FLOW CYTOMETRY RESOURCE

ABOUT

Bangs Labs, Inc. has supplied microspheres to diagnostic companies, instrument manufacturers, and researchers in the life sciences for over 35 years. From the acquisition of Flow Cytometry Standards Corporation (FCSC) in 2000, we have had the pleasure of providing innovative products for instrument calibration, validation, QC, and standardization.

We are here to answer your questions and contribute our expertise to flow cytometry users, assay developers, and instrument manufacturers. Contact us (info@bangslabs.com) and let us put our decades of real-world experience to work for you.

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QUALITY

Bangs Laboratories' Quality Management System has been certified by NQA to comply with **ISO 13485:2016** for the design, manufacture, processing and distribution of microspheres and related products.

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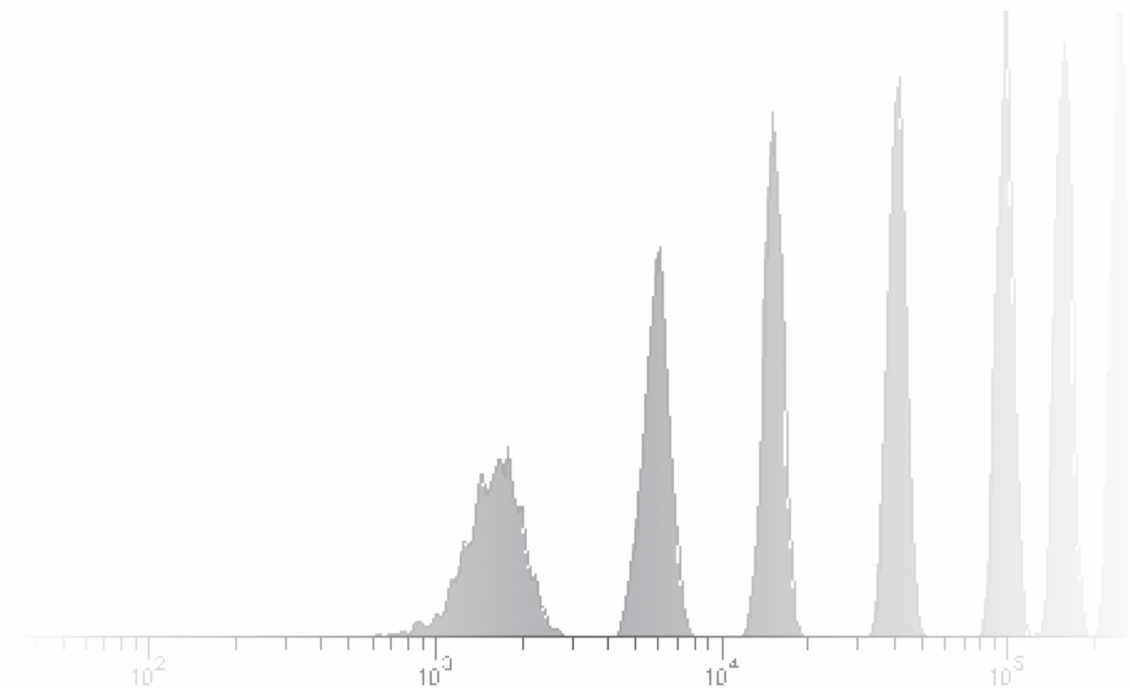


INTRODUCTION

Flow cytometry is a complex but highly informative technology that is used for lymphocyte immunophenotyping, cell cycle analysis, microvesicle analysis, phagocytosis studies, detection of apoptosis, immune cell activation studies, and gene expression analysis. Coupling flow cytometry with other methods such as microscopy, mass spectroscopy, or cell sorting capabilities expands its applications even further. So much so, that the technology is used in research centers, clinics, and spans molecular biology, pathology, immunology, virology, and many other disciplines.

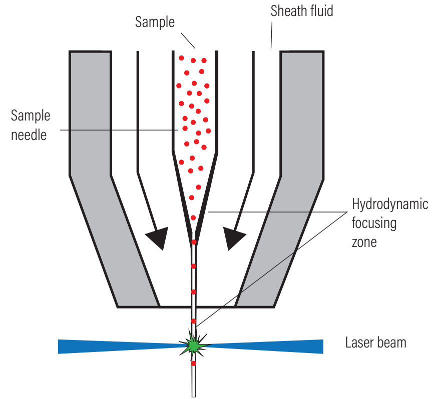


Figure 1: Alignment of particles with laser beam in the flow cell

INSTRUMENT DESIGN

The instruments that make these analyses possible house a sophisticated architecture of lasers, detectors, and fluidics that work in concert to provide detailed information about the samples that are analyzed *(Figure 1)*. Information regarding every particle that passes through the flow cell is collected, including relative size (forward scatter - FSC), internal complexity (side scatter - SSC), and fluorescence. Instruments are often equipped with 2 or more lasers and multiple detectors per laser, much like the configuration described in *Figure 2* and *Table 1*.

