SPHERO™ Technical Note **STN-8 Rev C. 060706**

CALIBRATION AND PERFORMANCE TRACKING OF FLOW CYTOMETERS USING SPHERO™ CALIBRATION PARTICLES

Introduction

The SPHERO™ Calibration Particles are versatile, stable, economical and convenient to use. These particles contain a mixture of fluorochrome which are spectrally similar to many of the fluorochromes used in flow cytometry. As a result, they are used for routine alignment, day-to-day performance verification and long term performance tracking of several channels of flow cytometers in one run. These particles are very stable since the fluorochromes are entrapped inside the particles instead of being located on the surface. They 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. These products and their uses are described briefly as follows:

Rainbow Calibration Particles (RCPs):

The RCPs are designed for the routine calibration of most available channels in any flow cytometer. For example, these particles are used to verify the instrument set up and to check the linearity and sensitivity of the instrument. If factory recommended procedures are used for instrument set up, we recommend that the RCPs are included into QC programs to track the long term and day-to-day performance.

The RCPs contain a mixture of similar size particles with different fluorescence intensities. Each particle has a mixture of fluorescent dyes entrapped inside. This allows the excitation and detection of the particles in most channels of any flow cytometer. For example, **Figure 1** shows the histograms of the Spherotech Cat. No. RCP-30-5 in the FITC, PE, ECD, PE-Cy5, and APC channels. In addition, the MEF (Molecules of Equivalent Fluorochrome) values of each peak in the RCP-30-5 have been assigned for the FITC, PE, ECD, PE-Cy5, and APC channels.

Since MEF values do not specify the fluorophore used or the intended channel in flow cytometer, Spherotech has decided to use more specific terms, namely: MEFL (Molecules of Equivalent Fluorescein), MEPE (Molecules of Equivalent PE) and MEPCY (Molecules of Equivalent RPE-Cy5), etc. However, the users are welcome to use whatever terms they prefer.

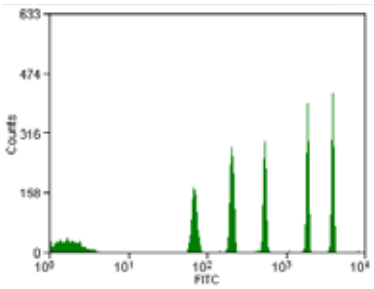
After obtaining the histograms, the Relative Channel Number of the RCPs vs. the MEF (ie. MEFL, MEPE or MEPCY) is plotted to obtain Calibration Graph in all channels as shown in **Figure 2.** These graphs are used to determine the linearity of the instrument and corresponding MEF values of stained cell samples according to the procedures described in Spherotech **STN-9**. The collection of the obtained data provides day-to-day performance verification. This data can be collected over several years for long term performance tracking of the instrument.

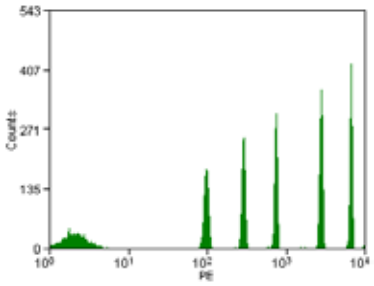
Ultra Rainbow Calibration Particles (URCPs):

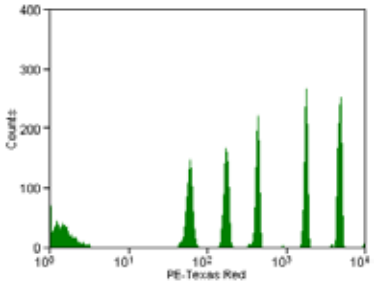
New flow cytometers with an increasing number of fluorescent channels are now available. As a result, the Ultra Rainbow Calibration Particles with enhanced UV and Far Red fluorescence intensity have been designed for performance tracking of flow cytometers with multiple lasers. For example, the Ultra Rainbow Calibration Particles have excellent resolution in the UV, violet, green, yellow, orange, red, far red, and IR channels of the flow cytometer. The URCPs are available in 6 peaks, 3.8μm or 5.1μm.

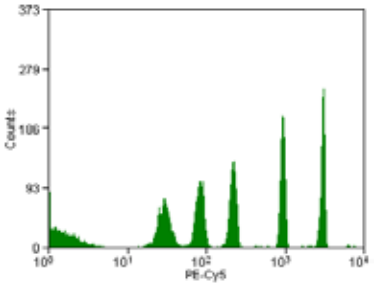
These particles are similar to the RCPs since a calibration graph for the URCPs is obtained using the same procedure. For example, **Figure 3** shows the histograms for the URCP-38-2K, while **Figure 4** shows the calibration curves. However, the URCP have a broader excitation wavelength range and improved resoltuion in the UV and Far Red Channels.

