

APPLICATIONS

- Fluorescence Quantitation for cellular expression analysis
- Instrument QC:
 - Threshold sensitivity
 - Resolution
 - Linearity
- Determination of antibody effective F:P ratio

INTRODUCTION

Quantum™ MESF (Molecules of Equivalent Soluble Fluorochrome) microsphere kits enable the standardization of fluorescence intensity units for applications in quantitative fluorescence cytometry. The beads are run on the same day and at the same fluorescence settings as stained cell samples to establish a calibration curve relating instrument channel values to standardized fluorescence intensity (MESF) units. Because Quantum™ MESF microspheres are surface labeled with the actual fluorochromes that are used to stain cells in flow cytometry, the standards are environmentally responsive (e.g. to pH, temperature, etc.), and quantitative assignments are truly relevant.

In addition to quantitative fluorescence cytometry, Quantum™ MESF kits may be used to assess the detection threshold, resolution, and linearity of the relevant detector. A free QuickCal® template is provided with each kit to aid in determining expression levels of cells, and for evaluating instrument linearity and detection threshold.

Quantum™ MESF standards may also be used in conjunction with a Simply Cellular® population to determine a labeled antibody's effective F:P (Fluorophore:Protein) ratio and conversion of results from MESF to ABC (Antibody Binding Capacity) units. See PDS 849, *Determination of Antibody Effective F:P Ratio*, for more information regarding this use.

MATERIALS

Quantum™ MESF Kit Components

- 1 – Blank microsphere population
- 4* – Microsphere populations surface labeled with increasing amounts of the specified fluorochrome. These populations are calibrated in MESF units. (** FITC-5 kits have 5 labeled populations.*)
- Access code for downloading an appropriate QuickCal® analysis template from the website

Microbeads are supplied in dropper bottles at a concentration of approximately $\sim 2 \times 10^6$ beads / mL.

Kits are available in three sizes:

| | | |
|---|---|---|
| A – 20 tests | B – 100 tests | C – 280 tests |
| 5 bottles x 1mL (standard kits); or | 5 bottles x 5mL (standard kits); or | 2 x (5 bottles x 7mL) (standard kits); or |
| 6 bottles x 1mL (FITC-5 standard kit); or | 6 bottles x 5mL (FITC-5 standard kit); or | 2 x (6 bottles x 7mL) (FITC-5 standard kit); or |
| 2 bottles x 1mL (FITC-5 premixed kit) | 2 bottles x 5mL (FITC-5 premixed kit) | 2 x (2 bottles x 7mL) (FITC-5 premixed kit) |

Standard MESF kits include individually packaged bead populations; premixed MESF kits include two bottles, one with the blank bead population and a second with all fluorescent bead populations combined.

| Cat. Code | Description |
|-----------|------------------|
| 823 | APC |
| 488 | Alexa Fluor® 488 |
| 647 | Alexa Fluor® 647 |
| 822 | Cy™5 |
| 555 | FITC-5 |
| 555p | FITC-5 premixed |
| 828 | PE-Cy™5 |
| 827 | PE |

PROCEDURES

Section 1: General

1. Investigators are advised to optimize the use of microsphere standards in any application. For Quantum™ MESF kits, this includes ensuring that the labeled cells and Quantum™ MESF beads appear in the same window of analysis (i.e. are on scale) when run at the same fluorescence (PMT and compensation) settings.
2. Remove the bottles from and return them to the refrigerator as quickly as possible (<5 minutes) to avoid the negative effects of repeated temperature cycling.
3. Prepare all suspensions immediately prior to use.
4. Protect from light to guard against photobleaching.
5. The standards must be analyzed on the same day, on the same instrument, and at the same fluorescence (PMT and compensation) settings as stained cell samples, although forward and side scatter settings (FSC, SSC) may be adjusted to optimize dot plot gating.
6. Bead populations may be run individually (standard kits) or as mixed populations. If the resolution of the detector is sub-optimal, running the populations individually will ensure best gating.

