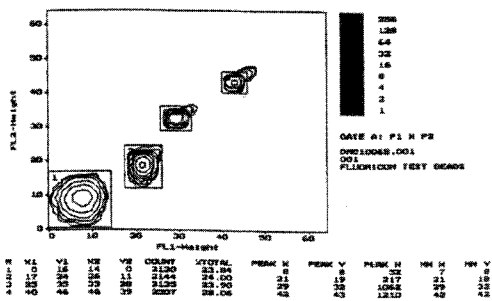


3. SPHERO™ Calibration Particles

SPHERO™ Calibration Particles are designed for routine calibration of flow cytometer and are used extensively by many laboratories for QC and long term performance tracking of the flow cytometer. They can also be used for routine alignment and calibration in fluorescence and confocal fluorescence microscopy.

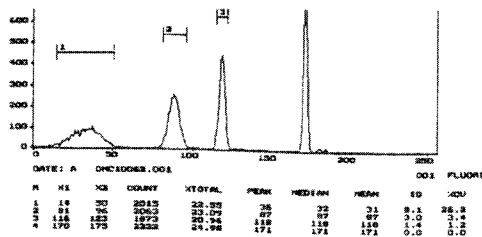
- (A) The **Rainbow Calibration Particles** contain a mixture of several similar size particles with different fluorescence intensities. Every particle contains a mixture of fluorophores that allows excitation at any wavelength from 365 to 650 nm. It enables the calibration of all channels in the flow cytometer with the same set of particles. The fluorophores used are very stable but non-spectral matching to the commonly used fluorophores such as, FITC, PE or PE-Cy5. Dilution of a few drops of the particles from the dropper bottle to 1 mL of a diluent will provide adequate particle concentration for flow cytometer calibration. The diluted Rainbow Calibration Particles remain stable following repeat freezing and thawing. Diluted particle suspension can be stored in the freezer and reused later.

Histograms showing individual peaks representing various fluorescence intensities in Rainbow Calibration Particles (Catalog # RCP-20-5) are shown in Figure 3 below (courtesy of Dr. David Coder of University of Washington, Seattle, WA).



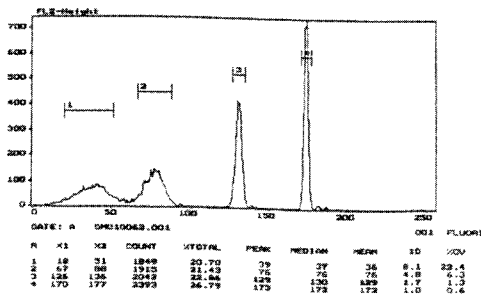
FL1 vs. FL2 Fluorescence Distributions

The contour plot displays the singlet bead distributions of doubly-labeled beads. A slight shoulder (upper right) may indicate the inclusion of some doublet beads not gated out. Gates defined on FS vs. FL1 for example, show how to easily resolve singlet beads. The FL2 brightness of population 2 is slightly less than the FL1 brightness of the same population.



Green Fluorescence Distribution of Singlet Beads

Histogram of gated FL1 distribution; the singlet beads of different intensity are clearly resolved.