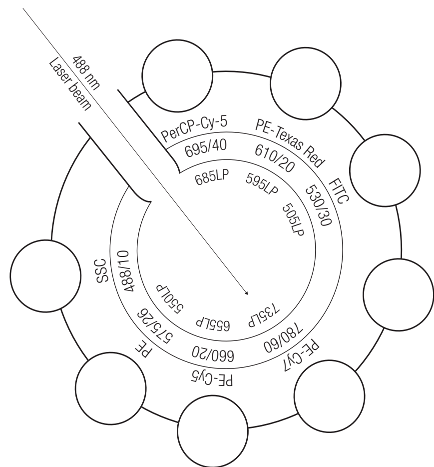


Figure 2: Concept of 488 nm optical array

Table 1: Sample configuration for a BD LSRII cytometer, including violet (405 nm) laser / detector add-on.

Laser	Detector	Dichroic mirror	Bandpass filter
405 nm (50mW)	VIO 450	-	450/50
	VIO 525	505 LP	525/50
488 nm (25mW)	SSC	-	488/10
	FITC	505 LP	530/30
	PE	550 LP	575/26
	PE-Texas Red	595 LP	610/20
	PerCP-Cy™5.5	685 LP	695/40
	PE - Cy™ 5	655 LP	660/20
	PE - Cy™ 7	735 LP	780/60
633 nm (20mW)	APC	-	660/20
	APC - Cy™7	735 LP	780/60

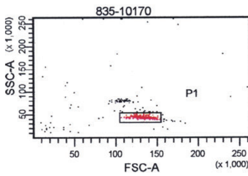
1. *Shapiro HM. (2003) Practical Flow Cytometry, Fourth Edition. John Wiley & Sons: Hoboken. (ISBN:0-471-41125-6)*

FLOW CYTOMETRIC ANALYSIS

Due to flow cytometry's utility across so many disciplines, the instruments have been improved successively year after year. There are highly expanded lists of fluorochromes, fluorescent proteins, and dyes in addition to instruments with advanced optics and electronics that increase the number of parameters that can be evaluated simultaneously.

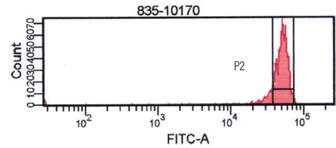
Data may be displayed in single- or multiparametric format with associated statistics, per the typical dual parameter dot plot (FSC/SSC) and single parameter fluorescence histogram in *Figure 3.*

3a. FSC / SSC dot plot with gated singlets population and associated statistics



Population	%Parent	FSC-A Median	FSC-A CV	SSC-A Median	SSC-A CV
P1	86.0	134,829	5.5	43,436	4.4

3b. Fluorescence (FITC) histogram of singlets from the FSC/SSC gate shown in 3a



Population	FITC-A Median	FITC-A CV
☒ P2	52,178	14.5

Figure 3: Single- or multiparametric flow cytometry plots

Though unstained cells will yield characteristic scatter patterns that can be readily identified in a FSC/SSC dot plot (*Figure 4a*), fluorescent reporters and stains are used individually or in combination to provide specific information about the expression of various surface or intracellular markers, metabolic state, membrane integrity, etc. In a classic immunophenotyping example, *Figure 4b* demonstrates the exclusion of granulocytes and monocytes, and the analysis of CD45 expressing lymphocytes stained with an anti-CD45-APC-Cy™7 antibody.

Figure 4a: FSC/SSC dot plot of unstained leukocytes (lysed RBC whole blood)

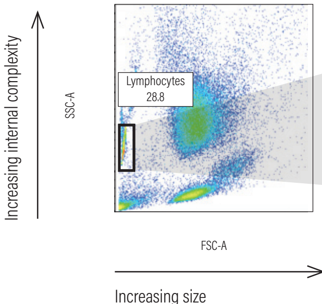


Figure 4b: SSC/APC-Cy™7 fluor dot plot of stained CD45+ lymphocytes

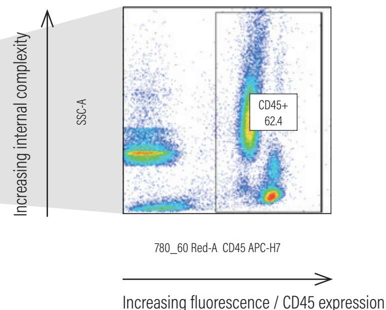


Figure 4: Fluorescent antibodies to antigens increase specificity of cell types.

