Vesicle Flow Cytometry Analysis Kit

FOR VESICLE COUNTING AND SIZING

For detection, enumeration and size determination of vesicles in samples

Introduction

Background

Sub-micron sized membrane vesicles and related nanoparticles are of broad academic and commercial interest. Biological systems use membrane vesicles tor transport cargo within and between cells and biologically-inspired synthetic vesicles are used as carriers for drugs and other industrial uses. Extracellular vesicles (EVs) are a diverse set of membrane-bound nanoparticles that include exosomes, microvesicles/ectosomes, enveloped viruses and virus-like particles, and others that are particularly interesting as potential biomarkers, vaccines, and therapeutics. The measurement of EVs and their cargo are essential to understanding their origins, potential targets, and possible functions.

Flow cytometry is an attractive technology to measure individual EVs, but their small size and low refractive limit the sensitivity and specificity of conventional light scatter-based detection approaches. Light scatter intensity from small particles decrease dramatically with size, and their low refractive index results in scatter intensities that are an order of magnitude (or more) lower than comparably sized polystyrene beads. In addition, light scatter from non-vesicle particulates and other components of the sample, reagents, and buffers can overwhelm the dim EV signals and limit assay sensitivity, accuracy, and precision.

Fluorescence-based detection offers several advantages for measurement of EVs and other dim particles, including specific detection using appropriate fluorescence stains, lower intrinsic background for improved sensitivity, and intensity standards and calibration approaches that enable quantitative reporting and sharing of data. Moreover, new flow cytometers with high quantum photodetectors provide significantly improved sensitivity compared to older instruments. Cellarcus has exploited these advantages to develop flow cytometry-based assays for single EV analysis that offer superior specificity, sensitivity, and reproducibility.

Assay Principle

The Cellarcus vFC™ Vesicle Analysis Kit uses a fluorogenic membrane probe to stain vesicles and enable their detection and size estimation using flow cytometry. The membrane stain formulation is optimized to measure vesicles in the range of $1x10^5$-$1x10^8$ vesicles/uL. Samples should be diluted if necessary. If the concentration of EVs in the sample is not known, a preliminary sample dilution series should be performed to establish the linear range. Some biofluids (culture media, urine) can have lower EV concentrations, in which case concentration may be required (via ultrafiltration, centrifugation or precipitation). Researchers interested in measuring extracellular vesicles (EVs) from human or other species should use our EV Analysis Kits, which include antibodies against EV cargo and EV standards that serve as positive controls.

Typical pre-analytical sample preparation from cell culture supernatants involves serial centrifugation (2,500 xg, 10 min, 2x) to pellet cells and large debris. vFC™ is compatible with samples that have been processed by ultracentrifugation, ultrafiltration, or precipitation. The processed supernatant is typically aliquoted and frozen (-80C) for later analysis.

For EV counting and sizing, samples are diluted in vFC™ Buffer and arrayed in a 96 well plate along with the Lipo100™ Standard and buffer controls. Staining solution containing vFluor Red Membrane Stain is added, and the samples mixed and incubated for > 30 minutes at ambient temperature. For EV cargo measurements, qualified fluorescent antibodies or other stains are also included. Stained samples are then diluted and measured on the flow cytometer. A standardized data analysis template is used for gating and reporting of essential EV metrics, including number, size, and cargo fluorescence.

vFC™ Kit Contents & Storage Conditions

The vFC™ Vesicle Analysis Kit is shipped with cold packs and components are stable at 4C. Do not use past printed expiration date.

Kit Component	Size	Storage Conditions	Notes
vFluor Red™, Membrane Stain (100x)	1 x 50 uL	Refrigerate (2-8C)	
Lipo100™ Standard (10x)	2 x 50 uL	Refrigerate (2-8C)	
EV Lysing Solution (1000x)	1 x 25 uL	Refrigerate (2-8C)	
VFC Staining Buffer (1x)	1 x 100 mL	Refrigerate (2-8C)	
Anti-TS PE Mix (10x)	1 x 20 tests or 1 x 100 tests	Refrigerate (2-8C)	When purchased as part of kit
EV standard (10x)	1 x 50 uL	Refrigerate (2-8C)	When purchased as part of kit

Limitations

- The vFC™ Vesicle Analysis Kit is intended for RESEARCH USE ONLY. The product is not intended for the diagnosis, prevention or treatment of a disease.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.

