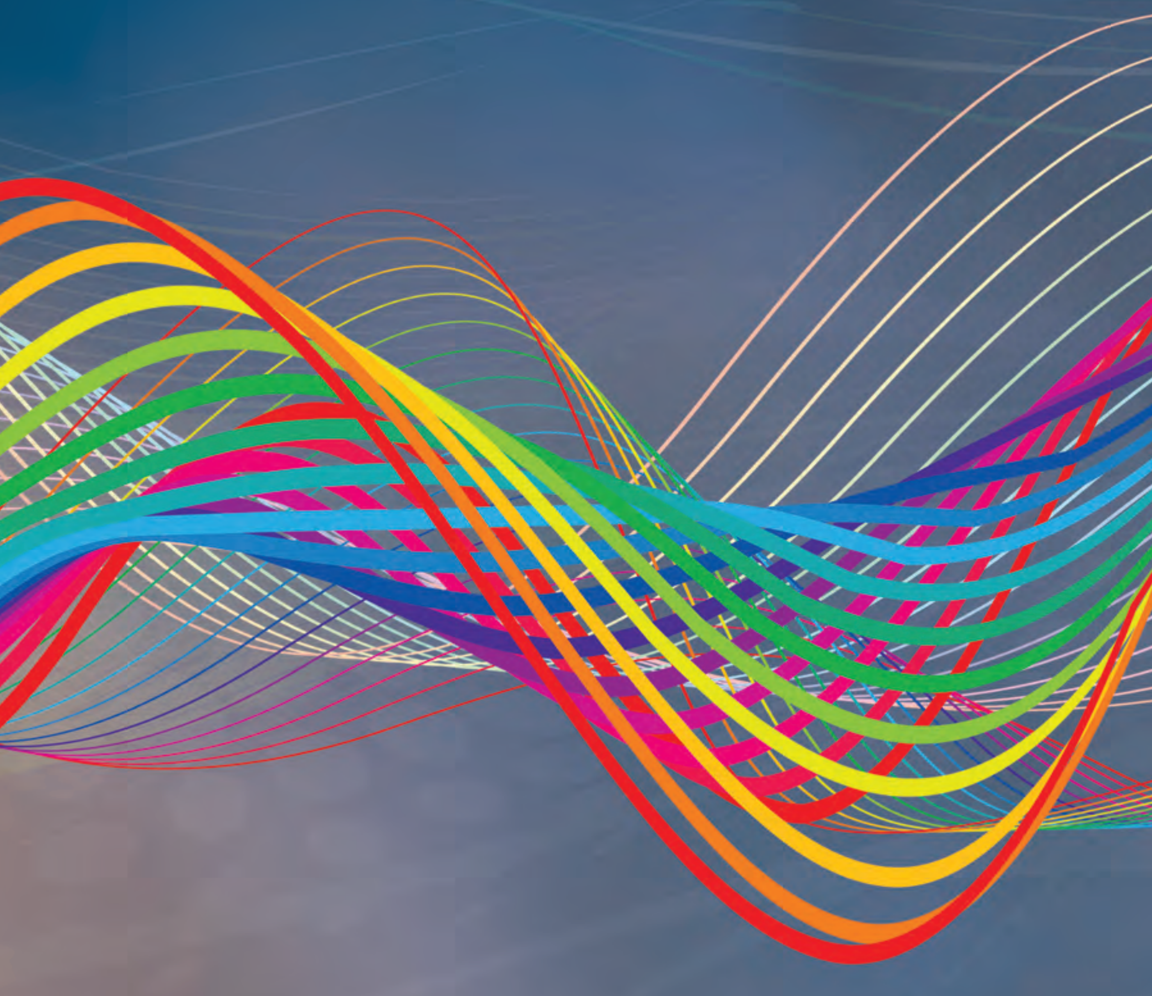
Polysciences, Inc.

FLOW CYTOMETRY RESOURCE GUIDE



ABOUT POLYSCIENCES INC.

Polysciences, Inc. is a world leader in the development of particle-based solutions for diagnostics, bioprocessing and instrument standardization. Our capabilities in life sciences, coupled with our expertise in specialty chemicals and electronics, allow us to address the unique requirements of advanced applications in medical devices, biosensors and nanotechnology. We are your source for organic, inorganic, biodegradable, magnetic, fluorescent, dyed and specific antibody and general protein coated particles.

