SpheroTECHNICAL NOTES

STN-8 Rev. B 101095

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

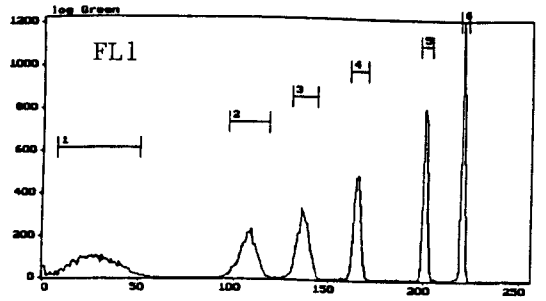
The SPHERO™ Calibration Particles are developed to provide the flow cytometer users with stable and easy to use particles for the routine alignment, day-to-day performance verification and long term performance tracking of flow cytometer. The SPHERO™ Calibration Particles have very small coefficients of variation in both size and fluorescence. They are available in sizes from 2 um to 7 um to suite the preference of the users. These products and their uses are described briefly as follows:

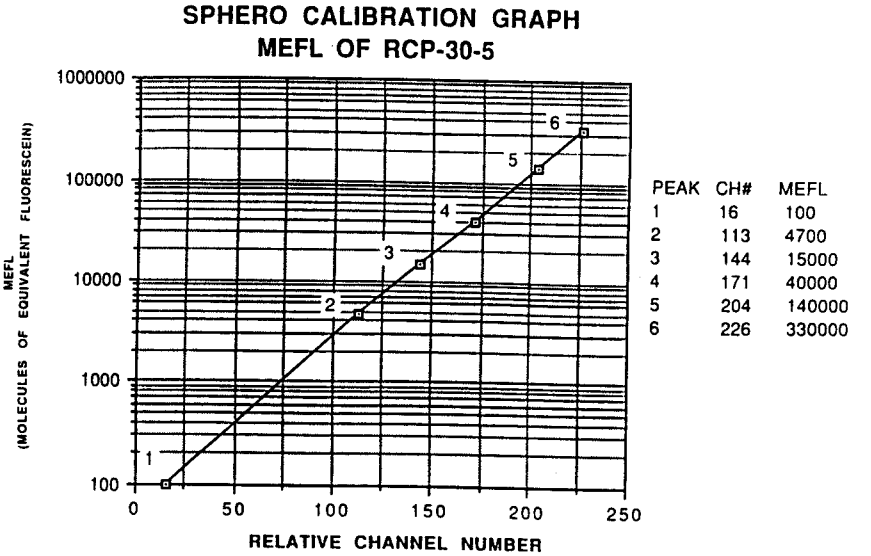
Rainbow Calibration Particles (RCPs):

The RCPs are designed for the routine calibration of all available channels in any flow cytometer; to verify the instrument set up; and, to check the linearity and sensitivity of the instrument. For those labs that already have QC program established which uses factory recomended procedure and particles for routine calibration, the RCPs are still very useful as a supplement to verify the day to day performance and to track the long term performance of the instrument. The RCPs contain a mixture of particles with similar size but with different fluorescence intensity. Each particle in the suspension contains a mixture of fluorescent dyes entraped inside the polystyrene particles which enable the particles to be excited and detected in all available channels of any flow cytometer. The MEF (Molecules of Equivalent Fluorochrome) values of each peak in RCP-30-5 have been determined in FL1, FL2 and FL3 channel (Fig 1a, b and c), which can be used to check the linearity of the instrument and to determine the corresponding MEF values of other RCP's or stained cell samples according to the procedures described in STN-9. 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.

Fig. 1

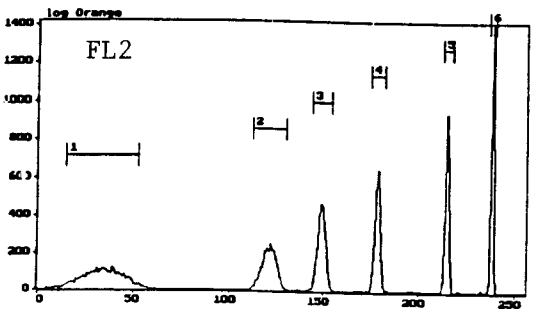
a.

