vFC™ Protocol 0.3 - Fluorescence Calibration using nanoCal™ Beads

Objective

Calibrate the relevant fluorescence channels to report intensity in absolute units of antibodies bound per vesicle (ABV).

Materials

- nanoCal™ Antibody Capture Beads (800 nm diameter polystyrene, $1x10^6$/mL)
  Note: Calibrated beads are available for each of mouse, rat, rabbit, and hamster antibodies.
- Fluorescent antibody conjugate(s) (10x)
- vFC™ Staining and Dilution Buffer
- 1.5 mL microfuge tubes for staining and washing
- v-bottom plate for measurement

Procedure

1. Vortex Capture Beads for 10 seconds.
2. Add one drop (~50 uL) of nanoCal™ antibody capture beads to a microfuge tube. You will need one tube for each fluorophore to be calibrated.
3. Add 5 uL of 10x fluorescent antibody conjugate to each tube. Mix well by vortex.
4. Incubate for 1 hour at RT in the dark.
5. Wash beads by two cycles of centrifugation and resuspension. To the stained beads add 1000 uL vFC™ staining buffer followed by centrifugation at 10,000 xg for 10 min (*note: there will not be a visible pellet*). Aspirate buffer carefully by removing buffer from the top of the tube and stopping when about 50uL remains. Repeat wash 1 more time. After second aspiration, add 200uL of vFC staining buffer to tube and resuspend beads by vortexing for 10 secs.
6. Transfer washed sample to v-bottom plate for flow cytometer measurement.
7. Measure using the vCal Bead Template at the same fluorescence channel gains as for vFC™ analysis.
8. Fill one well with 300uL of vFC buffer without beads
9. Measure a Buffer-only well to estimate the system background.
   On conventional flow cytometers, including the CytoFlex and Aurora, lower the FCS trigger channel threshold until the system is triggered by the background noise and then collect data file. On the CellStream and ImageStream, measure the buffer as you would measure beads.
10. Save data files with informative names that include the data and antibody conjugate used to stain the bead (eg. 20210704 nanoCal CD9 PE.fcs, 20210704 Buffer-only.fcs, etc).

Data Analysis

1. Open the nanoCal™ Bead analysis layout using FCS Express or FCS Express Reader and load the appropriate data files into each plot.
2. Use the bivariate plot of scatter parameters (eg VSSC vs SSC) to identify and gate on the population of single beads (Figure 2).
3. Use the univariate plot of marker fluorescence intensity in the appropriate channel to visually inspect the separation of three bead populations and set the markers to report the medians of the individual peaks.
4. Use the nanoCal Bead ABV assignments (from the lot-specific Certificate of Analysis) and the FCS Express Channel Calibration Tool to generate a calibration file (Tools>Channel Calibration) to estimate the number of FL-mAbs bound per EV (Figure 1).

