

## Protocol B. Fluorescence Calibration and Compensation

### Objective

Calibrate the intensity and spectral response of the instrument for relevant fluorescence channels to report intensity in absolute units of antibody binding capacity (ABC), vesicle size (diameter via mean equivalent surface area, MESA) and account for fluorescence spillover between channels.

### Materials

- nanoCal<sup>™</sup> Antibody Capture Beads (800 nm diameter polystyrene, 1x10<sup>6</sup>/mL)
  - o Prestained nanoCal beads, freeze-dried (CBS7Fluor-2T, where Fluor is the stain) or
  - User-stained nanoCal beads (see Appendix A)
- vFRed<sup>™</sup>-stained Lipo100<sup>™</sup> Vesicle Size Standard
  - o prepared and measured using Protocol B2 (Appendix B), or
  - o measured as part of Protocol 1 or Protocol 2
- vFC<sup>™</sup> Staining and Dilution Buffer
- Gloves
- Microwell plate (v-bottom: Sartstedt 82.1583.001, other plates or tubes may be suitable)
- vCal<sup>™</sup> Bead data acquisition template (created during **Protocol A Instrument QC and Qualification**)
- <u>Protocol B Fluorescence Calibration Layout</u> for <u>FCS Express Reader</u> or <u>FCS Express</u>
- Protocol B Vesicle Size Calibration Layout for FCS Express Reader or FCS Express
- Protocol B Fluorescence Compensation Layout for FCS Express Reader or FCS Express

### Procedure

- 1. Acquire calibration data for:
  - 1a. Immunofluorescence using nanoCal<sup>™</sup> beads and
  - 1b. Vesicle size using vFRed-stained Lipo100 Vesicle Size standard.
- 2. Generate and validate the intensity calibration for:
  - 2a. Immunofluorescence using nanoCal<sup>™</sup> beads and
  - 2b. Vesicle size using vFRed-stained Lipo100 Vesicle Size standard.
- 3. Determine the spillover matrix and create and validate compensation.



### B1. Data Acquisition

### B1a. nanoCal™ Beads

- 1. Reconstitute pre-stained nanoCal<sup>™</sup> beads in vFC buffer according to the instructions (typically 100 uL)
- 2. Place each nanoCal<sup>™</sup> bead in a different well of a 96-well plate for flow cytometer measurement.
- 3. Fill one well with 300uL of vFC buffer without beads
- 4. Measure using the vCal Bead Template at the same fluorescence channel gains as for vFC<sup>™</sup> analysis.
- Measure the 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.
- 6. 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).

### B1b. Lipo100 Vesicle Size Standard

Measurement of the Lipo100 Vesicle Size Standard is part of every vFC<sup>™</sup> Protocol and assay. In Protocol 1 Sample Dilution Series, Lipo100 serves as a vesicle positive control. In Protocol 2 and other Protocols that measure EV cargo, Lipo100 serves as a vesicle positive control and an immunofluorescence negative control.

For purposes of Fluorescence unmixing and Calibration, vFRed-stained Lipo100 fluorescence intensity data measured as part of Protocol 1 and Protocol 2 may be used. If you are performing Fluorescence Unmixing and Calibration prior to Protocols 1 and 2, use Protocol B2 (**Appendix B**) to prepare and measure vFRed-stained Lipo100.



### B2. Data Analysis – Fluorescence Calibration

### B2a. Data Analysis – Immunofluorescence Calibration

- 1. Open the vFC<sup>™</sup> Fluorescence Calibration analysis layout using FCS Express or FCS Express Reader and load the appropriate data files into each plot. Use the bivariate plot of scatter parameters (eg VSSC vs SSC) to identify and gate the population of single beads (**Figure 1**).
- 2. 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.