INSTRUMENT QUALIFICATION & QC

Within the life sciences, there is a heavy reliance on analytical instruments to make decisions related to research, manufacturing, and clinical applications. As this is important work that demands accurate, reliable, and relevant data, instruments must be thoughtfully selected, thoroughly qualified, and have capabilities verified throughout their active use. Qualification is a comprehensive process that is undertaken to ensure that each instrument meets expected capabilities and is suited to its intended use. It features thorough performance tests, which upon completion, will serve as a foundation for ongoing instrument QC and proficiency programs.

Following qualification, the instrument QC program is intended to provide an accurate picture of instrument status and provide confidence in resulting data. Specific QC tests should be relevant in type and frequency to the work being performed, and the maintenance and service history should also be considered. If certain components or subsystems have been shown to be less stable, these may warrant more rigorous surveillance.

Each day should begin with a general system check that provides an indication that subsystems and components are functioning. Additional tests should then be performed to address the specific use of the instrument.

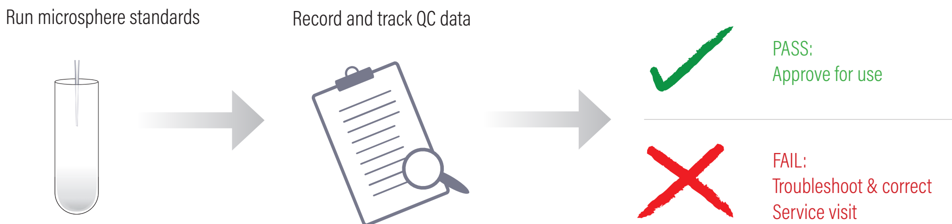


Figure 5: QC process

Table 2: Example of a basic QC program for a 2 laser cytometer

Frequency	Product & Catalog Code	Purpose	Coverage	Data
Daily	Full Spectrum (#885) or Quantum QC (#725)	Basic check of system; Laser alignment check	All lasers / detectors	Chart channel values; Record CVs
Daily for quantitative	Quantum MESF *(See page 14)*	Run at specific PMTs for quantitative expression analyses: Linearity, resolution, detection threshold, alignment	Specific detector	Confirm resolution; Record Linearity; Chart detection threshold and CV
Daily for quantitative; or Weekly	Quantum QC (#725)	For qualitative analyses; Linearity, resolution, detection threshold, alignment	All lasers/ detectors	Confirm resolution; Record Linearity; Chart detection threshold and CV
Weekly	Time Delay Standard (#830)	Time delay check	Delay between laser 1 (488nm) and laser 2 (635nm)	Confirm time delay

A basic program like the example in *Table 2* ensures surveillance of the complete system, i.e. the optics (lasers, detectors, flow cell alignment), fluidics (observation of flow rates, time delay confirmation), and associated computing. Recording values for certain parameters in Levey Jennings charts can readily confirm satisfactory performance, or aid in identifying both random errors (electronic noise, air bubbles, etc.), and systemic errors (bias, shifts and trends due to temperature fluctuation, laser deterioration, misalignment, etc.) so that corrective action may be taken. Thresholds may be developed for watchful monitoring (A) or intervention (B), *Figure 6*.

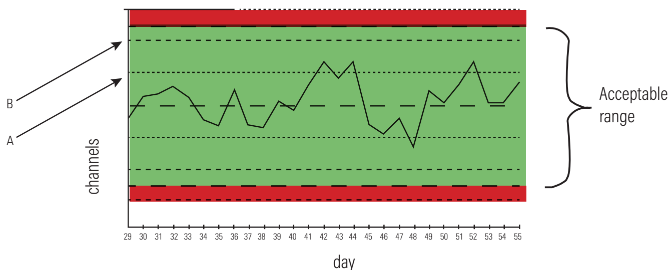


Figure 6: Sample Levey Jennings chart for a single fluorescence channel

1. *Green, CL, Brown, L, Stewart, JJ, Xu, Y, Litwin, V, & Mc Closkey, TW. (2011). Recommendations for the validation of flow cytometric testing during drug development: I instrumentation. J Immunol Methods, 363(2), 104-119. https://doi.org/10.1016/j.jim.2010.07.004*

2. *Perfetto, SP, Ambrozak, D, Nguyen, R, Chattopadhyay, PK, & Roederer, M. (2012). Quality assurance for polychromatic flow cytometry using a suite of calibration beads. Nat Protoc, 7(12), 2067-2079. https://doi.org/10.1038/nprot.2012.126*

3. *Turner, KL. Instrument Qualification, QC and Standardization. The Latex Course, September 2012.*

4. *United States Pharmacopeia, Chapter <1058>, Analytical Instrument Qualification, Rockville, USA, 2008.*

STANDARDIZED INSTRUMENT SET-UP

While the extremely sensitive nature of flow cytometers permits the analysis of micron-scale (or smaller) and dimly labeled fluorescent particles, it also makes them sensitive to even the most subtle changes in cell samples, instrument operation, and the laboratory environment. For these reasons, it is imperative that instrument configuration and operating conditions be standardized as much as possible, and that suitable reference materials are used for tests and assays.

