STN-9 Rev. C 071398

MEASURING MOLECULES OF EQUIVALENT FLUORESCEIN (MEFL), PE (MEPE) AND RPE-CY5 (MEPCY) USING SPHERO™ RAINBOW CALIBRATION PARTICLES

The Molecules of Equivalent Fluorochrome (MEF) value is a useful parameter for flow cytometer users. However, since the Quantum Yield of the most fluorochromes usually changes upon binding to the cells, the actual number of the fluorochrome molecules binding to the cells may not be exactly the same as MEF value indicated. The MEF value may also vary depending upon the method being used for measurement. There are several methods to measure the MEF value. The most commonly used method is to generate a standard curve with a fluorimeter using solutions of fluorochrome in various concentrations and then compare the fluorescence intensity of the labeled cells or particles to obtain the equivalent concentration of fluorochrome on the cells or particles. Knowing the number of cells or particles used and the equivalent fluorochrome concentration one can calculate the MEF value accordingly. The second method is by subtraction. If one uses a solution of fluorochrome with known concentration and measure the absorbance or fluorescence intensity. The decrease in absorbance or fluorescence intensity after binding to known number of cells or particles reflects the fraction of the fluorochrome bound to the cells or particles. Knowing the number of particles used and the amount of fluorochrome bound to the cells or particles, one can calculate the number of FLUOROCHROME PER PARTICLE (FPP). In theory, if the Quantum Yield of the fluorochrome does not change upon binding to the cells or the particles, the MEF value will be the same as FPP value. However, as mentioned previously, since the Quantum Yield of the fluorochrome changes upon binding to the cells or particles, the FPP value obtained by using the subtraction method will not be the same as the MEF value obtained by using the first method. If the Quantum Yield of the fluorochrome decreases upon binding to the cells or particles, which usually is the case, the FPP value will be higher than the MEF value obtained by using the first method. A third method of obtaining the FPP value, which is a variation of the subtraction method, is to use avidin coated cells or particles to titrate with biotinylated

fluorochrome and find out the binding capacity of the avidin coated cells or particles to the biotinylated fluorochrome. If enough avidin coated cells or particles are used to capture all of the biotinylated fluorochrome, one can easily calculate the FPP value. Similarly one can use biotin coated cells or particles and avidin-fluorochrome conjugate to obtain the FPP value. This method will only work for ligands such as avidin-biotin with very high binding constant to ensure 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 a more specific terms namely: MEFL (Molecules of Equivalent Fluorescein), MEPE (Molecules of Equivalent PE) and MEPCY (Molecules of Equivalent RPE-Cy5), etc. However, users are welcome to use whatever terms they prefer.

The SPHERO™ Rainbow Calibration Particles (RCPs) are developed to provide the flow cytometer users with products which are very stable, versatile, economical and convenient to use than any other similar products currently available. The RCPs contain a mixture of fluorochromes compatible spectrally but not identical with the commonly used fluorochromes used in flow cytometry. The fluorochromes are entrapped inside the particles instead of on the surface. As a result, the RCPs are very stable and can be stored at room temperature if preferred. The RCPs are packaged in a convenient dropper bottle to facilitate the dispensing and storage. The diluted particles can be stored for latter use if desired. The RCPs have very small coefficients of variation both in size and fluorescence and can be used in all channels of the any multi-laser flow cytometers. The RCPs also contain multiple peaks and the MEFL, MEPE and MEPCY of each peak of the 3 um Rainbow Calibration Particles, RCP-30-5, have been determined according to the first method described previously which allow the users to check the linearity of the instrument and the performance of the sorter. The calibration graph also enable the users to assign the MEFL, MEPE and MEPCY of the unknown sample easily by using the flow cytometer.

Since the RCPs are not prepared by using fluorophores commonly used for cell staining, there is no exact spectral matching between RCPs and stained cells. As a result, the assigned MEFL, MEPE and MEPCY values could vary somewhat on different instruments due to the difference in the specifications of the optical filters used. However, the slope of the Calibration Graph should remain the same

on different instruments. Due to the long term stability of the RCPs, the Calibration Graph once generated should not change on the same instrument.

