SPHERO™ Technical Note **STN-14 Rev B 011408**

DETERMINING PMT LINEARITY IN FLOW CYTOMETERS USING THE SPHERO™ PMT QUALITY CONTROL EXCEL TEMPLATE

Introduction

The fluorescence linearity of flow cytometers is affected by optical alignment, laser power, electronical offsets, and amplifier calibration[1]. In addition, it is important to monitor and validate flow cytometers' performance due to the nature of the information obtained during diagnostic testing. As a result, it is recommended that the linearity of the flow cytometer is determined on a monthly basis, after instrument repair, and after instrument relocation[2].

The SPHERO™ Calibration Particles and SPHERO™ PMT Quality Control Excel Template (PMT QC Template) are designed for linearity calibration and long term performance tracking of flow cytometers. They will help flow cytometer users verify the operation of their instruments. The PMT QC Template is a valuable tool for determining the linearity of log amplifies. The information acquired from this template should be implemented into flow cytometer calibration documentation. The user can determine a schedule for routine maintenance procedures and tolerance limits of linearity based on instrument trends or malfunctions using this template.

The SPHERO™ Rainbow Calibration Particles (RCPs) and Ultra Rainbow Calibration Particles (URCPs) contain a mixture of similar size particles with different fluorescence intensities. These products contain a mixture of fluorochrome compatible spectrally but not identical with the common fluorochromes used in flow cytometry such as FTIC, PE, PE-CY5, ECD and ACP. Each particle is assigned a Molecules of Equivalent value in multiple channels such as Fluorescein (MEFL), PE (MEPE), PE-CY5 (MECY), ECD (MEPTR), and APC (MEAP). In addition, they have very small coefficients of variation both in size and fluorescence[3]. As a result, the linearity for each channel of the flow cytometer can be determined using SPHERO™ Calibration Particles, the assigned Molecules of Equivalent values, and the PMT QC Template. Refer to STN#9 for information regarding Molecules of Equivalent Fluorescence.

Results and Discussion

In the following example the SPHERO™ Rainbow Calibration Particles Cat. No. RCP-30-5 and PMT QC Template are used to determine the logarithmic amplifier linearity of a Dako Cyan™ ADP. The following protocol is used to collect the data for the RCPs:

1. Set a live gate for the siglet population on the FSC vs SSC histogram to exclude aggregates. Since these beads are much smaller than blood cells, the FSC gain has to be increase to place the beads on scale in the light scatter plot.

NOTE: The Relative Channel Number of the initial dot display screen may look cluttered due to the number of the populations and the aggregates. However, after setting a live gate on the FCS vs SSC, the dot display screen is cleaned.

2. Set PMT voltages: Input the instrument settings normally used for specimens in your laboratory. In most instances, the number of peaks will correspond to the histograms as shown in the package insert.
3. Turn off compensation.
4. Collect the plots for your panel, for example: FSC vs. SSC, FL1-log, FL2-log, FL3-log, FL4-log, FL5-log, FL6-log, FL7-log, FL8-log, FL9-log
5. Count a minimun of 5000 events inside the gate.
6. Record the peak value and channels of separation between adjacent peaks.

The calibration plots for the fluorescence channels MEPE, MECY, MEFL, and MEAP using Spherotech Cat. No. RCP-30-5 are shown in Figure 1. These graphs were made by comparing the linear Relative Channel Number of each different fluorescent coated bead population against the MEF, i.e., MEFL, MEPE or MECY, and MEAP value. Originally, this data was collected in the 4 decade log scale in both 256 channel arithmetic/linear (Relative Channel Number) and Geometric (Mean Channel Number, i.e. Relative Brightness). These are the preferred scales for obtaining data when using the SPHERO™ PMT QC Template. However, the SPHERO™ PMT QC Template also provides tables to convert data from other log amplifiers used in flow cytometers. For example, Figure 2 shows the SPHERO™ PMT QC Template Table #2 for converting 1024 Mean Channel Number to 256 Relative Channel Numbers for Beckman Coulter instruments. BD FACS operators should use the SPHERO™ PMT QC Template Table #1 shown in Figure 3 to convert 1024 Relative Channel Number to the 256 Relative Channel Numbers, or the SPHERO™ PMT QC Template Table #3 show in Figure 3 to convert 10^4 Geometric Mean Channel Numbers to the 256 Relative Channel Numbers. However, many of the new instrument provide data in a 5 decade log scale. Figure 5 shows the SPHERO™ PMT QC Template Table #4 for converting 10^5 Geometric Mean Channel Numbers to the 256 Relative Channel Numbers.

