vFC™ Protocol 0

For vesicle detection and size calibration.

Objective

To test performance and calibrate the instrument for vFC™ counting and sizing. For Protocol 0 on an instrument that is appropriately configured, we would expect to see approximately 50,000 events with a diameter distribution that ranges between ~50 – 300 nm, and a median of 100-130 nm and mean of ~120-150 nm.

Materials

a. Gloves
b. Staining Buffer, 8 mL
c. Lipo100™ Standard (10x)
d. vFRed™ membrane stain (100x)
e. Microwell plate

Procedure

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

Prepare Working Solution

2. Prepare 10x vFRed™ working solution by adding 8 µL vFRed (100x) to 72 µL Staining Buffer.

Prepare Samples

3. Prepare EV samples (See Protocol 0 Plate Map below):
    a. Pipet 40 µL of Staining Buffer into wells A1-H1
    b. Add 5 µL Lipo100™ (10x) to wells C1, D1, G1, and H1.
    c. Add 5uL of staining buffer into wells A1, B1, E1, and F1
4. Add 5 µL 10x vFRed™ to all wells except A1 and E1.
5. Mix well, incubate 1 hour at RT in the dark.

Table 1. Staining reactions

Reagent	CytoFlex	CellStream
Buffer	40 uL	35 uL
Lipo100	5 uL	10 uL
vFRed	5 uL	5 uL
Total	**50 uL**	**50 uL**

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)

Table 2. Post-stain dilution and run

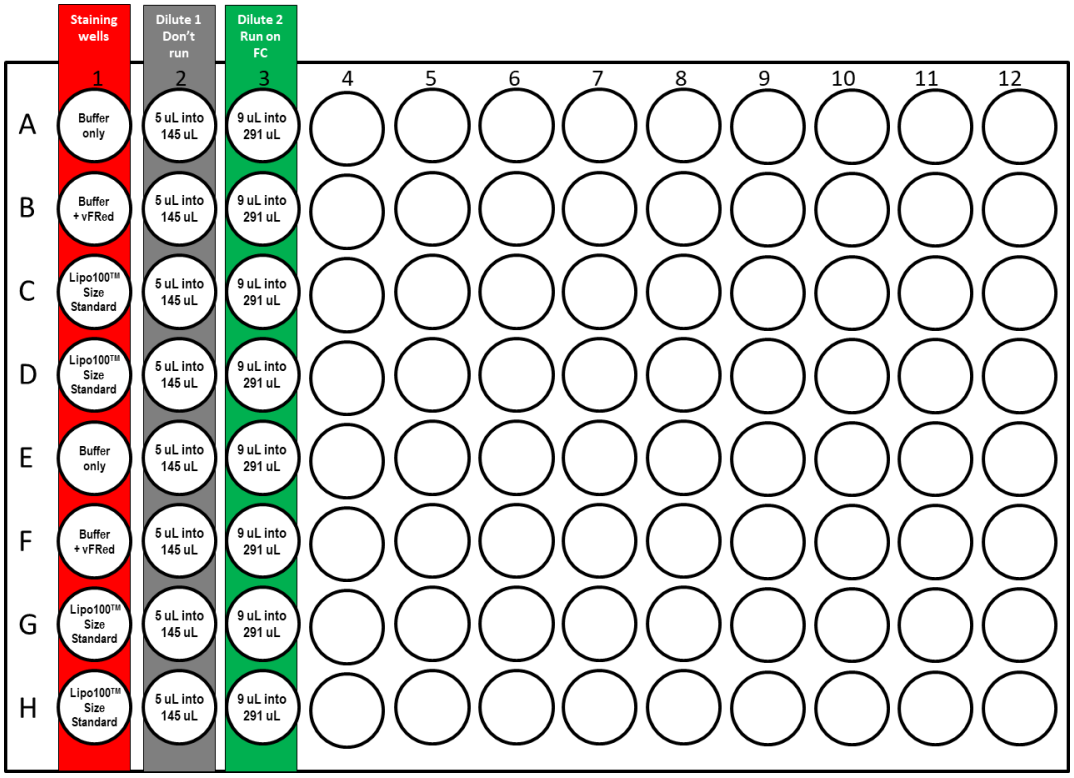
Reagent	CytoFlex	CellStream
Staining	50 uL	50 uL
Dilution 1	5 µL → 145 uL	1.5 µL → 300 uL
Dilution 2	9 µL → 291 uL	
Dilution factor	1000	200
Run speed	High 60 uL/min	Slow 3.7 uL/min

   For the CellStream®
    e. Add 298.5ul of staining buffer to row 2.
    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. From the Data List, click the Add File (+) and navigate to the data directory and select the Protocol 0 data files).
   a. Select a **Buffer + vFRed™** data file and click 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 click Change Data On All Plots. Inspect the Gating Plots. Adjust Gates as needed to select vFRed™-positive events.
3. Run the Vesicle Calibration Tool or Copy the Lipo100 vFRed™ intensity data to the clipboard, and paste into the vFC Size Calibration Tool located under the *Data Analysis* tab at www.cellarcus.com/products/vfc-base-kit. Enter the parameters from calibration results into the Surface Area parameter via Tools>Transforms>Parameter Math.
4. Inspect the resulting size distribution. The Lipo100 vesicle estimated size should range between ~50 – 300 nm, and a median 100-130 nm and mean of ~120-150 nm.
5. Export plots and statistics via Batch Processing.

Protocol 0 Plate Map



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Notes