I. SPHERO CALIBRATION GRAPH for RCP-30-5

Although Spherotech offers different sizes of RCPs, the 3 um RCPs (RCP-30-5) are being used as the reference standard to calibrate the other RCPs in order to obtain the consistent data on the same instrument no matter which set of RCPs are being used. Free RCP-30-5 sample is available upon request if other RCPs are being used routinly. The Calibration Graph of RCP-30-5 could be plotted manually or by using any computer softwares, such as Excel, Lotus 123, Quatro Pro, Sigma Plot, etc. 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). All peaks as shown in the package inserts should appear within the scale. 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. If other RCPs are being used, the brightest peak should be be within scale and in the lower to mid 4th decade. Plot the Relative Channel Number vs the MEF (ie. MEFL, MEPE or MEPCY). A linear Calibration Graph Should be obtained in all three channels as shown in Fig. 1.

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

$$\text{Mean Channel Number} = 0.1 \times 10^{(4 \times \text{Rel Ch\#}/R)}$$

$$\text{Relative Channel Number} = (R/4) \log (\text{Mean Ch\#} \times 10)$$

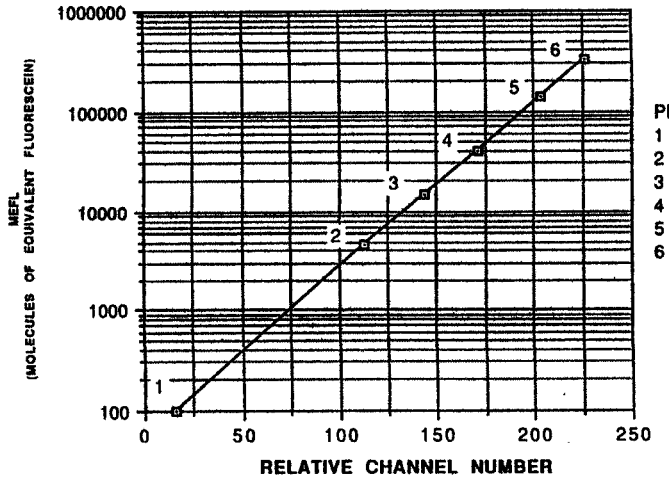
Where R = Resolution (ie. 256 or 1024)

Since the Mean Channel Number reflect the relative brightness of the particles, if both the unknown and the RCP-30-5 are run under the same instrument settings, the the MEF of the unknown could be calculated by the ratio of the Mean Channel Number of known vs unknown.