After converting the data into the Relative Channel Numbers these values were entered into the CH# Column on the PMT QC Template. An example of the PMT QC Template table for entering the Relative Channel Number for the MEFL channel is shown in Figure 6.

Another benefit of the SPHERO™ PMT QC Template is that can calculate the MEF values of unknowns. The Cross Calibration Table of the PMT QC Template is used to determine the number of related fluorophores for an unknown sample or other particles. Figure 7 shows an example of the Cross Calibration Table for the MEFL channel. The MEF calculation for samples is accurate if the calibration and the evaluated of the unknown samples or particles is performed using the same instrument settings. To perform this operation, first the Relative Channel Number of the unknown is converted into a 256 Relative Channel Number. Then enter the converted Relative Channel Number into the Cross Calibration Table. This table will use the regression equation to solve for the MPE value.

Conclusion

The SPHERO™ PMT QC Template is used to standardize and monitor the linearity of a flow cytometer. A linear Calibration Graph from the SPHERO™ PMT QC Template should be obtained in all channels as shown in Figure 1. This will help identify any problems with the flow cytometer. Important data to note from the Calibration Graphs are the Average Residual Percentage and the Regression Coefficient. The Average Residual Percentage, should be less than 5%. It determines the average percent difference between the data points and the regression line. The Regression Coefficient shows the linearity of the PMT. The Regression Coefficient should be collect over time and graphed on Levy Jennings plots to determine the acceptable instrument operation. Please see STN-8 for more details on performance tracking of flow cytometers. Due to the long-term stability of calibration particles with embedded fluorochromes, the Calibration Graph, once generated, should not change on the same instrument. Any drastic change in the Average Residual Percentage or the Regression Coefficient indicates an instrumental problem.

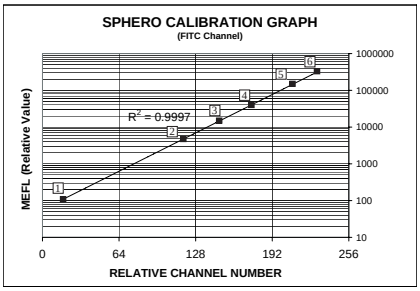
Even though significant instrument-to-instrument variations in the Relative Channel Numbers are expected, the slope of the Calibration Graph should remain the same on similar instruments. The slope of the Calibration Graph is useful when normalizing different instrument within the same laboratory. See STN-9 for more information regarding normalization of different instruments.

The Cross Calibration Table of the SPHERO™ PMT QC Template also enables the users to assign the MEAP, MEFL, MEPE and MEPCY of the unknown sample easily by using the flow cytometer.

References

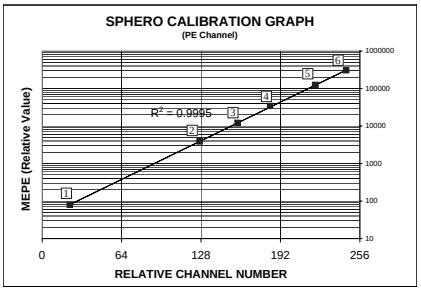
1. BD Biosciences. FACService. Vol 8, Jan 2002
2. Wadsworth Center: New York Department of Health. Online. Available: http://www.wadsworth.org/cellimmum/ci_questions.htm. 28 Feb 2002
3. Spherotech. Sphero TechNote. STN-9 Rev D, August 2007

