# vFC™ Protocol 2

*Measuring EV Surface Cargo*

## Objective

Protocol 2 measures individual EV number, size, and specific cargo using a fluorescent marker, such as a vTag™ antibody. 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 Reference Preparation (10x)
e. Fluorescent antibody (FL mAb, 10x)
f. EV lysing solution

## Materials to be provided by User

a. Gloves
b. Microwell plate (Sartstedt 82.1583.001)
c. Single channel and multichannel pipettes (5 uL – 300 uL)
d. Pipette tips
e. V-bottom plate

## 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. Thaw or reconstitute the EV Reference Preparation. For lyophilized EVs, reconstitute according to the product label instructions (typically in 25 uL of vFC Buffer for a 5-Test vial).
3. 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 | 50 | 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 Reference Prep | 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**

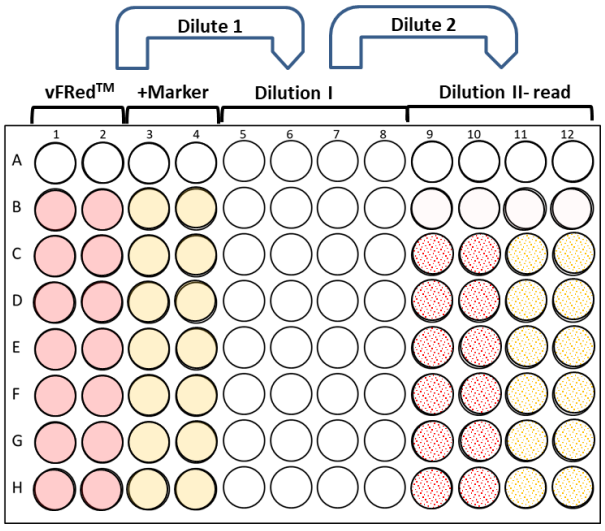
7. During the staining incubation, ready the flow cytometer to run load the vFC Assay data acquisition Template (prepared in Protocol A Instrument QC and Qualification).
8. If not previously done, set that optimal trigger threshold (Appendix A).
9. Dilute the staining reaction according to Table 2.2.
    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]
10. Run the wells in Columns 9-12 at the indicated sample flow rate and time (**Table 2.2**).

| **Table 2.2 Post-stain dilution and run** | | | |
|---|---|---|---|
| **Reagent** | **CytoFlex, Aurora** | **CellStream** | **ImageStream** |
| Staining | 50 uL | 50 uL | 50 uL |
| Dilute 1 | 8 µL → 245 uL | 8 µL → 105 uL | 5 µL → 145 uL |
| Dilute 2 | 8 µL → 245 uL | 8 µL → 105 uL | |
| **Dilution factor** | 1000 | 200 | 30 |
| **Run** | High, 120 s 60 uL/min | Slow, 120 s 3.7 uL/min | Fixed, 3 min |

**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 as above and read.

## 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 Compensation file (*.compensation) via Tools>Compensation and Unmixing>Created manually (**see Protocol B**).
3. Load the appropriate fluorescence intensity Channel Calibration file (*.cal) via Tools>Channel Calibration>Load (see **Protocol B**).
4. Load the appropriate Vesicle Size Calibration file (*.fcf) into the Surface Area parameter via Tools>Transforms>Parameter Math (See **Protocol B**).
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 Ref Prep +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).

## Developed, Manufactured, and Distributed By

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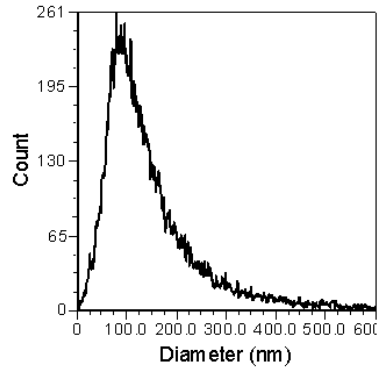
## Example Data

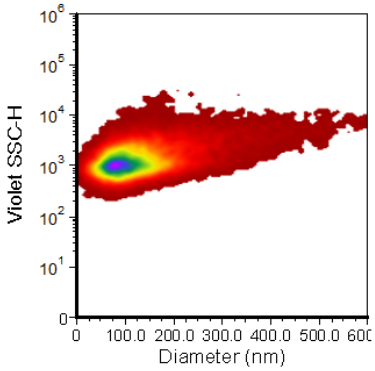
# vFC Report

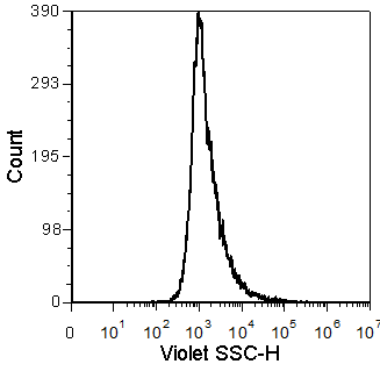
Experiment:
Well name: 03-20190917 TS mix PE-C12.fcs
Sample: EV Std PLT 190114
Volume:
Instrument: CytoFLEX LX AS38003

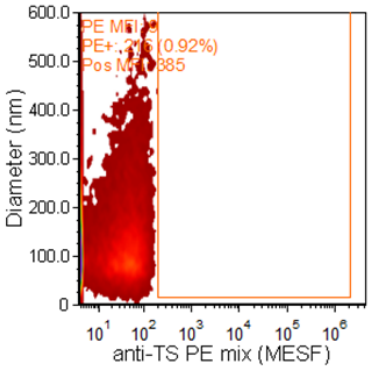
| Parameter | # of Events | Median | Arithmetic Mean | CV |
|---|---|---|---|---|
| Diameter (nm) | 32526 | 120 | 164 | 98 |

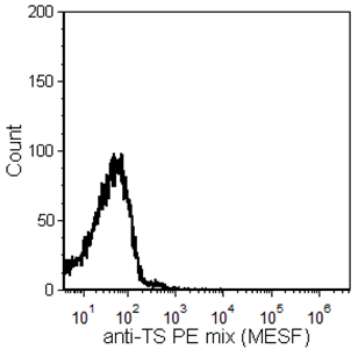
| Parameter | # of Events | Median | Arithmetic Mean | CV |
|---|---|---|---|---|
| Violet SSC-H | 32700 | 1225 | 2946 | 337 |

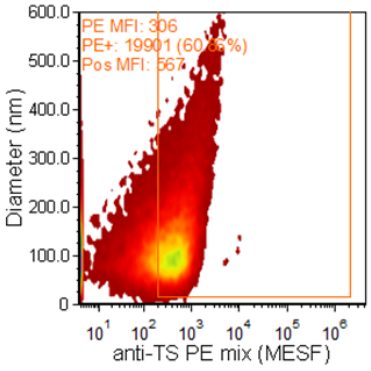


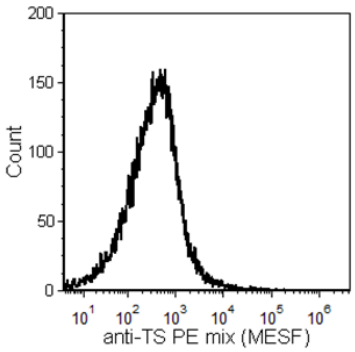



Notes

# 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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