SPHERO™ Technical Note **STN-9 Rev D. 041106**

MEASURING MOLECULES OF EQUIVALENT FLUOROCHROME (MEF) USING SPHERO™ RAINBOW AND ULTRA RAINBOW CALIBRATION PARTICLES

Introduction

The Molecules of Equivalent Fluorochrome (MEF) value is useful for flow cytometer users. However, the Quantum Yield of most fluorochromes usually changes upon binding to the cells. As a result, the actual number of the fluorochrome molecules binding to the cells may be different than the expected MEF value. In addition, the MEF value will vary depending upon the measurement method.

There are several methods for measuring the MEF value. Most commonly, a standard curve is generated with a fluorimeter using solutions of fluorochrome in various concentrations. The fluorescence of these solutions is then compared to the intensity of labeled cells or particles less the fluorescence of a blank particle or non-labeled cell. This provides the equivalent concentration of fluorochrome. The MEF value is then calculated by dividing the equivalent fluorochrome concentration by the number of cells or particles used.

Another procedure to determine the MEF value is the subtraction method. During this method, the absorbance or fluorescence intensity of a fluorochrome solution with known concentration is measured. Next, the absorbance or fluorescence intensity after binding to known number of cells or particles is measured. The decrease in absorbance or fluorescence intensity reflects the fraction of the fluorochrome bound to the cells or particles. The FLUOROCHROME PER PARTICLE (FPP) is then calculated by dividing the amount of fluorochrome bound to the cells or particles by the number of particles. In theory, if the Quantum Yield of the fluorochrome does not change upon binding to the cells or the particles, the MEF value is the same as the FPP value. However, since the Quantum Yield of the fluorochrome changes upon binding to the cells or particles, the FPP value obtained from the subtraction method is different than the MEF value from the standard curve method. Typically, the Quantum Yield of the fluorochrome decreases upon binding to the cells or particles. As a result, the FPP value from the subtraction method is greater than the MEF value obtained from the standard curve method.

A third method for obtaining the MEF value is available. This method is a variation of the subtraction method used to determine the FPP. During this method, avidin coated cells or particles are titrated with biotinylated fluorochrome. The binding capacity of the avidin coated cells or particles to the biotinylated fluorochrome is then determined. After all of the biotinylated fluorochrome is captured by avidin coated cells or particles, the FPP value can be calculated. Similarly, biotin coated cells or particles and avidin-fluorochrome conjugate can be used to obtain the FPP value. This method will only work for ligands such as avidin-biotin with very high binding constant. This ensures the complete binding of fluorochrome to the cells or particles.

Since MEF does not describe either the type of fluorochrome used or the intended channel of flow cytometer, Spherotech has decided to use more specific terms namely: MEFL (Molecules of Equivalent Fluorescein), MEPE (Molecules of Equivalent PE) and MECY (Molecules of Equivalent Cy5 (for RCP-30-5, 6 peaks)), etc. However, users are welcome to use whatever terms they prefer.

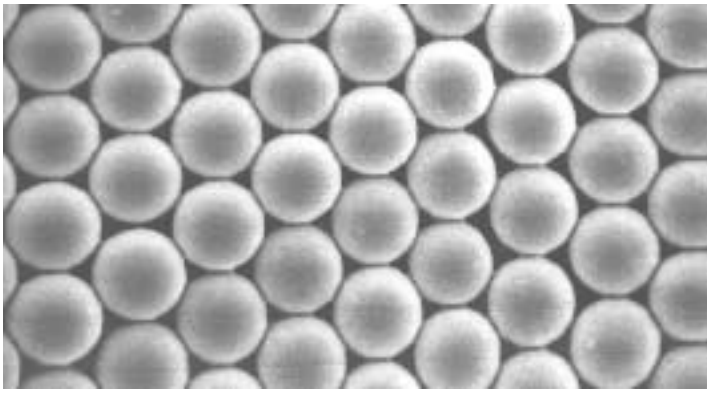
The SPHERO™ Rainbow Calibration Particles (RCPs) and Ultra Rainbow Calibration Particles (URCPs) are more versatile, stable, economical and convenient to use than other similar products available. For example, they contain a mixture of fluorochrome which are spectrally similar to many of the fluorochromes used in flow cytometry. As a result, they can be used to calibrate multiple channels of the flow cytometer in one run. In addition, these particles are very stable since the fluorochromes are entrapped inside the particles

instead of being located on the surface. Furthermore, the RCPs and URCPs are packaged in a convenient dropper bottle to facilitate the dispensing and storage. The diluted particles can be stored for later use if desired to reduce costs.