www.cellarcus.com



3. Use the nanoCal<sup>™</sup> Bead ABC 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 2**).

alibr	ation Informat	ion						Add	Calibration Informa	ation
D	Parameter	Equation	F:P	R	Min.	# Decades	Active	Edit	Create calibration for	r PE-A 🗸
	PE-A	y = 0.02x + 7.95	1	0.999	-9.34	0.0	$\checkmark$	Remove	Calculate regression	n
2	APC-A	y = 0.22x -21.47	1	0.998	-414.09	0.0	$\checkmark$	Remove	Calculate regres	ssion from 478 - Histogram - 02-20220309 nanoCe ~
	FITC-A	y = 0.03x + 14.79	1	0.998	-29.71	0.0	$\checkmark$	Save		5
	BV421-A	y = 0.03x + 11.71	1	1.0	-88.57	0.0	$\checkmark$			195 M 1 M 2
	BV510-A	y = 0.1x - 15.12	1	1.0	-104.57	0.0		Load		1975 11483 77704
;	PE594-A	v = 0.03x + 5.13	1	0.999	-21.21	0.0			l ā	98
,	PC7-A	y = 0.04x + 9.95	1	0.999	-20.9	0.0				*1 🗸 🖵 🖷 👘
5	BV605-A	y = 1.21x -112.93	1	0.998	-1287.42	0.0				$-10^2 10^3 10^4 10^5 10^6 10^7$
								OK		1 2-4
<							>	Cancel	Overlay Ov	verlay #1 - 02-20220309 nanoCal PE-C4.fcs (Parame >>
(775) (775)	ELLARC	US		vCal	™ nanoCa	l™ Antibod	y Capture-be	eads-Mouse	Number of peak Markers	ks to use for regression 3 🗘
505 C	ELLARC OSCIEN( Coast Blvd 5. Ste 409 L	US CES 9 Jolla, CA		vCal	™ nanoCa	l™ Antibod	y Capture-be vCal™ assa CBS7M	eads-Mouse ay calibrator Lot 220127	Number of peak Markers Marker 1 Marker 2 Marker 3	ks to use for regression 3 Molecules/Bead 47 270 1400
505 C	Coast Bird S. Ste 409 L	CUS CES a Jolie, ca Ca	ertifi	vCal	<sup>™</sup> nanoCa Analysi	I™ Antibod	y Capture-be vCal™ assa CBS7M	eads-Mouse ay calibrator Lot 220127	Number of peak Markers Marker 1 Marker 2 Marker 3	ks to use for regression 3 Molecules/Bead 47 270 1400 Calculate
BI 505 C	Coast Blvd 5. Ste 409 L	CUS CES a Jolio, CA Co Mouse Ig	ertifi	vCal	™ nanoCa Analysi	I™ Antibod	y Capture-b vCal™ assa CBS7M	eads-Mouse ay calibrator Lot 220127	Number of peak Markers Marker 1 Marker 2 Marker 3	ks to use for regression 3 Molecules/Bead 47 270 1400 Calculate
Sos concernance	ELLARC OSCIENC coast Blvd 5. Ste 409 L uct Description ficity intration	CUS CES a Jolio, CA Co Mouse lg 1x10° beads/ml	ertifi	vCal	™ nanoCa Analysi	I™ Antibod	y Capture-b vCal™ ass CBS7M	calter eads-Mouse ay calibrator Lot 220127	Number of peak Markers Marker 1 Marker 2 Marker 3 Dye molecules : Anti	ks to use for regression 3 Molecules/Bead 47 270 1400 Calculate bodies 1:1
505 C Specifi Conce	Coart Bird 5. Ste 409 L Lact Description Refly Intration	CUS CES a Jolio, CA Mouse Ig 1x10° beads/ml	ertifi	vCal	<sup>™</sup> nanoCa Analysi	I™ Antibod <sup>,</sup> is	y Capture-bd vCal™ assa CBS7M	calibrator Lot 220127	Number of peak Markers Marker 1 Marker 2 Marker 3 Dye molecules : Antil Calibrated suffix	ks to use for regression 3  Molecules/Bead 47 270 1400 Calculate bodies 1 : 1
BI 505 C	Coast Blvd 5. Ste 409 L uct Description ficity entration	LUS a Jolia, CA Mouse Ig 1x10° beads/ml	ertifi	vCal	™ nanoCa Analysi	I™ Antibod <sup>,</sup> S	y Capture-bø vCal™ assa CBS7M	ganter eads-Mouse ay calibrator Lot 220127	Number of peak Markers Marker 1 Marker 2 Marker 3 Dye molecules : Antii Calibrated suffix Use calibration	ks to use for regression 3  Molecules/Bead 47 270 1400 Calculate bodies 1: 1
Sos of Conce	ELLARC OSCIENC Coast Blvd 5: Ste 409 L uct Description ficity instration cations Cytometry	Mouse ig 1x10° beads/m1	ertifi	vCal	™ nanoCa Analysi	I™ Antibod	y Capture-bo vCal™ assa CBS7M	calter eads-Mouse ay calibrator Lot 220127	Number of peak Markers Marker 1 Marker 2 Marker 3 Dye molecules : Anti Calibrated suffix Use calibration Results	ks to use for regression 3  Molecules/Bead 47 270 1400 Calculate bodies 1: 1
BI 505 C Specifi Conce Flow C	ELLARC OSCIENC coast Bird 5 Ste 409 L uct Description ficity entration Cytometry Finding Sites	Mouse ig 1x10° beads/ml	ertifi	vCal cate of	™ nanoCa Analysi	I™ Antibod	y Capture-bo vCal™ assa CBS7M	Lairtei	Number of peak Markers Marker 1 Marker 2 Marker 3 Dye molecules : Antii Calibrated suffix VIse calibration Results Slope	ks to use for regression 3  Molecules/Bead 47 270 1400 Calculate bodies 1: 1 0.02 Correlation coefficient 0.9925
Sos of Conce	ELLARC OSCIENC coast Blvd 5. Ste 409 L uct Description ficity entration cations Cytometry Finding Sites Yeak 1	Mouse (g 1x10° beads/ml	ertifi	vCal cate of	™ nanoCa Analysi	I™ Antibod	y Capture-bo vCal™ assa CBS7M	calter eads-Mouse ay calibrator Lot 220127	Number of peak Markers Marker 1 Marker 2 Marker 3 Dye molecules : Antii Calibrated suffix Vuse calibration Results Slope	ks to use for regression 3  Molecules/Bead 47 270 1400 Calculate bodies 1 : 1 0.02 Correlation coefficient 0.99925 7 0602
Specifi Conce	ELLARC OSCIENC coast Blvd 5. Ste 409 L uct Description ficity entration Cytometry f Binding Sites Peak 1 Peak 2 Peak 2 Peak 3	Mouse Ig 1x10° beads/ml 50 ul/test (see proto 0 47 270	ertifi	vCal cate of	™ nanoCa Analysi	I™ Antibod	y Capture-bo vCal™ assa CBS7M	calibrator Lot 220127	Number of peak Markers Marker 1 Marker 2 Marker 3 Dye molecules : Antil Calibrated suffix Use calibration Results Slope Y Intercept	ks to use for regression 3  Molecules/Bead 47 270 1400 Calculate bodies 1: 1 0.02 Correlation coefficient 0.99925 7.9502

