vFC™ Protocol 1

Determining optimal sample dilution and EV concentration

Objective

Protocol 1 determines the optimal dilution and EV concentration for EV-containing samples, including culture media and fractionated plasma or other biofluids. The Protocol includes the necessary controls (buffer-only, reagent-only) and a sample serial dilution to determine assay dynamic range and lack of coincidence (swarm). This experiment also defines the optimal sample dilution (the dilution that produces ~50,000 Vesicle counts) for use in surface cargo measurement or other assays.

Materials

a. vFRed™ Membrane Stain (100x)
b. vFC™ Staining Buffer, 2 mL
c. Lipo100™ Vesicle Size Standard
d. Sample (concentrated culture media or fractionated biofluid)

To be provided by User

a. Gloves
a. v-bottom plate (Sartstedt 82.1583.001)
b. Pipettes (5 uL – 300 uL)
c. Pipette tips

Procedure

This Procedure is designed for two samples, for example conditioned media plus an untreated media control.

Prepare Working Solutions

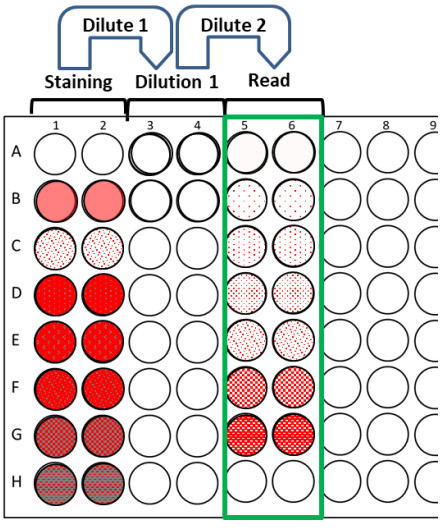
1. Prepare 10x vFRed™ stain solution (5 uL per well + 1 well)
    - For 2 samples plus controls (14 wells), 80 uL: add 8 uL vFRed™ (100x) to 72 uL vFC™ Staining Buffer.

Prepare Samples

2. Prepare serial dilutions of samples (Table 1 and Plate Map):
    a. Pipet vFC™ Staining Buffer into Rows A-H.
    b. Pipet 9 uL of EV samples into Row H and mix by pipetting up and down.
    c. Serially dilute EV samples up Rows G to D by transferring 45 uL from Row G to F, then 45 uL from Row F to E, and so on to row B. Discard 45 uL at row B.
3. Pipet 5 ul of Lipo100™ into Row C.
4. Add 5 uL 10x vFRed™ to all samples except Row A. Mix well, incubate 1 hour at RT.

Table 1. Staining reactions

	Row	Buffer	Sample	vFRed	Total
Buffer-only	A	50	0	0	50
Buffer +vFRed™	B	45	0	5	50
Lipo +vFRed™	C	40	5	5	50
Sample 1:160	D	45	45	5	50
Sample 1:80	E	45	45	5	50
Sample 1:40	F	45	45	5	50
Sample 1:20	G	45	45	5	50
Sample 1:10	H	81	9	5	50



Dilute and Read

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 A**).
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-stain dilution and run

Wells	**CytoFlex**	**CellStream**	**ImageStream**
Staining	50 uL	50 uL	50 uL
Dilution 1	8 µL → 245 uL	8 µL → 105 uL	5 ul→ 145 uL
Dilution 2	8 µL → 245 uL	8 µL → 105 uL	None
Post-stain Dilution	1000	200	30
Run	High 60 uL/min	Slow 3.7 uL/min	Slow x.x uL/min

Data Analysis

1. Open the vFC Analysis Layout with FCS Express Reader.
   Note: The vFC Analysis Layout has additional notes and tips to guide the data analysis.
2. Load the appropriate calibration results into the Surface Area parameter via Tools>Transforms>Parameter Math (See Protocol 0).
3. From the Data List, click the Add File (+) and select the Protocol 1 data files.
4. Order the files by time (by clicking on the $ETIM column in the Data List).
5. Select the file from Well A1, a **Buffer +vFRed** data file, and Change Data On All Plots. On the Gating Tab, inspect the Gating Plots. Adjust Time Gate, Pulse Gate, and Vesicle Gate as needed to minimize low- and high-scatter backgrounds.
6. Select a **Sample** data file and Change Data On All Plots. Inspect the Gating Plots. Adjust Gates as needed to select vFRed-positive events.
7. Export sample information and statistics using using Batch Processing.
8. On the Dilutions Tab, select a set of serial dilution data (eg Wells B1-B7) and load them into the overlay plots indicated. When data files are properly annotated, the plot titles and legend will be populated with the sample name and dilution, and the Vesicle Gate counts and Median Diameter will be plotted as a function of the dilution factor.
9. Copy the Batch Output data into the Data field of the vFC Protocol 1 Analysis template.