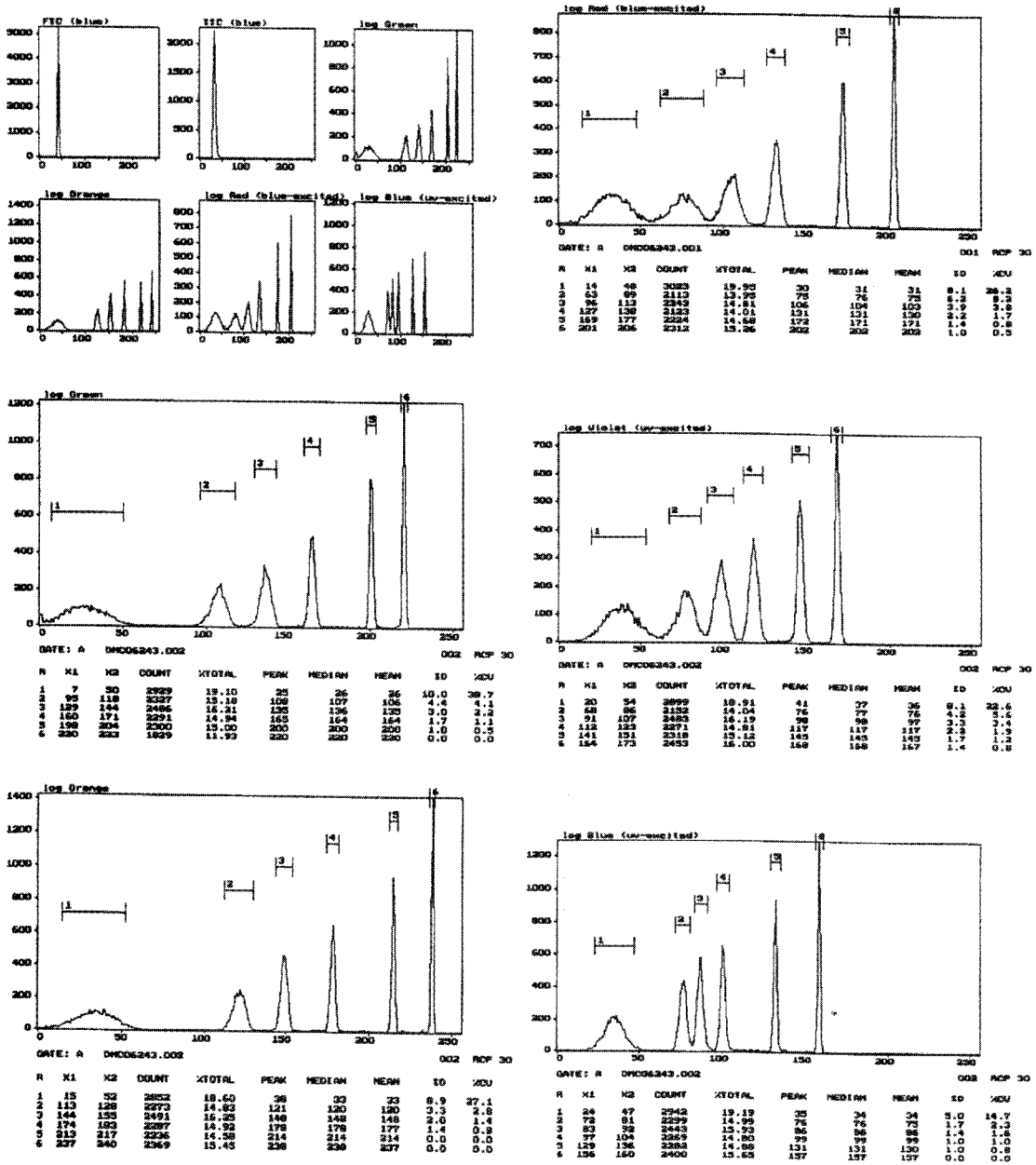


Yellow Fluorescence Distribution of Singlet Beads

Histogram of gated FL2 distribution; the singlet beads of different intensity are clearly resolved as are the yellow beads.

The FACStar Plus* data of the Rainbow Calibration Particles (Cat. No.: RCP-30-5) is shown below to illustrate the utility of Rainbow Calibration Particles in all channels of the flow cytometer.