#### Figure 2. FCS Express Fluorescence Channel Calibration using nanoCal<sup>™</sup> antibody capture beads.

- 4. Save the Channel Calibration file: Fluor Calibration Instrument YYMMDD Panel (eg. Fluor Calibration CytoFlex 220310 PEx3 BVx3 APC FITC.cal)
- 5. Load the Channel Calibration file (so its name will appear in the File Information text box) and inspect the recovery of standards on the nanoCal cal tab (**Figure 3**).



Sample: unstained

A. Gating

10

10

190-

143

95

Count

M1

9

-10

56

-10 102

Count

Violet SSC-H

# **Fluorescence Intensity Calibration** and Spectral Compensation

## vCal™ nanoCal Beads - CytoFlex

**Fluorescence Calibration Report** 

Instrument: CytoFLEX LX AS38003

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

nanoCals

10<sup>3</sup>

ssc-H

PE

M2

47

М3

10<sup>5</sup>

19925

File: 02-20220310 4pk bead 78mW blue laser-C1.fcs Date: 10-Mar-2022

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

194

146

97

Count

M2 М3

50 215

C. Calibrated Fluorescence

M4 1826

10<sup>5</sup> 10<sup>6</sup>

Ax488

10<sup>2</sup>

10<sup>3</sup> 10<sup>4</sup> FITC-A

Ax64

M2

36

90

67

22

Count

M1

-68

-**10<sup>2</sup>** 0

М3

225

M4

1649

10<sup>5</sup>

10<sup>4</sup>

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

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.





