

## Protocol 0.1 Instrument QC and Calibration with nanoRainbow Beads

### Purpose

Assess laser alignment and fluorescence resolution and calibrate instrument flow rate and fluorescence intensity axes.

### Materials

Kit Component	Size		Store
vCal™ nanoRainbow beads	2.5 mL		4°C
vCal™ nRB Report layout			

### Materials to be provided by User

Gloves

Microwell plate (Sartstedt 82.1583.001)

Pipettes (5 uL – 300 uL)

Pipette tips

FCS Express Reader software ([FCS Express Reader](#))

### Procedure

1. Create a New Experiment and Load the vCal Bead Settings\_YYMMDD.ist file created during Instrument Set-up.
2. Define save file name as YYMMDD\_ *Cytometername*\_ nanoRainbow.
3. Vortex nanoRainbow beads well.
4. Place two drops (~100 uL) undiluted nanoRainbow beads in a well and record for 30 seconds at the sample flow rate used for vFC™.

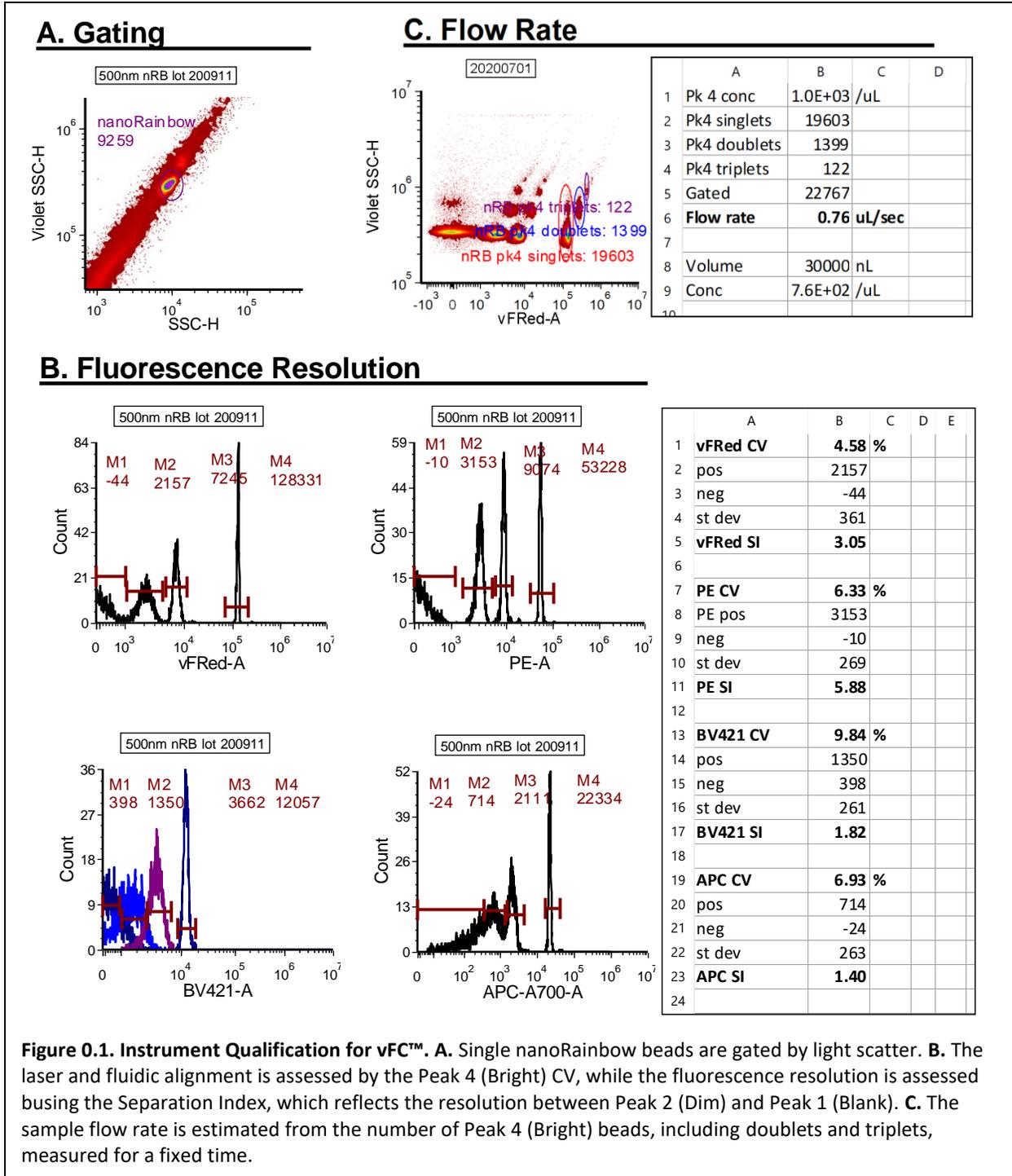
### Analysis

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

#### A. Laser Alignment and Fluorescence Resolution

1. Inspect the fluorescence histograms of a representative channel from each laser and adjust the markers to select each of the four bead populations.
2. Laser alignment is assessed by inspecting the CV of the bright bead population, which 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.
3. Fluorescence resolution is assessed via the Separation Index (SI), which reflects the resolution of dim signals from background by comparing the difference between the blank and dimmest bead to the standard deviation of the background. This semi-quantitative

metric is useful for an initial analysis of instrument performance, and the vFRed™ SI should be >3.0 to ensure efficient EV detection.



**Figure 0.1. Instrument Qualification for vFC™.** A. Single nanoRainbow beads are gated by light scatter. B. The laser and fluidic alignment is assessed by the Peak 4 (Bright) CV, while the fluorescence resolution is assessed by using the Separation Index, which reflects the resolution between Peak 2 (Dim) and Peak 1 (Blank). C. The sample flow rate is estimated from the number of Peak 4 (Bright) beads, including doublets and triplets, measured for a fixed time.