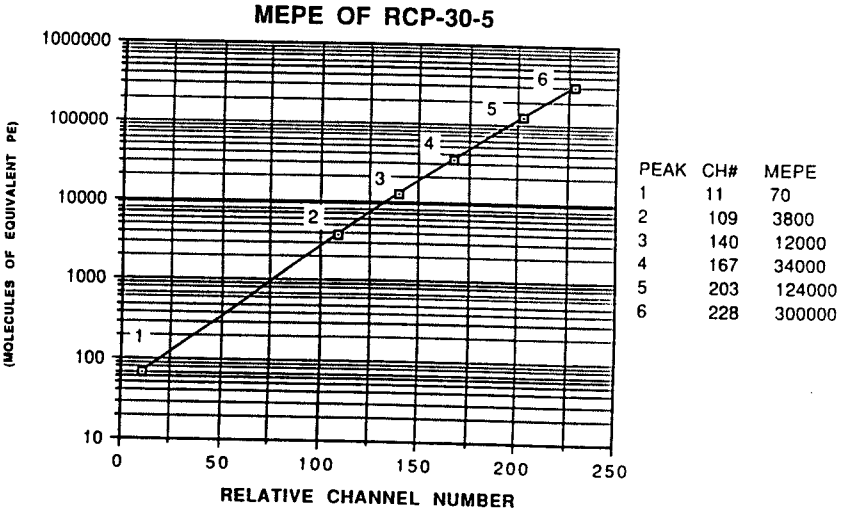




PEAK	CH#	MEFL
1	16	100
2	113	4700
3	144	15000
4	171	40000
5	204	140000
6	226	330000

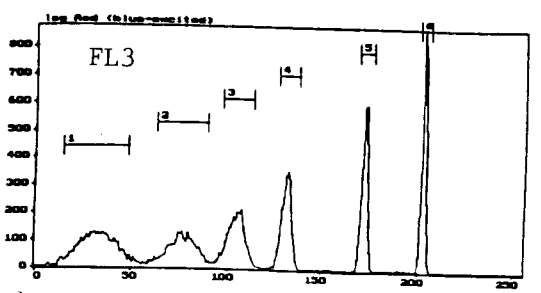
b.

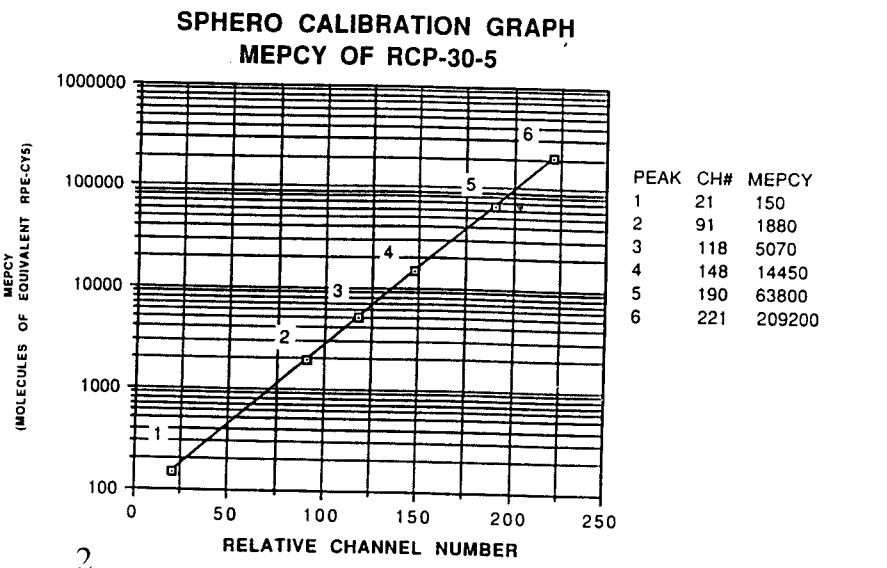




PEAK	CH#	MEPE
1	11	70
2	109	3800
3	140	12000
4	167	34000
5	203	124000
6	228	300000

c.