The use of reference beads can ameliorate differences in range, relative scale, and reporting units, as well as daily fluctuation due to electronic noise, ambient temperature, and humidity. For example, Quantum™ QC may be used to set up all detectors by positioning a specific peak at a relevant target channel value *(Figure 7)*.

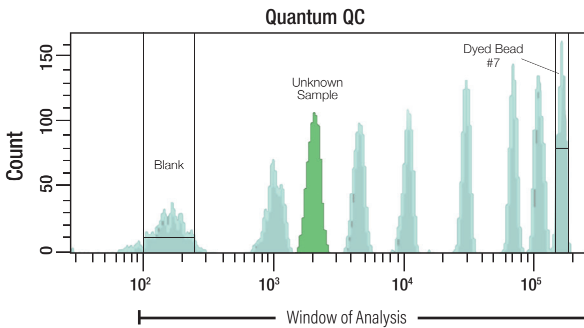


Figure 7: Use Quantum™ QC to define window of analysis i.e. upper & lower fluorescence limits

Table 3: Products for Instrument Set Up

Catalog #	PDS	Name	# beads	Fluorescence
725	725	Quantum™ QC	8	Full spectrum + Blank
885	885	Full Spectrum™	1	Full spectrum
512, 515, 518, 521	510	Right Reference Standards	1-3	FITC, PE, PE-Cy™5, or APC
See Table 5	890	Fluorescence Reference Standards	1	*See Page 13*

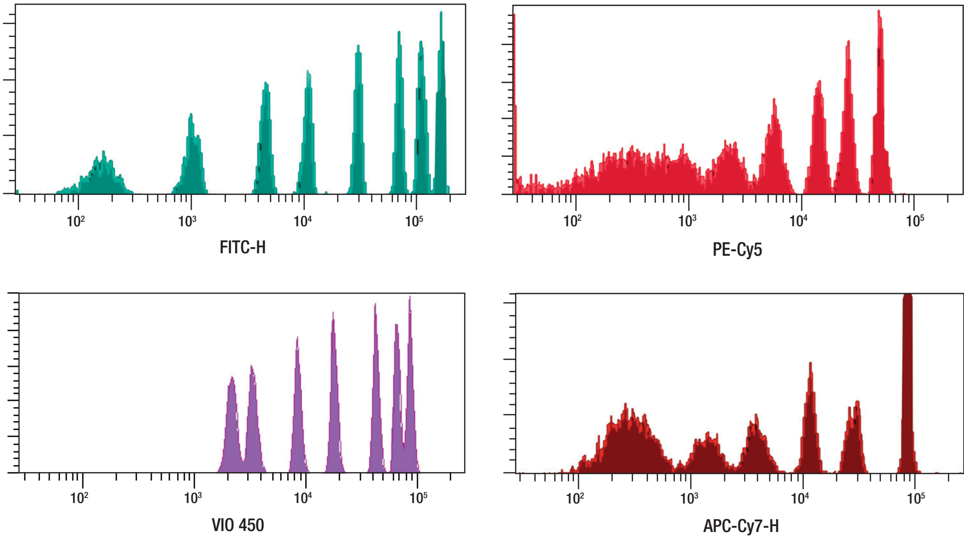


Figure 8: Quantum™ QC Histograms

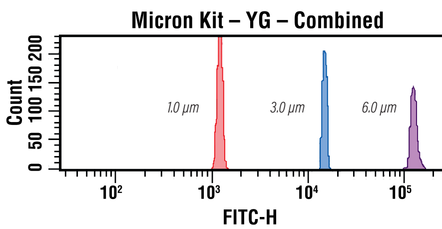
1. Purvis, N, & Stelzer, G. (1998). Multi-platform, multi-site instrumentation and reagent standardization. Cytometry, 33(2), 156-165. https://www.ncbi.nlm.nih.gov/pubmed/9773876

SEE COMPENSATION SECTION (PG 10)
FOR SET-UP RELATED TO COMPENSATION

INSTRUMENT SET-UP: SMALL PARTICLES

Current applications in flow cytometry extend beyond the analysis of lymphocytes and push cytometers to their limits of detection for particle size and fluorescence. Small particle analyses, including platelet and endothelial-derived microparticles, exosomes, microvesicles, or microbial species, require modified processes and specialized instrument set-up. Our fluorescent small bead calibration kits *(Figure 9, 10, and 11)* can aid in:

- Determining an instrument's limit of size detection
- Assessing background particulates and developing modified preparatory processes (e.g. fluid filtration)
- Small particle size calibration
- Refining instrument settings (threshold, PMT, windows extension)