Fig. 1

SPHERO CALIBRATION GRAPH

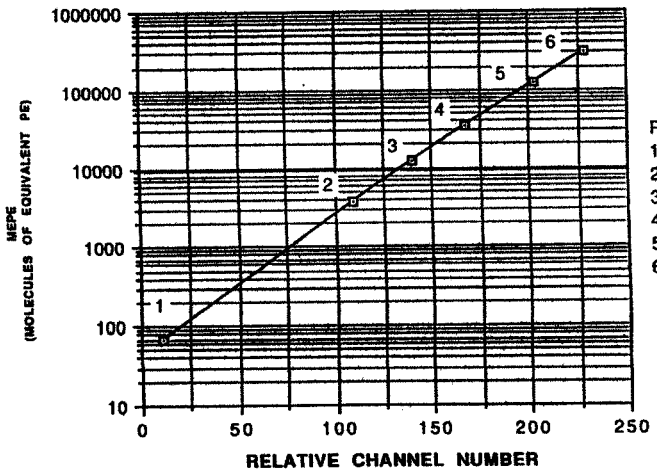
MEFL OF RCP-30-5



PEAK	CH#	MEFL
1	16	100
2	113	4700
3	144	15000
4	171	40000
5	204	140000
6	226	330000

DR. CODER'S DATA
30-MEFL-DC-041895A

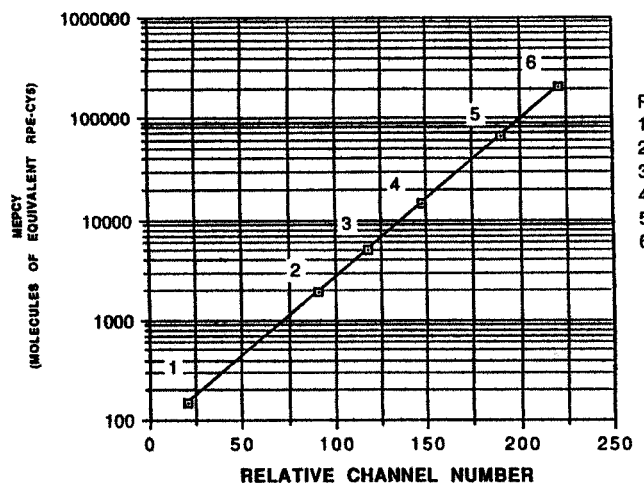
MEPE OF RCP-30-5



PEAK	CH#	MEPE
1	11	70
2	109	3800
3	140	12000
4	167	34000
5	203	124000
6	228	300000

DR. CODER'S DATA
30-MEPE-DC-G-041895A

MEPCY OF RCP-30-5



PEAK	CH#	MEPCY
1	21	150
2	91	1880
3	118	5070
4	148	14450
5	190	63800
6	221	209200

II. MEASURING MEF WITH RCPs

The MEF value of the unknown sample can be obtained as follows:

1. Run the 3.0 um RCPs (RCP-30-5) or other RCPs which have been calibrated against the RCP-30-5 and adjust the signal settings such as laser power, PMT voltage and amplifier gain to put all peaks in 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 SPHERO CALIBRATION GRAPH, a semi-log graph paper or on the computer to obtain calibration graph similar to Fig. 2.

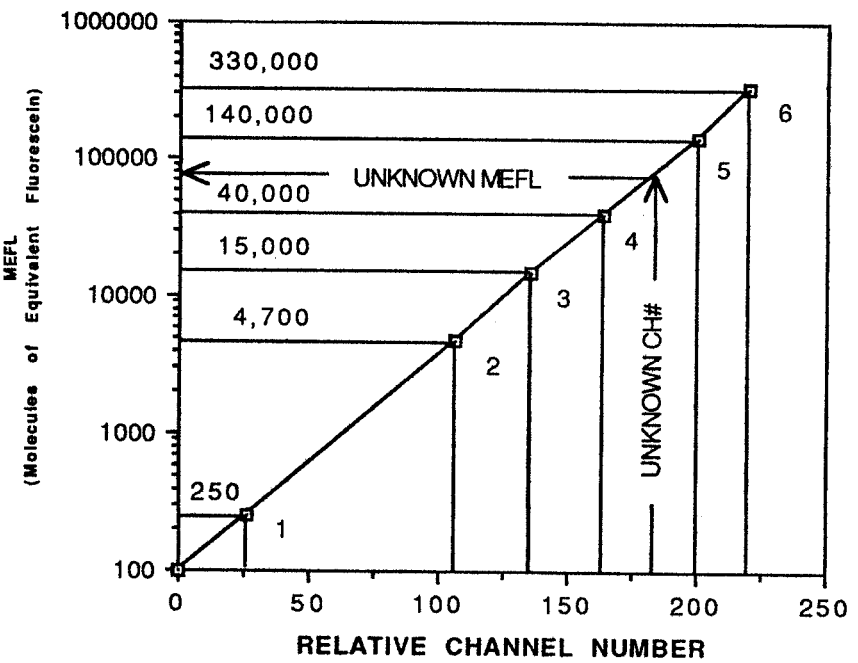
3. Run the unknown sample using exactly the same instrument settings and record the Relative Channel Number and Mean Channel Number of the sample.

4. Use Fig. 2 as example to determine the MEF value of the unknown sample by using the calibration graph or by calculation using the ratio of Mean Channel Number of brightest peak vs unknown.

Fig. 2

MEASURING MEF of UNKNOWN SAMPLE WITH RCP-30-5

SPHERO CALIBRATION GRAPH



PEAK	REL CH#	MEAN CH#
1	26	0.26
2	106	4.5
3	135	12.9
4	164	36.5
5	200	133.4
6	220	273.8

III. NORMALIZATION OF DIFFERENT INSTRUMENT

In order to ensure the consistency of the data. All instruments within the same lab should be normalized when RCPs are being used for routine QC of the instruments. The normalization procedures need to be performed only once. Thereafter the calibration graph for each instrument should remain the same as long as the same master lot of RCP-30-5 is being used.

1. Run RCP-30-5 in the 4 decade log scale in both Arithmetic/linear (Relative Channel Number) and Geometric (Mean Channel Number, ie. Relative Brightness) as described previously. Record the Relative Channel Number and Mean Channel Number of each peak.

2. Using the same instrument setting run cell or particle sample stained with fluorophores commonly used for cell staining, such as FITC, RPE or RPE-Cy5, etc.