Section 2: Fluorescence Intensity Calibration

1. Observe conditions specified in *Section 1: General*.
2. Manually shake the bottles to ensure uniform suspensions of microspheres. Do not vortex or sonicate the bottles.
3. Add one drop of the reference blank "B" to 400µL suspending solution. For cell-based analyses (i.e. fluorescence quantitation), the beads must be suspended in the same type of buffer or medium as stained cells.
4. Analyze the microspheres on the flow cytometer. Adjust the flow rate or suspension concentration such that the count rate is optimal for your instrument. A count rate of 100 beads / second is recommended.
5. Using the FSC vs. SSC dot plot, construct a live gate around the singlet population of the microspheres. (*Figure 1*).
6. Create a fluorescence histogram for the appropriate detector (e.g. FL1/ FITC), including only the events falling in the singlets gate of the FSC vs. SSC dot plot.
7. Verify that the reference blank population appears near the origin of the histogram (*Figure 2*).
8. Combine 1 drop of each of the fluorescence intensity populations to 400µL of the same type of buffer for analysis. Analyzing each intensity separately is also an option. We recommend running the reference blank separate from the labeled populations.
9. Analyze the microspheres on the flow cytometer, adjusting fluorescence settings (PMT voltages) as needed. Discernable fluorescence peaks should be observed (*Figure 2*), confirming the instrument's ability to resolve different intensities.
10. A standardized gating practice is recommended, i.e. full-width, half-height gating (*Figure 2*). This is the gating strategy that we use when making assignments to the microspheres, and will serve to both bring your practices into alignment with ours, and minimize variation between your runs.
11. When establishing a calibration plot, make no further adjustments to the instrument once you have begun collecting data. Record the instrument settings (e.g. amplifier gains, PMT voltages, compensation, etc.) Record the channel value (median or geo mean) for each of the microsphere populations.
12. **Go to www.bangslabs.com/quickcal** to download Bangs Laboratories' quantitative analysis template, QuickCal® v. 2.3. Using QuickCal®, you may:
 - a. Generate a calibration curve
 - b. Determine detection threshold
 - c. Determine linearity
 - d. Quantitate the fluorescence intensity of stained cell samples.
 To access this free service, you will need to use the QuickCal® access code affixed to your Quantum™ MESF kit. See Product Data Sheet (PDS) 819, *QuickCal® v. 2.3 Data Analysis Program*, for more information on use of the QuickCal® analysis template.
13. Enter the channel values (median or geo mean) of the labeled bead populations into QuickCal®. A calibration curve and Regression Coefficient (r²) value will appear (*Figure 3*).

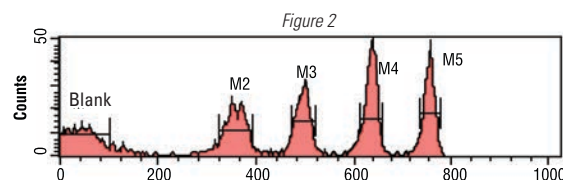
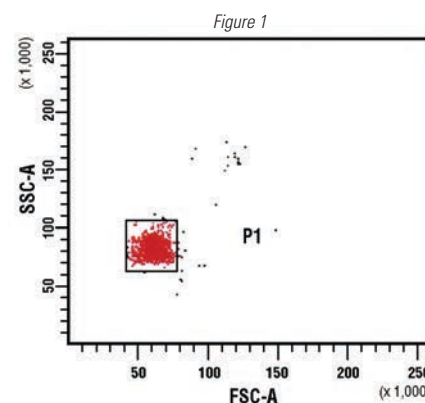


Figure 3

| Marker | % Gated | CV | Median |
|--------|---------|--------|--------|
| M1 | 21.73 | 117.64 | 11.00 |
| M2 | 16.07 | 4.38 | 360.00 |
| M3 | 15.67 | 2.21 | 493.00 |
| M4 | 21.83 | 1.44 | 634.00 |
| M5 | 16.98 | 1.10 | 752.00 |

The screenshot shows the QuickCal v 2.3 software interface. At the top, it says 'Bangs Laboratories, Inc. QuickCal v 2.3'. Below this, there are fields for 'Quantum™ MESF Lot# xxxxx' and 'Acquisition Date dd/mm/yy'. The main data entry table is as follows:

| Read | MESF/ABC | Channel |
|---------|----------|---------|
| Blank | | 11 |
| Read #1 | 4211 | 360 |
| Read #2 | 13018 | 494 |
| Read #3 | 53558 | 634 |
| Read #4 | 158861 | 752 |

Below the table, there are fields for 'Comments', 'Instrument', 'Make/Model', 'PMT Settings', 'Antibody Used', 'Linear Regression', and 'Detection Threshold'. A 'Sample' table is also present with columns for '#', 'Sample Name', 'Channel', and 'MESF/ABC'. To the right, a '1024 Scale Calibration Plot (Normalized to 256)' is shown, which is a linear graph of MESF/ABC vs Histogram Channels. A red arrow points from the 'Median' column of the table above to the 'Channel' column of the data entry table. Another red arrow points from the 'MESF (or ABC) assignment of stained cell samples' to the 'MESF/ABC' column of the data entry table.

Section 3: Threshold Sensitivity

1. After completing *Section 2: Fluorescence Intensity Calibration* and plotting the calibration curve using QuickCal®, determine and record the channel value (median or geo mean) of the reference blank. The MESF value of the blank bead will be read from the curve, and will appear in the “Detection Threshold” cell.