Figure 4



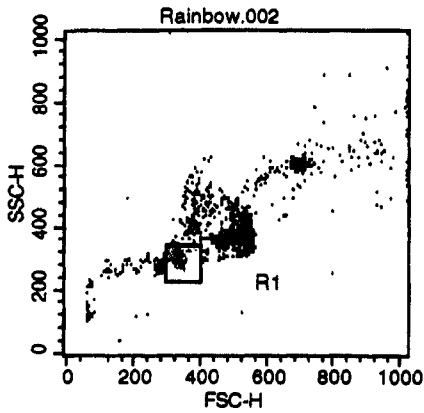
* FACStarPlus is a Trademark of BDIS, data provided by Dr. Coder, U. of Washington

The histograms of RCP-30-5 obtained from Becton Dickinson FACSCalibur in all four channels

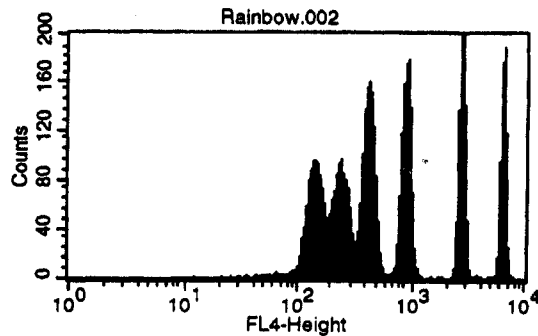
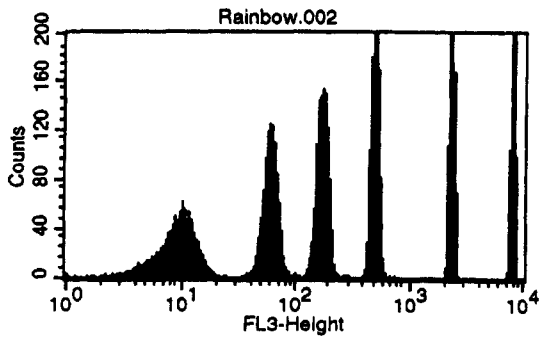
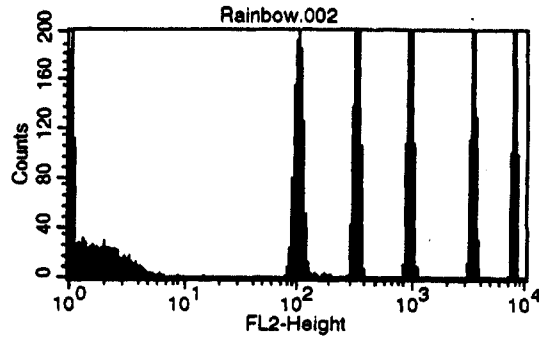
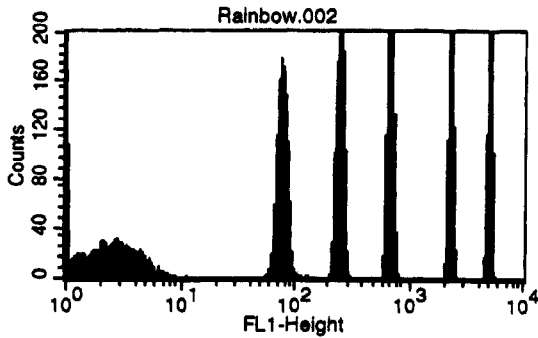
10/4/97

1:00:23 PM

BECTON
DICKINSON



SPHERO™
RAINBOW CALIBRATION PARTICLES
RCP-30-5



*FACSCalibur is a Trade Mark of BD Biosciences

The histograms of RCP-30-5 obtained from Beckman Coulter EPICS in all four channels