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

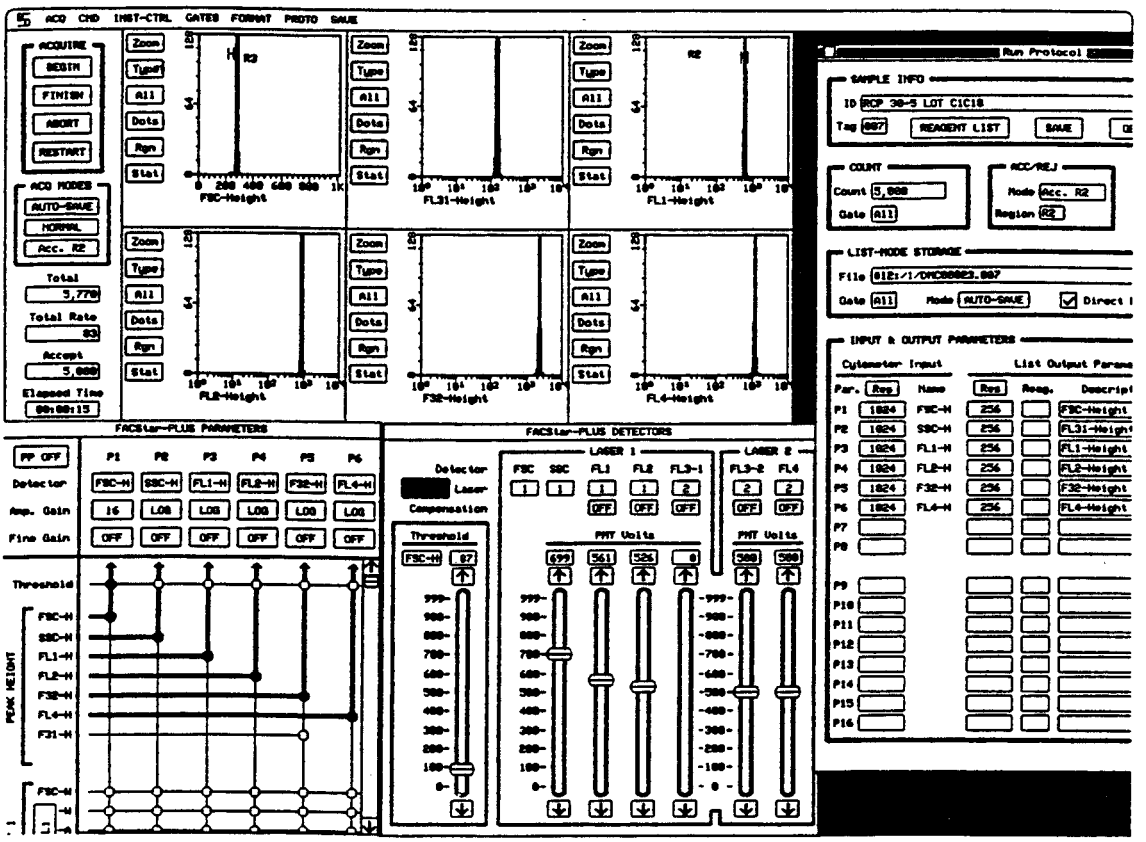
Rainbow Fluorescent Particles (RFPs):

The RFPs contain single population of Rainbow Particles to facilitate the alignment of optical system. The RFPs are the brightest peak of the corresponding RCPs with the exception of RFP-30-5A which is designed to have about the same fluorescence intensity as the stained cells in all channels of the flow cytometer, including the UV channel. A screen print of RFP-30-5A is shown in Fig. 2.

Fig. 2.

RFP-30-5A

RAINBOW FLUORESCENT PARTICLES



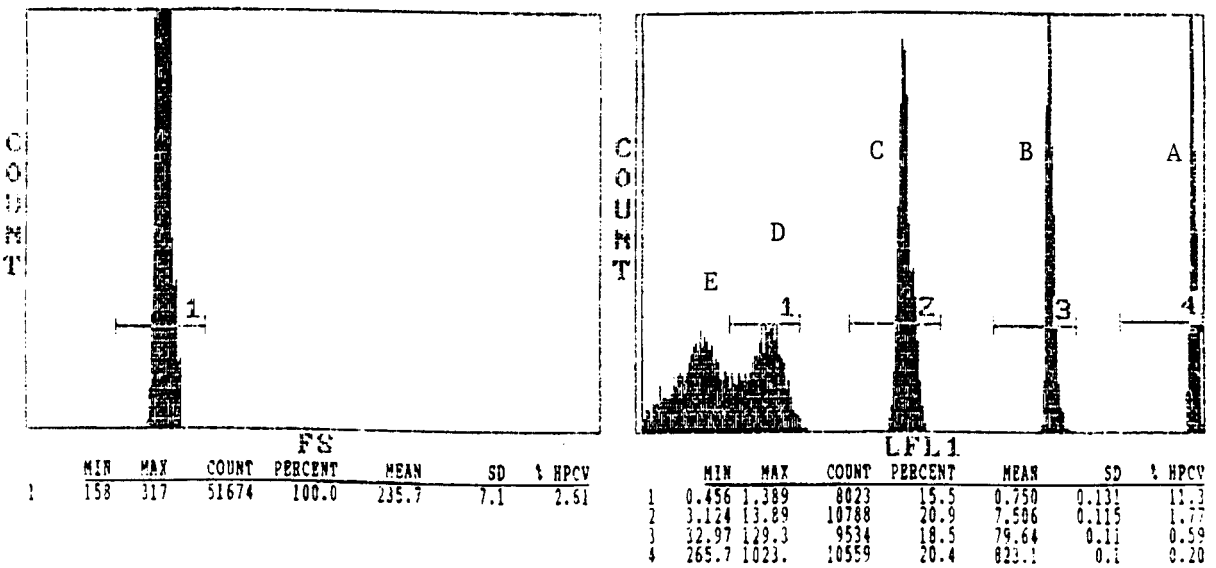
FSC: 488 nm SCATTER ; FL31: RED; FL1: GREEN;
FL2: YELLOW; F32: VIOLET; FL4: BLUE