### B2b. Data Analysis - Vesicle Size Calibration

- 1. Open the vFC<sup>™</sup> Analysis Protocol B Vesicle Size Calibration Layout with FCS Express (or FCS Express Reader). Note: The vFC Analysis Layout has additional notes and tips to guide the data analysis.
- 2. From the Data List, click the Add File (+) and navigate to the data directory and select the Protocol 0 data files.
  - a. Select a <u>Buffer + vFRed™</u> data file and click Change Data On All Plots. Inspect the Gating Plots. Adjust Time Gate, Area/Pulse Gate, and Vesicle Gate as needed to minimize background events.
  - b. Select a Lipo100<sup>™</sup> data file and click Change Data On All Plots. Inspect the Gating Plots. Adjust Gates as needed to select vFRed<sup>™</sup>-positive events (Figure 1A-C).



3. Open the **vFC<sup>™</sup> Vesicle Size Calibration Tab** and load (drag and drop) the Lipo100 Reference size data onto the Input Size Histogram Plot.



- 4. View the resulting Vesicle Size Calibration plot of vFRed Intensity vs Surface Area (Figure 2A) and the resulting regression equation and coefficients.
- 5. Open Transformations (Tools>Transformations) then select the Vesicle Size Calibration parameter math Transformation (**Figure 3**).





- 6. Edit the Surface Area Transformation by entering the values in the equation to the slope (mA) and intercept (y-int).
- Save Transformation by right-clicking on the box with the equations and saving as: Vesicle Size Calibration CytoFLEX – YYMMDD – Calibrator Filename (eg. "Size Calibration – Aurora – 220401 – Lipo100 B9.fcf"). This Transformation (.fcf file) will be loaded into the vFC Report Layout for analysis of vFC Assay results.
- 8. Save vFC<sup>™</sup> Layout as: vFC Protocol B Vesicle Size Calibration Instrument Date Samples (eg. "vFC Protocol B Vesicle Size Calibration Aurora 220401 lipo100 B9.fey."

<b>Fransformations</b>	×			
9 - 🗉 X 0 - 🖉 🔊				
Vesicle Size Calibration - U0481 - 240318 - C4 lipo100.fcf				
	Edit Sequence Item			×
	Parameter Information			
		◯ Simple		
Parameter Options	Output Parameter	First Input Parameter	Second Input Paran	neter
Template File: lipo100+vFR.fcs	Output Parameter Name	130-11 · ·	130-11	
Sate Options	Surface Area (nm2)			
Gate No Gate 🗸		O Formula		
Transformed Data Ontions		Custom Token: find_vFRed*28.	48-4581.5956	
Divelay Name Suffix Barameter Math applied				
Display Name Suffix Parameter Math applied		Evaluate tokens before adding to data		
Transformation Options			OK	Cancel
Formula Sequence			ÖK	cuncer
Surface Area (nm2) = P21*28.48-4581.5956				
Diameter (nm) = if P168 > 0 then 2*sqrt(P168/(4*3.14159)) else -2*sqrt(abs(P168)/(4*3.14159)) B6-A/B5-A = P84/P81				
New Edit Delete				



### B3. Data Analysis – Fluorescence Spectral Compensation

This step creates the spectral spillover and compensation matrices using the stained nanoCal<sup>™</sup> Beads and scattertriggered buffer data, together with the vFRed-stained Lipo100 data collected in step B2, or as part of Protocols 1 or 2).

#### Procedure

- 1. Open the vFC<sup>™</sup> Fluorescence Compensation data analysis Layout using FCS Express or FCS Express Reader
- 2. Load the data files from the stained nanoCal beads (plus the unstained bead control) into the Data List. Load the data files from vesicle spectral reference standards (vFRed-Lipo100 and plus buffer-only).
- 3. On the **nanoCal Tab**, use the bivariate plot of scatter parameters to identify and gate on the population of single beads. Load the single-color bead data into the appropriate individual histograms. Inspect the positive and negative populations in each histogram.
- 4. On the Gating Tab, load vFRed-Lipo 100 data into the appropriate histograms and inspect the data
- 5. Click on the **Tools** tab→**Compensation and Unmixing** command to open the **Compensation and Unmixing** navigator (Figure 1).
- 6. The **Compensation and Unmixing** navigator can be moved anywhere on the screen; it is <u>dockable and pinnable</u>, and you can move it around the screen or close and reopen as needed.
- 7. Click on the blue plus button, 🗢, to Add a new compensation (Figure 1, red outline).
- 8. A text box highlighted in blue will appear with the text **New Compensation** (Figure 1).