CORPORATE LOCATIONS

Our U.S. corporate headquarters and main manufacturing facilities are situated on 7 acres with 50,000 square feet of space located about 20 miles north of Philadelphia, as well as corporate locations around the world. We are ready to meet your global needs.

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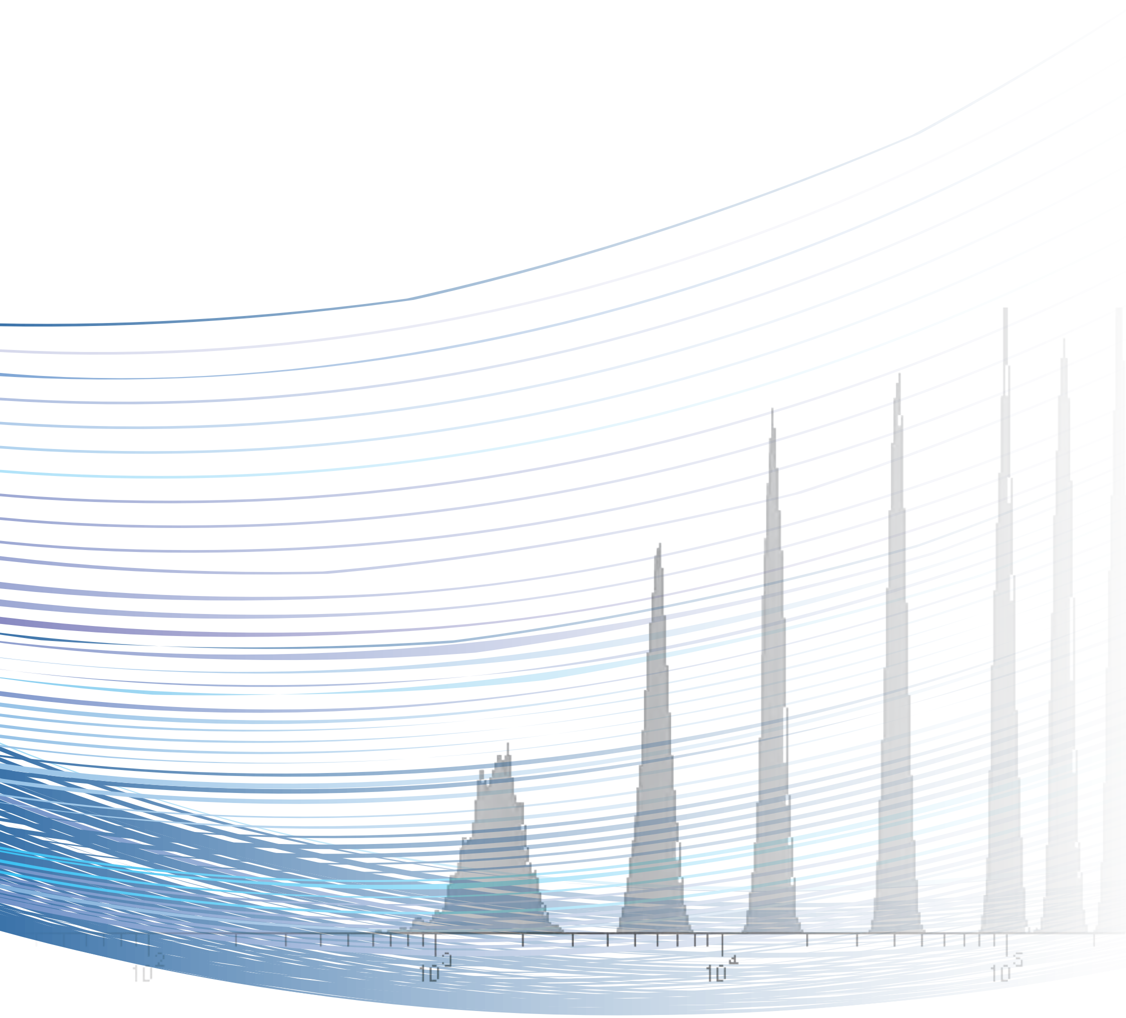
ISO CERTIFICATIONS

Polysciences has been certified by American Management Technology, Inc. as having demonstrated that our Quality Management System complies with the requirements of both **ISO 9001:2008** and **ISO 13485:2003** for the manufacture, processing, and distribution of microspheres and related products.