Yellow Calibration Particles (YCPs):

The YCPs are intended for the calibration of the FL1 channel of the flow cytometer. The MEFL values of the YCPs have also been determined. The MEFL value of the brightest peak (4.88×10^6) 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. A set of histograms obtained on EPICS Profile Analyzer and the MEFL value for all peaks are shown below (Fig. 4):

Fig. 3

YCP-70-5



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	158	317	51674	100.0	235.7	7.1	2.61

	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	0.456	1.389	8023	15.5	0.750	0.131	11.3
2	3.124	13.89	10788	20.9	7.506	0.115	1.77
3	32.97	129.3	9534	18.5	79.64	0.11	0.59
4	265.7	1023.	10559	20.4	823.1	0.1	0.20

PEAK	FL1 MEFL
A	4,880,000
B	472,000
C	44,500
D	4,450
E	~600

Blank Calibration Particles (BCPs):

The BCPs have about the same background fluorescence of the unstained cell and are used to set the fluorescence threshold of the instrument. The BCPs are the Dimmest peaks in the corresponding RCPs.

Procedure:

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

1. Dilute 3~5 drops of the particles to 1 ml of water or buffer. Sonicate or vortex the diluted particles briefly before using. Inclusion of small amount of detergent (0.001~0.01%) 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 used. 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 3~5 drops of particles to 1 ml of dilution buffer in a 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.

2. Use the BCPs 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.

3. Use stained cells or commercially available products to set the compensation for each channel of interest.

4. If available, use the single population particles such as RFPs or RFP-30-5A to align the optical system to obtain the tightest spot on the screen for all channels of interest and set live gate for the siglet population on the FSC vs SSC histogram. The fluorescence intensity of the RFP-30-5A has been adjusted to approximate the intensity of the stained cells. Use the histogram in Fig. 2 as a guide to adjust the Gain and High voltage so that the position of the FL1 peak is similar to the histogram. If other RFPs are used the position of the peak should be on the right side of the screen in the 4-decade log scale as shown in Fig. 1. Count a minimun of 5000 events inside the gate. Record the CVs, Gain, High Voltage and Relative Channel Number for FSC, SSC and all fluorescence channels of interest in the lab note book as shown in Table 1 or computer program such as Sigma Plot to generate the Levy Jennings graphs as shown Fig. 4 a-e. If the values on any parameter exceed those of day to day average or preset values, which are determined by at least one week worth of data, additional calibration or alignment procedure should be performed according to the instrument operation manual. If RFPs 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.

