vFC™ Protocol 2

Measuring EV Surface Cargo.

Objective

To measure the presence of 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. Gloves
b. Microwell plate
c. vFRed™ Membrane Stain (100x)
d. VFC Staining Buffer, 2 mL
e. Lipo100™ Standard (10x)
f. EV standard (10x)
g. Fluorescent antibody (FL mAb, 10x)
h. EV lysing solution

Materials to be provided by User

a. Gloves
b. Microwell plate (Sartstedt 82.1583.001)
c. Pipettes (5 uL – 300 uL)
d. 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 VFC Staining Buffer (for 4 samples plus controls)
2. Prepare 10x Vesicle Lysing Solution by adding 5 uL to 495 uL Staining Buffer (500 uL)

Prepare Samples

3. Dilute sample to between ~$1x10^6$ and $1x10^8$/uL in VFC Staining Buffer in a microfuge tube and mix well.
   Note: For new samples with unknown concentrations, see Protocol 1.
4. Place 35 uL of VFC Staining Buffer into individual wells (see Protocol 2 Plate Map).
5. Add 5 uL of FL mAb (or buffer for no mAb samples)
6. Add 5 uL of diluted samples and standards to designated wells.
7. Add 5 uL of 10x vFRed™ to each well (expect row H), mix by pipetting up and down.
8. Incubate for 60 minutes in the dark at RT.

Dilute and Read

9. Dilute the staining reaction according to Table 2.
   For the CytoFlex®
   a. Add 145ul of staining buffer to row 2 and 291uL of staining buffer to row 3.
   b. Transfer 5 µL of stained sample (row 1) into a well containing 145 µL of Staining Buffer,

Table 2. Post-stain dilution and run

Reagent	CytoFlex	CellStream
Staining	50 uL	50 uL
Dilution 1	5 µL → 145 uL	1.5 µL → 300 uL
Dilution 2	9 µL → 291 uL	
Dilution factor	1000	200
Run	High 60 uL/min	Slow 3.7 uL/min

c. mix well by pipetting up and down (this will be dilution 1). Be careful to avoid foaming.
d. Transfer 9 µL of Dilution 1 into a well containing 291 µL of Staining Buffer, mix well (this will be dilution 2).
e. Run Dilution 2 on CytoFlex for fixed time (120 seconds) at fixed flow rate (High, 60 µL/min)

