? Are gate or attractor adjustments, or both, necessary to bring the Lymphosum within range?
- What is the %CD3 difference between the tubes if a two-tube or multitube panel was run?
  NOTE: Typically the limit is a 5% difference, but this value should be determined by each facility.
  Do the CD3 attractors need to be adjusted? If so, this adjustment is usually necessary on all tubes of the panel.
- Are the TruCOUNT™* Control beads being used?
  If so, ensure that the reported counts are within the range specified in the product insert. TruCOUNT Control beads are quite useful as an absolute count control and for verifying pipetting accuracy. If the Control bead counts are out of range, check the pipette calibration, and process a replicate sample.
- Are the CD3 absolute counts within the reference range established by the facility? Do the attractors need adjusting or is a replicate sample processed to rule out pipetting or TruCOUNT Tube errors?
- Does the data, after reanalysis, meet the laboratory's established criteria for reporting a result? If not, restain or redraw the patient sample. Proceed to the next sample and repeat the process.

WEB LINKS

Case Studies in Clinical Flow Cytometry
http://www.flowcases.org/website/index.cfm

Troubleshooting

- Commercial process controls will frequently have dimmer than normal CD3 staining. Since the attractors are defined based on staining patterns observed with fresh whole human blood, the $CD3^+$ attractor might need to be adjusted on all tubes of a multitube panel. Verify that each subset is properly classified. See Figures 1a, 1b, and 1c. Note the inadequate resolution between the $CD3^+$ and $CD3^-$ lymphocytes in Figure 1b.

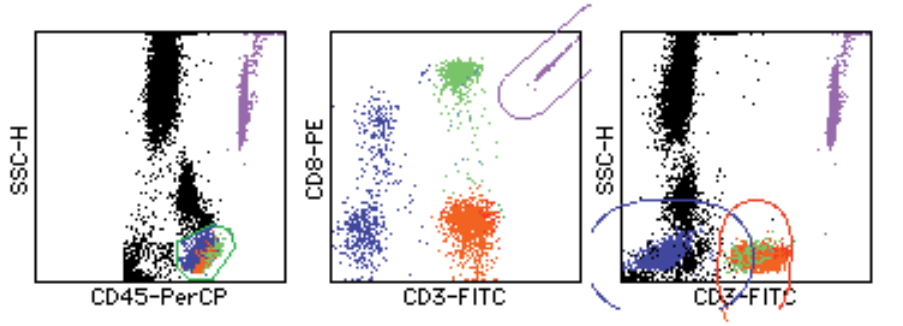


Figure 1a Fresh whole blood sample showing adequate resolution between the $CD3^+$ and $CD3^-$ lymphocytes