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









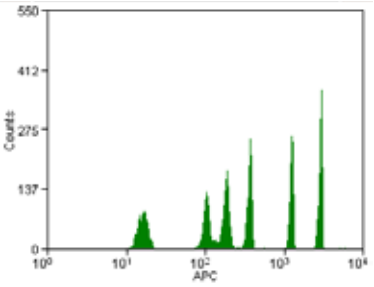
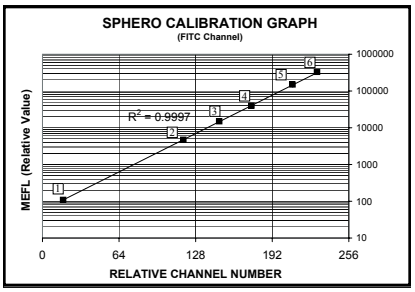
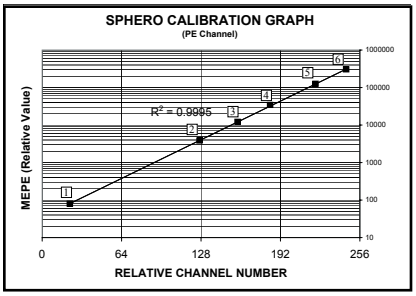


Fig. 2. SPHERO™ Cat. No. RCP-30-5 Calibration Graphs



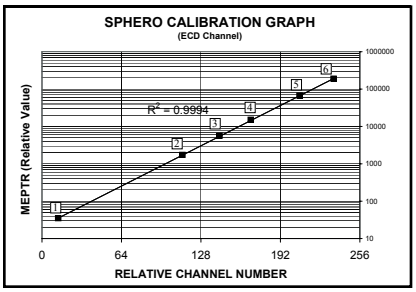
FITC Channel

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



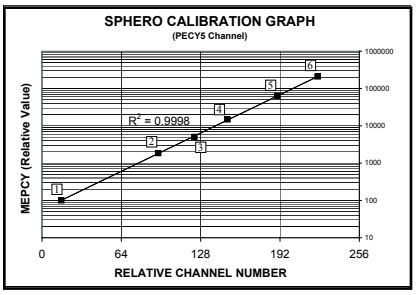
PE Channel

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



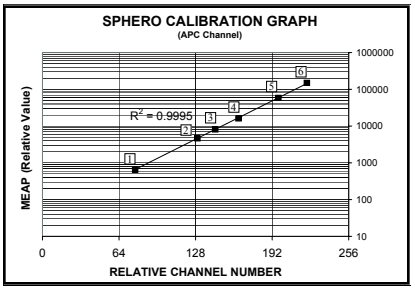
ECD Channel

PEAK	CH#	MEPTR
1	13	
2	115	1660
3	145	5560
4	171	15800
5	209	65940
6	237	190880



PE-CY5 Channel

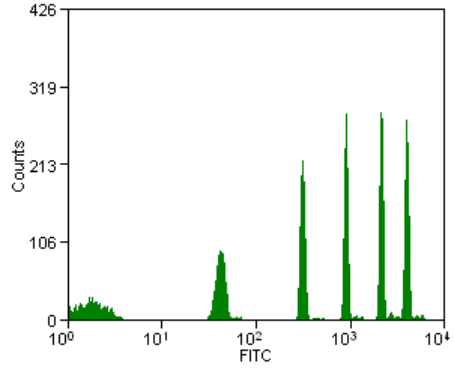
PEAK	CH#	MECY
1	21	
2	91	10000
3	118	27000
4	148	77000
5	190	340000
6	221	1115000

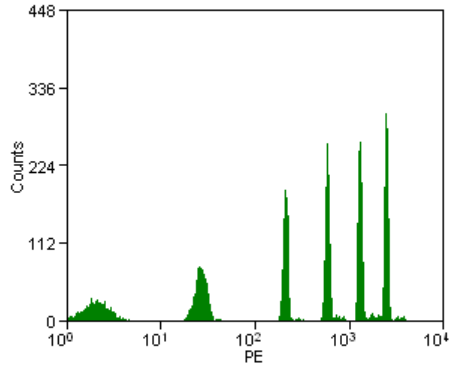


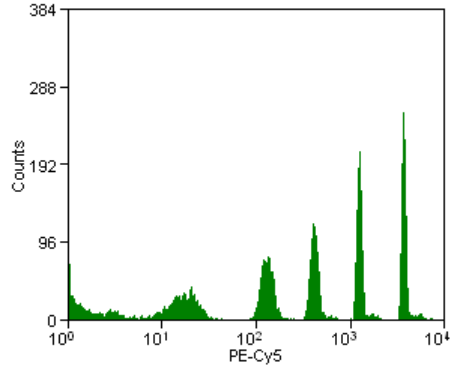
APC Channel

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

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







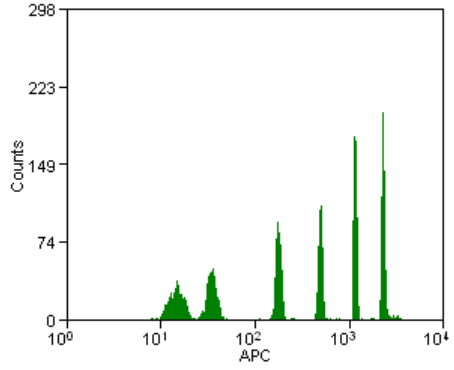
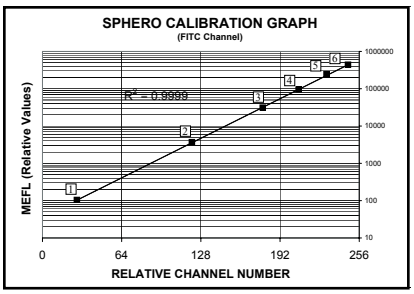
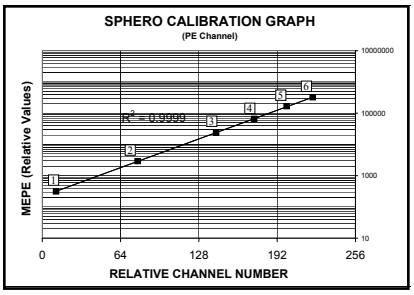