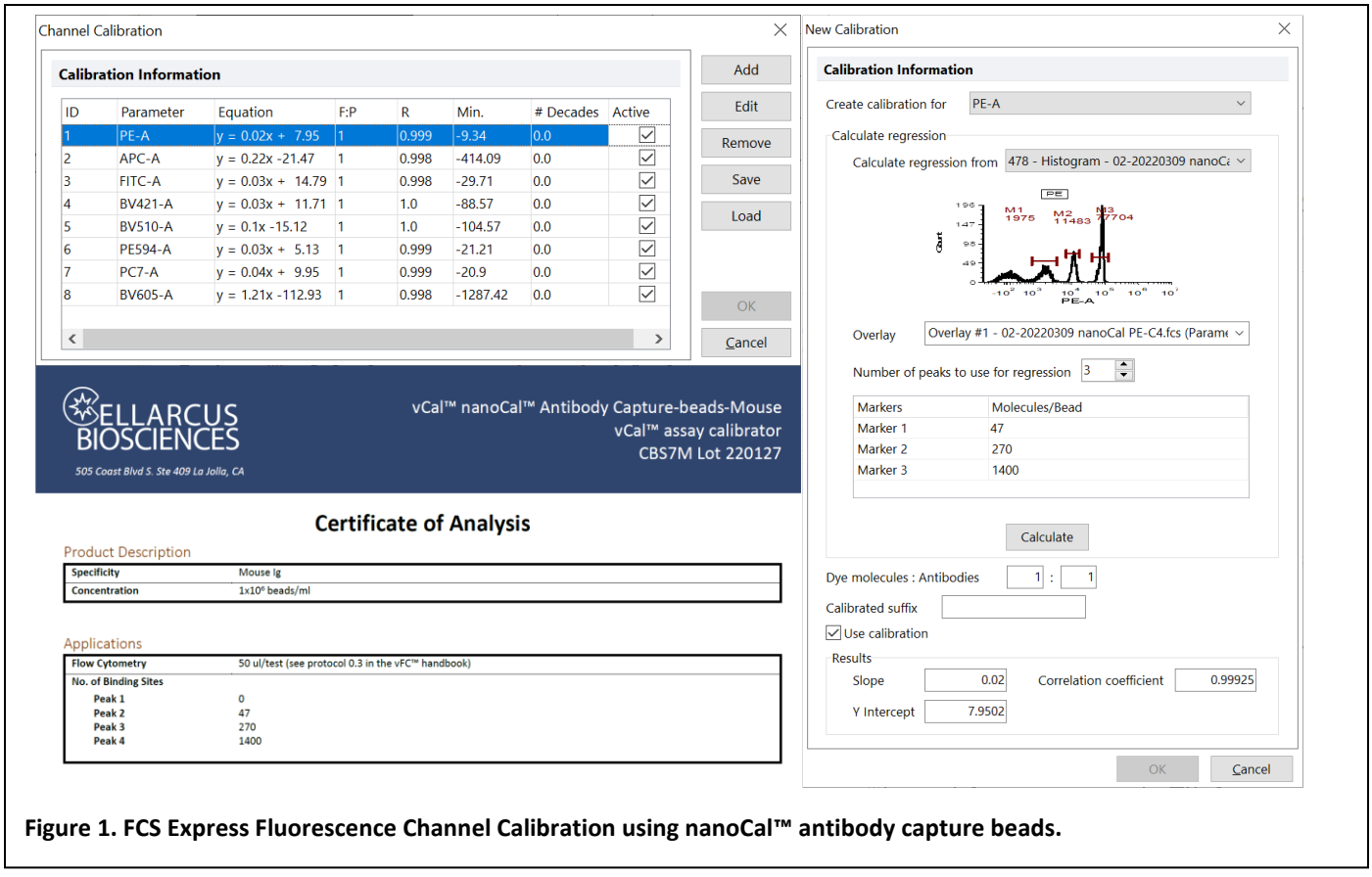


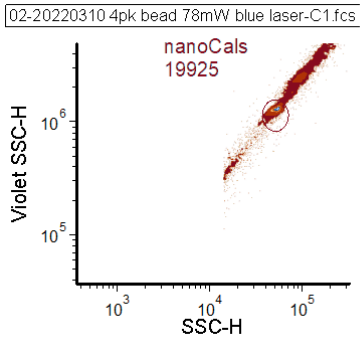
Figure 1. FCS Express Fluorescence Channel Calibration using nanoCal™ antibody capture beads.

5. Save the Channel Calibration file (nanoCal calibration – YYMMDD - fluors.cal), where "fluors" describes the fluorophores used in the calibration.
6. Load the Channel Calibration file (its name will appear in the File Information text box) and inspect the recovery of standards on the nanoCal - cal tab (Figure 3).

vCal™ nanoCal Beads - CytoFlex
Fluorescence Calibration Report

File: 02-20220310 4pk bead 78mW blue laser-C1.fcs **Date:** 10-Mar-2022
Sample: unstained
Instrument: CytoFLEX LX AS38003
C:\Users\Cellarcus\OneDrive - Cellarcus Biosciences\Webdata\CF\Prot 0.3 nanoCal Calibration\CF - 220310 PEx3 BVx3 R670.cal

A. Gating



B. Uncalibrated Fluorescence

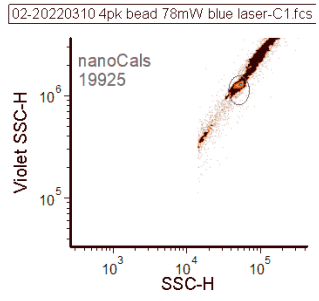
Figure 2. Fluorescence calibration using nanoCal™ beads. A. Single beads are gated by their light scatter. B. Individual peaks on each channel are gated and the median fluorescence intensity (MFI) values are used in the Channel Calibration dialogue in the vCal Bead Report layout.