TABLE 1

INSTRUMENT PARAMETERS

	CV	GAIN	H. V.	REL. CH#
FSC				
SSC				
FL1				
FL2				
FL3				
FL4				
FL5				

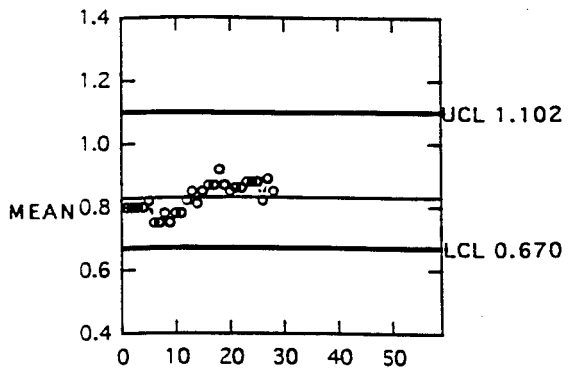
PARTICLES_____ Peak Number: _____

LOT _____ DATE_____

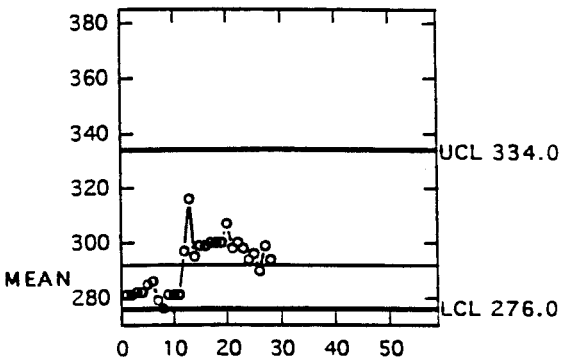
Fig. 4

LEVY JENNINGS GRAPHS OF INSTRUMENT PARAMETERS

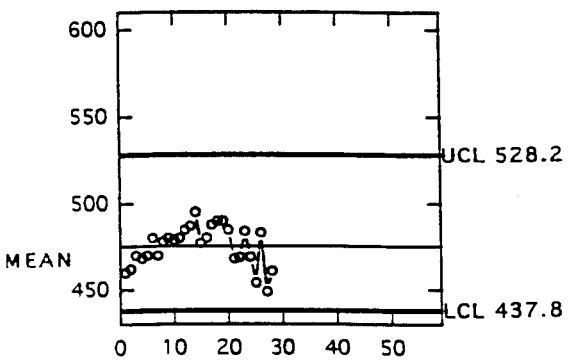
a). GAIN FOR FORWARD SCATTER JUL-SEP 1993



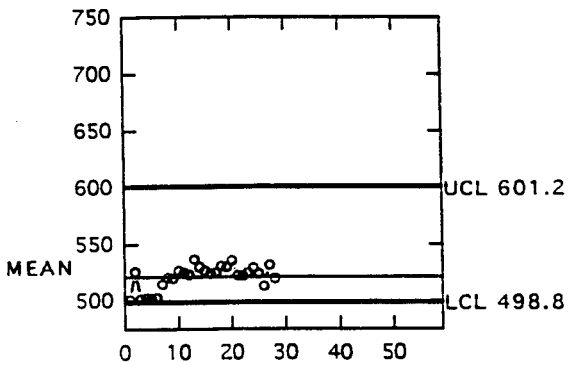
b) HIGH VOLTAGE FOR ORTHOGONAL SCATTER JUL-SEP 1993



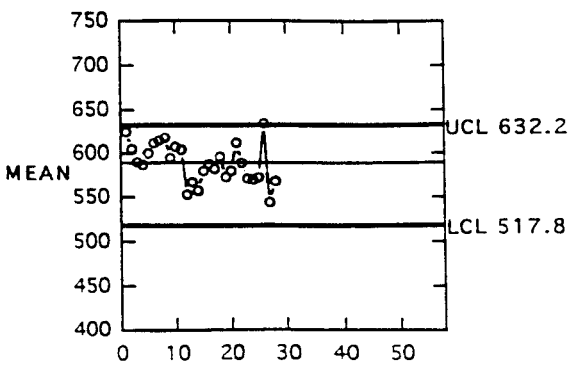
c) HIGH VOLTAGE FOR FITC (P4) JUL-SEP 1993



d) HIGH VOLTAGE FOR PE (P5) JUL-SEP 1993



e) HIGH VOLTAGE FOR CYANINE (P3) JUL-SEP 1993



5. Use the RCPs to verify the instrument settings and to check the sensitivity of the instrument. The Relative channel number of the initial dot display screen may look messy due to the number of the populations and the aggregates. However, after setting a live gate on either the FCS vs SSC or FSC vs FL1 the dot display screen should clean up as shown in Fig 5 and Fig. 6 below using RCP-30-5 and RCP-60-5 respectively as examples.

