Protocol 0.2. vFC™ Assay Calibration using Lipo100™ Vesicle Standard

Objective

Protocol 0 is designed to test instrument and assay performance using a minimal set of positive and negative controls. 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 detect approximately 50,000 events with a diameter distribution that ranges between ~50 – 300 nm, and a median diameter of ~100 nm.

Materials

a. Staining Buffer
b. Lipo100™ Standard
c. vFRed™ membrane stain
d. Microwell plate
e. Gloves

Procedure

Prepare Working Solution

1. Prepare vFRed™ membrane stain working solution (5 uL/well, +1 well)
   - 25 ul/5 wells: 2.5 µL vFRed™ (100x) into 22.5 µL Staining Buffer.

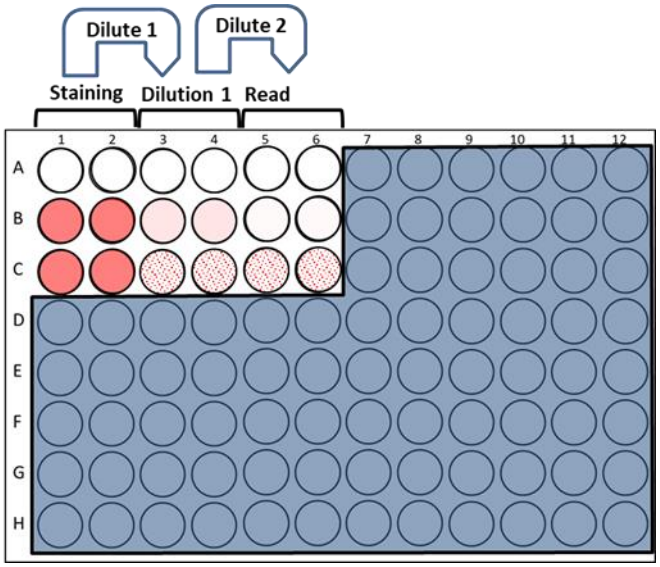
Prepare Samples

2. Prepare EV samples (Table 1 and Plate Map below):
   A. Buffer-only control
   B. Buffer +reagent control
   C. Lipo100™ vesicle size standard

Table 1.1 Staining reactions

Well	Buffer	Lipo100™	vFRed™	Total
A1,2	50	0	0	50 uL
B1,2	45	0	5	50 uL
C1,2	40	5	5	50 uL

3. Add 5 µL 10x vFRed™ to Rows B and C.
4. Mix well, incubate 1 hour at RT in the dark.



Dilute and Read

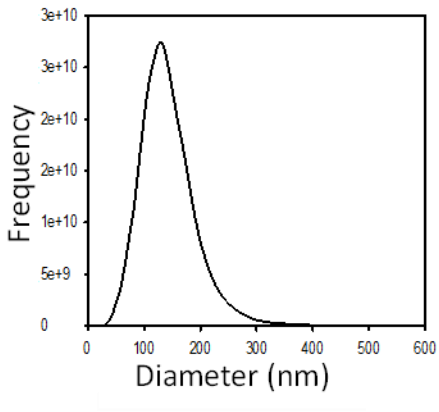
4. Dilute the staining reaction according to Table 1.2.
    a. Add the indicated amount of Staining Buffer to Columns 3-6
    b. Transfer the indicated amount of stained sample from Columns 1-2 to Columns 3-4, and mix well.
    c. Transfer the indicated amount of the Dilution 1 samples in Columns 3-4 to Columns 5-6, and mix well.
    d. Run the wells in Columns 5-6 at the indicated sample flow rate for 120 seconds each.

Table 1.2 Post-stain dilution and run			
Wells	**CytoFlex**	**CellStream**	**ImageStream**
Staining	50 uL	50 uL	50 uL
Dilution 1	8 µL → 245 uL	8 µL → 105 uL	5 ul→ 145 uL
Dilution 2	8 µL → 245 uL	8 µL → 105 uL	None
Post-stain Dilution	1000	200	30
Run	High 60 uL/min	Slow 3.7 uL/min	Slow x.x uL/min

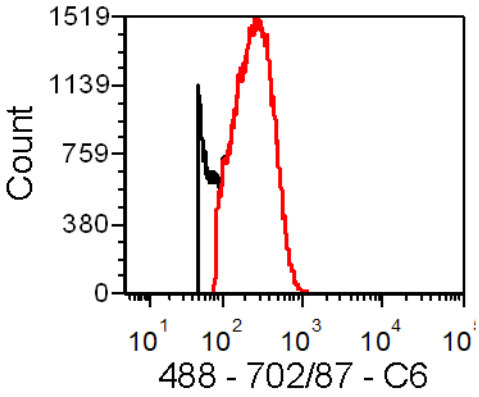
Data Analysis

1. Open the vFC™ Analysis layout with FCS Express Reader.
   Note: The vFC Analysis Layout has 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, Area/Pulse Gate, and Vesicle Gate as needed to minimize background events.
    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 population estimated diameter should range between ~80 – 250 nm, with a median 100-130 nm and mean of ~100 nm.

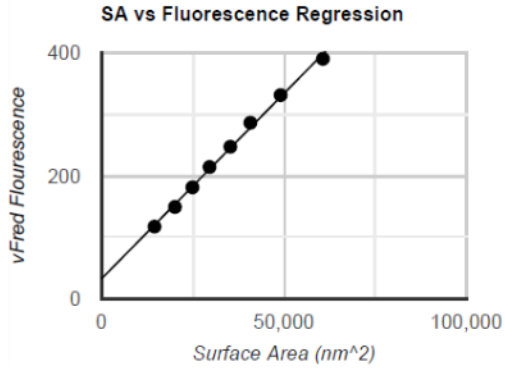
A. Lipo100 Diameter (NTA)



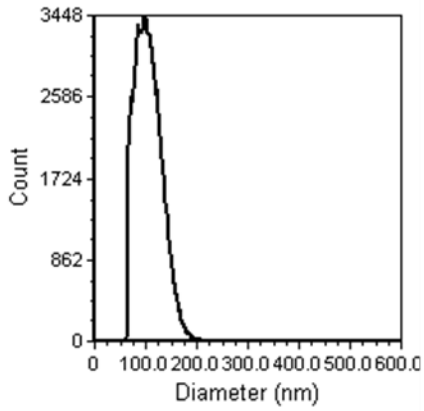
B. Lipo100 Fluorescence (FC)



C. Surface Area (NTA) vs vFRed



D. Lipo100 Diameter (vFC)



Export plots and statistics via Batch Processing.