Vesicle Flow Cytometry Analysis Kit

FOR VESICLE COUNTING AND SIZING

Protocol 5. Instrument characterization using vCal™ nanoRainbow Beads

Purpose

Assess vFRed fluorescence resolution, calibrate FITC and PE fluorescence channels, assess laser alignment, and calibrate instrument flow rate.

Materials

Kit Component	Size	Store
vCal™ nanoRainbow beads	1 mL	4°C
vFC™ Staining Buffer	100 mL	4°C

Materials to be provided by User

Gloves
Microwell plate (Sartstedt 82.1583.001)
Pipettes (5 uL – 300 uL)
Pipette tips

Procedure

1. Open vCal™ nanoRainbow Instrument Calibration experiment template
2. Place 100 ul of nanoRainbow Beads in a well and record 30 seconds of data under the same flow rate high:60 uL/min) and detector gains as for vFC (change trigger parameter to VSSC and trigger level to ~100,000)
3. Measure at same fluorescence channel gains as for EV analysis.
   Note: Scatter gains should be set to allow resolution of bead singlets from doublets and background

Analysis

1. Open the **vCal™ nRB Report Layout** and load the nanoRainbow Beads data file.
2. On the **Gating Tab**, inspect the time history and adjust the nanoRainbow gate on the population of single nanoRainbow Beads.

Assess vFRed Fluorescence Resolution and

3. On the **vFRed, FITC, PE Tab**, inspect the univariate plots of marker fluorescence intensity in the appropriate channel to visually evaluate the separation of bead populations.
4. Note the CV of the bright nRB bead (peak 4), which should be <6% or less, and the vFRed staining index calculated using the two dimmest peaks (peaks 1 and 2), which should be >18.

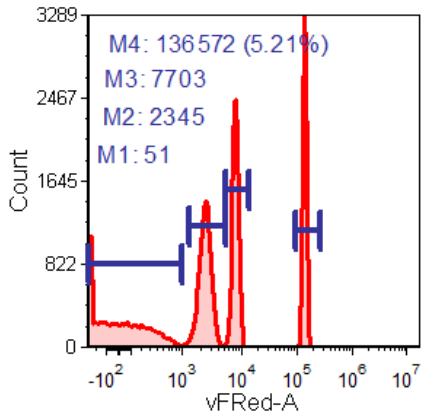

Figure 1. vFRed nanoRainbow beads

Table 1. vFRed SI Calculation

	A	B	C
1	Pos med	2370	
2	Neg med	57	
3	Neg sd	248	
4	**Sep Index**	**18.69**	

Calibrate FITC and PE Channels

5. Use the FCS Express Channel Calibration Tools to create a channel calibration file (Tools>Channel Calibration) using the nanoRainbow lot-specific MESF values (provided on the product data sheet).
6. Apply the calibration to the selected plots to validate calibration.

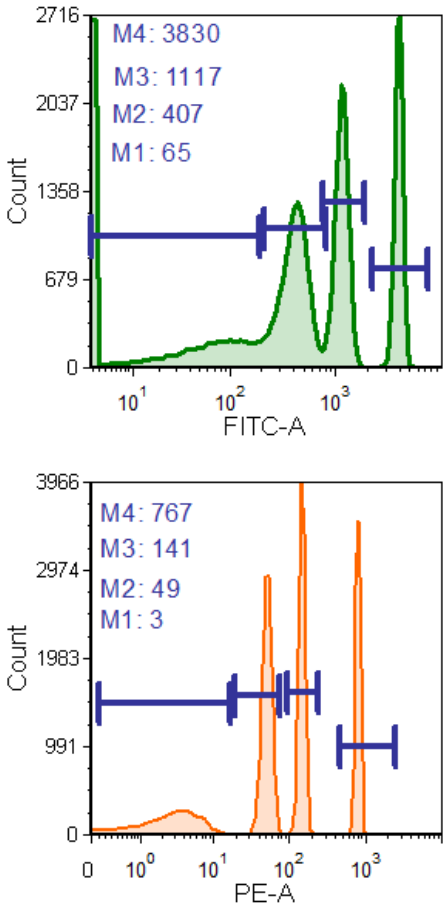

Figure 2. nanoRainbow bead FITC and PE channel intensity distributions plotted on calibrated axes.

Laser Alignment and Performance

7. On the **Lasers Tab**, Inspect the CV and peak shape of the brightest bead (Peak 4) in the representative histogram from each laser. CVs should be >8%. If CVs are higher, or there is an apparent leftward shoulder to the peak, alignment on that laser may be sub-optimal.

