vFC™ Protocol 1

Measuring EV Concentration and Optimal Dilution.

Objective

To determine the concentration of EVs and optimal dilution for running vFC™ on your samples. The Protocol includes the necessary controls (buffer-only, reagent-only, positive control) and a serial dilution of sample to determine concentration, dynamic range, and lack of coincidence (swarm). This experiment also defines the optimal dilution (the dilution that produces ~50,000 Vesicle counts) for use in surface cargo measurement or other assays.

Materials

a. Gloves
b. Staining Buffer, 2 mL
c. vFRed™ Membrane Stain (100x)
d. Four samples, diluted (Table 1.1)
e. Microwell plate

Table 1. Suggested initial sample dilution

Sample type	Initial dilution
Culture media	Neat
EVs enriched by ultracentrifugation, ultrafiltration, PEG precipitation	1:5

Procedure (for 4 unknowns)

1. Wear gloves for all manipulation of samples and reagents.

Prepare Working Solutions

2. 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)

Prepare Samples

3. Prepare serial dilutions of samples (See Protocol 1 Plate Map):
    a. Pipet 50 uL of Staining Buffer into wells A1-H4.
    b. Pipet 50uL of diluted EV samples into row G1-4 and mix by pipetting up and down.
    c. Serially dilute EV samples up Rows F to B, by transferring 50uL from row G to row E, then 50uL from Row E to row D, and so on to row B. Discard 50 uL at row B.
4. Add 5 uL 10x vFRed™ to all samples except Row H
5. Mix well, incubate 1 hour at RT.

Dilute and Read

6. 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, mix well by pipetting up and down (this will be dilution 1). Be careful to avoid foaming.
    c. Transfer 9 µL of Dilution 1 into a well containing 291 µL of Staining Buffer, mix well (this will be dilution 2).
    d. Run Dilution 2 on CytoFlex for fixed time (120 seconds) at fixed flow rate (High, 60 µL/min)
   For the CellStream®
    e. Add 298.5ul of staining buffer to row 2.

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

f. 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.
g. Run on CellStream for fixed time (120 seconds) at fixed flow rate (Low, 3.66 µL/min)

Data Analysis

1. Open the vFC Analysis Layout with FCS Express Reader.
   Note: The vFC Analysis Layout and Chapter 4 below have 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.
   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 **Sample** data file and Change Data On All Plots. Inspect the Gating Plots. Adjust Gates as needed to select vFRed-positive events.
4. Order the files by time (by clicking on the $ETIM column in the Data List) and select the first file.
5. Export plots and statistics via Batch Processing.
6. Copy the Batch Output data into the Data field of the vFC Protocol 1 Analysis template.

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Notes