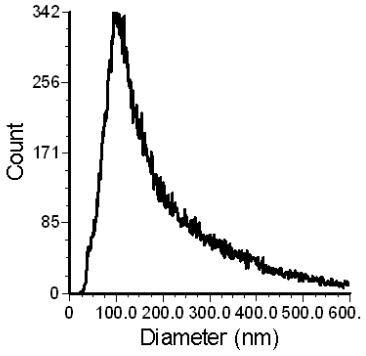
Example Data

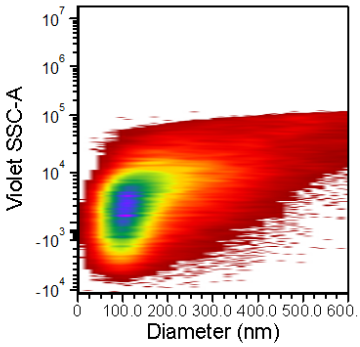
vFC™ Dilution Series - CytoFlex

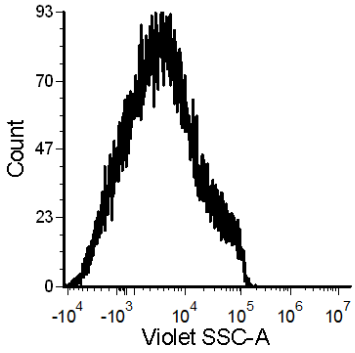
ELLARCUS BIOSCIENCES

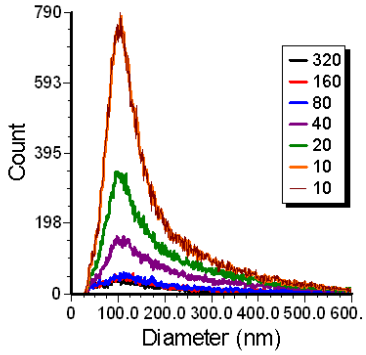
Project:
File name: 01-HEK high gluc 10% FBS 4_1-F11.fcs **Date:** 07-Apr-2022
Sample: 20
Concentration: 1.35E+7/uL
Instrument: CytoFLEX LX AS38003
Vol: 119999.4 **Tot**: 75100 **Aborts:** 562
Calibration:
C:\Users\Cellarcus\OneDrive - Cellarcus Biosciences\Webdata\CF\Prot 2 EV Cargo - PLT - TS mix\CF - 220310 PEx3 BVx3 R670.cal

Parameter	# of Events	Median	Arithmetic Mean
Diameter (nm)	51691	155	206

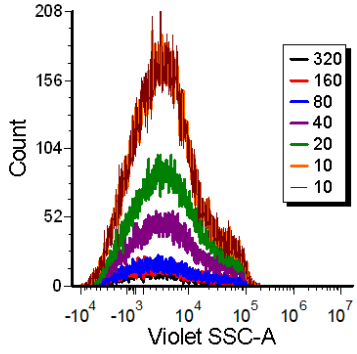


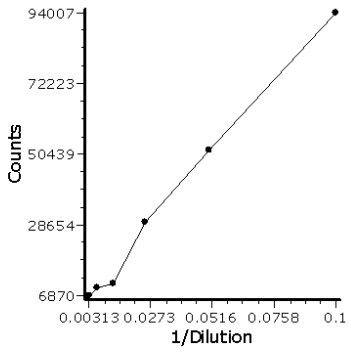


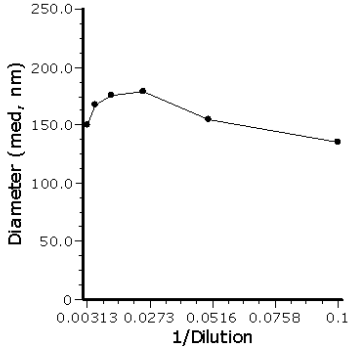




	A	B	C	D	E	F	G
1	Dilution	1/Dilution	Counts	Diameter (med, nm)	[EV] (/uL)		
2	320	0.003125	6870	150	2.20E+07		
3	160	0.00625	9330	167	1.49E+07		
4	80	0.0125	10713	175	8.57E+06		
5	40	0.025	29282	179	1.17E+07		
6	20	0.05	51691	155	1.03E+07		
7	10	0.1	94007	135	9.40E+06		
8	AVG				1.35E+07		
9							







Notes

Appendix A. Setting the Trigger Channel Threshold

Purpose

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

Procedure

1. Load a well of vFC™ 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.

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