Fig. 4. SPHERO™ Cat. No. URCP-38-2K Calibration Graphs



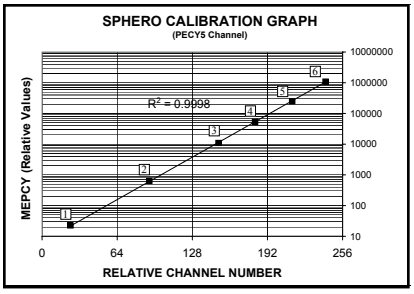
FITC Channel

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



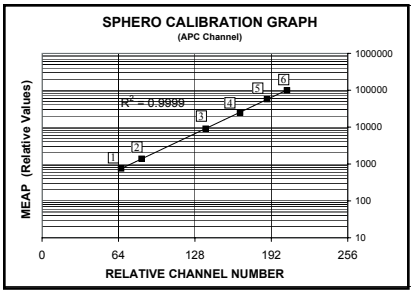
PE Channel

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

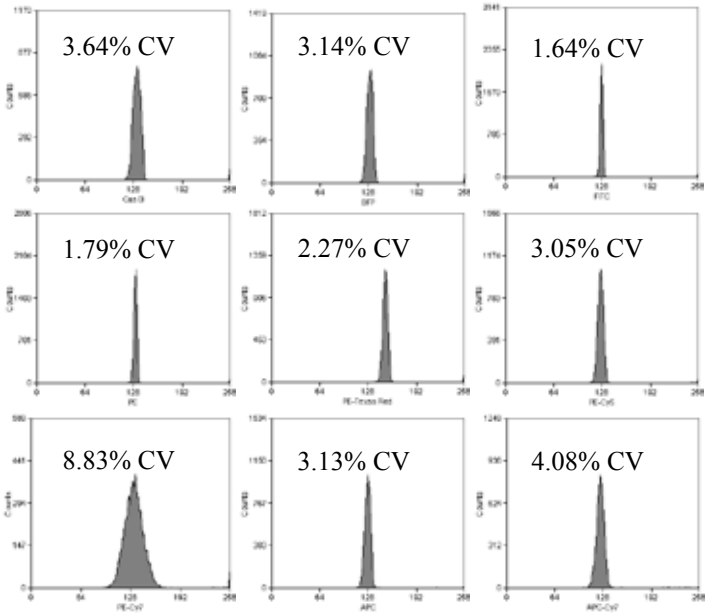
PEAK	CH#	MEAP
1	66	
2	83	1400
3	137	9000
4	168	25000
5	189	57000
6	205	101500

Rainbow Fluorescent Particles (RFPs)

The Rainbow Fluorescent Particles (RFPs) contain uniform size particles with a single intensity. The single peak of the RFP has low fluorescence and size coefficient of variation (CV). As a result, they are useful in the alignment of the optical system of the flow cytometer in multiple channels. For example, Spherotech Catalog Number RFP-30-5 is used to align the FITC, PE, PE-TR, PE-Cy5, and APC channels of the flow cytometer. See **Figure 5** for the histograms of Spherotech Cat. No. RFP-30-5.

The RFPs contain similar fluorophores to the Rainbow Calibration Particles. In addition, the RFPs have similar fluorescence intensities to the brightest peak of the corresponding Rainbow Calibration Particles with the exception of RFP-50-5, RFP-60-5, RFP-100-2 and RFP-30-5A. The RFP-30-5A has the fluorescence intensity similar to stained cells in all channels.

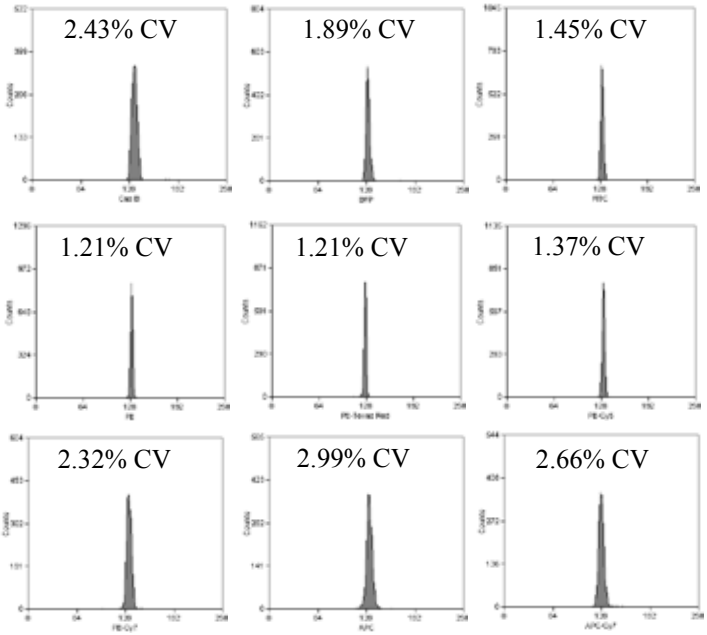
Fig. 5. SPHERO^{TM}
Cat. No. RFP-30-5 Histograms