vCal™ nanoCal Beads - CytoFlex
Fluorescence Calibration Report

CELLARCUS BIOSCIENCES

File: 02-20220310 4pk bead 78mW blue laser-C1.fcs **Date:** 10-Mar-2022
Sample: unstained
Instrument: CytoFLEX LX AS38003
C:\Users\Cellarcus\OneDrive - Cellarcus Biosciences\Webdata\CF\Prot 0.3 nanoCal Calibration\CF - 220310 PEx3 BVx3 R670.cal

A. Gating



C. Calibrated Fluorescence

Parameter	Marker	Median	Arithmetic Mean	95%-ile
FITC-A	M1	18	17	32
FITC-A	M2	50	52	76
FITC-A	M3	215	218	277
FITC-A	M4	1826	1765	2083

Parameter	Marker	Median	Arithmetic Mean	95%-ile
APC-A	M1	-68	-79	-29
APC-A	M2	36	41	117
APC-A	M3	225	239	389
APC-A	M4	1649	1638	2049

Parameter	Marker	Median	Arithmetic Mean	95%-ile
PE-A	M1	9	10	19
PE-A	M2	47	48	67
PE-A	M3	239	238	300
PE-A	M4	1575	1538	1798

Parameter	Marker	Median	Arithmetic Mean	95%-ile
PE594-A	M1	8	8	20
PE594-A	M2	48	49	73
PE594-A	M3	238	239	298
PE594-A	M4	1586	1555	1834

Parameter	Marker	Median	Arithmetic Mean	95%-ile
PC7-A	M1	10	9	22
PC7-A	M2	46	47	75
PC7-A	M3	235	237	310
PC7-A	M4	1612	1585	1895

Parameter	Marker	Median	Arithmetic Mean	95%-ile
BV421-A	M1	10	7	26
BV421-A	M2	48	53	97
BV421-A	M3	280	291	449
BV421-A	M4	1528	1541	2133

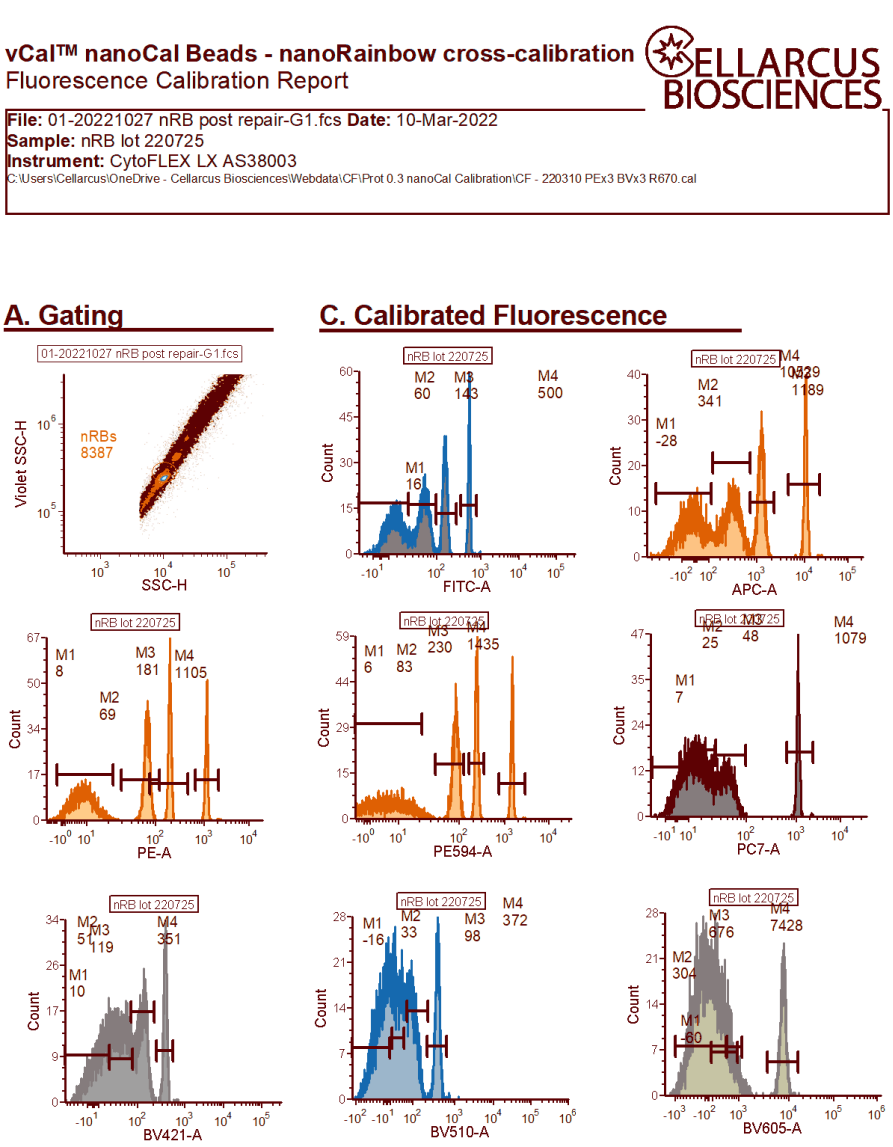
Parameter	Marker	Median	Arithmetic Mean	95%-ile
BV510-A	M1	-13	-17	6
BV510-A	M2	40	46	93
BV510-A	M3	251	252	385
BV510-A	M4	1447	1387	1786