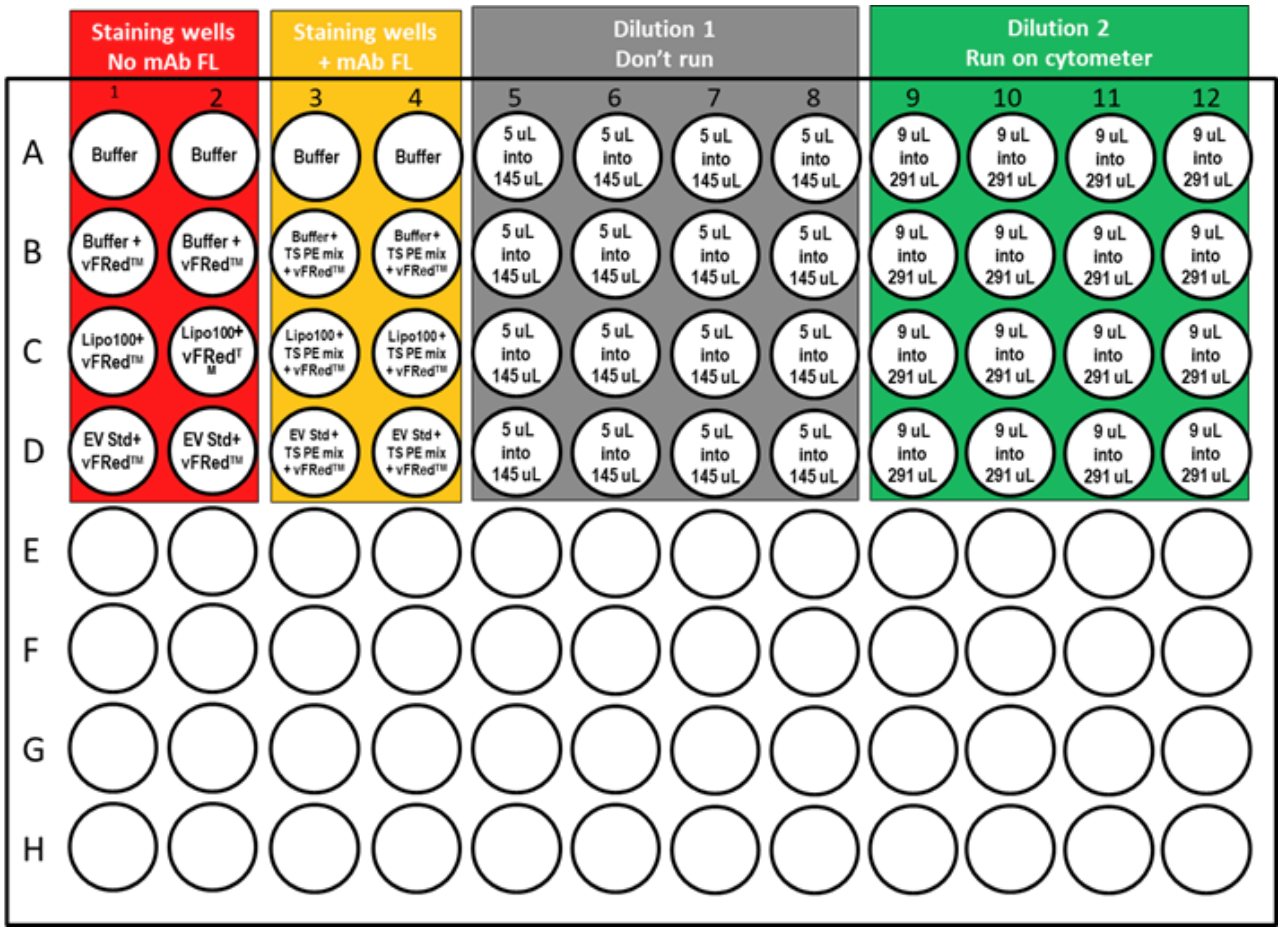
For the CellStream®

f. Add 298.5ul of staining buffer to row 2.
g. Transfer 1.5 µL of stained sample (row 1) into a well containing 298.5 µL of Staining Buffer, mix well by pipetting up and down. Be careful to avoid foaming.
h. Run on CellStream for fixed time (120 seconds) at fixed flow rate (Low, 3.66 µL/min)

Detergent Sensitivity

1. Following the first post-stain dilution (Step 10), 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 Analysis layout with FCS Express Reader.
   Note: The vFC Analysis Layout and Appendix A below have additional notes and tips to guide the data analysis.
1. Load the appropriate calibration results into the Surface Area parameter via Tools>Transforms>Parameter Math (See Protocol 0).
2. Load the appropriate fluorescence intensity Channel Calibration file (Tools>Channel Calibration>Load) (see Protocol 3 and/or 4).
3. vFC Analysis requires a calibrated instrument. If necessary, calibrate the relevant fluorescence channels using MESF Intensity Standard Beads (Protocol 3) or Antibody Binding Standard Nanobeads (Protocol 4).
4. From the Data List, click the Add File (+) and select the Protocol 2 data files.
   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.
5. Order the files by time (by clicking on the $ETIM column in the Data List) and select the first file.
6. Export plots and statistics via Batch Processing (Batch>Run)
7. Copy the Batch Output data into the Data field of the vFC Protocol 2 Analysis template.