Section 4: Fluorescence Quantitation

IMPORTANT:

- The standards must be analyzed on the same day, on the same instrument, and at the same fluorescence (PMT and compensation) settings as stained cell samples.
 - The fluorescence intensity of fluorochromes (e.g. FITC) can be highly dependent upon the pH, ionic strength, etc. of the suspending solution. For that reason, the beads must be suspended in the same type of buffer or medium as the stained cells when runs are performed.
 - For accurate MESF assignments, instrument linearity must be assured. A regression coefficient ≥ 0.9995 is generally desired.
 - Remove the bottles from and return them to the refrigerator as quickly as possible (<5 minutes) to avoid the cumulative negative effects of repeated temperature cycling.
1. After completing *Section 2: Fluorescence Intensity Calibration* and plotting the calibration curve using QuickCal®, analyze the stained cell samples on your flow cytometer. Suitable controls (e.g. unstained cells, isotype controls) should be run to aid in interpreting fluorescence intensity results.
 2. Record each sample’s channel value (median or geo mean) for the appropriate detector.
 3. Enter the channel values in the “Sample” section of the QuickCal® template. The MESF value of each will be determined automatically.

Section 5: Instrument QC

1. Quantum™ MESF kits may be utilized to monitor instrument performance as part of an ongoing QC program. Graphed data (e.g. Levey Jennings charts) can be extremely helpful in both understanding the instrument and environmental / seasonal effects, as well as highlighting outliers and trends.
2. After performing *Section 2: Fluorescence Intensity Calibration*, adjust PMT settings to a few different levels (low, medium, and high), while ensuring that all bead populations remain on scale.
3. Record the channel values of the bead populations for each different PMT setting, and complete a QuickCal® template for each.
4. The Linear Regression values should be comparable and should fall within the laboratory’s standard acceptance criteria at each PMT setting. Poor or disparate r^2 values may identify the need for further monitoring or a service visit.

Section 6: Troubleshooting

1. See PDS 818, *Quantum™ Simply Cellular® & Quantum™ MESF Tips and Techniques*.
2. Drain and fill the flow cell several times to eliminate air bubbles and debris.
3. Wash fluidics system by running a fresh solution of 10% household bleach. Follow manufacturer’s instructions.
4. Check system for pressure leaks.
5. Prepare fresh dilution of standards.
6. Check alignment of the instrument.
7. Ensure that the proper resolution has been selected when downloading the QuickCal® template.
8. Consult your service engineer.

REFERENCES AND FURTHER READING

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TRADEMARKS

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2. Cy™, including Cy5, is a trademark of GE Healthcare Limited. These products are manufactured under license from Carnegie Mellon University under U.S. Patent Number 5,268,486 and related patents.
3. Alexa Fluor® is a registered trademarks of Life Technologies Corporation.

RELATED TECHNICAL LITERATURE

1. PDS 818 – Quantum™ Simply Cellular® & Quantum™ MESF Tips and Techniques
2. PDS 819 – QuickCal®, v. 2.3 Data Analysis Program
3. PDS 849 – Determination of Antibody Effective F:P Ratio
4. Flow Quality Control and Standardization Brochure
5. Flow Cytometry Instrument Quality Assurance / Quality Control Brochure
6. Quantitative Cytometry Brochure

STORAGE AND HANDLING

Store at 2-8°C in the original opaque bottle. Protect from light. Do not freeze. Do not vortex or sonicate the stock suspensions as repeated treatment can be detrimental to the immobilized fluorochrome. Prepared samples may be vortexed briefly, if necessary to increase % singlets. Remove the bottles from and return them to the refrigerator as quickly as possible (<5 minutes) to avoid the cumulative negative effects of repeated temperature cycling. Do not store in a frost-free unit as temperature cycling may have negative effects on the product.

Expiration dating:

- Quantum™ FITC-5 MESF - six months from the date of purchase
- PE-Cy5 kits - full expiration is six months from the date of purchase (unopened bottle), 30 days upon opening
- all other kits - twelve months expiration date

SAFETY

Normal precautions for handling laboratory reagents should be followed. This particle suspension contains sodium azide. Sodium azide may react with lead and copper plumbing to form explosive metal azides. Upon disposal of material, flush with a large volume of water to prevent azide accumulation. Please consult the Safety Data Sheet for more information.

These products are for research use only and are not intended for use in humans or for *in vitro* diagnostic use.

ORDERING INFORMATION

| Cat. Code | Description | Sizes |
|-----------|--------------------------------|-------------------|
| 488 | Quantum™ Alexa Fluor® 488 MESF | 1mL, 5mL, or 14mL |
| 647 | Quantum™ Alexa Fluor® 647 MESF | 1mL, 5mL, or 14mL |
| 823 | Quantum™ APC MESF | 1mL, 5mL, or 14mL |
| 822 | Quantum™ Cy™5 MESF | 1mL, 5mL |
| 555 | Quantum™ FITC-5 MESF | 1mL, 5mL, or 14mL |
| 555p | Quantum™ FITC-5 MESF (Premix) | 1mL, 5mL, or 14mL |
| 828 | Quantum™ PE-Cy™5 MESF | 1mL |
| 827 | Quantum™ R-PE MESF | 1mL, 5mL, or 14mL |

Order online anytime at www.bangslabs.com.