vFC™ Protocol 2

Measuring EV Surface Cargo

Objective

Protocol 2 measures the presence of specific cargo using a fluorescent marker, such as a vTag™ antibody. EV surface cargo. This protocol includes the necessary controls (buffer-only, reagent-only, antigen-negative control, unstained control, positive control) to establish surface cargo staining specificity.

Materials

a. vFRed™ Membrane Stain (100x)
b. VFC™ Staining Buffer, 2 mL
c. Lipo100™ Standard (10x)
d. EV standard (10x)
e. Fluorescent antibody (FL mAb, 10x)
f. EV lysing solution
g. V-bottom plate

Materials to be provided by User

a. Gloves
b. Single channel and multichannel pipettes (5 uL – 300 uL)
c. Pipette tips

Procedure

Prepare Working Solutions

1. Prepare 200 uL 10x vFRed™ working solution (5 uL per well) by adding 20 uL vFRed™ (100x) to 180 uL Staining Buffer (for 4 samples plus controls, in duplicate)
2. Prepare 10x Vesicle Lysing Solution by adding 5 uL to 495 uL Staining Buffer (500 uL)

Prepare Samples

1. Dilute sample to between ~1x10^6 and 1x10^8/uL in Staining Buffer in a microfuge tube and mix well.
   Note: For new samples with unknown concentrations, see Protocol 1.
2. Place Staining Buffer into individual wells (see Table 2.1 and Protocol 2 Plate Map).
3. Add 5 uL of FL mAb (or buffer for no mAb samples)
4. Add 5 uL of diluted samples and standards to designated wells.
5. Add 5 uL of 10x vFRed™ to each well, mix by pipetting up and down.
6. Incubate for 60 minutes in the dark at RT.

Table 2.1. Staining

		vFRed™					vFRed™ +Marker				
	Row	Buffer	Marker	Sample	vFRed	Total	Buffer	Marker	Sample	vFRed	Total
Buffer only	A	50	0	0	0	50	45	0	0	0	50
Buffer +reagents	B	45	0	0	5	50	40	5	0	5	50
Lipo100™	C	40	0	5	5	50	35	5	5	5	50
EV standard	D	40	0	5	5	50	35	5	5	5	50
Sample 1	E	40	0	5	5	50	35	5	5	5	50
Sample 2	F	40	0	5	5	50	35	5	5	5	50
Sample 3	G	40	0	5	5	50	35	5	5	5	50
Sample 4	H	40	0	5	5	50	35	5	5	5	50

Dilute and Read

7. Dilute the staining reaction according to Table 2.2.
    a. Add the indicated amount of Staining Buffer to Columns 5-12
    b. Transfer the indicated amount of stained sample from Columns 1-4 to Columns 5-8, and mix well.
    c. Transfer the indicated amount of the Dilution 1 samples in Columns 5-8 to Columns 9-12, and mix well. [Not required for ImageStream]
    d. Run the wells in Columns 8-12 at the indicated sample flow rate for 120 seconds each.

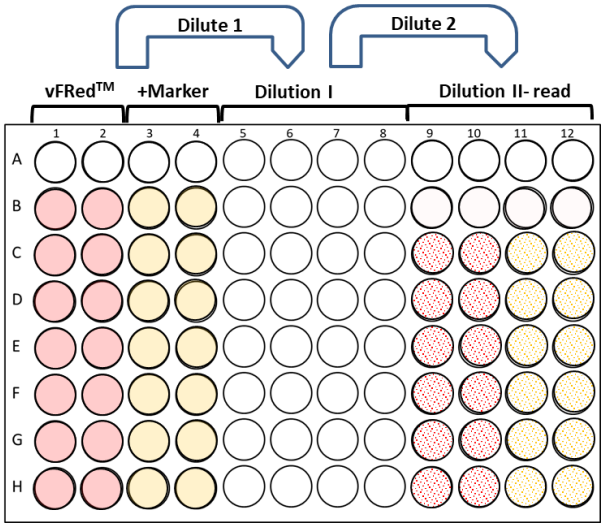
Table 2.2 Post-stain dilution and run

Reagent	**CytoFlex, Aurora**	**CellStream**	**ImageStream**
Staining	50 uL	50 uL	50 uL
Dilution 1	8 µL → 245 uL	8 µL → 105 uL	5 µL → 145 uL
Dilution 2	8 µL → 245 uL	8 µL → 105 uL	
Dilution factor	1000	200	30
Run	High 60 uL/min	Slow 3.7 uL/min	Fixed

Detergent Sensitivity

1. After the post-stain dilutions have been performed (Step 7), add 5 uL 10x Vesicle Lysing Solution to desired Staining Wells (eg wells A1-D4) and incubate 10 minutes.
2. Dilute and read as above.