The RCPs and URCPs are useful for flow cytometry users. They have very small coefficients of variation both in size and fluorescence. In addition, each bottle consists of multiple peaks with assigned MEF values for most channels of any multi-laser flow cytometer. These MEF values were determined according to the first method described previously. Since each peak is assigned a MEF value, a calibration curve can be made by comparing the Relative Channel Number of each bead population against the MEF, i.e., MEFL, MEPE or MEPCY value. The calibration curve is used to determine the linearity and the performance of flow cytometers and cell sorters. In addition, the calibration graph and cross-calibration techniques allows MEF values to be assigned to unknown sample.

The RCPs and URCPs are prepared using fluorophores not commonly used for cell staining. As a result, calculated MEF values could slightly vary on different instruments due to the difference in the optical filters. However, the slope of the Calibration Graph should remain the same on different instruments. Nonetheless, the long-term stability of the RCPs and URCPs generates a Calibration Graph that should not change on the same instrument.

The Scanning Electron Microscope (SEM) photo of SPHERO™ Cat. No. RCP-30-5



I. SPHERO CALIBRATION GRAPH for RCP-30-5 and URCP-38-2K

Although Spherotech offers different sizes of RCPs, the 3 µm RCPs (RCP-30-5) is used as the reference standard to calibrate the other RCPs. This insures consistent data is obtained on the same instrument when any RCP product is used.

As previously stated, most channels of any multi-laser flow cytometer can be used to generate data for the RCPs. Preferably, the data should be collected in 4 decade log scale in both 256 channel Arithmetic/ linear (Relative Channel Number) and Geometric (Mean Channel Number, ie. Relative Brightness). Furthermore, the 2nd brightest peak (peak #5) of RCP-30-5 should be near the beginning of the 4th decade or around 200 Relative Channel Number. As a result, all of the peaks should be within the scale as shown **Figure 1**., which shows the histograms of the RCP-30-5 in the FITC, PE, ECD, PE-Cy5, and APC channels. If other RCPs are used, the brightest peak should be within scale and in the lower to mid 4th decade. After data collection, the Relative Channel Number vs. the MEF (ie. MEFL, MEPE or MEPCY) can be plotted to obtain a linear Calibration Graph in all channels as shown in **Figure 2.** The Calibration Graph can be plotted manually or by using any computer software, such as Excel. A calibration graph for the URCP-38-2K can be obtained using the same procedure. **Figure 3** shows the histograms for the URCP-38-2K, while **Figure 4** shows its calibration curves.

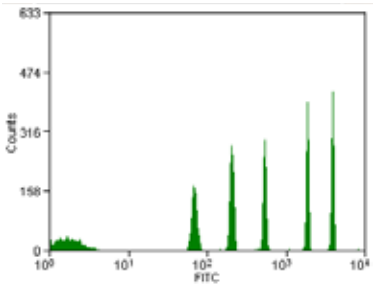
The formulas for converting the Relative Channel Number to Mean Channel Number (ie. Relative Brightness) or vice versa are as follows:

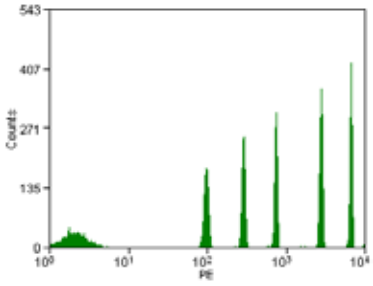
Mean Channel # = $10^{(4 \times \text{Rel Ch\#/R})}$