3. Use the assigned MEF value for the brightest peak to calculate the MEF of the stained cells or particles as described previously.

4. Run the same stained cells or particles and RCP-30-5 on a different instrument and use the MEF of stained cells or particles as obtained in step 3. to calculate the MEF of every peak.

5. Repeat step 4. on every instrument in the lab and plot the Calibration Graphs for every instrument.

IV. QUANTITATIVE FLOW CYTOMETRY USING RCP-30-5

If the normalization procedures as described in III. are performed by using stained cells or particles with MEF value obtained by using the first method as described previously, then the resulting Calibration Graph could be used for quantitative flow cytometry.

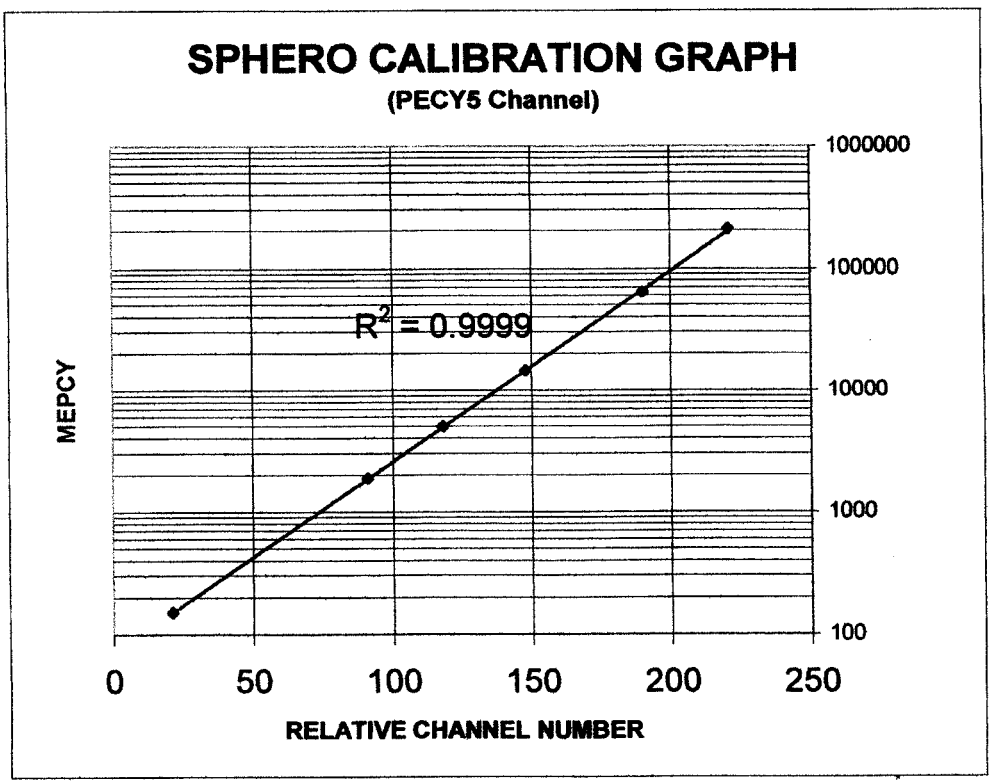
V. FUNCTIONAL TEST OF THE SORTER

Since the RCPs contain multiple populations in all channels of any flow cytomter. The RCPs could be used to check the performance of the sorter by gating on a particular population in any two dimentional dot plots. The sorted population could then be used check the purity and yield on flow cytometer.

A Template for MS Excel files, as shown below, is availble free of charge upon request. The template will allow the user to check and report the linearity of PMT in all channels easily by using RCP-30-5, RCP-30-5A, RQC-4K or ACP-30-5K.

PMT LINEARITY QC RECORD

PEAK #	CH #	MEPCY	CH/4	MEPCY LOG	CALC.	RESIDUAL	CALC. MEPCY
1	21	150	5.250	2.176	2.177	0.54%	150
2	91	1880	22.750	3.274	3.273	0.40%	1874
3	118	5070	29.500	3.705	3.695	2.58%	4960
4	148	14450	37.000	4.160	4.165	1.23%	14622
5	190	63800	47.500	4.805	4.822	3.64%	66430
6	221	209200	55.250	5.321	5.308	2.45%	203031



Rainbow Calibration Particles (RCP-30-5)	Lot No.:	File #
Acceptable:	By:	Date:
Action taken if not linear:		