Flow cytometry is a complex but highly informative technology that permits evaluation of cells, subcellular compartments / organelles and microparticles.

The instruments that make these analyses possible house a complex architecture of lasers, detectors and fluidics that work in concert to provide detailed information about the samples that are analyzed. Information regarding every particle that passes 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 2+ detectors per laser, much like the configuration described in *Table 1.*

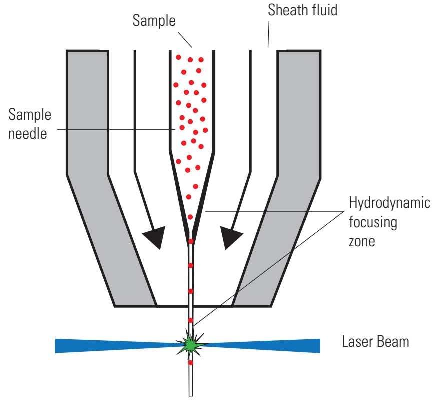


Figure 1: Alignment of particles with laser beam in the flow cell.

Laser	Detector	Dichoric mirror	Bandpass filter
405nm (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-TR	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 (20mW)	APC	-	660/20
	APC - CY™7	735 LP	780/60

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

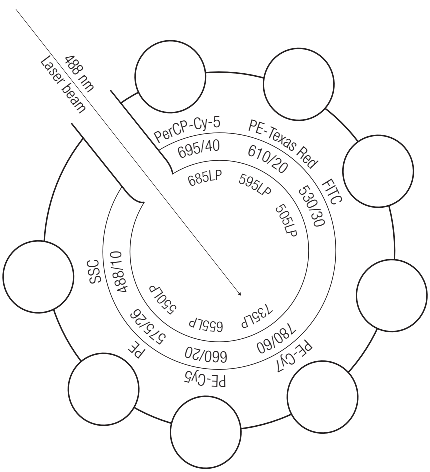


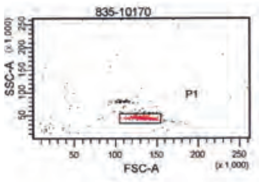
Figure 2: Concept of 488nm optical array

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

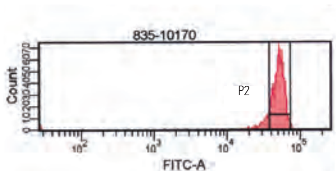
Data may be displayed in single- or multiparametric format with associated statistics, per the typical dual parameter dot plot (FSC / SSC) and single parameter fluorescence histogram in *Figure 3.*

Figure 3:

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



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



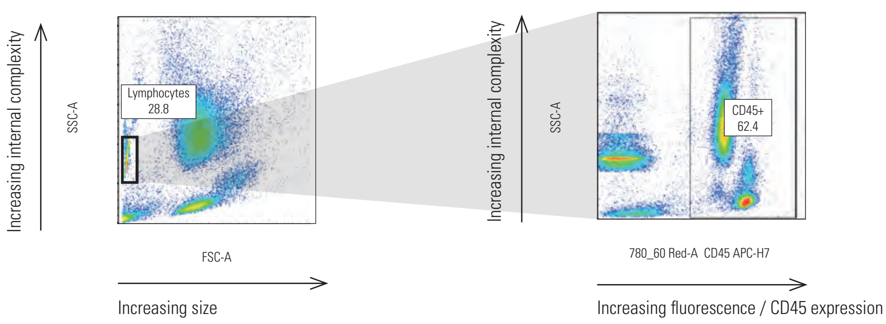
Population	%Parent	FSC-A Median	FSC-A CV	SSC-A Median	SSC-A CV
P1	86.0	134,829	5.5	43,436	4.4

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

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 Ab.

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.