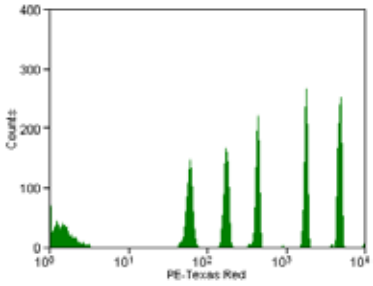
Relative Channel # = $(R/4) \log_{10}$ (Mean Ch#)

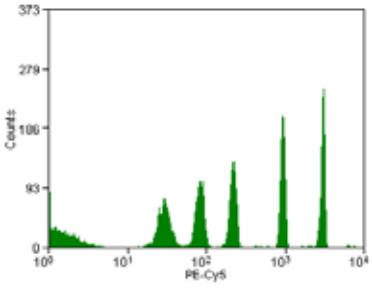
Where R = Resolution (ie. 256 or 1024)

Fig. 1. SPHERO^{TM} Cat. No. RCP-30-5 Histograms









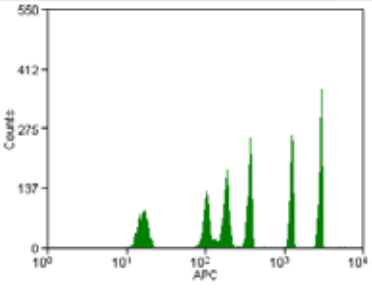
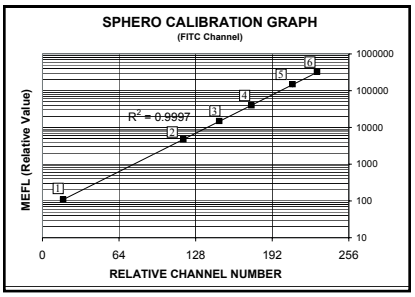
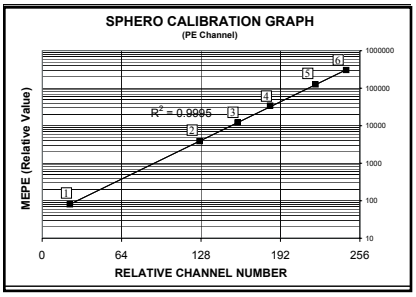


Fig. 2. SPHERO^{TM} Cat. No. RCP-30-5 Calibration Graphs



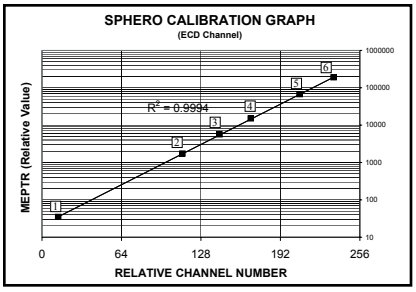
FITC Channel

PEAK	CH#	MEFL
1	16	
2	113	4700
3	144	15000
4	171	40000
5	204	140000
6	226	330000



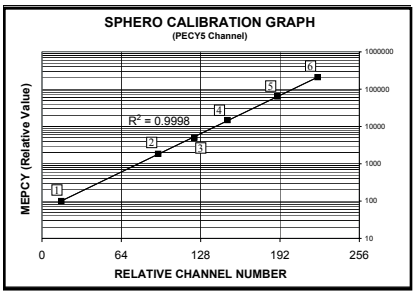
PE Channel

PEAK	CH#	MEPE
1	11	
2	109	3800
3	140	12000
4	167	34000
5	203	124000
6	228	300000



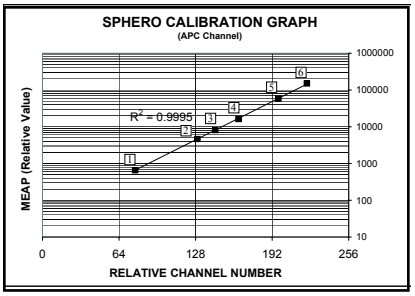
ECD Channel

PEAK	CH#	MEPTR
1	13	
2	115	1600
3	145	5560
4	171	15800
5	209	65940
6	237	190880



PE-CY5 Channel

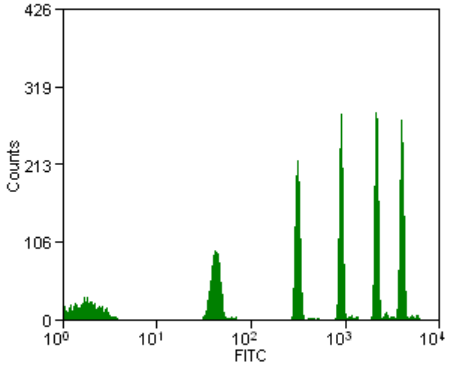
PEAK	CH#	MECY
1	21	
2	91	10000
3	118	27000
4	148	77000
5	190	340000
6	221	1115000