Compensation and Unn	nixing					
🕀 • 🔂   🗟 🖈	$\mathbf{T}$	🖉 🔎				
Loaded automati	cally with data files / sation					
Compensation Matrix	Spillover Matrix	Compensation Options	Automatic Compensation Setup			
Step 1 - Select sing	le-stained contr	ols	*			
DataFile	Paramet	er Negative con	trol			
Step 2 - Select Scat	ter Gate Parame	ters	*			
Step 3 - View Error	Step 3 - View Errors and Warnings 🛛 😵					
Step 4 - Adjust par	Step 4 - Adjust parameters (optional)					
Step 5 - Add extra	spillover targets	(optional)	*			
Step 6 - Calculate n	natrices		*			

Figure 1. Compensation and Unmixing navigator: use to add new compensations, edit definitions, create Automatic Compensations, and unmix spectral signatures

- 9. Click on the Add data for compensation button, <sup>□</sup>, in the **Automatic Compensation Setup**→**Step 1 Select single-stained controls** window (Figure 1, red arrow).
- 10. The Standard Open Data Dialog will appear (Figure 2).
- 11. Note: based on your <u>User Options</u>, the <u>Advanced Open Data Dialog</u> may appear. If the **Advanced Open Data**

dialog appears, please select the button to access the Standard Open Data Dialog.

- 12. Select and load the single stain and negative control data files appropriate for your instrument (Table 1).
- 13. Click Open file.



14. Check that all of the single-stained control files and the unstained control file have been brought into the Select single-stained control window. In some cases, the Parameters will need to be set manually. Assign each singlestained control the appropriate Negative Control (Table 1).

Table 1. Compensation Setup					
Data File	Parameter	Negative control			
Buffer	Negative control				
Lipo100	vFRed-A	Buffer-only			
Bead-FL1	FL1	Use self as negative or blank			
Bead-FL2	FL2	Use self as negative or blank			
Bead-FL3	FL3	Use self as negative or blank			
Bead-FLn	FLn-A	Use self as negative or blank			



Figure 2. Files for controls have been added to Step 1 in the Automatic Compensation Setup and are typically matched automatically to their respective parameters. Note: For some instruments this may need to be done manually (See instruments specific protocol 0).

- 15. Set the Scatter Gate Parameters (Step 2) to SSC-H and VSSC-H (Figure 3). FCS Express may regenerate charts each time something changes but this shouldn't affect the compensation results.
  - a. CytoFlex: <u>SSC-H</u> vs <u>VSSC-H</u>
  - b. CellStream: <u>Raw Max Pixel\_SSC 773/56 A1</u> vs <u>SSC 773/56 A1</u>
  - c. ImageStream: Raw Max Pixel MC CH12 vs Intensity MC CH12



Compensation and Unmixing		×
🔁 • 💁 🖪 💉 🔳 🗙		
Loaded automatically with a	data files	
Created manually		
🕀 🕍 New Compensation	Errors and warnings in step 3 often will	

disappear as you complete the other steps.

Compensation Matrix Spillove	Matrix Compensation	Options	Automatic Compensation Setup			
Step 1 - Select single-stained controls						
Step 2 - Select Scatter Gate	Parameters			*		
X Parameter for Scatter Gate	SSC - 773/56 - A1	~				
Y Parameter for Scatter Gate	Raw Max Pixel SSC - 77	3 🗸				
Step 3 - View Errors and Warnings						
Step 4 - Adjust parameters (optional) *						
Step 5 - Add extra spillover targets (optional) *						
Step 6 - Calculate matrices *						

#### Figure 3. Select scatter gate parameters

- 16. Once Step 1 and Step 2 of the FCS Express Compensation wizard are completed, charts and gates will be automatically generated for the compensation.
- 17. Step 3 View Errors and Warnings Don't worry about this step until the end. The errors should be resolved by the end of the protocol.
- 18. In the gate hierarchy (on gating tab) adjust so that the positive and negative control for vFRed are not gated (See diagram below).



19. Inspect the plots on the newly created pages (Figure 4). Inspect and adjust the Scatter Gate to select the population of single stained beads. For the antibody-stained beads, confirm that the positive and negative populations are correctly identified. Adjust positive and negative gates/markers as needed. The software should identify the brightest of the four peaks in the nanoCal bead set. The vFRed control charts will need to have their



gate adjusted as shown below (Figure 4). The chart for the stained vFRed control should be set to use the vesicle gate while the chart for unstained vFRed should be set to no gate.