Fig. 5

GATING OF RCP-30-5 SINGLET PARTICLES

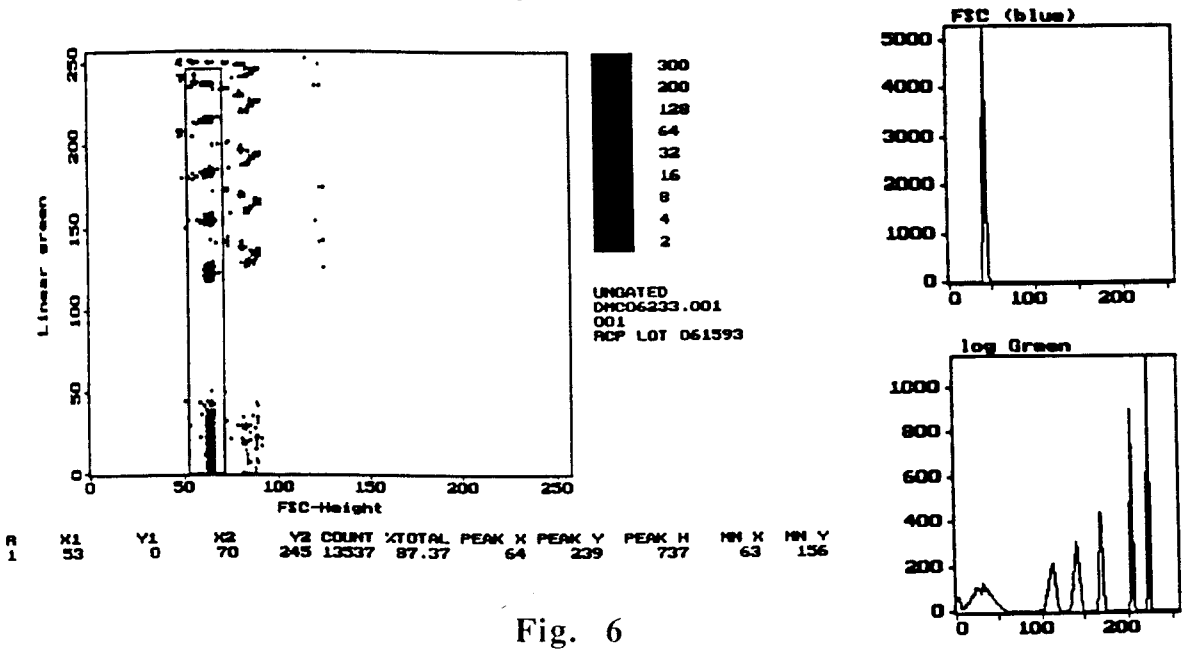
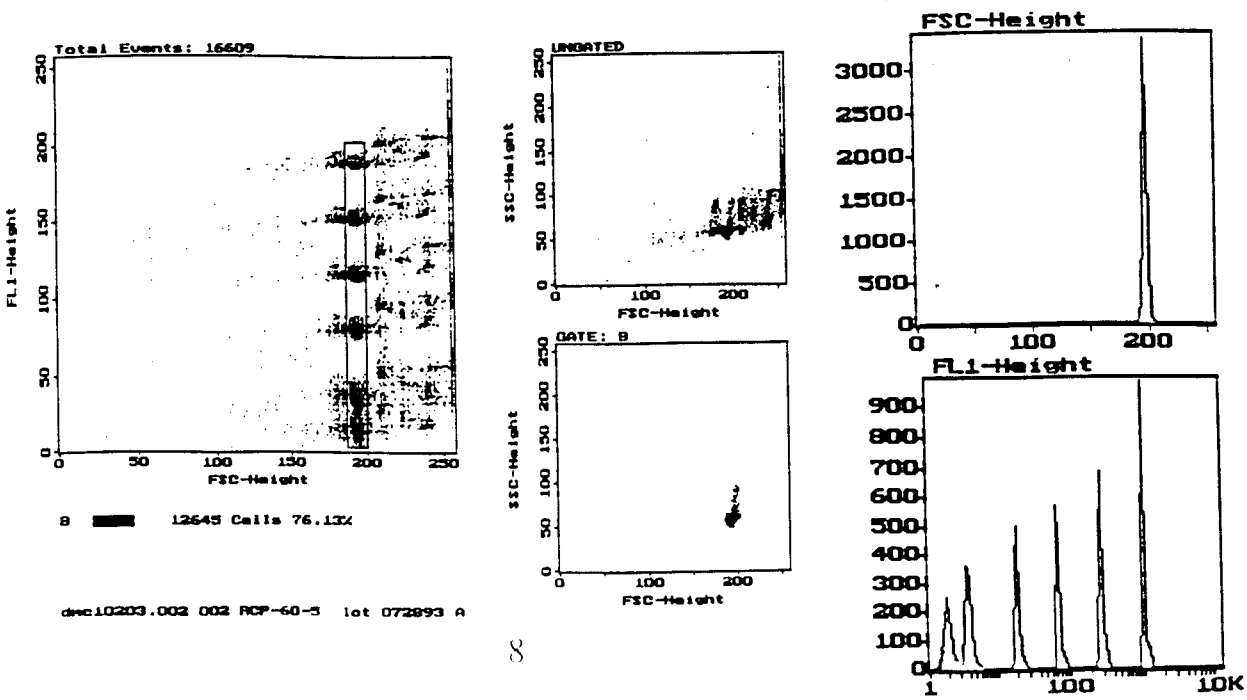