Ultra Rainbow Fluorescent Particles (URFPs)

The Ultra Rainbow Fluorescent Particles are similar to the Rainbow Fluorescent Particles in that they contain a single fluorescent peak designed for checking the optical alignment of any flow cytometer. They also have low fluorescence and size coefficient of variation (CV). However, they contain similar fluorophores to the Ultra Rainbow Calibration Particles. As a result, they are used in every fluorescent channel of the flow cytometer from UV to IR. See **Figure 6** for the histograms of Spherotech cat. No. URFP-30-5.

Fig. 6. SPHERO^{TM}
Cat. No. URFP-30-5 Histograms



NOTE: %CV is dependent on the flow rate, concentration, and the instrument used to evaluate the Rainbow Fluorescent Particles and Ultra Rainbow Fluorescent Particles.

Yellow Calibration Particles (YFPs)

The Yellow Calibration Particles (YCPs) are intended for the calibration of the FL1 channel of the flow cytometer. The MEFL values of the YCPs have also been determined. However, the MEFL value of the brightest peak (4.88 x 10^6) may be too bright for some users. In this case, the brightest peak can be put off scale to allow only four peaks to show on the screen. The histograms obtained on a CyAn™ ADP analyzer and the MEFL value for all peaks are shown below in **Figure 7**.

Allophycocyanin Calibration Particles (ACPs)

The **Allophycocyanin Calibration Particles** (Cat. No.: ACP-30-5K) contain a mixture of fluorescent particles with different intensities in the Allophycocyanin channel. They are excited by either a He-Ne laser at 632 nm or a diode laser at 635 nm. Similar to the other calibration particles, the MEAP values have been determined. As a result, the ACPs can be used to check the linearity of the instrument in the APC channel. Below in **Figure 8** are the histograms obtained on a CyAn™ ADP analyzer and the MEAP value for all peaks.