Figure 4. Adjust markers and gates on generated compensation plots (vFRed charts shown).

20. Click Calculate Matrices (Step 6) using the median fluorescence intensity values. The Spillover and Compensation matrices will be calculated (Figure 5).

#### Figure 5. The calculated Compensation matrix.

21. Export the compensation matrices as a \*.compensation file with an informative name (eg. CytoFlex - 220309 – buffer lipo100+vfred PEx3 BVx3 B525 R670.compensation", describing the particles and fluorophores used).



22. Copy the name of this file, and rename the New Compensation (right click, F2, Rename) by pasting.



Ð · 🔮		e Ti	X						Á.										
Loaded automatically with data files																			
Created manually																			
✓ ▲ CytoFlex - 220309 - Lipo_vFRed_nCal_buffer_A488_PEx3_BVx3 A647																			
		0	Com	pens	ation	Matri	ix												
		0	Spill	over	Matrix	ĸ													
			-			0.1					Spillover Matrix								
<ul> <li>Compensation Options</li> </ul>																			
		0	Com	pens	ation	Optic	ons												
		0	Auto	pens matio	ation c Con	optio	ons ation	Setu	p										
		0	Auto	omatio	c Con	optio	ation	Setu	р										
		0	Auto	matio	c Con	optio	ation	Setu	р										
Compenso	die= Matrix	Spillever	Auto	omatio		optio	ation	Setu	p										
Compenso Editor Ma	dies Matrix disc Edi 🔍	Spilever	Auto	matio		optio	ation	Setu	p										
Compenso Ration Ma Source	ation Matrix Atio Edi - N	Spillerer	Auto	omatio		optio	ation	Setu	p										
Compenso Ration Ma Source	dien Mateix discitation N APC-A scenario	Soffwer Kennen	Auto	omatio	ation c Con n Options	Adventor PCT-A	compercention	Setu	p rmes.A	4. 17	A. 17								
Compense Listor Ma Source APC-A BV271-A	APC-A	Spillever 6 8/421-A 6/20142 1.00888	Auto	eversatio	Target Price Addriss	Automatic PCT-A 0.053116	Compensation	PE-H -0.00167	P	-A. 57									
Compenso Editor Ma Source APC-A EVICT-A EVICT-A	dise Matrix dis Ed \v APC-A 3.00536 6.00116 6.00156	50/lever / / / / / / / / / / / / / / / / / / /	Auto	0matio	ation c Con n Cellens PTCA 4.0012 4.0012	PCT-A -0.05115 -0.00115 -0.00115 -0.00115	Compensation Compensation PESS4-A 4-008 4-0098 0.1125	Setu == Setup == Setup	P	4. ST 8. 75	# IT 15								
Compenso Editor Ma Source APC-A EVIZ1-A EVIZ1-A EVISTO-A	APC-A 400566 -0.00156 -0.00256	501/hver 2 2 20142 2 20142 2 20042 2 30042 2 30042 2 30042 2 30142 2 30142 2 3014 30142 3014 3014 3014 3014 3014 3014 3014 3014	Auto	00000000000000000000000000000000000000	ation c Con n Cellens Target PTC-A 4.00152 0.400152 0.400152 0.400152	PCT-A -0.02015 8.00446 -0.02015 8.00446 -0.02015	Compensation Compe	PE-H -0.00167 -0.00167 -0.00167 -0.00167 -0.00167 -0.00167 -0.00167	P	44, 817 8, 75 8, 8	# IT								