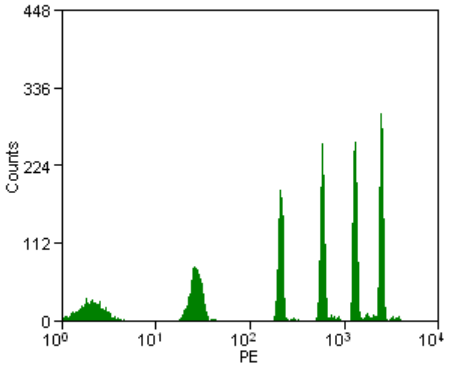


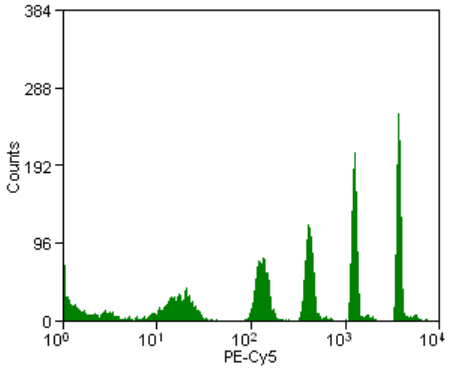
APC Channel

PEAK	CH#	MEAP
1	50	
2	84	4760
3	99	8160
4	118	16170
5	154	59050
6	180	150470

Fig. 3. SPHERO^{TM} Cat. No. URCP-38-2K Histograms







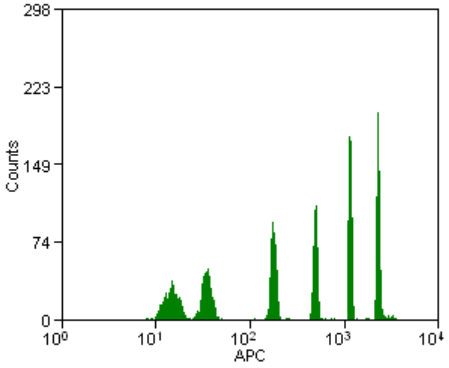
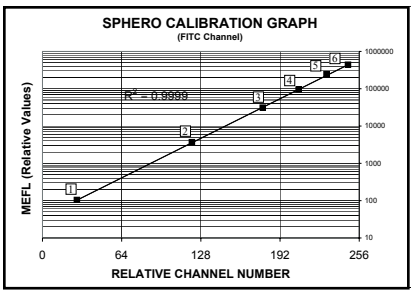
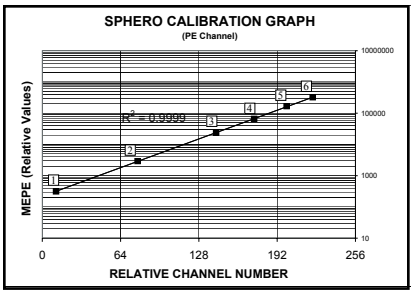