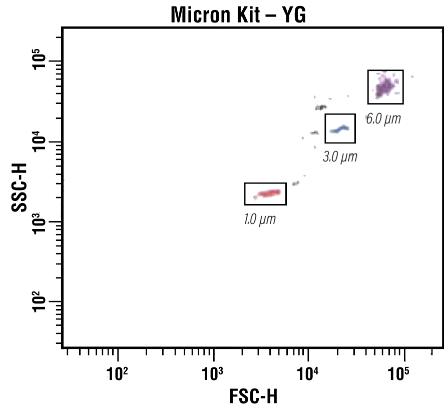
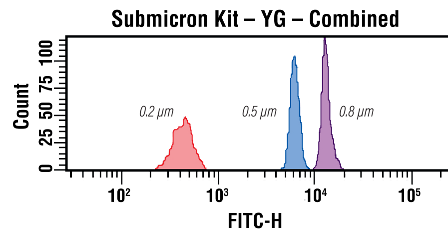


Figure 9: Micron Bead Calibration Kit - LSRII settings
FSC log 536 - Threshold 200
SSC log 247 - Threshold 200
FITC log 346 - Threshold 200



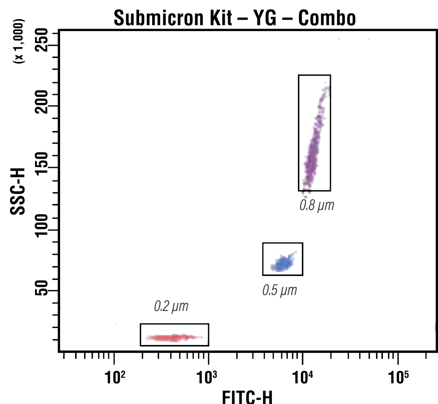


Figure 10: Submicron Bead Calibration Kit - LSRII settings
FSC log 500 - Threshold 200
SSC log 494 - Threshold 200
FITC log 587 - Threshold 200

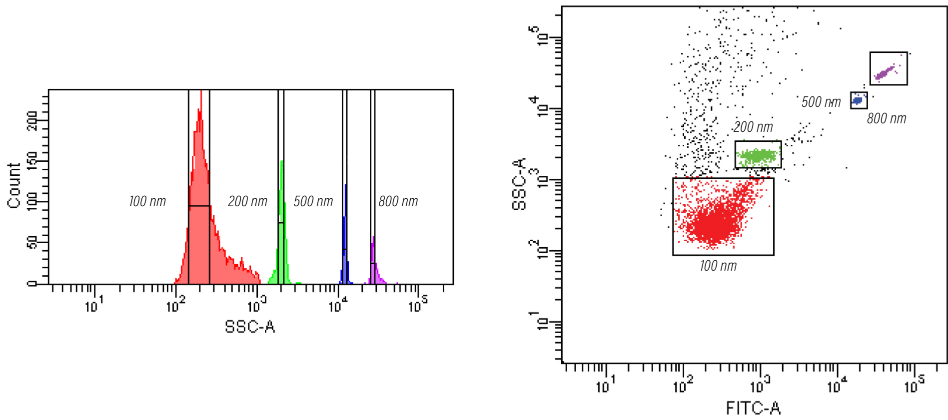


Figure 11: Nanobead (100 nm) & submicron bead calibration kits- BD FACSCanto II settings
SSC log 500 - Threshold 200
FITC log 650 - Threshold 300
Windows Ext. 2.0 Events: 5000

Table 4: Small Bead Calibration Kits

Catalog #	PDS	Name	Nominal Diameters
833	832	Micron Bead Calibration Kit	1.0 µm, 3.0 µm, 6.0 µm
832	832	Submicron Bead Calibration Kit	0.2 µm, 0.5 µm, 0.8 µm
834	834	Nanobead Calibration Kit	50 nm, 100 nm

1. Arraud, N, Gounou, C, Turpin, D, & Brisson, AR. (2016). Fluorescence triggering: A general strategy for enumerating and phenotyping extracellular vesicles by flow cytometry. Cytometry A, 89(2), 184-195. https://doi.org/10.1002/cyto.a.22669

2. Kong, F, Zhang, L, Wang, H, Yuan, G, Guo, A, Li, Q, & Chen, Z. (2015). Impact of collection, isolation and storage methodology of circulating microvesicles on flow cytometric analysis. Exp Ther Med, 10(6), 2093-2101. https://doi.org/10.3892/etm.2015.2780

INSTRUMENT SET-UP: COMPENSATION

Due to cytometer sensitivity, differing filter sets, and broad emission bands from fluorophores, fluorescence typically spills over into regions beyond that covered by the intended detector *(Figure 12)*. The most pronounced carryover tends to be into longer wavelengths (i.e. is red-shifted), though it can often be observed to a lesser extent at shorter wavelengths.

Multicolor analyses necessitate the correction of spectral overlap for each fluorochrome and detector. Compensation is performed by electronically subtracting the percentage of fluorescence signal that is equivalent to the carryover.