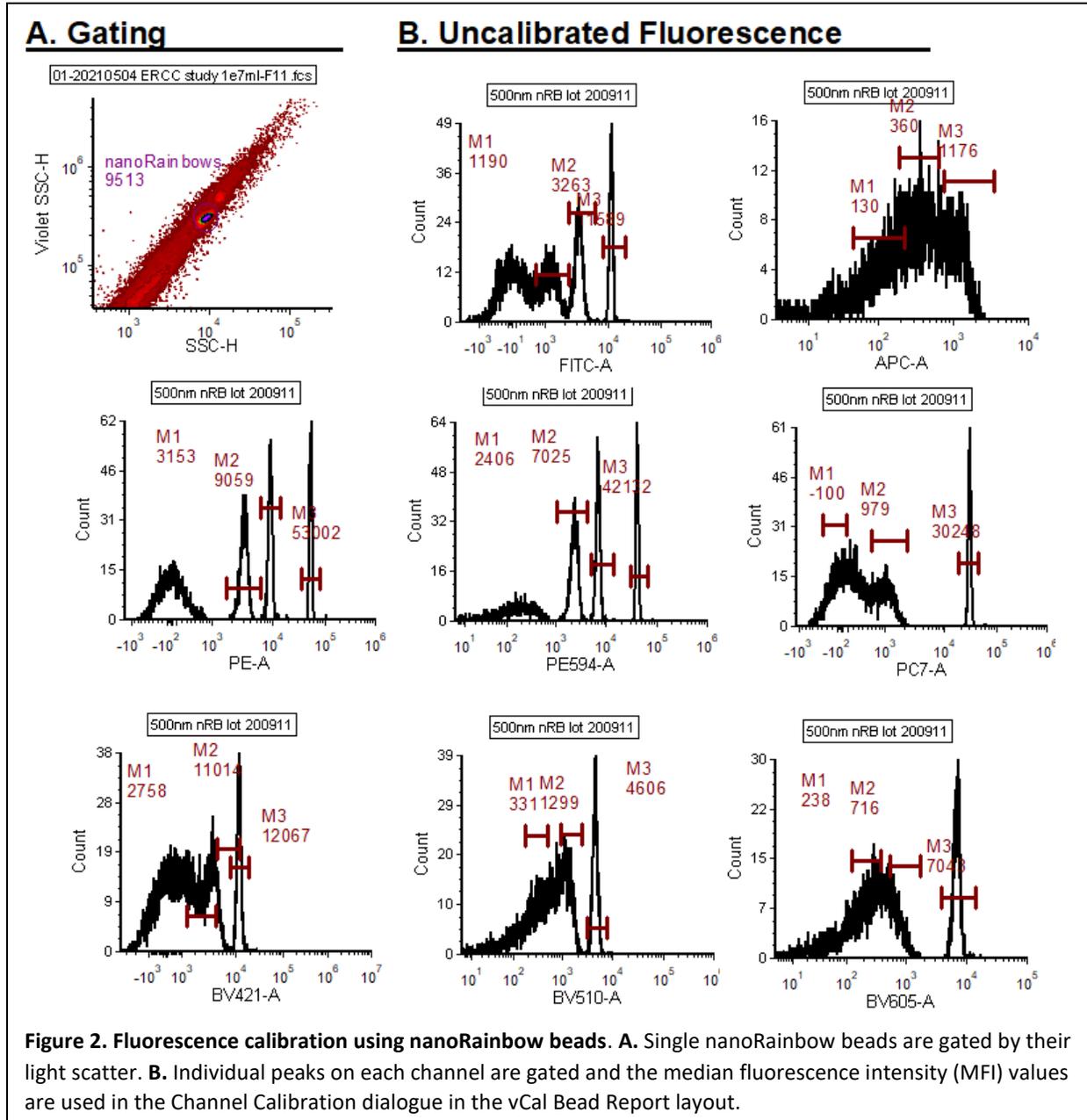
### B. Calibrate Sample Flow Rate

1. 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.