Safety

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. Samples that are potentially infectious, including samples prepared from human or animal biofluids, should be handled at BSL Level 2 and Universal Precautions observed.

Protocol Overview

Materials and Equipment to be Provided by User

- Disposable gloves
- Single channel and multichannel pipets
- Sterile pipet tips
- 1.5 mL microfuge tubes
- 0.65 mL microfuge tubes
- 96 well v-bottom plate, Sarstedt

Protocol Notes

DO NOT use colored, opaque, or silanized tubes.

Wear gloves when handling samples and reagents. Humans can be a significant source of background contamination.

Instrument Configuration and Setup

Beckman Coulter CytoFlex™

Filter configuration. The CytoFlex filters should be configured for the Violet Side Scatter (VSSC) detection option, as detailed in the CytoFlex Operation Manual.

Acquisition template. Load the appropriate *.xitm template file, which contains file and group naming rules applied to file names and Sample IDs. Frequently used plate layouts can be saved and used as templates for future experiments.

Gains and Calibration. Gains for all fluorescence channels should be set at 1000, 488 nm scatter (FSC and SSC) at 500, 405 nm scatter (VSSC) at 100. Run 8 peak Rainbow beads and cross calibrate against MESF beads (eg Quantum FITC from Bangs, PE-Quantibrite from BD, etc).

Trigger and Threshold. The instrument should be set to trigger from the 488x-690/50-H channel fluorescence channel, and the threshold should be set by running a sample of Dilution Buffer and adjusting until ~2-4 event/second is detected (100 events over 100 seconds) at the High sample rate (60 uL/min). Apply this threshold to all wells and save template file (.xitm) for future use. In general, once optimized the threshold does not need to be changed from day to day.

Amnis Image Stream

Protocol 0. Protocol testing and vesicle size calibration

Objective

Test performance of the instrument for VFC counting and sizing.

Materials

a. Gloves
b. Staining Buffer, 8 mL
c. Lipo100™ Standard (10x)
d. vFluor Red™ Membrane Stain (100x)
e. Microwell plate

Procedure

1. Wear gloves for all manipulation of samples and reagents.
2. Prepare 10x vFluor Red™ Membrane Stain working solution by adding 8 uL Membrane Stain (100x) to 72 uL Staining Buffer
3. Prepare EV samples (See Protocol 0 plate layout on p7):
    a. Pipet 40 uL of Staining Buffer into wells A1-H1.
    b. Add 5 ul Lipo100™ (10x) to wells C1, D1, G1, and H1.
4. Add 5 uL Membrane Stain to all wells except A1 and E1.
5. Mix well, incubate 1 hour at RT in the dark.
6. Transfer 5 uL of stained sample into a new well containing 145 uL of Staining Buffer, mix well by pipetting up and down (Dilution 1). Be careful to avoid foaming.
7. Transfer 9 uL of Dilution 1 into a new well containing 291 uL of Staining Buffer, mix well (Dilution 2).
8. Run Dilution 2 on CytoFlex for fixed time (120 seconds) at fixed flow rate (High, 60 uL/min).

Notes

For analysis on the Amnis ImageStream, run samples after Dilution 1.

Protocol 0. Example Layout

	Staining wells	Dilute 1 Don't run	Dilute 2 Run on FC									
	1	2	3	4	5	6	7	8	9	10	11	12
A	Buffer only	5 uL into 145 uL	9 uL into 291 uL									
B	Buffer+ Membrane Stain	5 uL into 145 uL	9 uL into 291 uL									
C	Lipo100™ Size Standard	5 uL into 145 uL	9 uL into 291 uL									
D	Lipo100™ Size Standard	5 uL into 145 uL	9 uL into 291 uL									
E	Buffer only	5 uL into 145 uL	9 uL into 291 uL									
F	Buffer+ Membrane Stain	5 uL into 145 uL	9 uL into 291 uL									
G	Lipo100™ Size Standard	5 uL into 145 uL	9 uL into 291 uL									
H	Lipo100™ Size Standard	5 uL into 145 uL	9 uL into 291 uL									