Within the life sciences, there is a heavy reliance on analytical instruments to make decisions related to research, manufacturing and, for clinical applications, patient care. 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 lives. 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. In particular, more stringent QC is required for quantitative assays.

Figure 5: QC process

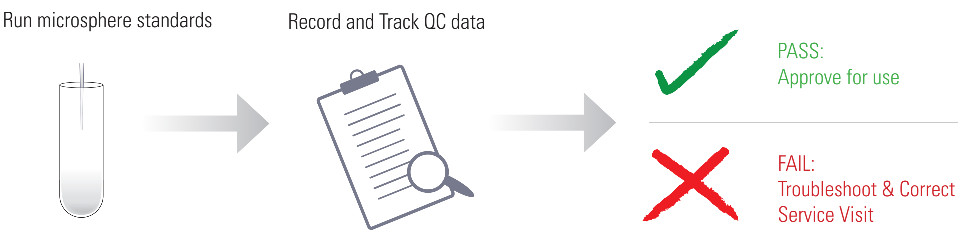
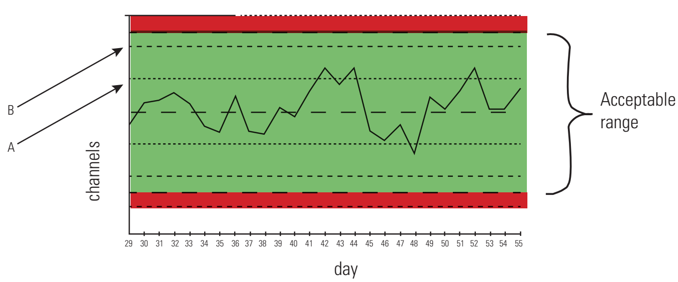


Figure 6: Example of a basic QC program for a 2 laser cytometer

Frequency	Product & Catalog Code	Purpose	Coverage	Data
Daily	Full Spectrum (#BLI885) or Quantum QC (#BLI725*)*	Basic check of system; Laser alignment check	All lasers / detectors	Chart channel values; Record CVs
Daily for quantitative	Quantum MESF *(See page 10)*	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 (#BLI725)	For qualitative analyses; Linearity, resolution, detection threshold, alignment	All lasers/ detectors	Confirm resolution; Record Linearity; Chart detection threshold and CV
Weekly	Time Delay Standard (#BLI830)	Time delay check	Delay between laser 1 (488nm) and laser 2 (635nm)	Confirm time delay

A basic program like the example in *Figure 7* 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 7.*

Figure 7: Sample Levey Jennings chart for a single fluorescence channel



1. *Green CL, Brown L, Stewart JJ, Xu Y, Litwin V, McCloskey TW. (2011) Recommendations for the validation of flow cytometric testing during drug development: I instrumentation. J Immunol Methods; 363(2):104-119.*

2. *Perfetto SP, Ambrozak D, Nguyen R, Chattopadhyay P, Roederer M. (2012) Quality assurance for polychromatic flow cytometry using a suite of calibration beads. Nature Protocols; 7(12):2067-2079.*

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.*

While the extremely sensitive nature of flow cytometers permits the analysis of micron-scale (or smaller) and dimly 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, and ambient temperature and humidity. As one example, Quantum™ QC may be used to set up all detectors by positioning a specific peak at a relevant target channel value.

Figure 8: Use Quantum™ QC to define window of analysis i.e. upper & lower fluorescence limits

