

LLARCUS SCIENCES VFCTM Protocol 0.3 Fluorescence Calibration using Antibody Capture Nanobeads

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, 1x10⁶/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 FLmAbs bound per EV (Figure 1).

| libra | ation Informat | ion | | | | | | Add | Calibration Information |
|--|---|---|--------|---------|---------------------|-------------|--------------------------|-------------------------------|--|
|) | Parameter | Equation | F:P | R | Min. | # Decades | Active | Edit | Create calibration for PE-A v |
| | PE-A | y = 0.02x + 7.95 | 1 | 0.999 | -9.34 | 0.0 | \checkmark | Remove | Calculate regression |
| | APC-A | y = 0.22x -21.47 | 1 | 0.998 | -414.09 | 0.0 | | Kennove | Calculate regression from 478 - Histogram - 02-20220309 nanoCa 🗸 |
| | FITC-A | y = 0.03x + 14.79 | 1 | 0.998 | -29.71 | 0.0 | \checkmark | Save | |
| | BV421-A | y = 0.03x + 11.71 | 1 | 1.0 | -88.57 | 0.0 | \checkmark | | 195 M1 |
| | BV510-A | y = 0.1x - 15.12 | 1 | 1.0 | -104.57 | 0.0 | | Load | 1975 M2 11483 77704 |
| | PE594-A | y = 0.03x + 5.13 | 1 | 0.999 | -21.21 | 0.0 | | | 98 |
| | PC7-A | y = 0.04x + 9.95 | | 0.999 | -20.9 | 0.0 | | | |
| | BV605-A | y = 1.21x - 112.93 | | 0.998 | -1287.42 | 0.0 | \checkmark | | $-10^2 10^3 10^4 10^6 10^6 10^7$ |
| | | , | | | | | | OK | PE-A |
| | | | | | | | | | |
| | | | | | | | > | <u>C</u> ancel | Overlay Dverlay #1 - 02-20220309 nanoCal PE-C4.fcs (Parame > |
| ~ | | | | vCal | ™ nanoCa | I™ Antibody | y Capture-b vCal™ ass | beads-Mouse say calibrator | Number of peaks to use for regression 3 Markers Molecules/Bead Marker 1 47 |
| R S Bl | ELLARC OSCIEN(Coast Bivd 5. Ste 409 L | LES | | vCal | ™ nanoCa | l™ Antibody | y Capture-b vCal™ ass | eads-Mouse | Number of peaks to use for regression 3 🗘 |
| ₩ 9 BI 505 C | Coast Blvd S. Ste 409 L | CES a Jolla, CA | ertifi | | ™ nanoCa Analysi | | y Capture-b vCal™ ass | beads-Mouse say calibrator | Number of peaks to use for regression 3 Markers Molecules/Bead Marker 1 47 Marker 2 270 |
| BI 505 C | Coast Blvd 5. Ste 409 L | CES a Jolia, CA Co | ertifi | | | | y Capture-b vCal™ ass | beads-Mouse say calibrator | Number of peaks to use for regression 3 Markers Molecules/Bead Marker 1 47 Marker 2 270 Marker 3 1400 Calculate |
| BI 505 C | Coast Blvd 5. Ste 409 L | CES a Jolla, CA | ertifi | | | | y Capture-b vCal™ ass | beads-Mouse say calibrator | Number of peaks to use for regression 3 Markers Molecules/Bead Marker 1 47 Marker 2 270 Marker 3 1400 |
| BI 505 C | Coast Blvd 5. Ste 409 L Locat Description ficity | CES a Jolia, CA Co Mouse Ig | ertifi | | | | y Capture-b vCal™ ass | beads-Mouse say calibrator | Number of peaks to use for regression 3 Markers Molecules/Bead Marker 1 47 Marker 2 270 Marker 3 1400 Calculate |
| | Coast Blvd 5. Ste 409 L Locast Blvd 5. Ste 409 L Loct Description Ricity entration | CES a Jolia, CA Co Mouse Ig | ertifi | | | | y Capture-b vCal™ ass | beads-Mouse say calibrator | Number of peaks to use for regression 3 Markers Molecules/Bead Marker 1 47 Marker 2 270 Marker 3 1400 Calculate Dye molecules : Antibodies 1 : 1 Calibrated suffix 1 |
| BI 505 C | Coast Blvd 5. Ste 409 L uct Description ficity entration | CES a Jolio, CA C Mouse Ig 1x10° beads/ml | | cate of | Analysi | | y Capture-b vCal™ ass | beads-Mouse say calibrator | Number of peaks to use for regression Markers Markers Molecules/Bead Marker 1 47 Marker 2 270 Marker 3 1400 Calculate Dye molecules : Antibodies 1 Calibrated suffix Vuse calibration |
| odu oplic | Coast Blvd 5. Ste 409 L uct Description ficity entration cations Cytometry | CES a Jolia, CA Co Mouse Ig | | cate of | Analysi | | y Capture-b vCal™ ass | beads-Mouse say calibrator | Number of peaks to use for regression Markers Marker 1 47 Marker 2 270 Marker 3 1400 Calculate Dye molecules : Antibodies 1 Calibrated suffix Use calibration Results |
| sos c codu pecifi ioncei low C | Coart Bird 5. Ste 409 L Lact Description ficity Intration cations Cytometry Binding Sites | Mouse ig 1x10° beads/ml | | cate of | Analysi | | y Capture-b vCal™ ass | beads-Mouse say calibrator | Number of peaks to use for regression Markers Markers Molecules/Bead Marker 1 47 Marker 2 270 Marker 3 1400 Calculate Dye molecules : Antibodies 1 Calibrated suffix Vuse calibration |
| odu sos c oplic low C | Coast Blvd 5. Ste 409 L uct Description ficity entration cations Cytometry | CES a Jolio, CA C Mouse Ig 1x10° beads/ml | | cate of | Analysi | | y Capture-b vCal™ ass | beads-Mouse say calibrator | Number of peaks to use for regression Markers Marker 1 47 Marker 2 270 Marker 3 1400 Calculate Dye molecules : Antibodies 1 Calibrated suffix Use calibration Results |

