



## Abstract

Historically, the characteristics of a cytometer have had an inherent influence on experimental results. Recent work at the **National Institute for Standards and Technology (NIST)** has established the first reference standards in an effort to improve quantitative fluorescence measurements. Although this problem is far from solved, there are now well-established protocols to monitor the sensitivity and precision of an instrument.

The most common terms for characterizing detector performance are known as **Q&B**. Q is the measure of detector efficiency. B is the the background light level, the number of photons measured when no cells are present. It is expressed in **Molecules of Equivalent Soluble Fluorophores (MESF)**, and both influence the width of the fluorescence peak. A linear analysis of bead data can easily determine Q&B and thereby provide a mechanism to monitor data across multiple cytometers, or across multiple settings of the same cytometer.

## General Information

Flow Cytometry is a qualitative assay, and for that reason, flow results describe the nature of a phenomenon but not necessarily with a *hard number*. Samples acquired by flow cytometers are measured in a dimensionless unit called *Fluorescence Intensity*. The most common use of this unit is in the *Median Fluorescent Intensity (MFI)*. For samples acquired on different instruments, the use of a dimensionless unit means sample MFIs from one instrument are not directly comparable to sample MFIs from a different cytometer.

When acquired along with the other sample controls, the standard curve of known fluorescent intensity provides information about instrument-to-instrument non-equivalency: high backgrounds and inadequate sensitivities. By examining the unique background (B) and sensitivity (Q) values generated for any given parameter on a cytometer, Q and B calculations make it possible for a flow researcher to generate hard numbers describing staining in terms of MESF.

The Q for an instrument is affected by all the different ways a cytometer can be impaired in detecting fluorescence from a sample. The most obvious contributors to low Q are fluorochrome choice, excitation power, and other optical components. The B represents how much background noise is generated by an instrument. B can be affected by how well a stained sample is washed, the autofluorescence of cells, spectral overlap, and other factors.

Values for Q and B can be calculated using beads acquired on a cytometer. Flow cytometers measure fluorescent signals from samples and report values using a relative unit called intensity, commonly referred to as MFI. Calibration must occur to convert this relative intensity to an absolute unit of fluorescence. Absolute units can be described by the terms MESF or Molecular Equivalents of Fluorescent Labeling (MEFL). The beads used for the calibration must possess a known amount of fluorescent molecules. The fluorescent intensity of a calibration bead standard then allows the relative MFI to be converted to MESF (or MEFL). In addition to the absolute intensity, the calibration bead also provides reference coefficients of variance necessary for the Q and B calculation.



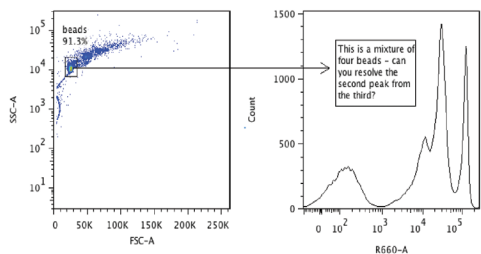
# Instrument Sensitivity

Several factors influence a cytometer's ability to distinguish separate populations. Laser output, choice of filters/detectors, event rate, etc. can all change the sensitivity and performance of the instrument.

Beads (*microspheres*) are small particles manufactured within published specifications for size and optical properties. Measuring beads on a regular basis will provide valuable standards for evaluating your experimental results.

Use of these particles in flow cytometry enables calibration (converting relative fluorescence to absolute units), capture (detecting soluble targets), compensation, and instrument sensitivity.

Beads used for instrument sensitivity assessment are usually a mixture of same-sized particles with varying (but known) fluorescence intensity. Beads produces a sharp spike in FSC x SSC plots. The events outside this spike are either bead debris or bead aggregates. Both should be gated out. In fluorescence plots, beads often show multiple populations (peaks):



Beckman Coulter provides IMMUNOBRITE beads. <https://www.beckmancoulter.com/eCatalog/CatalogItemDetails.do?productId=12991>

Bangs Labs provides MESF bead products. [http://www.bangslabs.com/products/flow\\_cytometry\\_products#mesf](http://www.bangslabs.com/products/flow_cytometry_products#mesf)