Fig. 4. SPHERO^{TM} Cat. No. URCP-38-2K Calibration Graphs



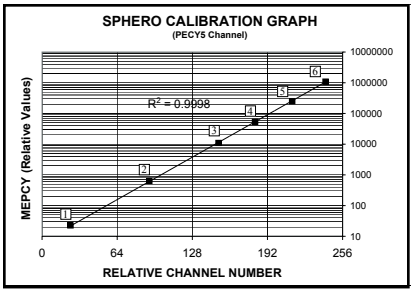
FITC Channel

PEAK	CH#	MEFL
1	28	
2	121	3635
3	178	31180
4	207	93455
5	230	237290
6	247	437385



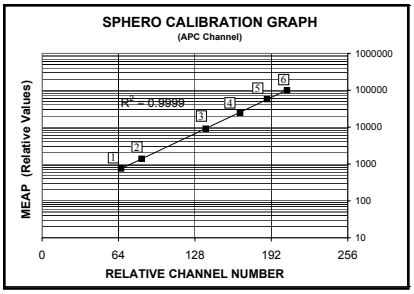
PE Channel

PEAK	CH#	MEPE
1	13	
2	89	2870
3	148	23850
4	177	67430
5	201	163085
6	218	319420



PE-CY5 Channel

PEAK	CH#	MECY
1	25	
2	69	630
3	126	10900
4	158	53125
5	216	250350
6	245	1109525



APC Channel

PEAK	CH#	MEAP
1	66	
2	83	1400
3	137	9000
4	168	25000
5	189	57000
6	205	101500

II. MEASURING MEF WITH RCPs or URCPs

The Mean Channel Number reflects the relative brightness of the calibration particles. As a result, the MEF value of unknown samples is obtained by comparing its Mean Channel Number vs. the Mean Channel Number of the RCPs or URCPs using the same instrument settings.

The MEF value of a unknown sample is obtained as follows:

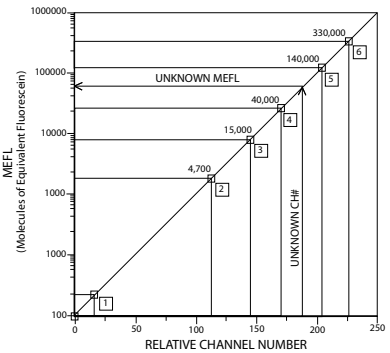
1. Run the 3.0 µm RCPs (RCP-30-5), 3.8 µm URCPs (URCP-38-2K), or other RCPs or URCPs, which the MEF are known. Adjust the signal settings such as laser power, PMT voltage and amplifier gain to place all peaks within the 4-decade log scale. Record the fluorescence Relative Channel Number and Mean Channel Number (ie. relative brightness) for each peak.

2. Plot the assigned MEF value for each peak vs the Relative Channel Number on the SPHERO PMT QC Template to obtain a Calibration Graph similar to **Fig. 2** or **Fig. 4**.

3. Run the unknown sample using the same instrument settings. Record the Relative Channel Number and Mean Channel Number of the sample.

4. Calculate the MEF of the unknown by cross-calibrating its Mean Channel Number against the Calibration Graph of the RCPs or URCPs. See Figure 5.

Fig. 5. MEASURING MEF of UNKNOWN



PEAK #	CH #	MEFL
1	16	-------
2	113	4700
3	144	15000
4	171	40000
5	204	140000
6	236	330000

III. NORMALIZATION OF DIFFERENT INSTRUMENTS

In order to ensure the consistency of the data, all instruments within the same lab should be normalized. The RCPs or URCPs can be used to normalize different instruments. After normalization, the calibration graph for each instrument is operable as long as the same master lot of calibration particles are used.

1. Run the RCP or URCP in the 4 decade log scale in both Arithmetic/linear (relative Channel Number) and Geometric (Mean Channel Number, ie. Relative Brightness) on all instruments.

2. Enter the 256 Relative Channel Number of the calibration particles for one of the instruments into the CH# Column for each peak of the Spherotech PMT QC Template. See **Fig.6** for an example of the Spherotech PMT QC Template used during the Normalization process.