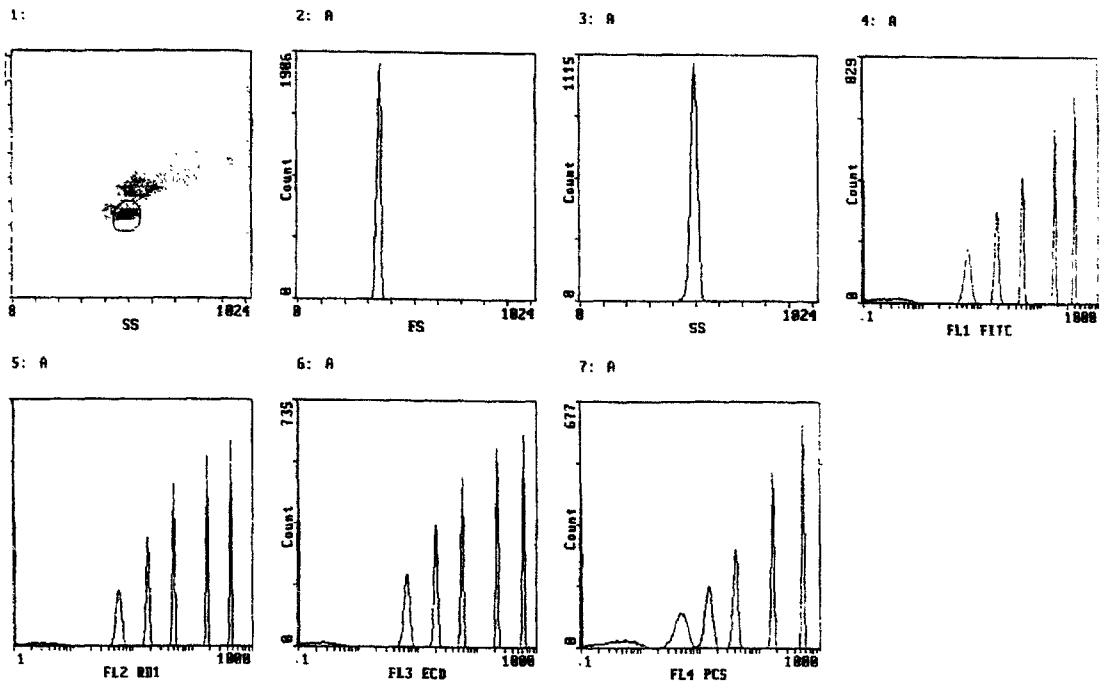
COULTER CORPORATION

COULTER(R) EPICS(R) Acquisition Flow Cytometry Report

4Oct97 17:39:07
3COLOR SUBSET PANEL CD45 GATED Z0000325
rainbow
126 seconds, 45236 events
Manual Stop

ID: FLOW

Flow cytometry. from prot. spherotech



Normalized, Listgating: Disabled

Region ID	%	Count	Mn X	Mn Y	PkPosX	PkPosY	PkCnt	FPCVX	FPCVY
A A	85.5	38670	487.9	339.9	480.0	336.0	7286	3.22	3.16

FL1 **FITC**
FL2 **PE**
FL3 **PE-TR**
FL4 **PE-Cy5**

SPHERO™
RAINBOW CALIBRATION PARTICLES
RCP-30-5

*EPICS is a Trade Mark of Beckman Coulter Corporation

The relative number of fluorophores per particles has been determined for every peak of RCP-30-5 in FL1 (FITC, MEFL), FL2 (RPE, MEPE), FL3 (RPE-Cy5, MEPCY) and FL4 (APC, MEAP) channels of flow cytometer to plot the calibration graph as shown below. The calibration graph can be used to check the linearity of the PMT in each channel. The relative number of fluorophores can be cross calibrated with cells or particles stained with known number of spectral matching fluorophores, such as FITC, PE, RPE-Cy5, to estimate the number of fluorophores on stained cells. The RCP-30-5A, which is identical to RCP-30-5 with the exception of two more peaks between the blank peak and the dimmest peak of RCP-30-5 to give a total of 8 peaks as shown in Figure 6. The RCP-30-5A is very useful in checking the sensitivity and resolution of the flow cytometer.

A Template for MS Excel files, as shown on page 28, is available 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.