Proper compensation requires reference materials that represent the *actual* fluorophore combinations of stained cells. Bangs offers both fluorophore-matched microspheres and microspheres with capture antibodies or functional groups for labeling with reactive fluorophores or fluorescent antibody conjugates. *Figure 13* illustrates the use of microsphere standards to develop a compensation matrix.

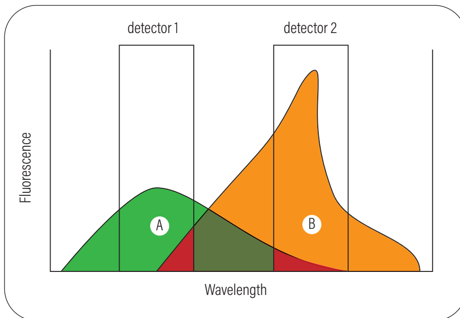


Figure 12: Fluorescence carryover

Using compensation, carryover fluorescence is electronically "subtracted" from unintended detectors so that the measured signal only represents the targeted fluorophore. This figure illustrates the carryover of Fluorophore A into the Fluorophore B detector, as well as the carryover from Fluor B into the Fluor A detector. A compensation matrix might be:

Fluor A – 2% Fluor B

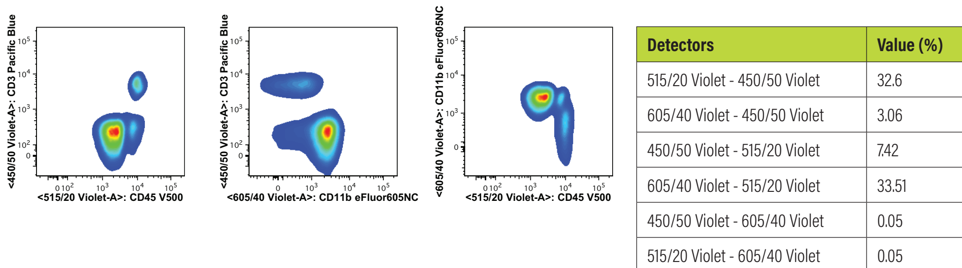
Fluor B – 1% Fluor A

Table 5: Compensation Standard Products

Catalog #	PDS	Product	Populations
820	820	FITC/PE Compensation Standard	4 populations (1 blank, 1, FITC, 1 PE, and 1 FITC/PE)
See pg. 13	890	Fluorescence Reference Standards	Single-fluorophore populations
550-552, 556	850	Simply Cellular® Compensation Standards (anti-mouse, rat, or human IgG)	1 or 2 populations (high binding capacity and/or low binding capacity)
835	835, 850	Simply Cellular® anti-Mouse for Violet Laser	1 blank and 1 high binding capacity
553-554	854	Protein A, Protein G Antibody Binding Beads	Single populations
450-451	853	Viability Dye Compensation Standards	Single population for amine-reactive fluorophores

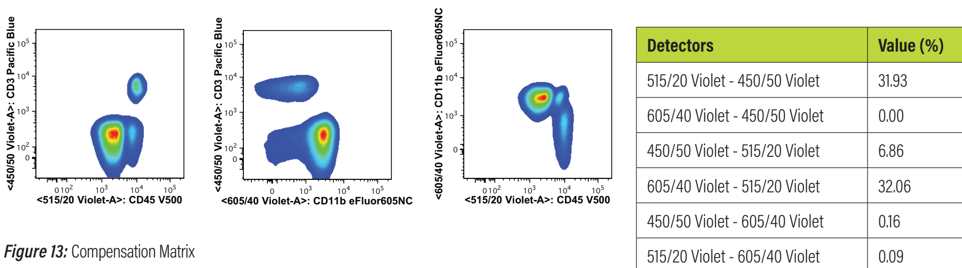
Simply Cellular® anti-Mouse IgG Bead for Violet Laser produces comparable data when compared to cells.

Compensated with: Stained Violet Beads



Detectors	Value (%)
515/20 Violet - 450/50 Violet	32.6
605/40 Violet - 450/50 Violet	3.06
450/50 Violet - 515/20 Violet	7.42
605/40 Violet - 515/20 Violet	33.51
450/50 Violet - 605/40 Violet	0.05
515/20 Violet - 605/40 Violet	0.05

Compensated with: Stained Cells



Detectors	Value (%)
515/20 Violet - 450/50 Violet	31.93
605/40 Violet - 450/50 Violet	0.00
450/50 Violet - 515/20 Violet	6.86
605/40 Violet - 515/20 Violet	32.06
450/50 Violet - 605/40 Violet	0.16
515/20 Violet - 605/40 Violet	0.09