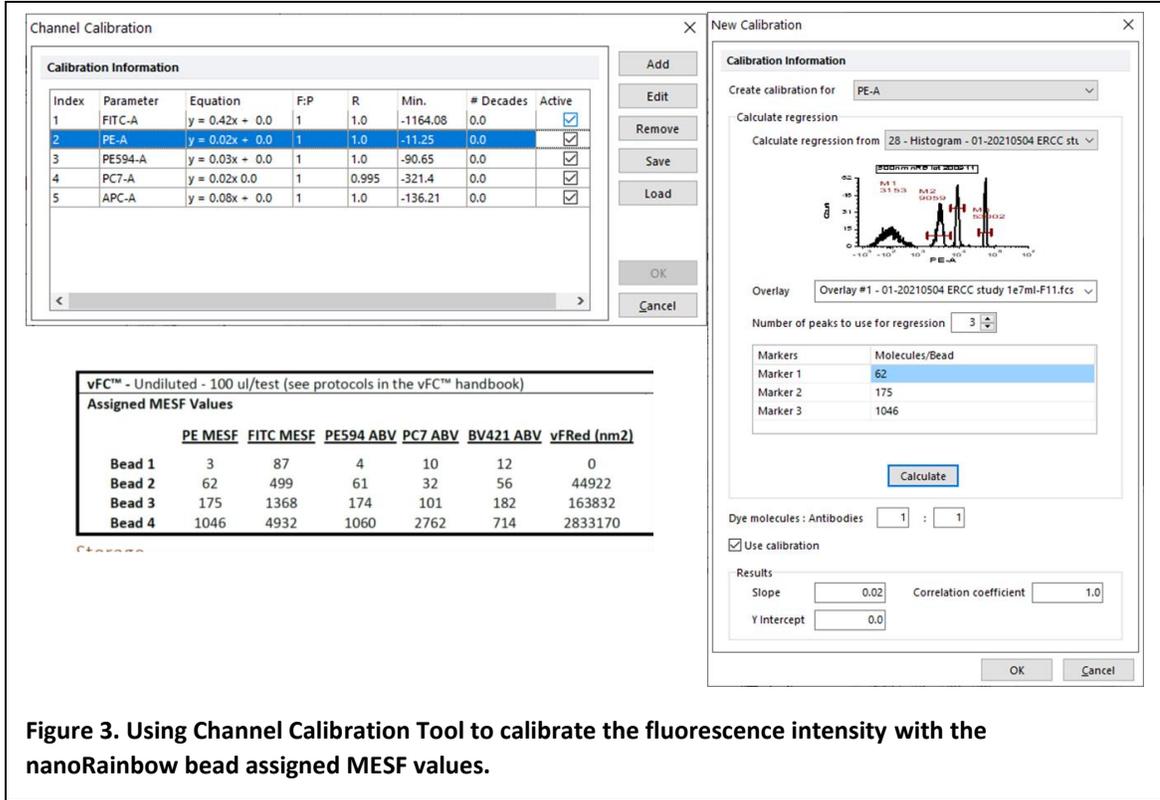
### C. Calibrate Fluorescence Channels

1. On the **nRB FL – all Tab** adjust the markers on each channel to calculate the median fluorescence intensity (MFI) for the three brightest beads in each channel.

*(If the brightest three beads cannot be resolved from background, calibrate using vCal™ AbCap beads stained with the desired fluorescence-conjugated antibody, as detailed in Protocol 0.3).*



- Use the FCS Express Channel Calibration Tool (Tools>Channel Calibration to create a channel calibration file) using the nanoRainbow lot-specific MESF/ABV values (provided on the product data sheet).



The screenshot shows two windows from the Channel Calibration tool. The 'Channel Calibration' window displays a table of calibration parameters:

Index	Parameter	Equation	F:P	R	Min.	# Decades	Active
1	FITC-A	y = 0.42x + 0.0	1	1.0	-1164.08	0.0	<input checked="" type="checkbox"/>
2	PE-A	y = 0.02x + 0.0	1	1.0	-11.25	0.0	<input checked="" type="checkbox"/>
3	PES94-A	y = 0.03x + 0.0	1	1.0	-90.65	0.0	<input checked="" type="checkbox"/>
4	PC7-A	y = 0.02x + 0.0	1	0.995	-321.4	0.0	<input checked="" type="checkbox"/>
5	APC-A	y = 0.08x + 0.0	1	1.0	-136.21	0.0	<input checked="" type="checkbox"/>

Below this table is a table of assigned MESF values for nanoRainbow beads:

	PE MESF	FITC MESF	PES94 ABV	PC7 ABV	BV421 ABV	vFRed (nm2)
Bead 1	3	87	4	10	12	0
Bead 2	62	499	61	32	56	44922
Bead 3	175	1368	174	101	182	163832
Bead 4	1046	4932	1060	2762	714	2833170

The 'New Calibration' window shows the configuration for a new calibration for parameter 'PE-A'. It includes a histogram of the data, a regression equation, and the number of peaks used for regression (3). The 'Markers' table is also visible:

Markers	Molecules/Bead
Marker 1	62
Marker 2	175
Marker 3	1046

The 'Results' section shows a slope of 0.02 and a correlation coefficient of 1.0.

**Figure 3. Using Channel Calibration Tool to calibrate the fluorescence intensity with the nanoRainbow bead assigned MESF values.**

- Apply the calibration to the selected plots to validate calibration.
- On the **nRB FL – cal Tab** examine the recovery of the standard values on plots displaying calibrated axes.
- Save the Channel Calibration file (Tools>Channel Calibration> Save) as Instrument\_nRB FL Calibration\_YYMMDD.cal