Fig. 6

GATING OF RCP-60-5 SINGLET PARTICLES



Count a minimum of 5000 events inside the gate. The number of peaks should correspond to the histograms as shown in the package insert. To ensure data consistancy, always use the same laser power and adjust the gain or high voltage to reset relative channel number of the designated peak. In some instrument, the dimmest two peaks may not resolve well enough, or the dimmest peak may be absent due to the intrinsic sensitivity of the instrument. Collect and print the data for all fluorescence channels of interest as shown in Fig. 7 using RCP-20-5. Record the peak value and channel separation between adjacent peaks in the lab note book or a computer program as shown in table 2 to generate the Levy Jennings graphs for the long term performance tracking of the instrument as shown in Fig. 8a-c which are kindly provided by Teresa Duling of The University of Iowa using RCP-20-5 as example. The channel separation between adjacent peaks should be whithin the upper and lower cut off limit (UCL & LCL) on the day to day basis. If not, additional QC protocols or alignment should be performed according to the instrument operation manual.

Fig. 7

HISTOGRAM OF RCP-20-5

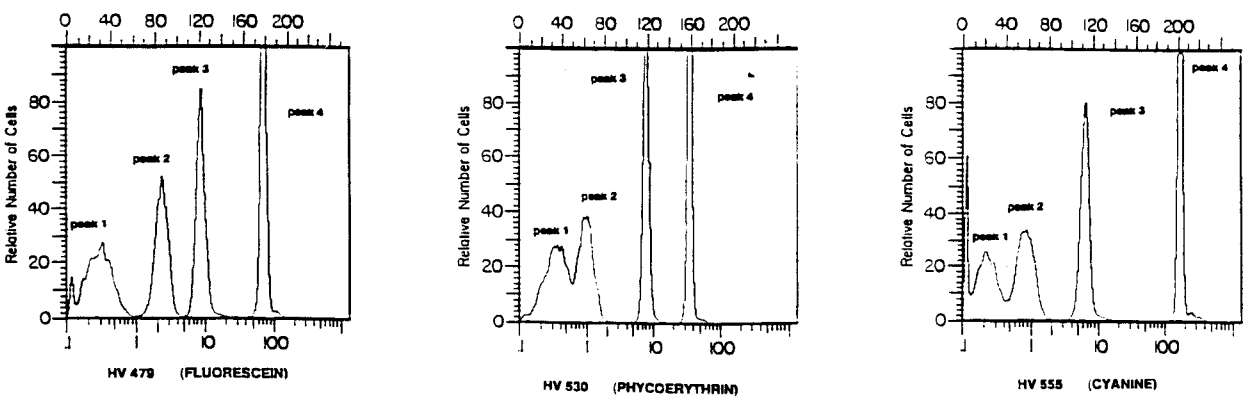


Table 2

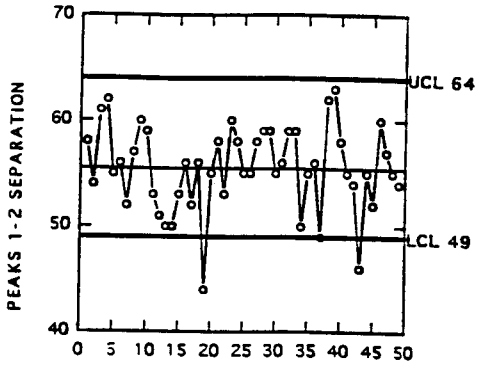
PEAK CHANNEL SEPARATION

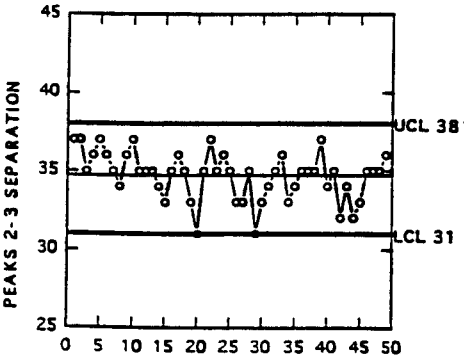
	FL1	FL2	FL3	FL4 (RED)	FL5 (UV)
PEAK 1-2					
PEAK 2-3					
PEAK 3-4					
PEAK 4-5					
PEAK 5-6					