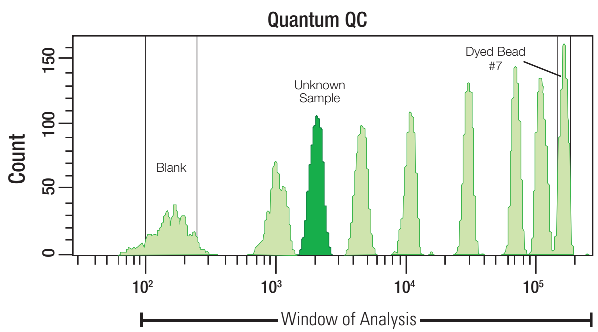
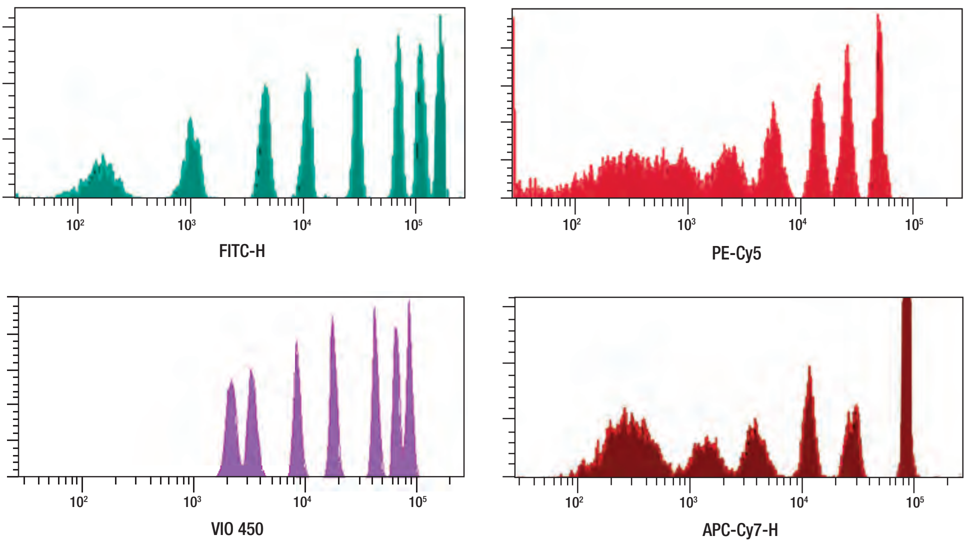


Table 2: Products for Instrument Set Up

Catalog Code	PDS	Name	# beads	Fluorescence
BLI725	725	Quantum™ QC	8	Full spectrum + Blank
BLI885	885	Full Spectrum™	1	Full spectrum
BLI512, 515, 518, 521	510	Right Reference Standards	1-3	FITC, PE, PE-Cy™5, or APC
See Table 3	892	Fluorescence Reference Standards	1	See Page 7

Figure 9: Quantum™ QC Histograms



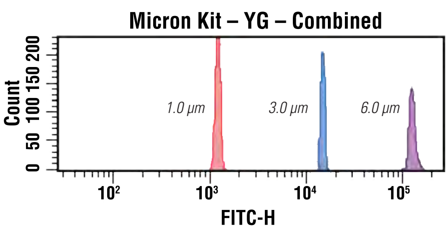
1. *Purvis N, Stelzer G. (1998) Multi-platform, multi-site instrumentation and reagent standardization. Cytometry; 33(2):156-65.*

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

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, microvesicles or microbial species, require modified processes and specialized instrument set-up. Our small bead calibration kits 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 10: Micron Bead Calibration Kit - LSRII settings
FSC log 536 - Threshold 200
SSC log 247 - Threshold 200
FITC log 346 - Threshold 200



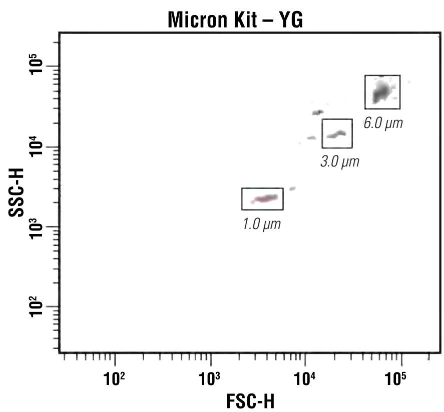
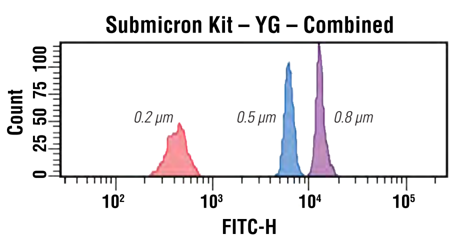


Figure 11: Submicron Bead Calibration Kit - LSRII settings
FSC log 500 - Threshold 200
SSC log 494 - Threshold 200
FITC log 587 - Threshold 200