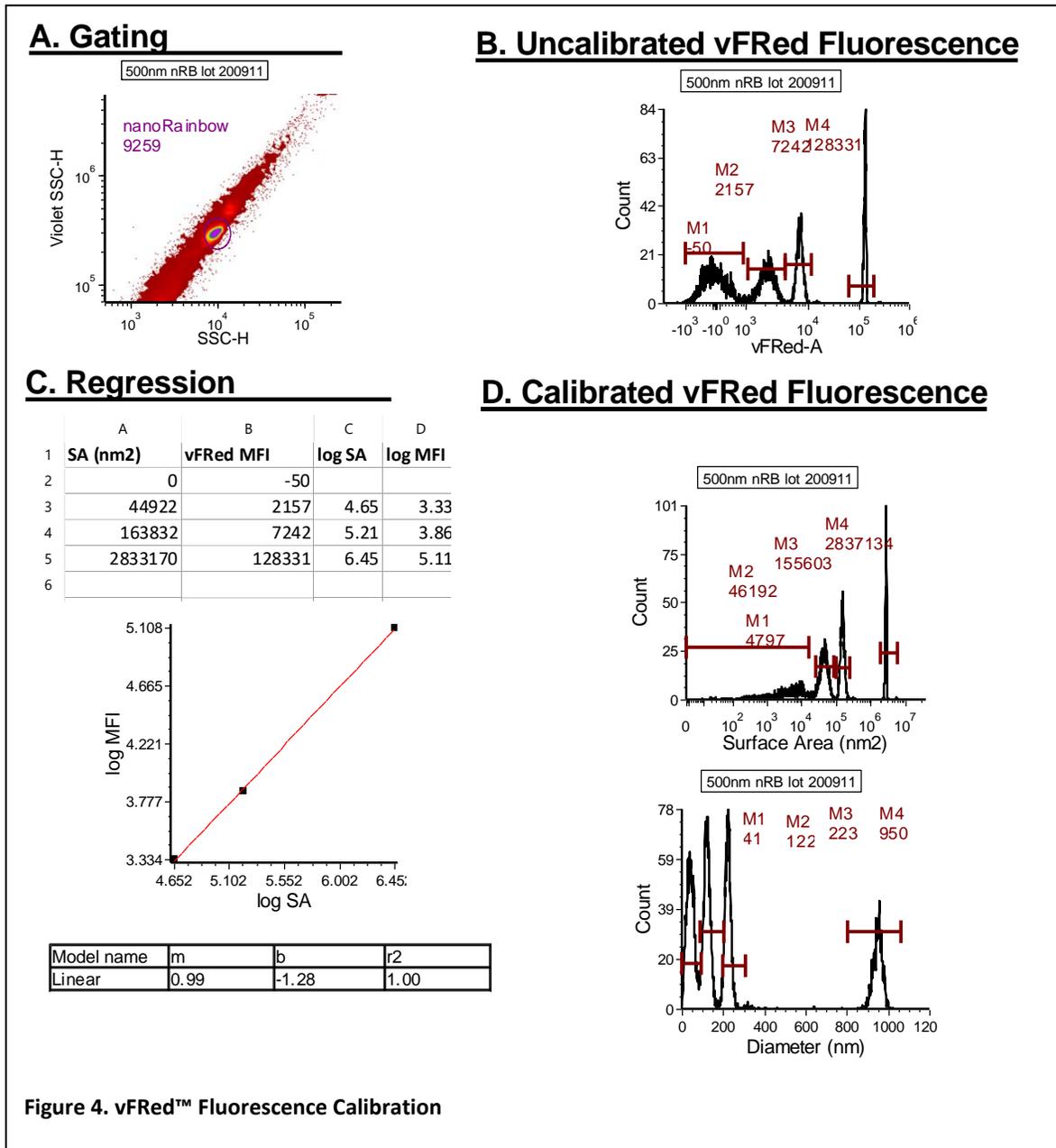
eg. **FC01\_nRB FL Calibration\_210704.cal**