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).



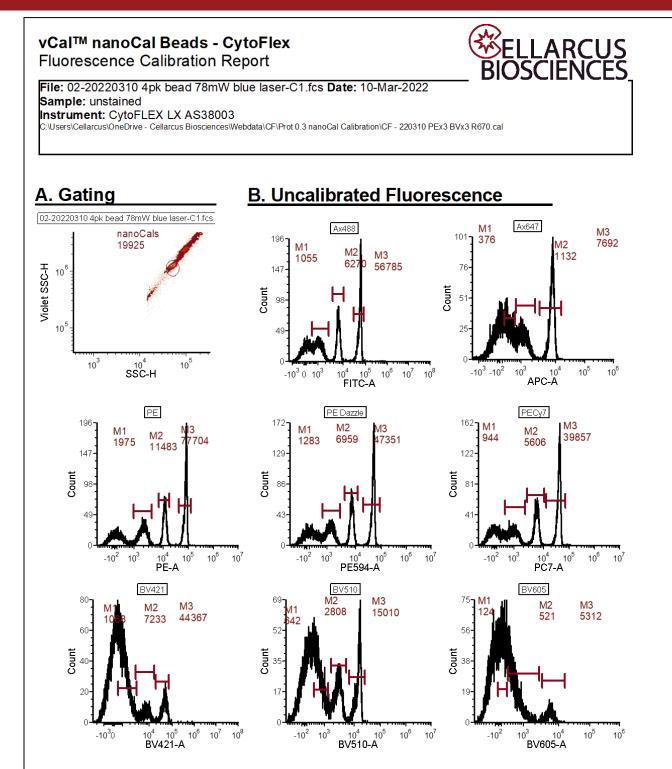


Figure 2. Fluorescence calibration using nanoCal™ beads. A. Single beads are gated by their light scatter. B. Individual peaks on each channel are gated and the median fluorescence intensity (MFI) values are used in the Channel Calibration dialogue in the vCal Bead Report layout.