Fig. 7. SPHERO™
Cat. No. YFP-70-5 Histograms

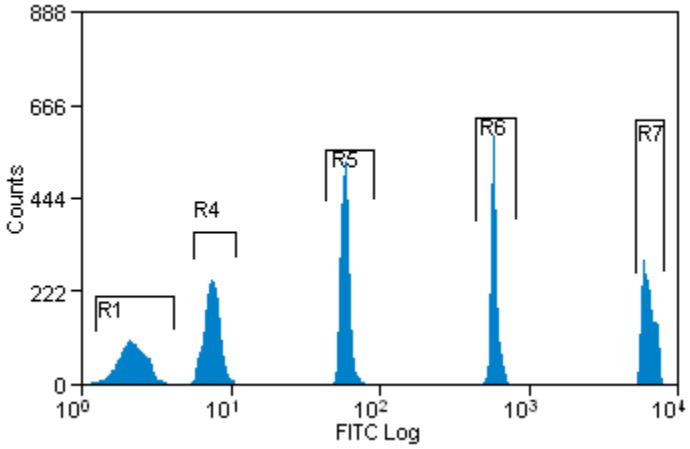


Fig. 8. SPHERO™
Cat. No. ACP-30-5K Histograms

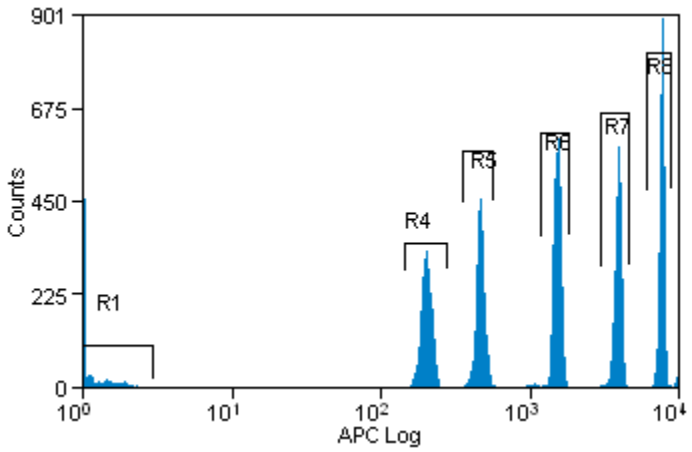


Table 1

YFP-70-5 MEFL Values

YFP-70-5 Peak Number	MEFL
1	~ 600
2	4,250
3	44,500
4	472,000
5	4,880,000

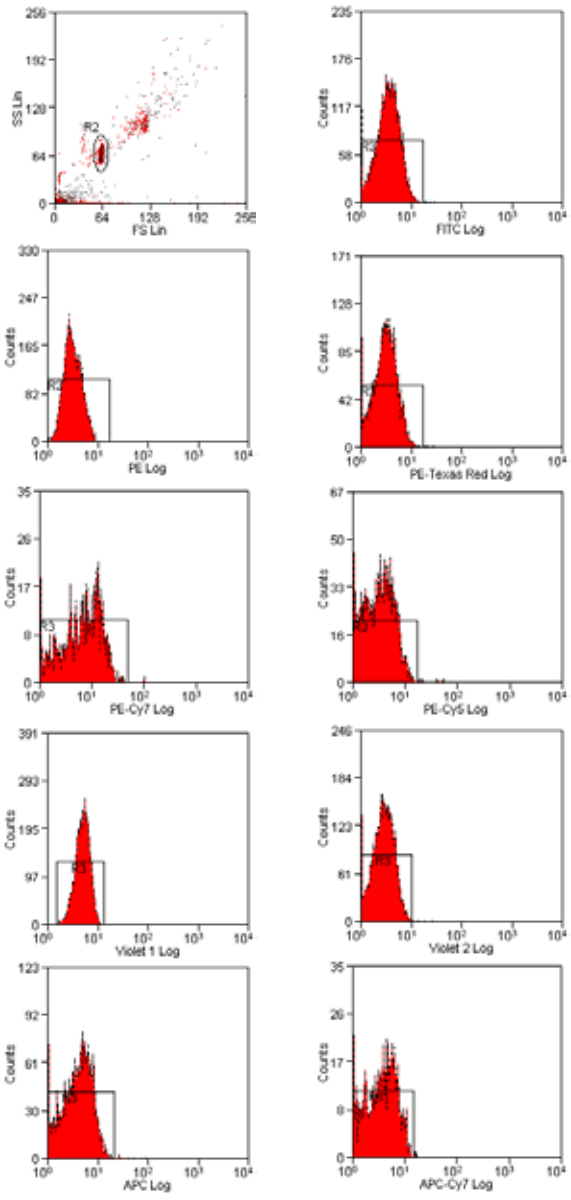
Table 2

ACP-30-5K MEAP Values

ACP-30-5K Peak Number	MEAP
1	N/A
2	7,880
3	17,400
4	53,100
5	130,000
6	208,000

Blank Calibration Particles (BCPs)

The BCPs have similar background fluorescence to unstained cells. They are used to set the fluorescence threshold of the instrument. The BCPs are the dimmest peaks in the corresponding RCPs. Histograms of the BCP-30-5 (3 micron) are shown is **Figure 9**.



Recommended Flow Cytometry QA Procedure

REAGENTS:

1. Sphero Blank Calibration Particles, Cat # BCP-30-5
2. Sphero Ultra Rainbow Fluorescent Particles, Cat. # URFP-30-2
3. Sphero Ultra Rainbow Calibration Particles, Cat. # URCP-38-2K
4. Sphero COMPtrol Compensation Particles, Cat. # CMIgP-50-3K

INSTRUCTIONS FOR USE:

Users are encouraged to modify the procedure described here to fit the QC needs of individual lab or personal preference.

A. Preparation of Particles

1. Vortex the particles vigorously.
2. Add 2-3 drops of calibration and fluorescent beads to 1 mL of sheath fluid. The inclusion of a small amount of detergent (~0.01%) in the dilution buffer will increase the percentage of the singlet population which usually vary from ~75% to 90% depending upon the size of the particles, concentration of the particles and the dilution buffer. The unused portion of the diluted particles suspension can be stored in the refrigerator for future use. If sterility is needed, the particles can be washed once with 70% alcohol or 3% hydrogen peroxide by centrifugation and resuspension as follows:
   a. Add 2-3 drops of particles to 1 mL of dilution buffer in 1.5 mL microfuge tube.
   b. Centrifuge, remove the supernatant and resuspend the particles in 1 mL of 70 % alcohol or 3% hydrogen peroxide by vortexing.
   c. After 5 mins, centrifuge, remove the supernatant and resuspend in 1 mL of sterile dilution buffer.
   d. Vortex, centrifuge, remove the supernatant and resuspend in 1 ml of sterile dilution buffer.
3. Vortex the diluted calibration and fluorescent particles briefly.

B. Daily Alignment

If available, use the single population particles such as RFP-30-5, RFP-30-5A, URFP-30-2, URFP-38-2, URFP-38-5A or other single intensity fluorescent particle to determine the quality of the optical system alignment. To determine the optical alignment of the system perform the following:

1. Set a live gate for the siglet population on the FSC vs SSC histogram to exclude aggregates.
2. Adjust the Gain and High voltage so that the mean channel number of the peak is in a predetermined position on each histogram of interest. **Fig. 5** or **Fig. 6** can be used as a guide if the RFP-30-5 or URFP-30-2 are used.
3. Count a minimum of 5000 events inside the gate.
4. Record the CV, Gain, High Voltage and Relative Channel Number for FSC, SSC and all fluorescence channels of interest as shown in **Table 3**. In addition, a computer program such as Excel can be used to generate the Levy Jennings graphs as shown **Fig. 11 and Fig 12**.