This Channel Calibration file will be loaded into the vFC Report layouts

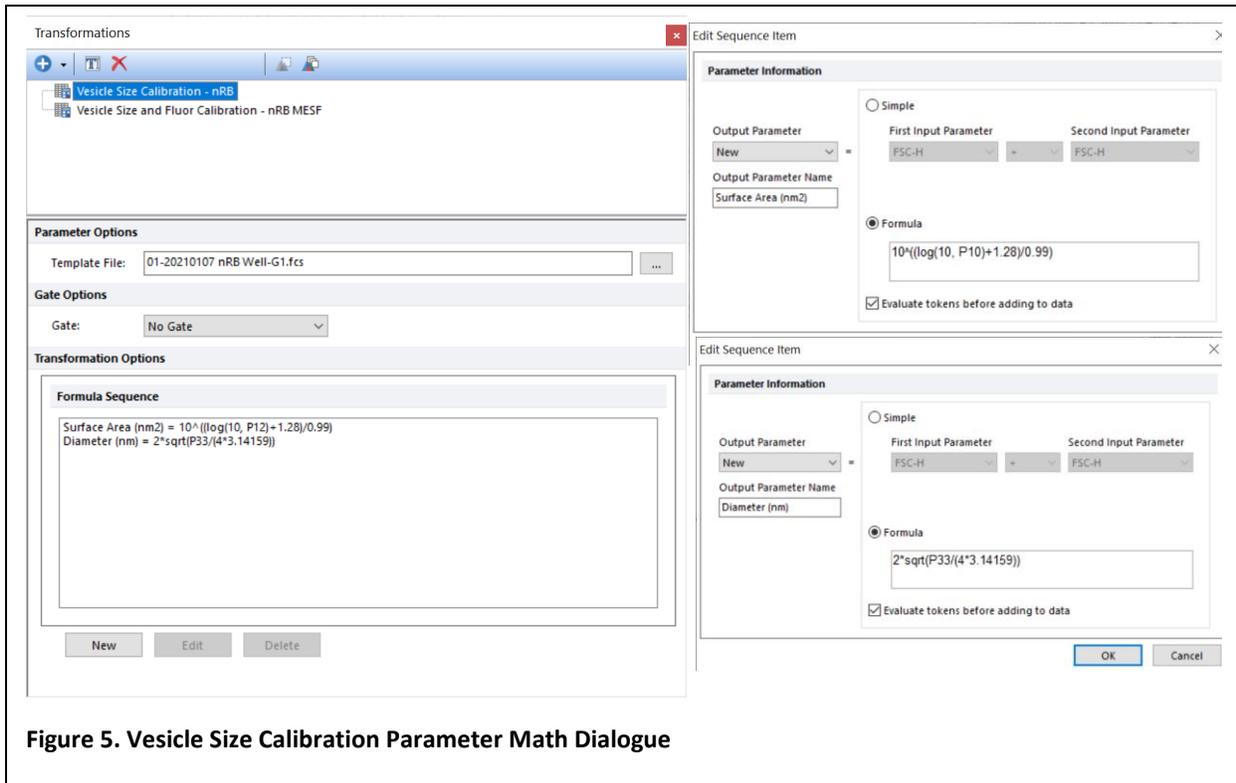
*Note that these MESF assignments are nominal and are likely to be similar across similarly configured instruments (lasers, filters, detectors, etc.), but may vary when configurations are dissimilar. When the highest accuracy is required, the instrument response and nRB assignments should be determined using appropriate MESF and/or antibody capture standards as described in Protocol 0.3.*

## D. vFRed™ Vesicle Size Calibration

1. On the nRB – Size Tab, adjust the markers to calculate the vFRed MFI for the three brightest peaks and inspect the resulting regression fit and results.



2. Use the FCS Express Transforms Tool (Tools<Transforms<nRB Size Calibration) to edit the nRB Size Calibration Parameter Math estimation of Surface Area (nm<sup>2</sup>) by inputting the slope and intercept values from the regression fit.



**Figure 5. Vesicle Size Calibration Parameter Math Dialogue**

3. Save the updated Parameter Math formula sequence file (right click<Save ) as Instrument\_nRB Size Calibration\_YYMMDD (eg. CytoFlex\_nRB Size Calibration\_200704.fcf). This formula sequence file will be loaded into the vFC Report layouts used in the vFC Assays.
4. Close the Transforms dialogue box. Inspect the recovery of the standards in the resulting Surface Area histograms.
5. Save the vCal™ Bead Report layout (vCal Bead Report\_CytoFlex\_nRB FL and Size Calibration\_200704.fey).

*Note that these Surface Area assignments are nominal and are likely to be similar across similarly configured instruments (lasers, filters, detectors, etc.), but may vary when configurations are dissimilar. When the highest accuracy is required, the instrument response and nanoRainbow Bead assignments should be determined using appropriate vesicle size standards as described in Protocol 0.2.*

Notes

Developed, Manufactured, and  
Distributed By  
**Cellarcus Biosciences, Inc.**  
Telephone: +1 (858) 239-2100  
Customer Care: [cellarcus@cellarcus.com](mailto:cellarcus@cellarcus.com)  
Technical Support:  
[technical@cellarcus.com](mailto:technical@cellarcus.com)