Fluorescence Calibration using Antibody Capture Nanobeads

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

02-20220310 4pk bead 78mW blue laser-C1.fcs

nanoCals

10³

ssc-H

PE

M2

47

М3

10² 10 PE-A

BV421 M4

 10^{3}

M4 239

10

19925

A. Gating

10

10

190-

143

95

Count

M1

9

-10

56

-10 10²

Count

Violet SSC-H

:/Users/Cellarcus/OneDrive - Cellarcus Biosciences/Webdata/CF/Prot 0.3 nanoCal Calibration/CF - 220310 PEx3 BVx3 R670.cal

194

146

97

.10

Count

M2 М3

50 215

C. Calibrated Fluorescence

M4 1826

10⁵

10⁶

Ax488

10²

10³ 10⁴ FITC-A

Ax64

M2

36

90

67

22

Count

M1

-68

-**10²**0

М3

225

M4

1649

10⁵

10⁴

APC-A

| 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 |

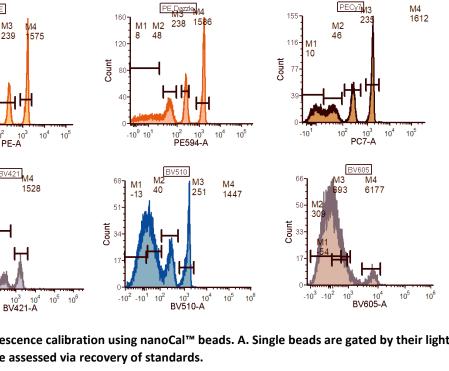
| Param | eter Markei | r Median | Arithmeti Mean | c 95%-ile |
|-------|-------------|----------|-------------------|-----------|
| PC7-A | M1 | 10 | 9 | 22 |
| PC7-A | M2 | 46 | 47 | 75 |
| PC7-A | M3 | 235 | 237 | 310 |
| PC7-A | M4 | 1612 | 1585 | 1895 |

| 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.





Fluorescence Calibration using Antibody Capture Nanobeads

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).

| vCal™ nanoCal Beads - nanoRainbow cross-calibration Fluorescence Calibration Report | |
|--|---|
| File: 01-20221027 nRB post repair-G1.fcs Date: 10-Mar-2022 | 1 |
| Sample: nRB lot 220725 | |
| Instrument: CytoFLEX LX AS38003 | |
| C:\Users\Cellarcus\OneDrive - Cellarcus Biosciences\Webdata\CF\Prot 0.3 nanoCal Calibration\CF - 220310 PEx3 BVx3 R670.cal | |

| 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 |

-33

351

1195

103

576

1496

-28

341

1189

APC-A

APC-A

APC-A

105

M4 1079

M1

M2

M3

M4

| A. Gating | C. Calibrated Fluores | cence |
|--|--|--|
| $H_{SS}^{10^{0}}$ | $m_{\text{EB}} to 220725$ M2 M8 M4 60 143 500 45 45 45 45 45 45 45 45 45 45 45 45 45 | $\begin{array}{c} \begin{array}{c} & & & & & \\ & & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ $ |
| $\mathbf{H}_{\mathbf{A}}^{\mathbf{A}} = \mathbf{H}_{\mathbf{A}}^{\mathbf{A}} = \mathbf{H}_{\mathbf$ | 59 44 59 59 44 59 59 59 59 59 59 59 59 59 59 | $\begin{array}{c} \begin{array}{c} & & & & \\ & & & & \\ & & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & $ |
| $u_{10}^{34} - \frac{10^{10}}{10^{10}} + 10^{$ | $u_{1}^{20} - \frac{10^{10}}{10^{2}} - \frac{10^{10}}{10^{$ | 10 ³ -10 ² 10 ³ 10 ⁴ |

APC-A 10529 10591 12178 Arithmetic Mean 95%-ile Parameter Marker Median 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 | МЗ | 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 |
| | | 070 | 070 | 454 |
| BV510-A | M4 | 372 | 373 | 454 |
| BV510-A | M4 | 372 | 3/3 | 454 |
| Parameter | M4 Marker | Median | Arithmetic Mean | 95%-ile |
| Parameter | | | Arithmetic | |
| | Marker | Median | Arithmetic Mean | 95%-ile |
| BV605-A | Marker M1 | Median -60 | Arithmetic Mean -69 | 95%-ile 429 |

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.