Figure 13: Compensation Matrix

1. *Perfetto, SP, Chattopadhyay, PK, Lamoreaux, L, Nguyen, R, Ambrozak, D, Koup, RA, & Roederer, M. (2006). Amine reactive dyes: An effective tool to discriminate live and dead cells in polychromatic flow cytometry. J Immunol Methods, 313(1-2), 199-208. https://doi.org/10.1016/j.jim.2006.04.007*

2. *Turner, K, Isaiah, S, Schretzenmair, R, Tijerina, J, Bantly, A. (2011) Novel compensation standard for the violet laser. CYTO, Baltimore, MD, May 21-25, 2011. (www.bangslabs.com)*

STANDARDIZED SAMPLE PREP

Immunophenotyping identifies specific immune cell populations by surface and/or intracellular markers. The technique has evolved from basic research applications to clinical applications that diagnose, monitor, and identify minimal residual disease (MRD) states. Sample preparation for immunophenotyping varies depending upon the application, yet the basic steps include:

1. Collecting a sample of fluid (blood, spinal fluid, etc.) or tissue.
2. (optional) Depleting or enriching the sample using density centrifugation, RBC lysis, magnetic particle isolation, etc.
3. Fixation and/or permeabilization (for intracellular markers).
4. Staining with fluorescently labeled antibodies.

Though the specific steps may be routine, sample preparation should be thoughtfully designed and standardized as cellular processes, expression of certain markers, cell viability, microvesicle counts, and size distribution may be sensitive to temperature, fixatives, lysing agents, etc. Changes in reagents, handling, or storage conditions may result in alterations in samples and resulting data.

As an additional note on sample preparation, flurophore selection is an important factor. Markers with low abundance should be labeled with bright fluorochromes, and those that express at high levels should be labeled with dimmer reporters. Consideration should also be given to the size of the fluorescent reporter in the context of potential steric effects (e.g. PE MW 260,000; FITC MW 389), stability, nonspecific binding, and spectral overlap. *(See Table 6)*

1. Aasebo, E, Mjaavatten, O, Vaudel, M, Farag, Y, Selheim, F, Berven, F, Bruserud, O, & Hernandez-Valladares, M. (2016). Freezing effects on the acute myeloid leukemia cell proteome and phosphoproteome revealed using optimal quantitative workflows. J Proteomics, 145, 214-225. https://doi.org/10.1016/j.jprot.2016.03.049

2. Stewart, JC, Villasmil, ML, & Frampton, MW. (2007). Changes in fluorescence intensity of selected leukocyte surface markers following fixation. Cytometry A, 71(6), 379-385. https://doi.org/10.1002/cyto.a.20392

3. Carter, PH, Resto-Ruiz, S, Washington, GC, Ethridge, S, Palini, A, Vogt, R, Waxdal, M, Fleisher, T, Noguchi, PD, & Marti, GE. (1992). Flow cytometric analysis of whole blood lysis, three anticoagulants, and five cell preparations. Cytometry, 13(1), 68-74. https://doi.org/10.1002/cyto.990130111

4. Kong, F, Zhang, L, Wang, H, Yuan, G, Guo, A, Li, Q, & Chen, Z. (2015). Impact of collection, isolation and storage methodology of circulating microvesicles on flow cytometric analysis. Exp Ther Med, 10(6), 2093-2101. https://doi.org/10.3892/etm.2015.2780

5. Panel design. BD Biosciences. Retrieved 11/21/2024 from https://www.bdbiosciences.com/en-us/resources/panel-design?tab=Overview

FLUOROPHORE MOLECULAR WEIGHTS & EXCITATION/EMISSION WAVELENGTHS

Single-color Fluorescence Reference Standards are labeled with specific fluorochromes to exhibit the same spectral characteristics as labeled cells. They may be used to QC a specific path of the optical system, to optimize filter sets for fluorophores, and to establish a test-specific Target Channel Value for instrument set-up.

Visible Spectrum

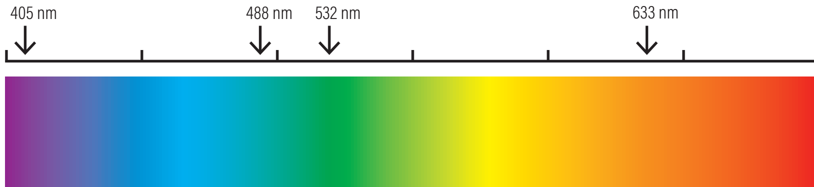


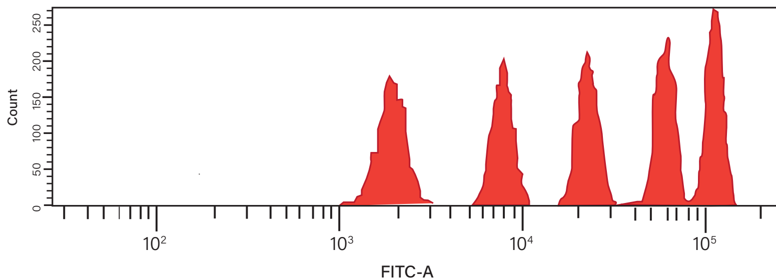
Table 6: Fluorescence Reference Spectrum Products