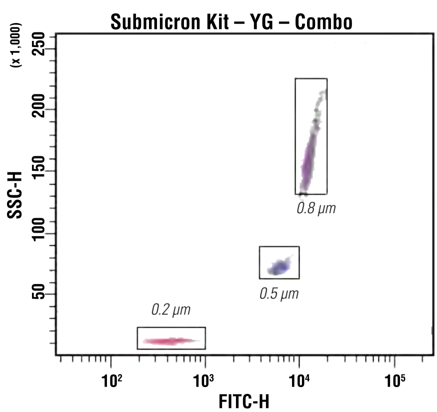


Figure 12: Nano Bead (100nm) & submicron bead calibration kits- BD FACSCanto II settings
SSC log 500 - Threshold 200
FITC log 650 - Threshold 300
Windows Ext. 2.0 Events: 5000

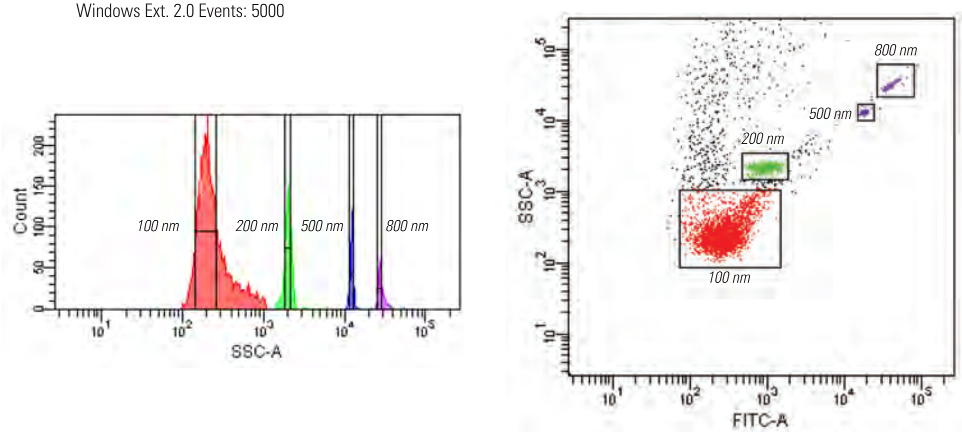


Table 3: Small Bead Calibration Kits

Catalog Code	PDS	Name	Nominal Diameters
BLI833	916	Micron Bead Calibration Kit	1.0µm, 3.0µm, 6.0µm
BLI832	916	Submicron Bead Calibration Kit	0.2µm, 0.5µm, 0.8µm
BLI834	916	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; 89(2):184-95.*

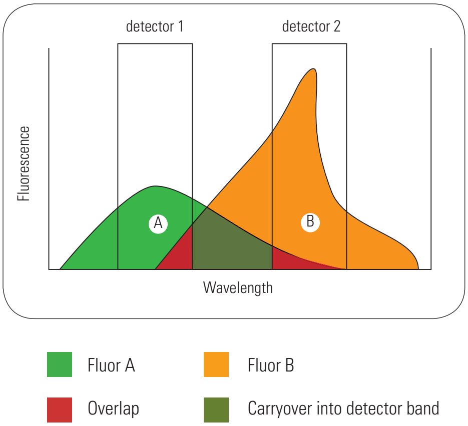
2. *Kong F, Zhang L, Wang H, Yuan G, Guo A, Li Q, Chen Z. (2015) Impact of collection, isolation and storage methodology of circulating microvesicles on flow cytometric analysis. Exp Ther Med; 10(6):2093-2101.*

Due to the nature of the cytometer (sensitive detection, specific filter sets) and the fluorophores themselves (broad emission bands), fluorescence typically spills over into regions beyond that covered by the intended detector. 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. Polysciences offers both fluorophore - matched microspheres and microspheres with capture Abs 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 13: Fluorescence Carryover



Using compensation, carryover fluorescence is electronically "subtracted" from unintended detectors so that the measured signal is as pure as possible. 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