Developed, Manufactured, and Distributed By
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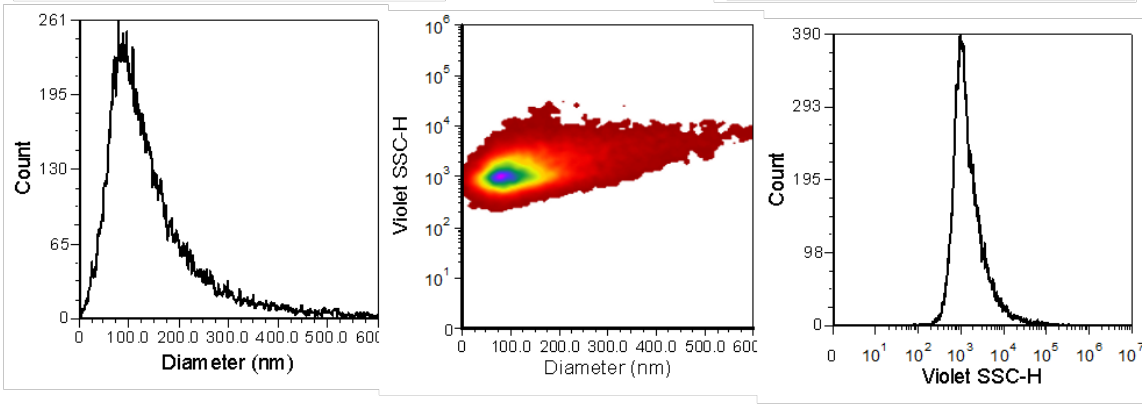
Example Data

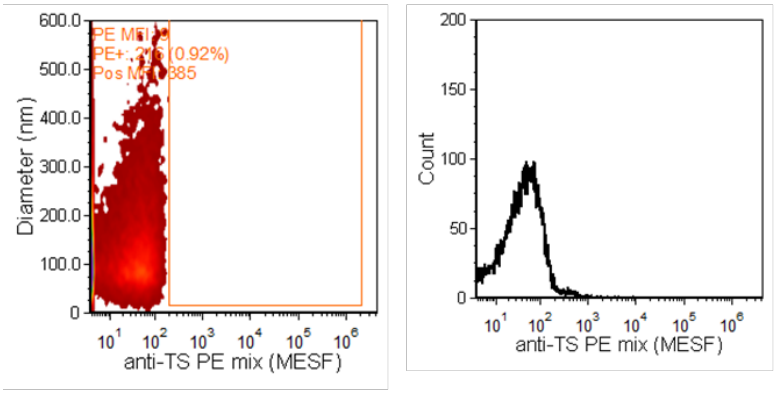
vFC Report

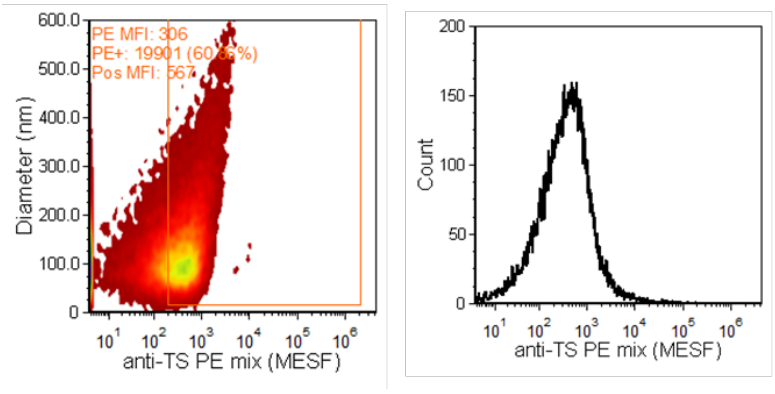
Experiment:
Well name: 03-20190917 TS mix PE-C12.fcs
Sample: EV Std PLT 190114
Volume:
Instrument: CytoFLEX LX AS38003

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