Fig. 5

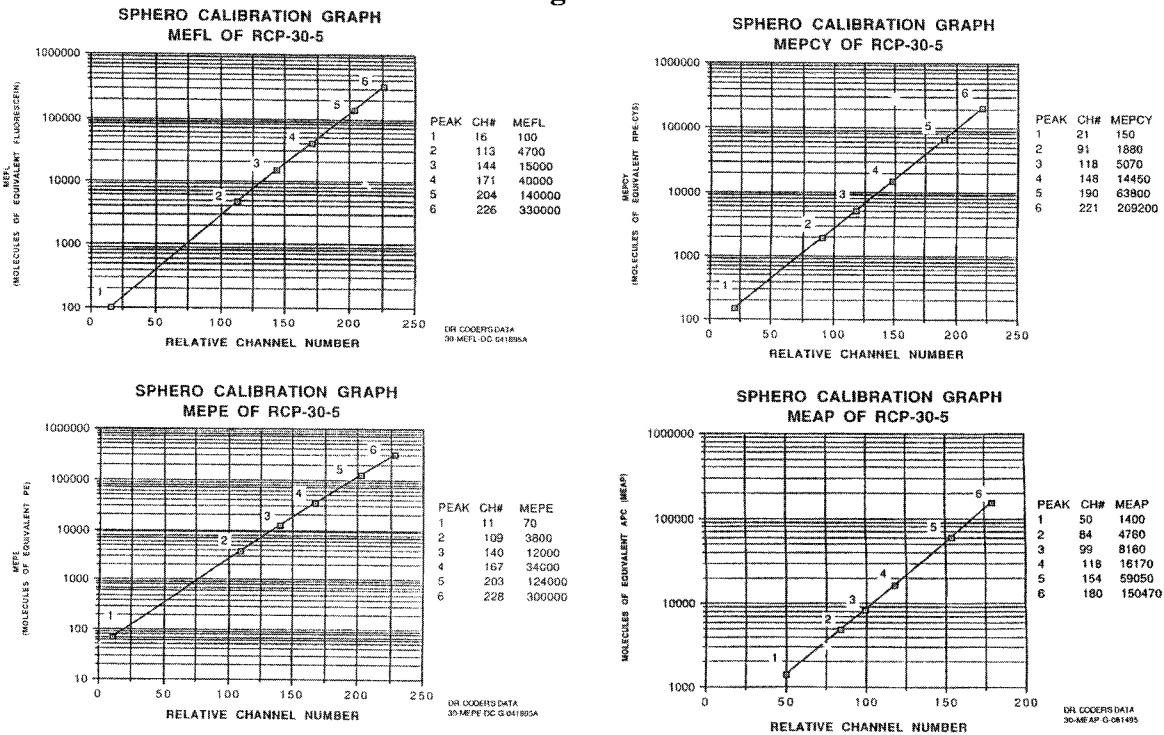
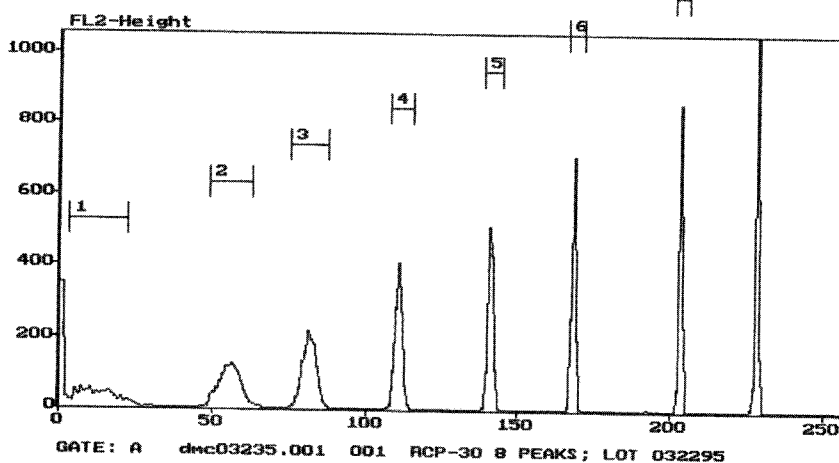