Catalog #	Description	MW	Excitation (nm)	Emission (nm)	Purpose
890	Certified Blank™				reference
897	Acridine Orange	265	500	526	DNA/RNA
886	Alexa Fluor® 488	643	499	519	conjugate
887	Alexa Fluor® 647	1300	652	668	conjugate
901	Allophycocyanine (APC)	104k	650	660	conjugate
898	Chlorophyll (*a* + *b*)	8014 (a) 907 (b)	430,453	642,662	plant pigment
895	Cy™5	792	649	666	conjugate
906	DAPI	277	350	470	DNA (A-T)
913	Far-Out Red	-	475,590	663	reference
891	Fluorescein	389	495	519	conjugate
894	Hoechst 33342	616	346	375,390	dsDNA
916	Pacific Blue™	339	410	455	conjugate
899	PE (R-phycoerythrin)	240k	480, 565	578	conjugate
908	PE-Cy™5	240k	480,565,650	670	conjugate
892	Propidium Iodide	668	536	617	DNA intercalator
905	T.M. Rhodamine (TRITC, TAMRA)	430	557	576	conjugate
893	Texas Red® (Sulforhodamine)	625	589	615	conjugate
915	Violet Laser (Glacial Blue)	-	360	450	reference

QUALITATIVE & QUANTITATIVE ASSAYS

Many immunophenotyping assays are qualitative in nature. For these types of studies, cells are stained for a certain marker, and the shift over an unstained population is used to determine relative expression (low, mid, high) or presence of the marker in general (positivity). In these types of studies, bead standards can be used to define the window of analysis, and to serve as reference points for a comparison of results. *(see pg. 6-7)*

Some applications require true quantitation of cell surface markers or intracellular proteins. Flow cytometry has become the gold standard for quantification of CAR-T cells and other adoptive T cell therapies, where it is used for evaluating delivery and persistence during treatments. For these types of expression studies, kits such as Quantum™ MESF and Quantum™ Simply Cellular® (QSC) permit the quantitation of fluorescence signal, and by extension, determination of antibody binding to the surface marker or expressed protein *(Figure 14)*. More information on qualitative and quantitative assays can be found in the support section of www.bangslabs.com, under Flow Cytometry Resources.



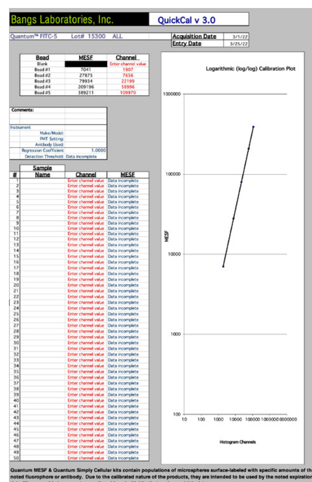


Figure 14: Quantum MESF histogram and QuickCal analysis template

Table 7: Quantitative Cytometry Products

Catalog #	Description	Fluorophore	MW
488	Quantum MESF Alexa Fluor® 488	Alexa Fluor® 488	643
555, 555p	Quantum MESF FITC-5	FITC	389
827	Quantum MESF PE	R-PE	240k
647	Quantum MESF Alexa Fluor® 647	Alexa Fluor® 647	1300
823	Quantum MESF APC	APC	104k
Catalog #	**Description**	**Capture Antibody**	**Binds**
815	QSC anti-Mouse IgG (Fc)	anti-Mouse IgG (Fc-specific)	Mouse mAb (Fc)
816	QSC anti-Human IgG (Fc)	anti-Human IgG (Fc-specific)	Human mAb (Fc)
817	QSC anti-Rat IgG (Fc)	anti-Rat IgG (Fc-specific)	Rat mAb (Fc)

1. Maecker, HT, & Trotter, J. (2006). Flow cytometry controls, instrument setup, and the determination of positivity. Cytometry A, 69(9), 1037-1042. https://doi.org/10.1002/cyto.a.20333

2. Randlev, B, Huang, LC, Watatsu, M, Marcus, M, Lin, A, & Shih, SJ. (2010). Validation of a quantitative flow cytometer assay for monitoring her-2/neu expression level in cell-based cancer immunotherapy products. Biologicals, 38(2), 249-259. https://doi.org/10.1016/j.biologicals.2009.12.001

NOTES

Some other products that may be of interest:

Cell Cycle Analysis

Microparticle Analysis

Size Estimation

Imaging Standards

Cell Viability Standards

Concentration Standards

Particulate Contamination Standards

Let us know how we can help.

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