Compensa Editor Ma Source APC-A BVE21-A BV510-A BV510-A FRIC-A	APC-A 400586 -0.0016 E.00256 -0.01254 E.00254	30/1/2/1.A 2.02/42 1.0008 2.12/42 0.12/10 0.00013	Auto	00000000000000000000000000000000000000	ation c Con n Optiano Target PIC-A -0.0152 0.400152 0.400152 0.400152 0.400152 0.400152	PCT-A -0.05716 -0.05216 -0.05216 -0.05216 -0.05216 -0.05216 -0.05216	Emperador Compensation Compensa	PE-H -0.00187 -0.00187 -0.0012 6.0524 -0.42173 -0.0048	P	14 BT 8 75 8	# IT 1 15 1 19								
Compenso Editor Ma Source APC-A BVIC1-A BVIC1-A BVIC2-A FIEC-A RC7-A	APC-A 100586 -0.00796 8.00296 -0.02996 8.00296 8.00296 8.00296 8.002979	5arilever 5arilever 4.100442 1.00089 4.10466 4.10466 4.000715 4.00025	Auto	84603-4 0.0043 0.0143 0.0143 0.0143 0.0156	ation c Con n Optiens Target PIC-4 4.00152 0.400152 0.400152 0.400152 1.40001 4.00306	PCT-A -0.05116 -0.05116 -0.05156 -0.05156 -0.05156 -0.05156 -0.05156 -0.05156 -0.05156 -0.05156 -0.05156 -0.05156 -0.05156 -0.05116 -0.0516 -0.0516	Compensation Compe	PE-H -0.00167 -0.0042 8.0514 -0.42133 -0.0042 -0.0043 -0.0043 -0.0043	P										
Compense Searce APCA BVI21-A BVI21-A BVI21-A BVI21-A RUCA FIECA PUISA-A	APC-A 1.00556 0.0254 0.0274	5011ever 5011ever 6.00142 1.00808 6.10486 6.10486 6.02013 6.00013 6.00013 6.00013	Auto Auto Mare 0 00002 0.00014 0.00014 0.00014 0.00014 0.00014	Press.4 0.00143 0.00143 0.00143 0.00143 0.00143 0.00143 0.00190 0.00190 0.00190	ation c Con regeters Prc.4 4.00546 0.80212 4.00546 0.80212 1.80016 4.00306 4.00306	PCT-A -0.025716 -0.00015 -0.00000000000000000000000000000000000	Engeneration Compensation Compe	PE-H 4.00167 4.00167 4.00167 4.00167 4.00167 4.00167 4.00167 4.00167 4.00167 4.00167 4.00167	P +FRed A -0.94987 0.00005 -0.91907 -0.91907 -0.91907 -0.91907 -0.91907	44 BT 15 B 19 BT 19 BT									
Compensa Seurce APC-A BVI21-J BV510-A BV105-A FRC-A RC7-A RC	APC-A APC-A V00256 -0.00254 -0.00254 -0.00254 -0.00254 -0.00254 -0.00279 -0.00179 -0.00471	50Hever 8421.A 4.00142 7.000142 6.10005 6.00005 0.00062	Auto Auto 94510.4 6.0002 4.00014 4.00019 4.00019 4.00019 4.00019	Evero.4 0.0043 0.01 0.0043 0.01 0.0043 0.01 0.0043 0.01 0.00540 0.00540 0.00540 0.00540 0.00540 0.00540 0.00540 0.00540 0.00540 0.00540 0.00540 0.00540 0.00540 0.00540 0.00540 0.00540 0.00540 0.00540 0.005400 0.00540000000000	ation c Con n Cellero PIC-A 4.00152 0.400152 0.400152 0.400152 0.400152 0.400152 0.400152 0.400152 0.400152 0.400152 0.400152 0.400152 0.400152	PCT-A -0.02716 0.00015 0.0000000000	PESSI-A 4008 40095 4000 4000	PE-H 40,00187 40,00187 40,00187 40,00188 40,00188 40,00188 40,00188 40,00188 40,00187 40,00087 40,0000	P +FRed.A -0.94937 0.00007 -0.29679 -0.29679 -0.29679 -0.29679 -0.29679 -0.29679 -0.29679 -0.29679 -0.29679 -0.29679	14. 617 75 79 81									