Fig. 6 RCP-30-5A

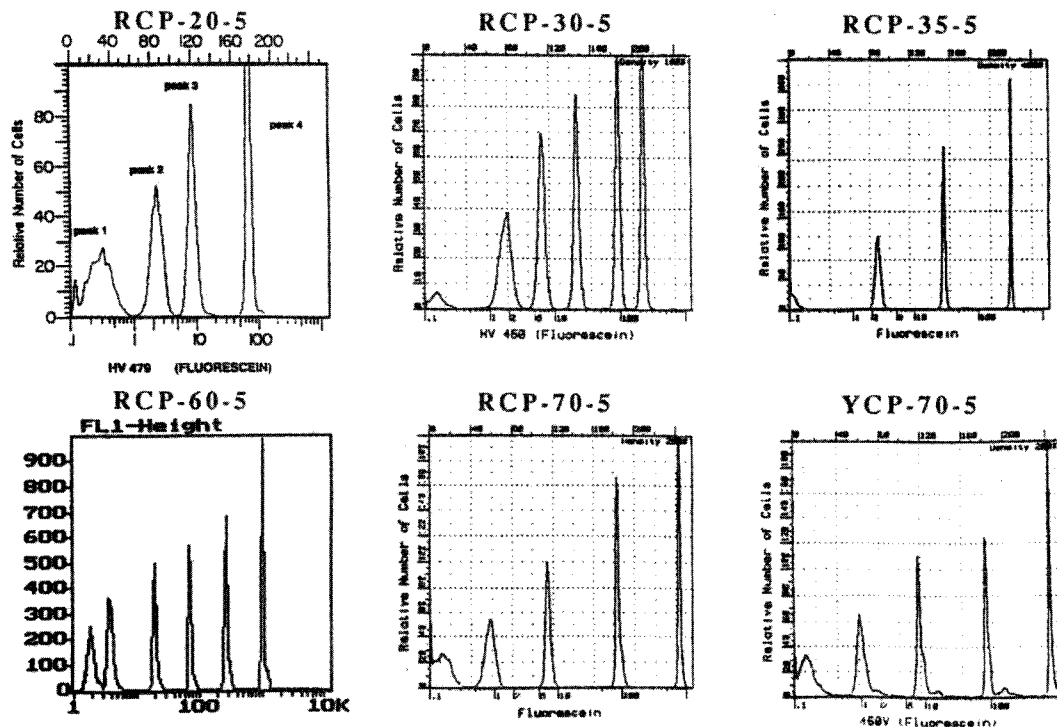


The Rainbow Calibration particles are available in size of 2 to 7 μm as shown in Figure 7 to suit various applications.

Figure 7

SPHERO™ CALIBRATION PARTICLES

FL1 Histograms



- (B) The **Rainbow Fluorescent Particles** are similar to Rainbow Calibration Particles as described before except these represent single peak, uniform size particles for use alone or in combination with others. The Rainbow Fluorescent Particles are usually the brightest peak of the corresponding Rainbow Calibration Particles as shown in Figure 7 with the exception of RFP-50-5, RFP-100-2 and RFP-30-5A. The RFP-30-5A has the fluorescence intensity similar to the stained cells in all channels. The RFP-50-5 can be used as internal standards to estimate the number of cell counts with flow cytometer. Since the Rainbow Fluorescent Particles contain single peak with very small fluorescence and size CV, they are very useful in the alignment of the optical system of the flow cytometer in all channels.

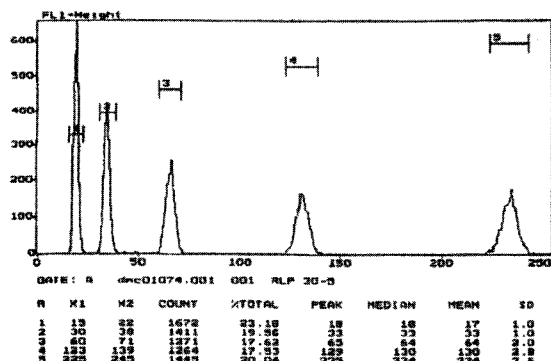


Go for the best!

Use Rainbow Calibration Particles for routine flow cytometer calibration. Regular use of Rainbow Calibration Particles will monitor the performance of your flow cytometer and improve the consistency of your data.