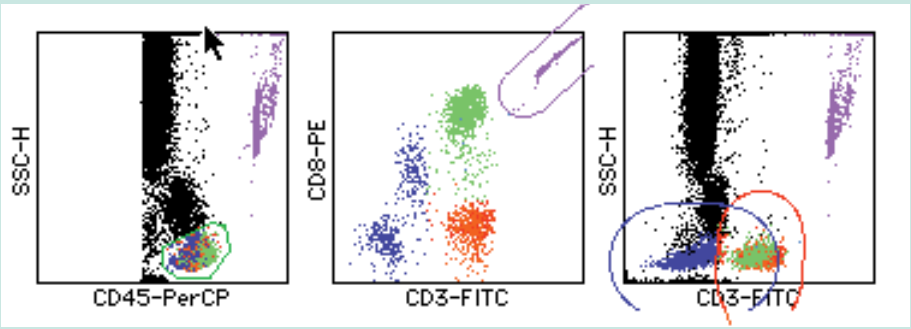


Figure 1b Preserved process control *before* the $CD3^+$ attractor correction

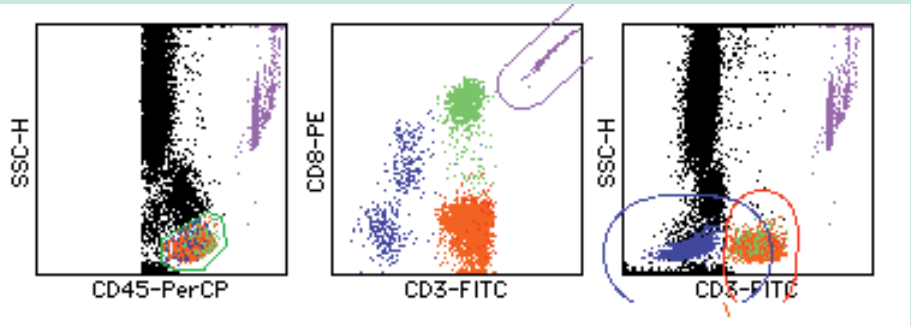


Figure 1c Preserved process control *after* the $CD3^+$ attractor correction

- In lipemic specimens, the lymphocytes, monocytes, and granulocytes appear compressed along the SSC axis. It is extremely difficult to re-gate such samples as the valley between the lymphocytes and monocytes can disappear. See Figure 2. The recommended remedy is to wash the sample twice in PBS with 0.1% azide before staining. In these instances, only percentages can be reported unless a validation of this wash procedure has been done.

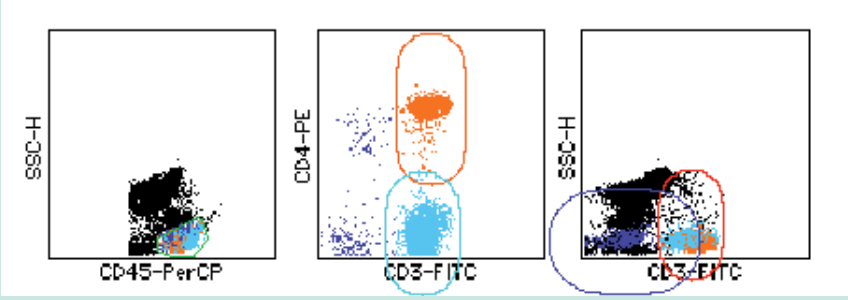


Figure 2 MultiSET analysis of a lipemic specimen showing SSC compression

- Specimens demonstrating a relative monocytosis can be inaccurately gated. The inclusion of monocytes in the lymphocyte gate is determined by the presence of $CD3^{-}CD4^{+}$ cells since monocytes express CD4. It is important to inspect all of the plots on the Laboratory Report to verify the accuracy of data. See Figure 3.

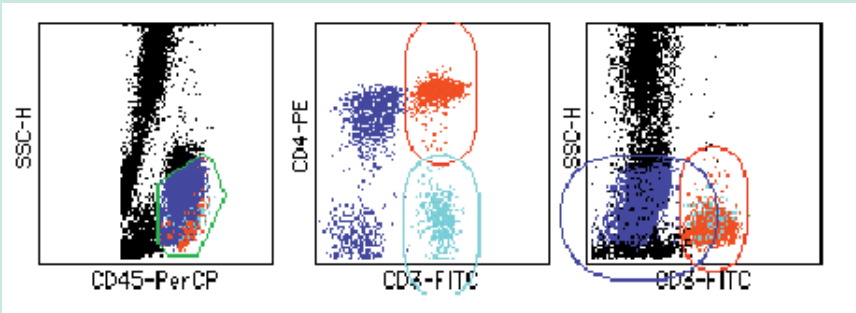


Figure 3 Inclusion of monocytes using MultiSET software

- Do not perform MultiSET analysis on specimens from patients receiving antigen-binding immunotherapeutic agents, such as OKT3, unless you are using a user-defined MultiSET Reagent file that does not contain a gate for the antigen. These drugs will hinder antigen labeling by the fluorochrome-conjugated antibody, resulting in either minimal or no positive staining. See Figure 4. These types of specimens can be labeled with alternative antibodies such as Anti–TCR α/β, and analyzed in CellQuest™ or CellQuest Pro software.