Detailed Protocol 1. Determining EV Concentration and Size Distribution

Materials

a. Gloves
b. Staining Buffer, 2 mL
c. vFluor Red™ Membrane Stain (100x)
d. Vesicle Lysing Solution (1000x)
e. Four samples, diluted (Table 1.1)
f. Microwell plate

Table 1.1. Suggested initial sample dilution	
Sample type	Initial dilution
Culture media, neat	Neat
EVs enriched by ultracentrifugation, ultrafiltration, PEG precipitation	1:20

Procedure (for 4 unknowns)

1. Wear gloves for all manipulation of samples and reagents.
2. Prepare 10x vFluor Red™ Membrane Stain working solution (5 uL per well)
3. Prepare 10x Vesicle Lysing Solution by adding 5 uL to 495 uL Staining Buffer (500 uL)
4. Prepare serial dilutions of samples (See Protocol 1 Example Layout):
    a. Pipet 50 uL of Staining Buffer into all wells A1-F4.
    b. Pipet 100 uL of diluted EV samples into row F1-4 and mix by pipetting up and down.
    c. Serially dilute EV samples up Rows F→B, by transferring 50uL from row F to row E, then 50uL from Row E to row D, and so on to row B. Discard 50 uL at row B.
5. Add 5 uL 10x vFluorRed™ to all samples except Rows G and H.
6. Mix well, incubate 1 hour at RT
7. Add 2 uL 10x Vesicle Lysing Solution to wells G1-4.
8. Transfer 18 uL of one stained sample from column of the dilution series (Row F-B) to Row G, pipette up and down to mix. Incubate 10 minutes.
9. Place 145 uL Staining Buffer into wells in columns 5-8, 291 ul into wells in columns 9-12.
10. Transfer 5 uL of stained sample From Rows A1-G4 into a new well containing 145 uL of Dilution Buffer, mix well by pipetting up and down (Dilution 1). Be careful to avoid foaming.
11. Transfer 9 uL of Dilution 1 into a new well containing 291 uL of Dilution Buffer, mix well (Dilution 2).
12. Run on Dilution 2 on CytoFlex for fixed time (120 seconds) at fixed flow rate (High, 60 uL/min).
13. Note for ImageStream analysis: Run samples after Dilution 1.

Notes

For analysis on the Amnis ImageStream, run samples after Dilution 1.

Protocol 1. Example Layout (for four unknown samples)

	Staining wells				Dilute 1 Don't run				Dilute 2 Run on cytometer			
	1	2	3	4	5	6	7	8	9	10	11	12
A	Buffer + Membrane stain	Buffer + Membrane stain	Buffer + Membrane stain	Buffer + Membrane stain	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
B	Sample 1 Dilute 32-fold	Sample 2 Dilute 32-fold	Sample 3 Dilute 32-fold	Sample 4 Dilute 32-fold	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
C	Sample 1 Dilute 16-fold	Sample 2 Dilute 16-fold	Sample 3 Dilute 16-fold	Sample 4 Dilute 16-fold	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
D	Sample 1 Dilute 8-fold	Sample 2 Dilute 8-fold	Sample 3 Dilute 8-fold	Sample 4 Dilute 8-fold	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
E	Sample 1 Dilute 4-fold	Sample 2 Dilute 4-fold	Sample 3 Dilute 4-fold	Sample 4 Dilute 4-fold	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
F	Sample 1 Dilute 2-fold	Sample 2 Dilute 2-fold	Sample 3 Dilute 2-fold	Sample 4 Dilute 2-fold	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
G	2 uL TX100 + 18 uL Sample	2 uL TX100 + 18 uL Sample	2 uL TX100 + 18 uL Sample	2 uL TX100 + 18 uL Sample	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
H	Buffer only	Buffer only	Buffer only	Buffer only	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL

Detailed Protocol 2. Measuring EV Surface Cargo

Materials

a. Gloves
b. Microwell plate
c. vFluorRed™ Membrane Stain (100x)
d. VFC Staining Buffer, 2 mL
e. Lipo100™ Standard (10x)
f. EV standard (10x)
g. Fluorescent antibody (FL mAb, 10x)

Procedure (for 4 samples)