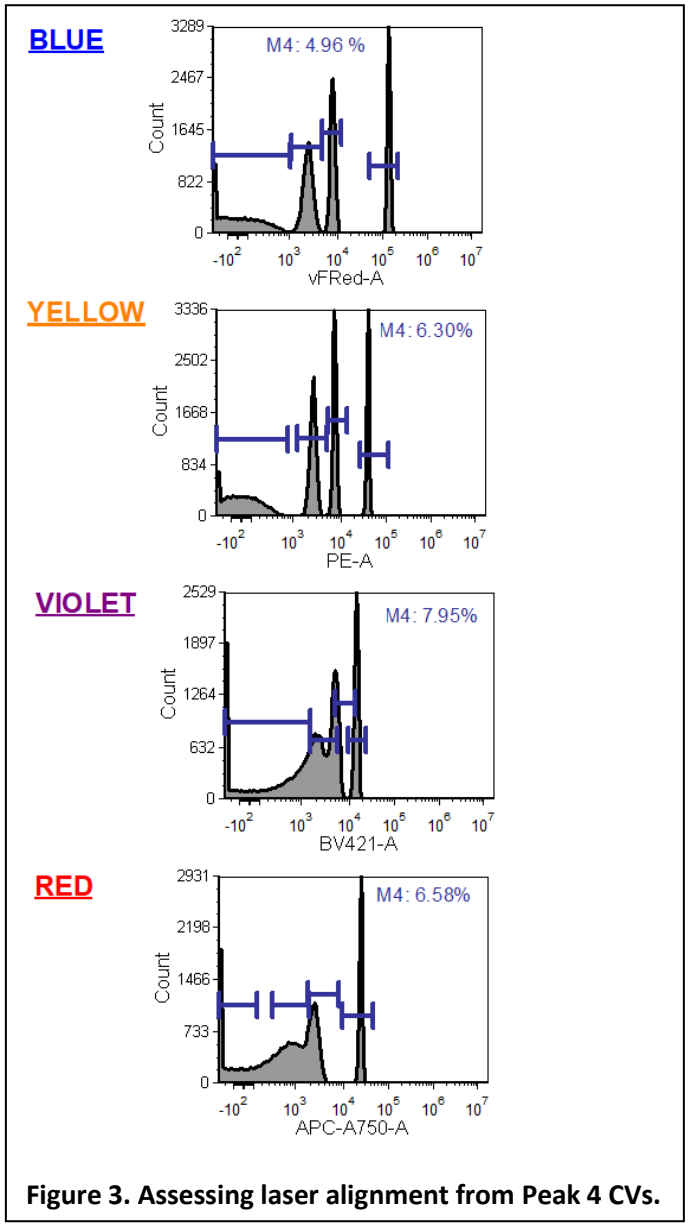

Figure 3. Assessing laser alignment from Peak 4 CVs.

Instrument Flow Rate Calibration

8. On the **Flow Rate Tab**, adjust the gates selecting the Peak 4 singlets, doublets and triplets. The Layout will calculate the sample flow rate using the known concentration of the nanoRainbow beads (1e7/mL). The Layout will also calculate the apparent Peak 4 bead concentration, assuming the instrument-reported volume estimate is correct.

Notes

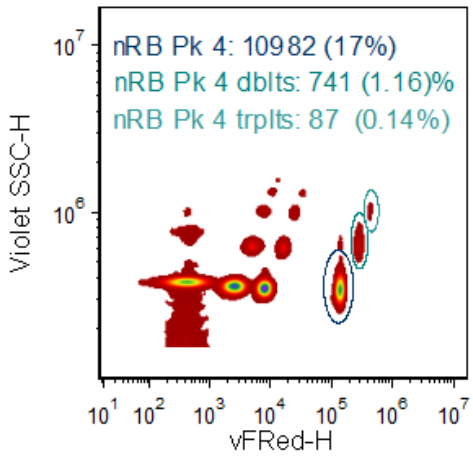

Figure 3. Assessing flow rate Peak 4 counts.

Table 2. Flow rate and concentration calculations

	A	B	C
1	Pk 4 conc	1.00E+04	/uL
2	Pk4 singlets	10982	
3	Pk4 doublets	741	
4	Pk4 triplets	87	
5	Gated	12725	
6	Flow rate	1.27	uL/sec
7			
8	Volume	5275	nL
9	Conc	2.41E+03	/uL