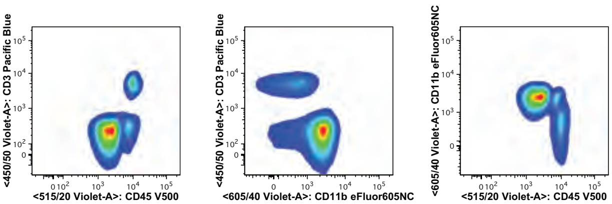
Fluor B – 1% Fluor A

Table 5: Compensation Standard Products

Catalog Code	PDS	Product	Binds
BLI820	820	FITC/PE Compensation Standard	Pre-labeled with FITC/PE
See pg. 13	890	Fluorescence Reference Standards	Pre-labeled with designated fluor pg.13
BLI550-552, 556	850	Simply Cellular® Compensation Standards	IgG from Mouse, Rat or Human, as noted
BLI835	835, 850	Simply Cellular® anti-Mouse for Violet Laser	IgG from Mouse
BLI553-554	854	Protein A, Protein G Antibody Binding Beads	See *PDS 854* for IgG affinities
BLI450-451	853	Viability Dye Compensation Standards	Amine-reactive dyes

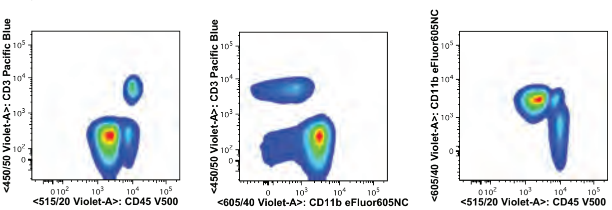
Figure 14: Compensation Matrix - **Simply Cellular® anti-Mouse IgG Bead for Violet Laser** produces comparable data when compared to cells.

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

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.*

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)*

Classic immunophenotyping involves fairly straightforward sample preparation. Following collection of the blood sample, there may be a depletion or enrichment step (e.g. via density centrifugation, RBC lysis, antibody-coated magnetic particles [e.g. BioMag® anti-leukocyte particles]), in addition to fixation and staining. 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, where markers with low expression are labeled with bright fluorochromes, and those that express at high levels are 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 4.)*

1. *Aasebo E, Mjaavatten O, Maudel M, Farag Y, Selheim F, Berven F, Bruserud O, Hernandez-Valladares. (2016) Freezing effects on the acute myeloid leukemia cell proteome and phosphoproteome revealed using optimal quantitative workflows. J Prote. Mics; Epub Apr. 20.*

2. *Stewart JC, Villasmil ML, Frampton MW. (2007) Changes in fluorescence intensity of selected leukocyte surface markers following fixation. Cytometry A; 71:379-385.*

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.*

4. *Kong F, Zhang L, Wang H, Tuan 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.*

5. *Edinger M. Multicolor Flow Cytometry: Principles of Panel Design. BD Biosciences.*

6. *Maecker H, Trotter J. Selecting Reagents for Multicolor Flow Cytometry, Application Note. BD Biosciences, 2009.*