If the values on any parameter exceed those of day-to-day average or preset values, which are determined by at least one months worth of data, additional calibration or alignment procedures should be performed according to the instrument operation manual.

If RFPs or URFPs are not available, align the optical system with RCPs and record all parameters of the instrument and the relative channel number of brightest peak or a designated peak in RCPs.

C. Setting the Threshold

Use the Blank Calibration Particles (Spherotech Cat. No. BCP-30-5) or unstained cells to adjust the forward scatter and to set the threshold. Place a live gate around the singlet population on the forward vs side scatter histogram.

D. Verification of Setting and Validation of Logarithmic Amplifier Linearity and Sensitivity

Use the RCPs or URCPs to verify the instrument settings and to check the sensitivity of the instrument.

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, increase the FSC gain to place the beads on scale in the light scatter plot. Set a gate around the singlet bead population. 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:
   Forward Scatter-linear vs. Side Scatter-linear
   FL1-log, FL2-log, FL3-log, FL4-log, FL5-log, FL6-log, FL7-log, FL8-log, FL9-log
   FL1-log vs FL2-log
5. Count a minimun of 5000 events inside the gate.
6. Record the peak value and channels of separation between adjacent peaks in a lab note book or on a computer program as shown in **Table 4**. Other important data that should be collected include the Calculated Linear MEF, Slope, Intercept, Percent Residual, and R^2. See Spherotech **STN-9** for information regarding these parameters. In addition, the sensitivity can be determined and recorded by cross-calibrating the channel number of the blank particles against the regression line created by the URCP-38-2K fluorescent peaks. The cross-calibration procedure is found in the Spherotech **STN-9.** Collection of the recommended parameters will provide data to generate Levy Jennings graphs for the long term performance tracking of the instrument. An example can be seen in **Figure 13**. These parameters should have values within the upper and lower cut off limit on a day-to-day basis. The upper and lower cut off limits should be determined based on the collection of at least one months collection of data after the instrument has recently beeen serviced. If the instruments performance does not fall within the cut off limits, additional QC protocols or alignment should be performed according to the instrument operation manual.

E. Adjust Compensation

In order to correct for "spillover" or spectra overlapping of fluorescent molecule, compensation should be performed.. In order to use the Spherotech COMPtrol particles for compensation the following procedure has been developed.

1. The compensation tube must consist of particles that are unstained as well as particles that are stained with the fluorescent probes of interest. The preparation of the COMPtrol particles is as follows:
   a. Vortex COMPtrol Particles thoroughly before use.
   b. Add 1 full drop (approximately 50µl) of each of the COMPtrol High Binding Capacity Particles , COMPtrol Low Binding Capacity Particles and Negative Control Particles to a 1.5mL microfuge tube.
   c. Add 10µl of the fluorescent monoclonal antibody conjugate (diluted to a concentration optimal for staining 10^6 cells) to the tube.
   d. Vortex the tube well.
   e. Incubate 15 – 30 minutes at room temperature. Protect from exposure to direct light.
   f. Add 1-2mL of staining buffer to the tube.
   g. Centrifuge the particles at 1500 x g for 1 minute and remove the supernatant.
   h. Resuspend the particles in 500µl of staining buffer to the tube.
   i. Vortex the tube well.
2. Set-up the flow cytometer PMT voltage settings using the target tissue for the experiment. The PMT voltages must be set high enough to guarantee that the COMPtrol negative population is off of the axis in every channel.
3. Gate on the singlet bead population based on FSC and SSC characteristics. Set the analysis gate so that only the COMPtrol particles are included.
4. Create a dot plot for the given fluorchrome-conjugated antibody as appropriate.
5. Perform compensation procedures if necessary by aligning the centers of the COMPtrol particles populations by matching the median fluorescences as shown in **Figure 10**.

Figure 10. Compensation performed on the FITC and APC channels using COMPtrol Particles Cat. No. CMIgP-50-3K

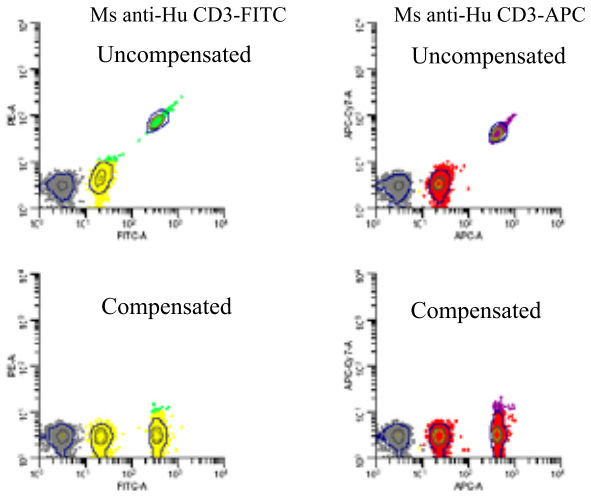


Table 3. Spherotech URFP-38-5A DAKO CyAn™ ADP Data

INSTRUMENT PARAMETERS												
Parameter	**FSC**	**SSC**	**FITC**	**PE**	**PE-TR**	**PE-Cy5**	**PE-Cy7**	**APC**	**APC-Cy7**	**Violet 1**	**Violet 2**	**DATE**
CV	1.83	5.88	2.33	2.59	3.23	4.87	12.81	5.5	4.68	2.66	4.16	7/3/06
GAIN	4.4	1	2	1	2	9.5	20	5	5	1	2	
H.V.	N/A	450	770	826	808	831	894	887	882	665	898	
REL. CH#	64.41	58.88	126.03	127.01	124.45	124.97	126.98	130.9	128.17	124.79	125.68	
PARTICLES: URFP-38-5A, 1:10 dilution												
LOT # : V01												