Spherotech provides broad spectrum and fluorochrome-specific calibration beads. <http://www.spherotech.com/CalibrationParticles.htm>

Dako provides cell surface antigen quantitation beads and broad-spectrum beads. [http://www.dakousa.com/index/prod\\_search/prod\\_products.htm?productareaid=45](http://www.dakousa.com/index/prod_search/prod_products.htm?productareaid=45)

Duke Scientific makes a broad-spectrum dyed bead. <http://www.brookhaven.co.uk/products/zeta-potential/microspheres/mffc.html>

Becton Dickinson markets the **Cytometer Setup and Tracking** software for managing its digital cytometers, including bead assays. [http://www.bdbiosciences.com/documents/BD\\_Cytometer\\_Setup\\_Tracking.pdf](http://www.bdbiosciences.com/documents/BD_Cytometer_Setup_Tracking.pdf)

*This section contains the bead manufacturer's published Antibody Binding Capacity, measured in Molecules of Equivalent Soluble Fluorophores (MESF).*

*Each lot of beads may have different (MESF) values.*

*Running the beads on your cytometer yields a set of peaks at a measured intensity and variance. Drag your bead sample to this window to fit the data and enter the peaks.*

*Intermediate calculations show the MESF and variance of the peaks.*

*To show the linearity of the instrument, the slope is fit across all bead peaks and across the two dimmest peaks.*

FlowJo's Q&B platform performs the regression analysis to calculate the relation between fluorescence and the variance of the same population. These are expected to be proportional.

Adding a Q&B platform to a population will automatically identify the major peaks in the distribution and calculate the variance of the populations. These data are compared to the manufacturer's provided reference information to compute the Q & B values. These values should be tracked over time to monitor the performance of the instrument. Use FlowJo's Quality Control plot to show these statistics over time.

The reference values are stored in your preferences, so they will reappear in subsequent tests. Whenever you change bead lots, be sure to re-enter the appropriate reference values for that specific set of beads.

Detection Efficiency and Noise Calibration (Q&B)

This will perform regression analysis on bead signals to calculate the detection efficiency (Q) and background noise (B) of your instrument. Use a set of 3 beads, sufficiently dim in fluorescence such that CVs are broadened by photoelectron statistics and background light.

**Calibration Input**

Calibration Bead Mean: 40008 Linear Fluorescent Units  
 Calibration Bead MESF: 570 MESF  
 Bright Bead CV: 1.82 %

**Bead Data**

Bead number	Mean (linear units)	CV (%)
1	13063	17.04
2	77088	10.5
3	224491	6.1

**Calculated Values**

MESF	Photoelectron SD Squared
471.1	6369.4
1098.3	12899.1
3198.4	34675.5

**Q & B Derivation**

Fit All Beads

1/Q	B/Q	Q =	B =
10.37641	1490.8	0.0964	144

Fit only two dimmest beads

1/Q	B/Q	Q =	B =
10.41043	1465.5	0.0961	141

Photoelectron SD Squared vs F (MESF) plot showing a linear regression with R: 1.000, Slope: 10.4, and Intercept: 1491.

*Linear regression of the fluorescence level (F) and the variance of the photoelectron count*



## Glossary of Acronyms and Terms

Q	The number of photoelectrons per fluorochrome, the detection efficiency of the photomultiplier
B	The background light level or noise. Noise is measured by the CV of the dimmest control bead.
CS&T	Becton Dickinson's Cytometer Setup and Tracking software
NIST	National Institute of Standards & Technology, the agency establishing fluorescent standards
MESF	Molecules of Equivalent Soluble Fluorophores
MEFL	Mean Equivalent Fluorochrome Labeling, interchangeable with MESF
ERF	Equivalent Reference Fluorophores
ABC	Antibody Bound to Cell
ABD	Assigned BD units, Becton Dickinson's proprietary fluorescence unit
Sensitivity	How well the instrument can resolve dim populations from each other and from background
Efficiency	The portion of photons emitted by the fluorophore that are measured by the detector
Precision	Narrowness of the distribution of the bead fluorescence
Linearity	How the signal-to-noise ratio compares on bright particles vs. dimmer particles
Noise	How many photons of light are detected when no events are present

## Links and References

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