1. Wear gloves for all manipulation of samples and reagents.
2. Prepare 200 uL 10x vFluorRed™ working solution (5 uL per well) by adding 20 uL vFluorRed™ (100x) to 180 uL VFC Staining Buffer (for 4 samples plus controls)
3. Prepare 25 uL 10x sample (~5e6/uL, as determined by serial dilution, see Protocol 1) in VFC Staining Buffer in a microfuge tube and mix well.
4. Place 35 uL of VFC Staining Buffer into individual wells or tubes (see Protocol 2 Example Layout).
5. Add 5 uL of appropriately diluted samples and standards to designated wells.
6. Add 5 uL of FL mAb (or buffer for no mAb samples)
7. Add 5 uL of 10x vFluorRed™ to each well, mix by pipetting up and down.
8. Incubate for 60 minutes in the dark at RT.
9. Transfer 5 uL of stained sample into a new well containing 145 uL of VFC Buffer, mix well by pipetting up and down (Dilution 1).
10. Transfer 9 uL of Dilution 1 into a new well containing 291 uL of VFC Buffer, mix well (Dilution 2).
11. Run on Dilution 2 on Cytoflex for fixed time (120 seconds) at fixed flow rate (High, 60 uL/min).

Notes

For analysis on the Amnis ImageStream, run samples after Dilution 1.

Protocol 2. Example Layout (for four unknown samples)

	Staining wells No mAb FL		Staining wells + mAb FL		Dilution 1 Don’t run				Dilution 2 Run on cytometer			
	1	2	3	4	5	6	7	8	9	10	11	12
A	Buffer + vFluorRed	Buffer + vFluorRed	Buffer + vFluorRed TS PE mix	Buffer + TS PE mix	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
B	Lipo100 + vFluorRed	Lipo100 + vFluorRed	Lipo100 + vFluorRed TS PE mix	Lipo100 + vFluorRed TS PE mix	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
C	EV Std + vFluorRed	EV Std + vFluorRed	EV Std + vFluorRed TS-PE mix	EV Std + vFluorRed TS-PE mix	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
D	Sample 1+ vFluorRed	Sample 1+ vFluorRed	Sample 1+ vFluorRed TS PE mix	Sample 1+ vFluorRed TS PE mix	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
E	Sample 2+ vFluorRed	Sample 2+ vFluorRed	Sample 2+ vFluorRed TS PE mix	Sample 2+ vFluorRed TS PE mix	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
F	Sample 3+ vFluorRed	Sample 3+ vFluorRed	Sample 3+ vFluorRed TS PE mix	Sample 3+ vFluorRed TS PE mix	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
G	Sample 4+ vFluorRed	Sample 4+ vFluorRed	Sample 4+ vFluorRed TS PE mix	Sample 4+ vFluorRed TS PE mix	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
H	Buffer only	Buffer only	Buffer only	Buffer only	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL

Data Processing and Analysis

FCS Express

Load Files:

1. Open the VFC Analysis Layout in FCS Express or FCS Express Reader. Load FCS data files.
2. Load Channel Calibration. The Channel Calibration file converts the Fluorescence intensity axes from arbitrary channel numbers to MESF units.
3. Export Vesicle Stain Fluorescence Intensity histogram data. This and the NTA size distribution is used to generate vesicle size calibration parameters using the vFC™ Calibration script.
4. Load Parameter Math. The Parameter Math file contains equations and parameters to convert Vesicle Stain Fluorescence Intensity to units of Surface Area (nm2) and Diameter (nm) and to calculate fluorescence Surface Density and the 690A/610A parameter.

Adjust and Apply Gates:

- The Time Gate eliminates spurious events observed in the first 15 uL of sample delivery [CytoFlex].
- The Vesicle Gate is a polygonal gate on the VSSC vs PECy5-A (Vesicle Stain fluorescence) bivariate plot that can eliminate some sources of background that have distinctive scatter and fluorescence profiles that allow them to be distinguished from vesicles.
- The Marker Positive Gates are polygonal gates on the Marker Fluorescence vs Diameter bivariate plots that are used to estimate the number and brightness of individual vesicles. These gates are set using the appropriate negative control samples.

Output Data:

Define the File Name and destination of Excel, PowerPoint, and/or PDF files produced by the Batch Processing Analysis.

Execute Batch Processing:

The Batch Processing in the provided Analysis Layout exports selected keywords and statistics from plots in the layout for each file.