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

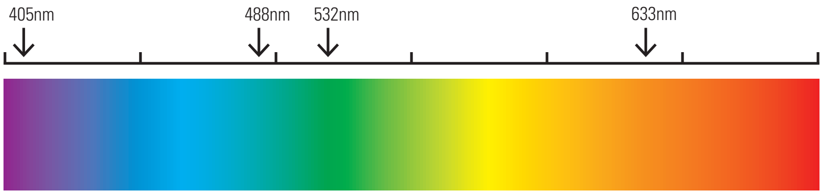


Table 4: Fluorescence Reference Spectrum Products

Catalog Code	Description	MW	Excitation (nm)	Emission (nm)	Purpose
BLI890	Certified Blank™				reference
BLI897	Acridine Orange	265	500	526	DNA/RNA
BLI886	Alexa Fluor® 488	643	499	519	conjugate
BLI887	Alexa Fluor® 647	1300	652	668	conjugate
BLI901	Allophycocyanine (APC)	104k	650	660	conjugate
BLI914	APC-Cy™7	104k	650	767	conjugate
BLI898	Chlorophyll (*a* + *b*)	8014 (a) 907 (b)	430,453	642,662	plant pigment
BLI895	Cy™5	792	649	666	conjugate
BLI906	DAPI	277	350	470	DNA (A-T)
BLI913	Far-Out Red	-	475,590	663	reference
BLI891	Fluorescein	389	495	519	conjugate
BLI894	Hoechst 33342	616	346	375,390	dsDNA
BLI916	Pacific Blue™	339	410	455	conjugate
BLI899	PE (R-Phyoerythrin)	240k	480, 565	578	conjugate
BLI908	PE-Cy™5	240k	480,565,650	670	conjugate
BLI889	PE-Cy™7	240k	480	767	conjugate
BLI909	PE-TR	240k	480,565,650	670	conjugate
BLI892	Propidium Iodide	668	536	617	DNA intercalator
BLI905	T.M. Rhodamine (TRITC, TAMRA)	430	557	576	conjugate
BLI893	Texas Red® (Sulforhodamine)	625	589	615	conjugate
BLI915	Violet Laser (Glacial Blue)	-	360	450	reference

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, intracellular proteins, etc., as with pharmaceutical trials that determine changes in cellular marker expression levels or distribution in response to administration of a particular drug. 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. Read more about these systems in our literature on Quantitative Fluorescence Cytometry.

Figure 15: Quantum MESF histogram and QuickCal analysis template

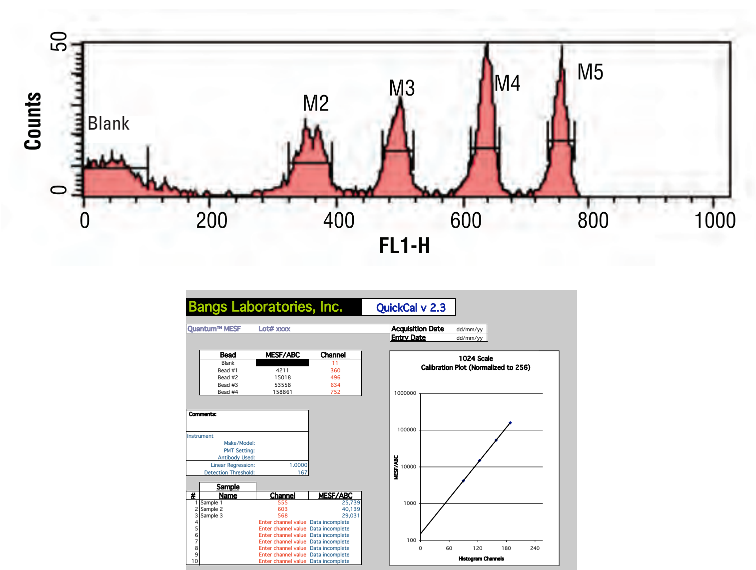


Table 6: Quantitative Cytometry Products

Catalog Code	Description	Fluorophore	MW
BLI488	Quantum MESF Alexa Fluor® 488	Alexa Fluor® 488	643
BLI555, 555p	Quantum MESF FITC-5	FITC	389
BLI827	Quantum MESF R-PE	PE	240k
BLI828	Quantum MESF PE-Cy™5	PE-Cy™5	240k
BLI822	Quantum MESF Cy™5	Cy™5	792
BLI647	Quantum MESF Alexa Fluor® 647	Alexa Fluor® 647	1300
BLI823	Quantum MESF APC	APC	104k
Catalog Code	**Description**	**Capture Antibody**	**Binds**
BLI815	QSC anti-Mouse IgG (Fc)	anti-Mouse IgG (Fc-specific)	Mouse mAb (Fc)
BLI816	QSC anti-Rat IgG (Fc)	anti-Rat IgG (Fc-specific)	Rat mAb (Fc)
BLI817	QSC anti-Human IgG (Fc)	anti-Human IgG (Fc-specific)	Human mAb (Fc)

1. *Maecker HT, Trotter J. (2006) Flow cytometry controls, instrument setup and the determination of positivity. Cytometry; 69A:1037-1042.*

2. *Randlev B, Huang L-C, Watatsu M, Marcus M, Lin A, Shih S-J. (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.*

NOTES

Some other products that may be of interest:

Cell Cycle Analsis

Microparticle Analysis

Size Estimation

Imaging Standards

Cell Viability Standards

Concentration Standards

We are here for you; let us know how we can help.

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