Figure 11. Levy Jennings Graphs for the %CV of the URFP-38-5A at the 128 Channel Number on the DAKO CyAn™ ADP

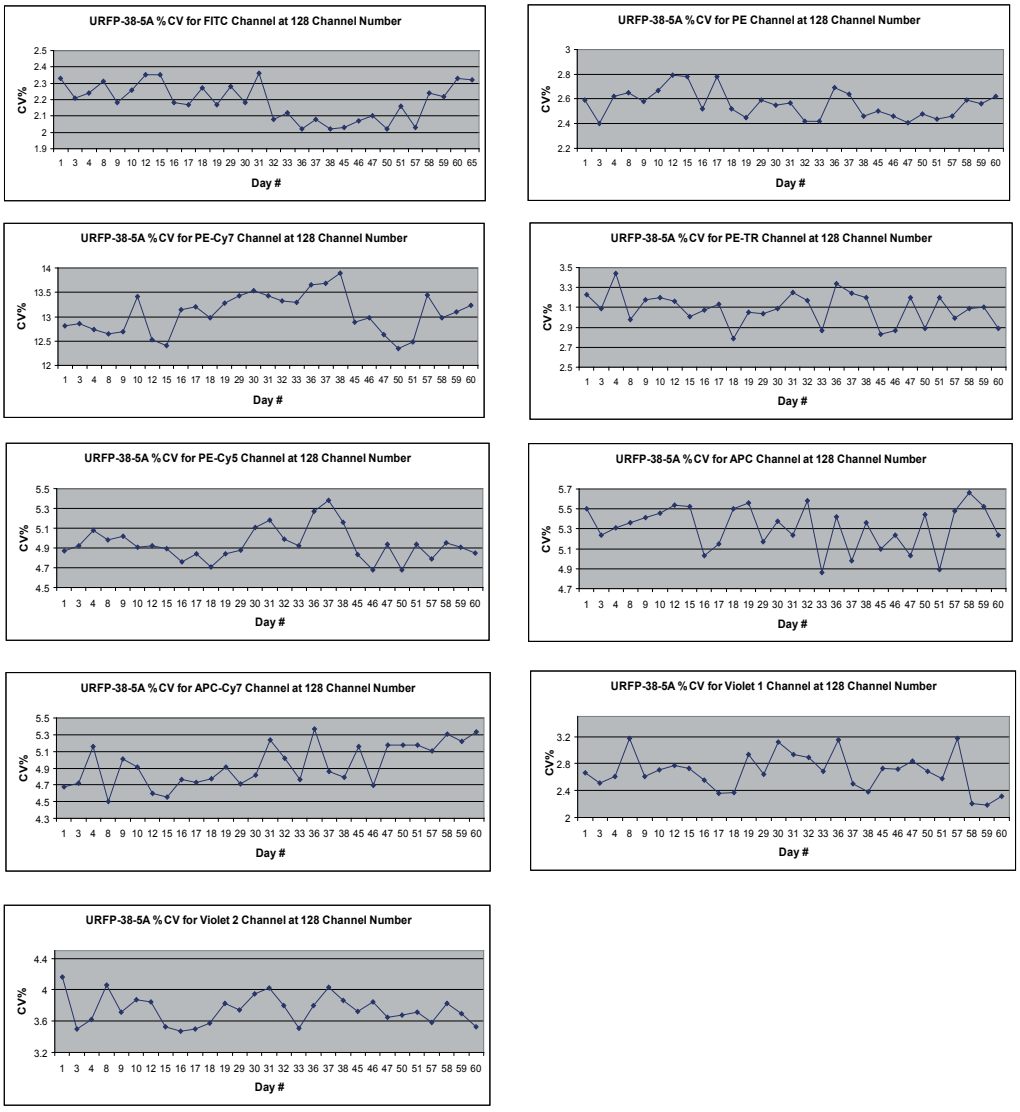


Figure 12. Levy Jennings Graphs for the Voltage Setting used to set the URFP-38-5A at the 128 Channel Number on the DAKO CyAn™ ADP