23. Save the Layout with a similarly informative name (eg "vFC Protocol 0.4d Compensation - CytoFlex - 220309 – buffer lipo100+vFRed PEx3 BVx3 B525 R670.fey") as documentation of the calculations that produced the Spillover and Compensation matrices.

### Notes

For more information about using FCS Express for Compensation and Spectral Unmixing, see the FCS Express manual, Tutorials, and videos at denovosoftware.com.

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



### Appendix A. Staining nanoCal<sup>™</sup> Antibody Capture Beads

Cellarcus nanoCal<sup>™</sup> Antibody Capture Beads have calibrated antibody binding capacities that can be stained with fluorescent antibody conjugates of interest and used to calibrate the intensity and spectral responses of an instrument. Cellarcus offers pre-stained, freeze-dried nanoCal<sup>™</sup> beads, as well as unstained for use with custom conjugates or antibodies from venders other than Cellarcus.

### Materials

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

### Procedure

- 1. Vortex nanoCal<sup>™</sup> Antibody Capture Beads for 10 seconds.
- 2. Add one drop (~50 uL) of nanoCal<sup>™</sup> 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<sup>™</sup> 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.



### Appendix B. Protocol B2. vFRed<sup>™</sup>-stained Lipo100<sup>™</sup>

### Objective

Protocol B2 calibrates the relationship between vesicle membrane surface area and vFRed<sup>™</sup> fluorescence using Lipo100<sup>™</sup>, a synthetic vesicle size standard whose size distribution has been determined using orthogonal methods, including nanoparticle tracking analysis (NTA) and resistive pulse sensing (RPS). Lipo100<sup>™</sup> will be stained with vFRed<sup>™</sup>, measured on the flow cytometer, and linear regression performed to determine the fluorescence brightness per unit surface area (F/nm2). These regression coefficients are used to estimate the size of individual EVs.

### Materials

- a. vFC<sup>™</sup> Staining Buffer
- b. Lipo100<sup>™</sup> Standard
- c.  $vFRed^{TM}$  membrane stain
- d. Microwell plate (Sartstedt 82.1583.001)
- e. Gloves
- f. vFC<sup>™</sup> Vesicle Size Calibration Layout (FCS Express)

### Procedure

#### Prepare Working Solution

- 1. Prepare vFRed<sup>™</sup> membrane stain working solution (5 uL/well, +1 well)
  - a. 25 ul/5 wells: 2.5 µL vFRed<sup>™</sup> (100x) into 22.5 µL Staining Buffer.

#### **Prepare Samples**

- 2. Prepare EV samples (Table 1 and Plate Map below):
  - A. Buffer-only control
  - B. Buffer +reagent control
  - C. Lipo100<sup>TM</sup> vesicle size standard

Table 1.1 Staining reactions						
Well	Buffer	Lipo100 <sup>TM</sup>	vFRed <sup>TM</sup>	Total		
A1,2	50	0	0	50 uL		
B1,2	45	0	5	50 uL		
C1,2	40	5	5	50 uL		

- 3. Add 5  $\mu$ L 10x vFRed<sup>TM</sup> to Rows B and C.
- 4. Mix well, incubate 1 hour at RT in the dark.

 Dilute 1
 Dilute 2

 Staining Dilution 1 Read

 A

 1

 2

 3

 4

 5

 6

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 0

 <



- 4. During the staining incubation, ready the flow cytometer to run load the vFC Assay data acquisition Template (prepared in Protocol A Instrument QC and Qualification).
- 5. If not previously done, set that optimal trigger threshold (Appendix C).

#### **Dilute and Read**

- 6. Dilute the staining reaction according to Table 1.2.
  - a. Add the indicated amount of Staining Buffer to Columns 3-6
  - b. Transfer the indicated amount of stained sample from Columns 1-2 to Columns 3-4, and mix well.
  - c. Transfer the indicated amount of the Dilution 1 samples in Columns 3-4 to Columns 5-6, and mix well.
- 7. Run the wells in Columns 5-6 at the indicated sample flow rate for 120 seconds each.

Table 1.2 Post			
Wells	CytoFlex	CellStream	ImageStream
Staining	50 uL	50 uL	50 uL
Dilution 1	$8 \mu\text{L} \rightarrow 245$	$8~\mu L  ightarrow 105$	5 ul→ 145 uL
	uL	uL	
Dilution 2	$8~\mu L  ightarrow 245$	$8~\mu L  ightarrow 105$	None
	uL	uL	
Post-stain	1000	200	30
Dilution			
Run	High	Slow	Slow
	60 uL/min	3.7 uL/min	x.x uL/min



# Appendix C. Setting the Trigger Channel Threshold

### Purpose

This Protocol instructs on the setting of an optimal detection trigger threshold for vFRed-based EV detection.

### Procedure

- 1. Load a well of vFC<sup>™</sup> Staining Buffer and allow system to run for 15 seconds on high flow rate (60ul/min).
- 2. Ensure that the blue laser-excited, B6-H (vFRed) fluorescence channel is set at a threshold of 1100, then adjust the manual threshold until you get 10-15 events/second in a Buffer Only sample.
- 3. Save the new Settings file (.expt) for future use. In general, once optimized the threshold does not need to be changed every time you run an experiment.

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