Parameter	Marker	Median	Arithmetic Mean	95%-ile
BV605-A	M1	-54	-64	424
BV605-A	M2	309	353	782
BV605-A	M3	693	728	1046
BV605-A	M4	6177	6280	9102

Figure 3. Fluorescence calibration using nanoCal™ beads. A. Single beads are gated by their light scatter. C. The fluorescence axes calibrations are assessed via recovery of standards.

Cross-calibrate nanoRainbow Beads

1. On the nRB x-cal Tab, select all plots on the Layout Tab, select the nanoRainbow bead data file from the Data List, and Change Data on Selected Plots.
2. Use the bivariate plot of scatter parameters (eg VSSC vs SSC) to identify and gate on the population of single nanoRainbow beads (Figure 4).
3. Adjust the markers on each bead population on each channel. Ensure the appropriate Channel Calibration file (*.cal) is loaded and displayed in the Layout header.
4. The median fluorescence intensities (MFIs) , which should be valid for each bead in each channel represent the instrument-specific intensity assignments, which we be valid as long as there are no major changes to the instrument (eg, laser wavelengths and power, optical filters, etc).



Parameter	Marker	Median	Arithmetic Mean	95%-ile
FITC-A	M1	16	15	30
FITC-A	M2	60	60	81
FITC-A	M3	143	144	168
FITC-A	M4	500	498	543

Parameter	Marker	Median	Arithmetic Mean	95%-ile
APC-A	M1	-28	-33	103
APC-A	M2	341	351	576
APC-A	M3	1189	1195	1496
APC-A	M4	10529	10591	12178

Parameter	Marker	Median	Arithmetic Mean	95%-ile
PE-A	M1	8	8	16
PE-A	M2	69	69	82
PE-A	M3	181	170	202
PE-A	M4	1105	1103	1209

Parameter	Marker	Median	Arithmetic Mean	95%-ile
PE594-A	M1	6	5	17
PE594-A	M2	83	84	104
PE594-A	M3	230	230	261
PE594-A	M4	1435	1433	1567

Parameter	Marker	Median	Arithmetic Mean	95%-ile
PC7-A	M1	7	6	13
PC7-A	M2	25	25	33
PC7-A	M3	48	50	71
PC7-A	M4	1079	1087	1222

Parameter	Marker	Median	Arithmetic Mean	95%-ile
BV421-A	M1	10	7	26
BV421-A	M2	51	52	77
BV421-A	M3	119	119	162
BV421-A	M4	351	350	400

Parameter	Marker	Median	Arithmetic Mean	95%-ile
BV510-A	M1	-16	-20	6
BV510-A	M2	33	34	53
BV510-A	M3	98	103	148
BV510-A	M4	372	373	454

Parameter	Marker	Median	Arithmetic Mean	95%-ile
BV605-A	M1	-60	-69	429
BV605-A	M2	304	350	763
BV605-A	M3	676	721	1040
BV605-A	M4	7428	7461	9168

Figure 4. Cross-calibration of nanoRainbow beads. A. Single nanoRainbow beads beads are gated by their light scatter. **C.** The nanoCal fluorescence axes calibrations are applied to the nanoRainbow beads to provide instrument-specific assignments.