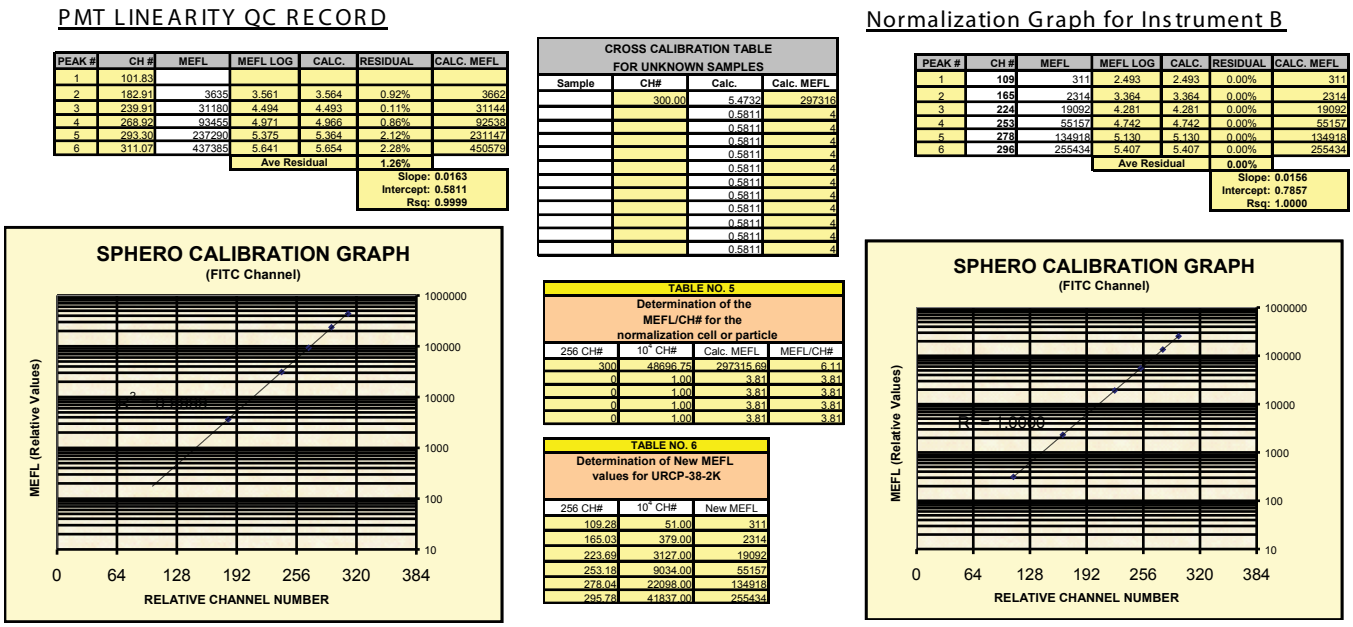
3. Using the same settings, obtain the 10^4 Channel Number for cells or particles stained with fluorophores commonly used for cell staining such as FITC, RPE, or RPE-Cy5 on all instruments. Convert this 10^4 channel number to the 256 Relative Channel Number using Table # 3 of the PMT QC Template.

4. Enter the 256 Relative Channel Number of the stained cells or beads into the Cross Calibration Table for Unknown Samples obtained by the same instrument as in Step 3.

5. Enter the 256 Relative Channel Number for the RCP or URCP from the second instrument into Table #5. If the data is collected as a 10^4 Channel Number, convert it to a 256 Relative Channel Number using Table #3.

6. Normalization of the different instruments is complete.

Fig. 6. Spherotech PMT QC Template for the Normalization of Two Instruments Using URCP-38-2K



PMT LINEARITY QC RECORD

PEAK #	CH #	MEFL	MEFL LOG	CALC.	RESIDUAL	CALC. MEFL
1	101.83					
2	182.91	3635	3.561	3.564	0.92%	3662
3	239.91	31180	4.494	4.493	0.11%	31144
4	268.92	93455	4.971	4.966	0.86%	92539
5	293.30	237290	5.375	5.364	2.12%	231147
6	311.07	437385	5.641	5.654	2.26%	450579
			Ave Residual		1.26%	
					Slope: 0.0163	
					Intercept: 0.5811	
					Rsq: 0.9999	

CROSS CALIBRATION TABLE FOR UNKNOWN SAMPLES

Sample	CH#	Calc.	Calc. MEFL
	300.00	5.4732	297316
		0.5811	4
		0.5811	4
		0.5811	4
		0.5811	4
		0.5811	4
		0.5811	4
		0.5811	4
		0.5811	4
		0.5811	4
		0.5811	4
		0.5811	4
		0.5811	4

SPHERO CALIBRATION GRAPH
(FITC Channel)

MEFL (Relative Values)