Protocol 2 Plate Map



Data Analysis

1. Open the vFC Protocol 2 Analysis layout with FCS Express or FCS Express Reader.
   Note: The vFC Analysis Layout and Appendix A below have additional notes and tips to guide the data analysis.
2. Load the appropriate Vesicle Size Calibration file (*.fcf) into the Surface Area parameter via Tools>Transforms>Parameter Math (See Protocol 0.1 and/or 0.2).
3. Load the appropriate fluorescence intensity Channel Calibration file (*.cal) via Tools>Channel Calibration>Load (see Protocol 0.1 and/or 0.3).
4. Load the appropriate Compensation file (*.compensation) via Tools>Compensation and Unmixing>Created manually (see Protocol 0.4)
5. From the Data List, click the Add File (+) and select the Protocol 2 data files.
6. Order the files by time (by clicking on the $ETIM column in the Data List) and select the first file.
7. Inspect the negative and positive controls and adjust gates if necessary
   a. Select a **Buffer +vFRed** data file and Change Data On All Plots. Inspect the Gating Plots. Adjust Time Gate, Pulse Gate, and Vesicle Gate as needed to minimize low- and high-scatter backgrounds.
   b. Select a **Lipo100** data file and Change Data On All Plots. Inspect the Gating Plots. Adjust Gates as needed to select vFRed-positive events.
   c. Select a **Sample** data file and Change Data On All Plots. Inspect the Gating Plots. Adjust Gates as needed to select vFRed-positive events and eliminate background events. Inspect the Report Plots. Adjust the Fluorescence Gates to the edge of the negative (unstained) sample distribution so as to gate on Positive events.
   d. Select a **Lipo100 +TS Mix PE** data file and Change Data On All Plots. Inspect the Gating Plots. Adjust Gates as needed to select vFRed-positive events and eliminate background events. Note any positive fluorescence events that may be due to antibody/fluorophore aggregates.
   e. Select an **EV Std +TS Mix PE** data file and Change Data On All Plots. Adjust the Fluorescence Gates to the edge of the negative (unstained) sample distribution so as to gate on Positive events. Note the number and brightness of positive events.
   f. Select and inspect **Sample** data files.
8. Export plots and statistics via Batch Processing (Batch>Run)
9. Copy the Batch Output data into the Data field of the vFC Protocol 2 Analysis template.

Developed, Manufa buzas.edit@med.semmelweis-univ.huctured, and Distributed By
Cellarcus Biosciences, Inc.
Telephone: +1 (858) 239-2100
Customer Care: cellarcus@cellarcus.com
Technical Support: technical@cellarcus.com

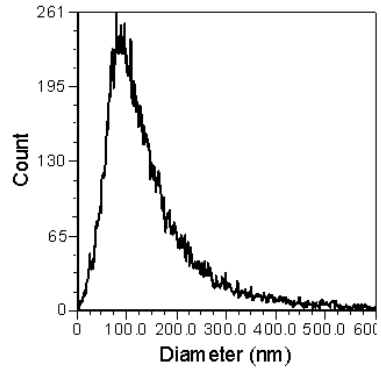
Example Data

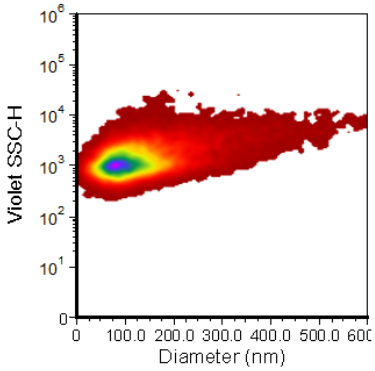
vFC Report

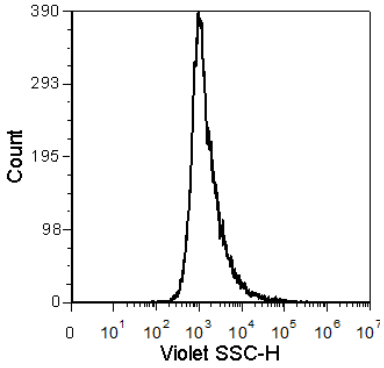
Experiment:
Well name: 03-20190917 TS mix PE-C12.fcs
Sample: EV Std PLT 190114
Volume:
Instrument: CytoFLEX LX AS38003

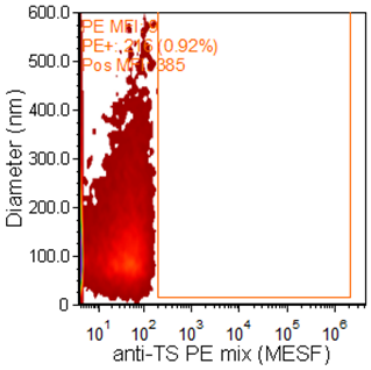
Parameter	# of Events	Median	Arithmetic Mean	CV
Diameter (nm)	32526	120	164	98

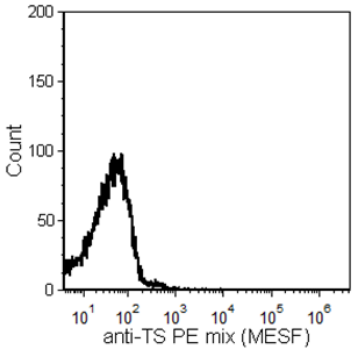
Parameter	# of Events	Median	Arithmetic Mean	CV
Violet SSC-H	32700	1225	2946	337

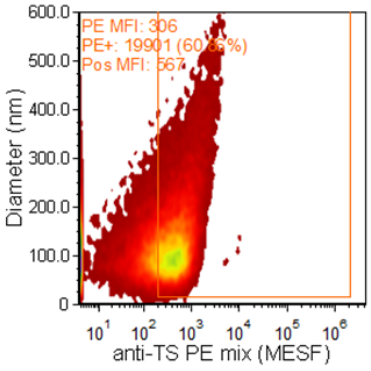


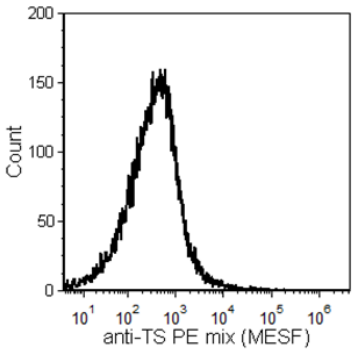












Notes