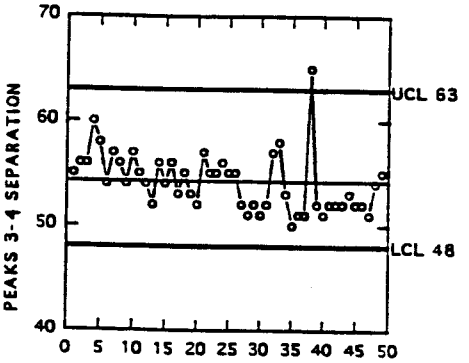
Fig. 8

LEVY JENNINGS GRAPHS OF RCP-20-5 PEAK CHANNEL SEPARATION

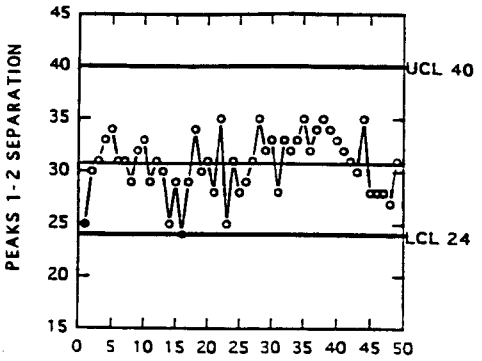
a) FITC (P4) PEAK SEPARATION 1993

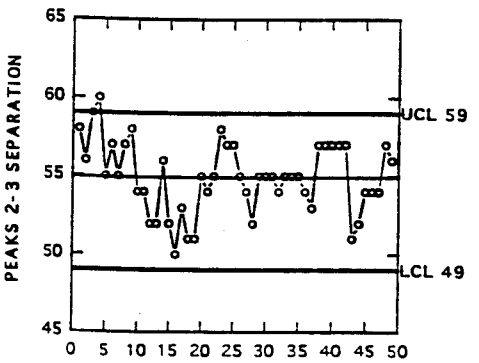


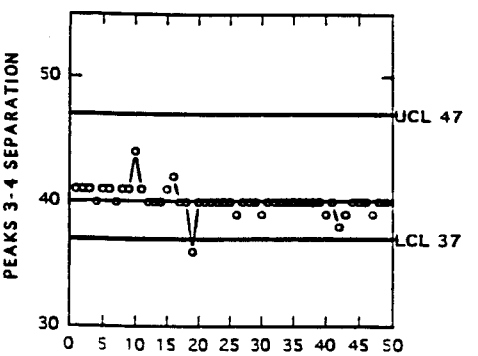




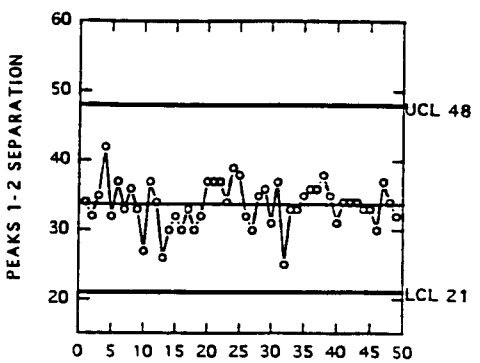
b) PE (P5) PEAK SEPARATION 1993

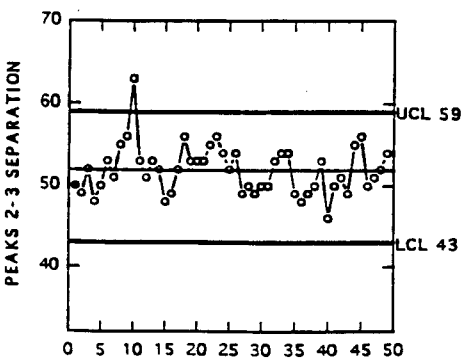


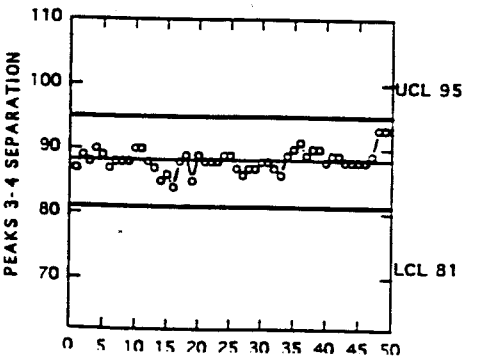




c) CYANINE (P3) PEAK SEPARATION 1993







Spherotech, Inc.
1840 Industrial Dr., Suite 270, Libertyville, IL 60048

Phone: 800-368-0822 or 847-680-8922; Fax: 847-680-8927; E-mail: jwsphero@aol.com
Visit us on the web at *http://www.spherotech.com*