1000000
100000
10000
1000
100
10

0 64 128 192 256 320 384

RELATIVE CHANNEL NUMBER

TABLE NO. 5
Determination of the MEFL/CH# for the normalization cell or particle

256 CH#	10^{x} CH#	Calc. MEFL	MEFL/CH#
300	48696.75	297315.69	6.11
0	1.00	3.81	3.81
0	1.00	3.81	3.81
0	1.00	3.81	3.81
0	1.00	3.81	3.81

TABLE NO. 6
Determination of New MEFL values for URCP-38-2K

256 CH#	10^{x} CH#	New MEFL
109.28	51.00	311
165.03	379.00	2314
223.69	3127.00	19092
253.18	9034.00	55157
278.04	22098.00	134918
295.78	41837.00	255434

Normalization Graph for Instrument B

PEAK #	CH #	MEFL	MEFL LOG	CALC.	RESIDUAL	CALC. MEFL
1	109	311	2.493	2.493	0.00%	311
2	165	2314	3.364	3.364	0.00%	2314
3	224	19092	4.281	4.281	0.00%	19092
4	253	55157	4.742	4.742	0.00%	55157
5	278	134918	5.130	5.130	0.00%	134918
6	296	255434	5.407	5.407	0.00%	255434
			Ave Residual		0.00%	
					Slope: 0.0156	
					Intercept: 0.7857	
					Rsq: 1.0000	

SPHERO CALIBRATION GRAPH
(FITC Channel)

MEFL (Relative Values)

1000000
100000
10000
1000
100
10

0 64 128 192 256 320 384

RELATIVE CHANNEL NUMBER

IV. QUANTITATIVE FLOW CYTOMETRY USING RCPs or URCPs

The amount of fluorescent conjugate bound per cell is determined using quantitative flow cytometry. Typically, calibrated spectral matching surfaced-dyed particles with different levels of fluorescence are used to cross-calibrate unknown samples in order to determine the absolute number of fluorophore bound per cell.

The **Easy Calibration Fluorescent Particles** consist of a mixture of particles with intensities calibrated in terms of Molecules of Equivalent Fluorochrome (MEF) units. Spherotech offers **Easy Calibration Fluorescent Particles** with three different fluorochromes including FITC, PE, and PE-Cy5.

Since each **Easy Calibration Fluorescent Particle** population is assigned a MEF unit, linearity calibrations can be performed as described in **I. SPHERO CALIBRATION GRAPH for RCP-30-5 and URCP-38-2K.** As a result, quantitative determinations for stained cells can be performed when their obtained channel numbers are cross calibrated against the regression line. Refer to **II. MEASURING MEF WITH RCPs or URCPs** for more information regarding the cross calibration of unknowns.

However, unlike the RCPs and URCPs, the **Easy Calibration Fluorescent Particles** are surfaced labeled with fluorescent conjugates. Since fluorescent conjugates are thermally and photolytically unstable in the long term, these beads have a limited shelf -life.

In comparison, the RCPs and URCPs have enhanced stability over surfaced labled particles. They provide a more convenient, economical, and reliable way to perform quantitative determination in the long term. In order to use the RCPs or URCPs for quantitative determination, new MEF values based on the **Easy Calibration Fluorescent Particles** must be assigned to the RCPs or URCPs.

New MEF values can be assigned to the RCPs or URFPs using the procedure in **II. MEASURING MEF WITH RCPs or URCPs**. During the reassignment of the

MEF values, the **Easy Calibration Fluorescent Particles** are used as the baseline to cross-calibrate the RCPs or URCPs, which are treated as unknowns. See **Figure 7** for the Calibration Graph of the Spherotech Cat. No. ECFP-F2-5K (PE Easy Calibration Fluorescent Particles) and the Cross-Calibration of the Spherotech Cat. No. RCP-30-5. As a result, new MEF values based on the spectral matching fluorophores are assigned to the RCP or URCPs. A new Calibration Graph is then created with the new RCP or URCPs MEF values. This new calibration graph can then be used during **II. MEASURING MEF WITH RCPs or URCPs** to perform quantitative determination of unknown samples with confidence. **Figure 8** shows the new Calibration Graph of the Spherotech Cat. No. RCP-30-5 after the Cross-Calibration against the surface-labeled, spectral matching particles (ECFP-F2-5K). In addition, **Table 1** shows a comparison of the new MEF values assigned to Spherotech Cat. No. RCP-30-5 after the Cross-Calibration against Spherotech Cat. No. ECFP-F2-5K on three instruments.