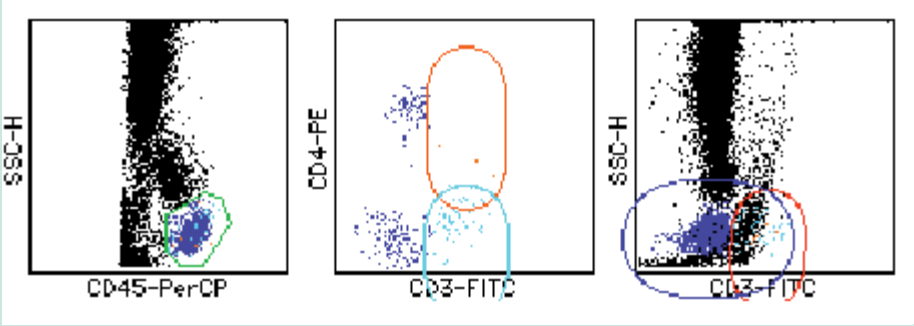


Figure 4 Inhibition of CD3 staining due to OKT3 treatment

* For In Vitro Diagnostic Use.

The following products are covered by the listed patents held or licensed by BD Biosciences.

PE	US Patent Nos. 4,520,110; 4,859,582; 5055,556; European Patent No. 76,695; Canadian Patent No. 1,179,942
PerCP	US Patent No. 4,876,190
Cy5.5	US Patent Nos. 5,268,486; 5,486,616; 5,569,587; 5,569,766; and 5,627,027
FACS Lysing Solution	US Patent Nos. 4,654,312; 4,902,613; and 5,098,849

WEB LINKS

Conference on Guidelines for Laboratories Performing HIV-related CD4 T-Cell Determinations
http://www.phppo.cdc.gov/mlp/cd4.asp

Upcoming Courses and Workshops 2002

Workshops	Intracellular Cytokine Detection	Techniques in Cell-Cycle Analysis	CellQuest	Sorting Review and Options FACSVantage SE
Course Content	• In vitro activation of whole blood • Cell permeabilization and labeling techniques • Flow cytometric analysis of cytokine-producing lymphocyte subsets • Troubleshooting data and sample preparation	• Paraffin-embedded tissue for single-color DNA analysis • BrdU-labeled cells for two-color DNA analysis • Cells for two-, three- or four-color DNA analysis	• Data acquisition menu features • Data analysis using one- and two-parameter plots • Logical gating strategies • Histogram overlays • Design of Experiment Documents • Annotation features • Exporting data	• Sorting theory including drop-delay optimization, sort modes, and sterile sort setup • Sorting options including TurboSort Option™, large-particle sorting using MacroSORT™ Plus, sorting into wells, or onto slides using CloneCyt™ Plus software. Because this course is customized for participants, the sorting options covered can vary.
Prerequisites	A basic understanding of immunology and completion of an operator course, or equivalent experience that includes data acquisition and analysis using CellQuest software	Completion of an operator course, or equivalent experience that includes proficiency with CellQuest and ModFit™ *LT* software	Basic Macintosh® skills and a basic understanding of flow cytometry, and the analysis of flow cytometry data	Completion of a FACS Vantage™ SE operator course, or equivalent experience
Who Should Attend	Users proficient in FACSCalibur/ FACScan™/FACSort™ operation and who want to gain ex- perience in preparing and analyzing specimens used in studying immune function	Users proficient in FACSCalibur/FACScan/ FACSort operation and those who want to expand their repertoire of DNA analysis techniques	Users who have upgraded to FACStation™, users who deal mainly with data analysis rather than cytometer operation, or users who are familiar with CellQuest but require more in-depth training	Users who have a working knowledge of the FACS Vantage SE instrument and who want to obtain more experience with cell sorting.
Duration	1 day	2 days Contact hours [a] - 13	1 day	2 days
Schedule	Feb 28, Mansfield, MA May 16, San Jose, CA	Feb 26–27, Mansfield, MA May 14–15, San Jose, CA	Feb 25, Mansfield, MA May 13, San Jose, CA	Feb 25–26, Mansfield, MA May 13–14, San Jose, CA
Tuition	$495	$990	$495	$990

[a] BD Biosciences is approved as a provider of continuing education programs in the clinical laboratory sciences by the ASCLS P.A.C.E.

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23-5711-02