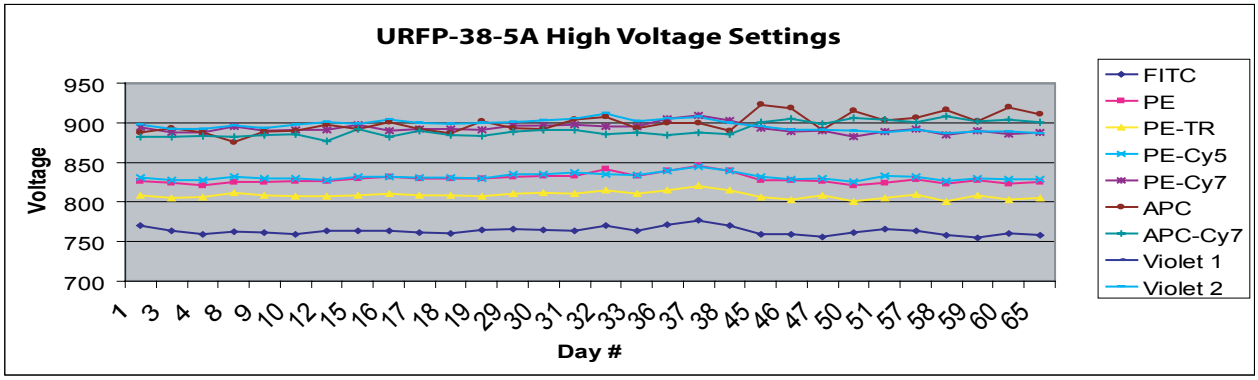


Table 4 . Spherotech URCP-38-2K DAKO CyAn™ ADP Data

Channel	Peak #	Mean CH #	Channels of Separation	Assigned MEF Value	MEF Log	Calc. Log MEF	Calc. Linear MEF	Slope	Intercept	% Residual	R^2	Sensitivity
FITC	2	74.72	N/A	3636	3.561	3.562	3652	0.0161	1.6287	0.81	1.0000	177
	3	592.83	517.11	31180	4.494	4.491	30971					
	4	1735.62	1142.79	93455	4.971	4.972	93864					
	5	4193.97	2458.35	237290	5.375	5.368	233350					
	6	7817.90	3623.93	437385	5.641	5.647	443796					
PE	2	59.18	N/A	2870	3.458	3.462	2899	0.0155	1.7001	1.03	0.9999	173
	3	482.19	423.01	23850	4.377	4.368	23339					
	4	1413.34	931.15	67430	4.829	4.832	67992					
	5	3391.2	1977.86	163085	5.212	5.210	162332					
	6	6752.71	3361.51	319420	5.504	5.508	321980					
PE-TR	2	44.77	N/A	7480	3.874	3.882	7615	0.0153	2.2617	1.61	0.9999	355
	3	374.4	329.63	62600	4.797	4.787	61200					
	4	1152.87	778.47	184935	5.267	5.266	184517					
	5	3168.68	2015.81	508840	5.707	5.697	497625					
	6	7659.69	4491.01	1149085	6.060	6.073	1183174					
PE-Cy5	2	29.33	N/A	630	2.799	2.797	626	0.0212	0.8088	0.70	1.0000	24
	3	242.41	213.08	10900	4.037	4.039	10941					
	4	781.94	539.53	53125	4.725	4.728	53468					
	5	2458.32	1676.38	250350	5.399	5.402	252349					
	6	7271.85	4813.53	1109525	6.045	6.040	1096641					
PE-Cy7	2	10.84	N/A	2725	3.435	3.446	2790	0.0150	2.4526	1.86	0.9999	1536
	3	86.11	75.27	21300	4.328	4.309	20390					
	4	332.70	246.59	73485	4.866	4.872	74479					
	5	1351.26	1018.56	286175	5.457	5.456	285721					
	6	5415.78	4064.52	1073175	6.031	6.034	1082234					
APC	2	36.20	N/A	1400	3.146	3.146	1398	0.0145	1.7004	0.46	1.0000	194
	3	271.35	235.15	9000	3.954	3.957	9053					
	4	808.38	537.03	25000	4.398	4.396	24909					
	5	1959.31	1150.93	57000	4.756	4.753	56604					
	6	3701.72	1742.41	101500	5.006	5.009	102103					
APC-Cy7	2	40.55	N/A	1755	3.244	3.240	1737	0.0161	1.5876	1.13	0.9999	64
	3	302.61	262.06	13655	4.135	4.137	13694					
	4	962.64	660.03	43800	4.641	4.653	44966					
	5	2442.64	1480.00	117260	5.069	5.068	117049					
	6	5178.48	2735.84	257610	5.411	5.404	253312					
Violet 1	2	25.96	N/A	211	2.324	2.316	207	0.0147	0.9821	2.34	0.9998	75
	3	184.81	158.85	1267	3.103	3.119	1317					
	4	518.96	334.15	3519	3.546	3.542	3486					
	5	1409.36	890.40	8917	3.950	3.951	8941					
	6	3393.05	1983.69	20718	4.316	4.311	20474					
Violet 2	2	14.89	N/A	608	2.784	2.786	612	0.0155	1.6193	0.79	1.000	427
	3	100.58	85.69	4104	3.612	3.612	4092					
	4	283.99	183.41	11561	4.060	4.060	11495					
	5	755.63	471.64	30735	4.483	4.483	30437					
	6	1827.38	1071.75	72366	4.865	4.865	73287					

Figure 13. Levy Jennings Graphs for the R^2 of the URCP-38-2K on the DAKO CyAn™ ADP