Fig. 1: SPHERO CALIBRATION GRAPHS for RCP-30-5



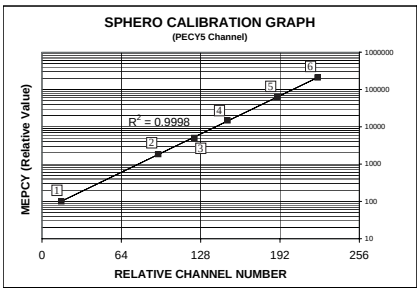
FITC Channel

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



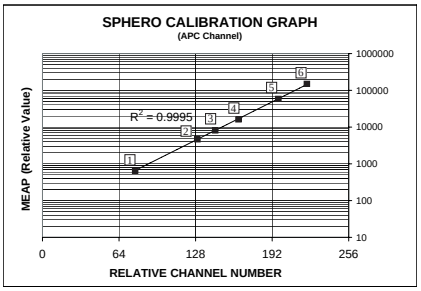
PE Channel

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



PE-CY5 Channel

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



APC Channel

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

TABLE NO. 1	
1024 REL. CH# to 256 REL. CH# CONVERSION	
1024 CH#	256 CH#
64	16
452	113
576	144
684	171
816	204
904	226
	0
	0

Figure 2: Table for Beckman Coulter users to convert 1024 Relative Channel Numbers to 256 Relative Channel Numbers

TABLE NO. 2	
1024 MEAN CH# to 256 REL. CH# CONVERSION	
1024 CH#	256 CH#
0.178	16.03
5.83	113.00
17.78	144.00
46.98	171.00
154	204.00
339	225.93
	#NUM!
	#NUM!

Figure 3: Table for BD FACS users to convert 1024 Relative Channel Number to 256 Relative Channel Numbers

TABLE NO. 3	
10^4 MEAN CH# to 256 REL. CH# CONVERSION	
10^4 CH#	256 CH#
1.78	16.03
58.3	113.00
177.83	144.00
469.8	171.00
1540	204.00
3398	226.00
	#NUM!
	#NUM!

Figure 4: Table for BD FACS users to convert 10^4 Geometric Mean Channel Numbers to 256 Relative Channel Numbers

TABLE NO. 4	
10^5 MEAN CH# to 256 REL. CH# CONVERSION	
10^5 CH#	256 CH#
2.18	15.99
246	112.96
1117	144.01
4164	171.00
20800	204.01
60750	226.00
	#NUM!
	#NUM!

Figure 5: Table for BD FACS to convert 10^5 Geometric Mean Channel Numbers to 256 Relative Channel Numbers

Figure 6: SPHERO™ PMT QC Template table for entering the relative Channel Number obtained for the calibration particles for the MEFL channel

PEAK #	CH #	MEFL	MEFL LOG	CALC.	RESIDUAL	CALC. MEFL
1	16.00					
2	113.00	4700	3.672	3.668	1.15%	4654
3	144.00	15000	4.176	4.173	0.71%	14898
4	171.00	40000	4.602	4.613	2.41%	41039
5	204.00	140000	5.146	5.151	0.96%	141599
6	226.00	330000	5.519	5.510	1.61%	323323
			Ave Residual		1.37%	
					Slope: 0.0163	
					Intercept: 1.8261	
					Rsq: 0.9999	

Figure 7: SPHERO™ CROSS CALIBRATION TABLE for determining the MEF of umknowns

CROSS CALIBRATION TABLE FOR UNKNOWN SAMPLES			
Sample	**CH#**	**Calc.**	**Calc. MEFL**
	13.92	0.8080	6
	103.19	2.2638	184
	162.20	3.2261	1683
	190.67	3.6904	4902
	215.75	4.0993	12568
	236.73	4.4415	27638
	248.28	4.6298	42641
		0.5811	4
		0.5811	4
		0.5811	4
		0.5811	4
		0.5811	4