(C) The **Rainbow Linear Calibration Particles** (Cat. No.: RLP-30-5) are designed to check the linearity of the PMT in the linear scale of all available channels in the flow cytometer. RLP-30-5 are also useful for the quantitation of the DNA content of cells stained with either Hoechst dyes, DAPI, Ethidium Bromide, Propidium Iodide, YOYO or TOTO. The representative histograms of RLP-30-5 in various channels are shown below.

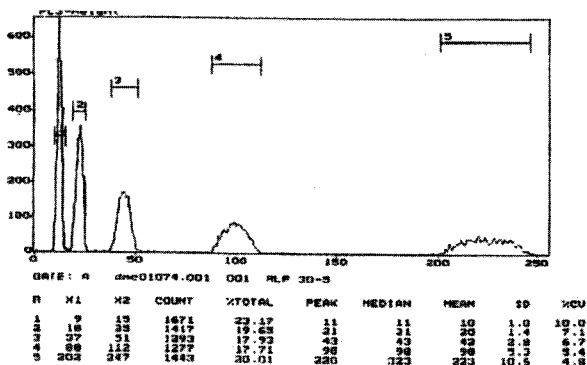
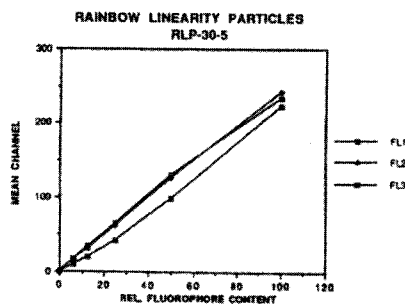
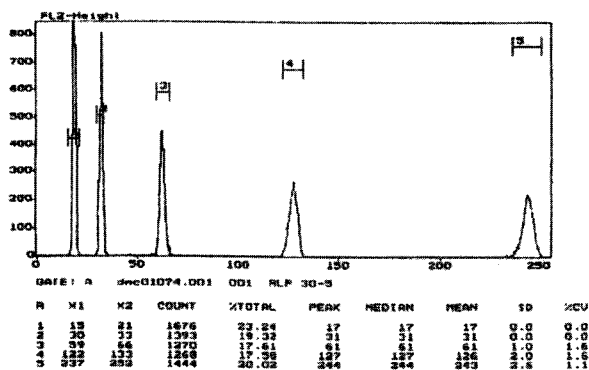
Figure 8



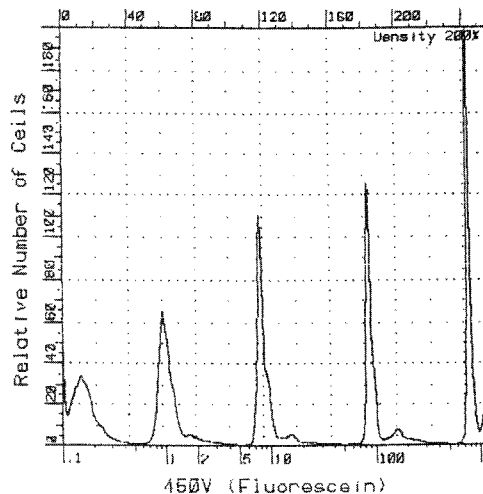
RLP 30-5 beads with linear amplification

Gated on singlet beads; all 5 populations are present for each color group.

Color	Bead Pair	Ratio
FL1	1:2	1.83
	2:3	1.94
	3:4	2.03
	4:5	1.90
FL2	1:2	1.82
	2:3	1.97
	3:4	2.07
	4:5	1.93
FL3	1:2	2.00
	2:3	2.10
	3:4	2.33
	4:5	2.28



- (D) **Yellow Calibration Particles** (Cat. No.: YCP-70-5) are designed for routine calibration of the Fluorescein channel in the flow cytometer. They contain a set of similar size Yellow Particles with different fluorescence intensities. The Yellow Calibration Particles, like other Fluorescent Particles, are more stable than particles that are surface labeled with FITC. A histogram of Yellow Calibration Particles (courtesy of Dr. Parks of Stanford University) is shown below.