Fig. 7. Spherotech PMT QC Template for the Cross-Calibration of RCP-30-5 Against the PE Easy Calibration Fluorescent Particles

PMT LINEARITY QC RECORD

PEAK #	CH #	MEPE	MEPE LOG	CALC.	RESIDUAL	CALC. MEPE
1	15.00	97		1.977		95
2	95.00	1680	3.225	3.228	0.86%	1691
3	125.00	5067	3.705	3.697	2.05%	4979
4	159.00	16604	4.220	4.229	2.03%	16936
5	180.00	36390	4.561	4.557	0.83%	36073
			Ave Residual		1.44%	
					Slope: 0.0156	
					Intercept: 1.7426	
					Rsq: 0.9999	

CROSS CALIBRATION TABLE FOR UNKNOWN SAMPLES			
Sample	CH#	Calc.	Calc. MEPE
RCP-30-5-1	16.80	2.0053	101
RCP-30-5-2	118.76	3.5996	3966
RCP-30-5-3	148.98	4.0722	11767
RCP-30-5-4	174.27	4.4676	29230
RCP-30-5-5	211.74	5.0535	112521
RCP-30-5-6	235.42	5.4238	263831
		1.7426	55
		1.7426	55
		1.7426	55
		1.7426	55
		1.7426	55
		1.7426	55

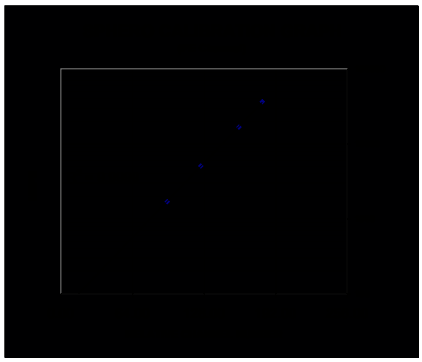


Fig. 8. Spherotech PMT QC Template for the RCP-30-5 after Cross-Calibration Against the PE Easy Calibration Fluorescent Particles (ECFP-F2-5K)

Normalization Graph for Instrument B

PEAK #	CH #	MEPE	MEPE LOG	CALC.	RESIDUAL	CALC. MEPE
1	16.80	101		2.005		101
2	118.76	3966	3.598	3.598	0.00%	3966
3	148.98	11767	4.071	4.071	0.00%	11767
4	174.27	29230	4.466	4.466	0.00%	29230
5	211.74	112521	5.051	5.051	0.00%	112521
6	235.42	263831	5.421	5.421	0.00%	263831
			Ave Residual		0.00%	
					Slope: 0.0156	
					Intercept: 1.7428	
					Rsq: 1.0000	

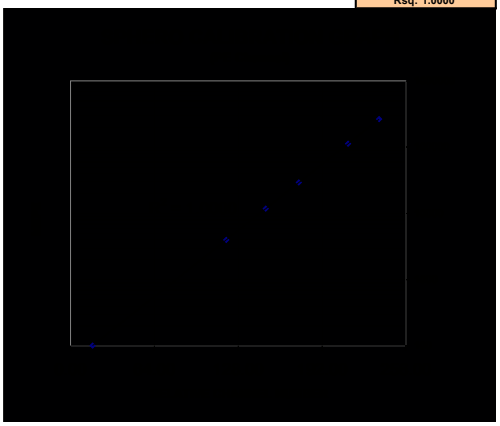


Table 1

RCP-30-5 MEF Values Obtained By Cross-Calibration with PE Easy Calibration Fluorescent Particles (ECFP-F2-5K)

	Instrument			
RCP-30-5 Peak Number	Dako Cyan™LX	Ortho Cytoron Absolute™	Beckman Coulter Cell Lab Quanta™	Original Assigned MEPE Value
1	101	125	N/A	70
2	3966	2977	3643	3800
3	11767	9328	10562	12000
4	29230	24258	26019	34000
5	112521	102255	100612	124000
6	263831	267751	260526	300000