Figure 9

- (E) The **Blank Calibration Particles** are the blank peak of the corresponding Rainbow Calibration Particles as shown in Figure 7. The Blank Calibration Particles can be used to set the threshold of the instrument in each channel.
- (F) The **Allophycocyanin Calibration Particles** (Cat. No.: ACP-30-5K) contain a mixture of 3.0 μm fluorescent particles with different intensities in Allophycocyanin channel and can be excited with either He-Ne laser at 632 nm or a diode laser at 635 nm. The number of Allophycocyanin per particles has been determined as shown in Figure 10.

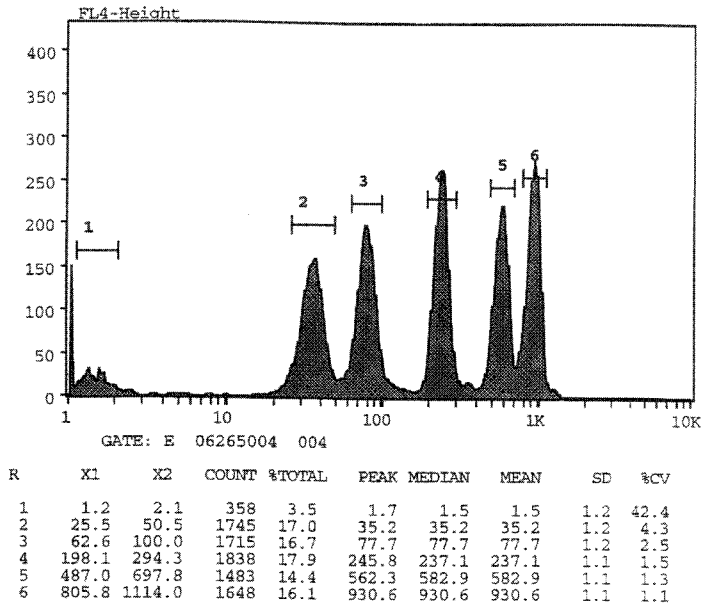


Spherotech carries a wide selection of Rainbow Calibration Particles offering:

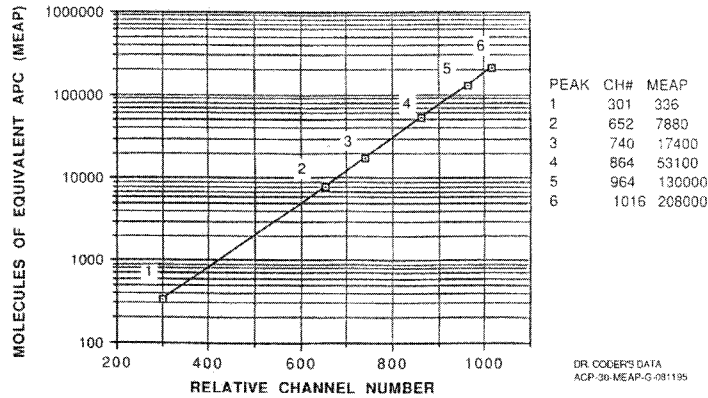
- Multiple peaks
- Multiple channels
- Long term stability
- Cost saving
- Convenience

A Template for MS Excel files, as shown on page 28, is available 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.

Fig. 10
ACP-30-5K



SPHERO CALIBRATION GRAPH
MEAP OF ACP-30-5K



Due to the stability of SPHERO™ Rainbow Calibration Particles, these particles are very useful for long term tracking of instrument performance as well as for routine calibration of the flow cytometer. Advantages of using water insoluble fluorescent dyes to obtain fluorescent particles as compared to surface labeled microparticles are listed below.

The SPHERO™ Fluorescent Particles and Calibration Particles are:

- Very stable and may be used for several years when stored properly.
- Can withstand freeze-thaw cycles; diluted particles can be stored frozen for later use.
- Available with multiple fluorophores incorporated in the same particle; may be used in different channels of the flow cytometer.
- Available with different fluorescent intensities on the same size particles.
- Can be sanitized by treating with 70% ethanol or other antibiotic agents.
- Economical.