Vesicle Flow Cytometry Analysis Kit

FOR VESICLE COUNTING AND SIZING

Summary

Prior to running the vFC™ Assay, it is necessary to configure, characterize, and calibrate the instrument.

Instrument Setup involves configuring the instrument with the appropriate filters, parameter names, and lasers and detector settings, and creating Data Acquisition Templates for running vCal™ Calibration Beads and vFC™ EV Analysis Assays.

Instrument Calibration involves using these Data Acquisition Templates with Calibration Protocols to measure the vCal™ bead and vesicle standards, and to calibrate instrument performance using the accompanying Data Analysis Layouts.

> **Protocol 0.1 - Instrument Characterization using vCal™ nanoRainbow Beads** characterizes several critical performance metrics that enables evaluation of laser alignment, fluorescence resolution, and sample flow rate.
>
> **Protocol 0.2 - vFC™ Assay Calibration using Lipo100™ Vesicle Standard** qualifies the instrument and plasticware for vFC™, and calibrates the vFRed™ fluorescence response in terms of vesicle size (surface area).
>
> **Protocol 0.3 - Fluorescence Calibration using vCal™ Antibody Capture Beads** calibrates fluorescence response in units of MESF or ABV, and generates spectral reference standards for compensation and/or spectral unmixing.
>
> **Protocol 0.4 – Fluorescence Compensation using vCal™ Antibody Capture Beads** determines the spectral spillover matrix used to compensate signals for individual detector channels for the spectral overlaps between dye emission and detector emission band passes.
>
> **Protocol 0.5 – Fluorescence Unmixing using vCal™ Antibody Capture Beads** determines the fluorophore reference spectra to be used in spectral unmixing to determine the contributions of individual fluorophores to the measured emission spectra.

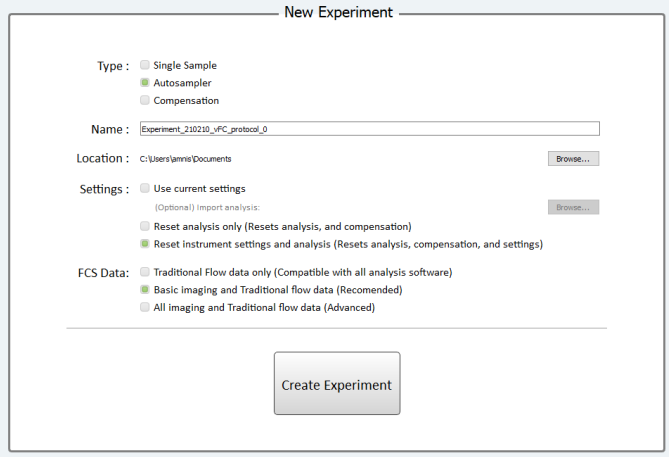
Once properly configured, characterized, and calibrated, the instrument is ready for use with the vFC™ EV Analysis Assay Kit to measure EV concentration and size (Protocol 1) and EV surface markers (Protocol 2).

Instrument Setup – Luminex CellStream

1. Create New Experiment and Settings Files

Before running vFC™ Protocols, it is necessary to create Settings files (*.ist), which contain the data acquisition settings for vFC assays. We will create one Settings file for analysis of vCal calibration beads, and a second for vFC assays.

After initializing the instrument, create a New Experiment (File> New experiment) and make the following selections:



Type: Autosampler

Name: Append a descriptive name after the date.

Settings: Reset instrument setting and analysis

FCS Data: Basic imaging and Traditional flow data

and then click “Create Experiment”.

2. Enable Small Particle Detection

The “Small Particle Detection” mode operates the detector at high gain, increasing sensitivity for measurement of dim particles. Choose this option:

Instrument>Advanced>Set Up Small Particle Detection

3. Acquisition Settings

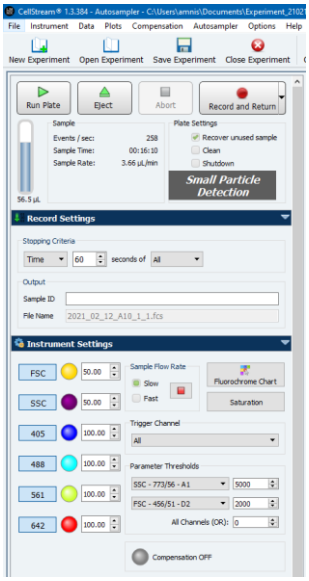
vFC™ assays using the CellStream are performed with all fluorescence excitation lasers at maximum power (100%), and the FSC and SSC lasers turned Off, all thresholds set to “none” (Table 1). If your instrument is equipped with 375 nm and/or 532 nm lasers, set these to 0% power.

Table 1. Acquisition settings

	vCal™ Calibration Beads		vFC™ Assay	
Laser	**Power (%)**	**Threshold**	**Power (%)**	**Threshold**
FSC	5	2000	5	none
SSC	1	5000	1	none
375	0	none	0	none
405	100	none	100	none
488	100	none	100	none
532	0	none	0	none
561	100	none	100	none
642	100	none	100	none
730	OFF	none	OFF	none

vCal™ Calibration particles are measured using the same fluorescence settings, but with the FSC and SSC lasers at 50% power, and thresholds set to 2000 and 5000 respectively.

Define vCal™ Beads Settings



In **Record Settings**:

Set Stopping Criteria to: Time, 60 seconds in All gates.

In **Instrument Settings**:

Set the FSC and SSC Laser Power at 50%, and

Set all other enabled lasers to 100%.

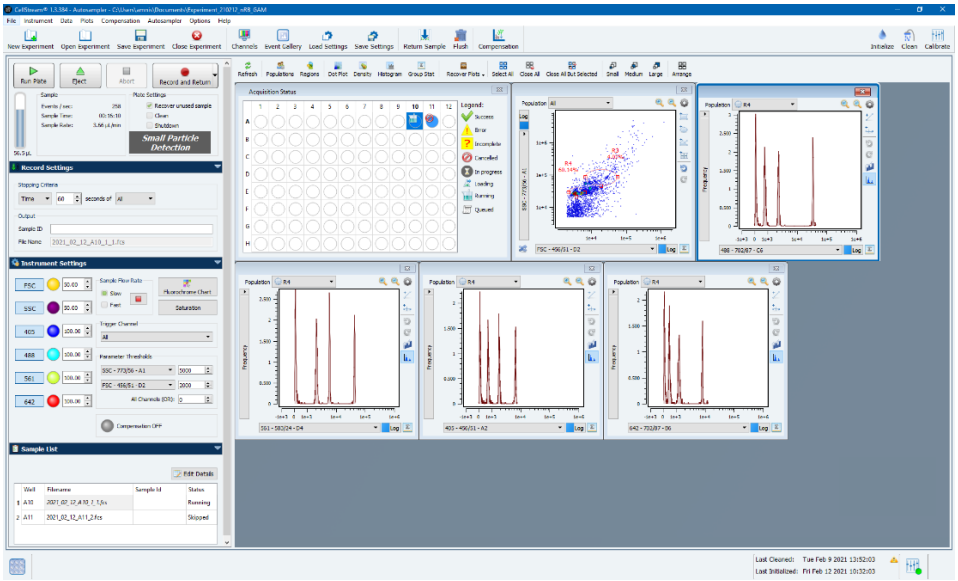
Set the Trigger Channel to All.

Set the Parameter Thresholds to FSC (2000) and SSC (5000).

Set the Sample Flow Rate to Slow (3.66uL/min)

Define acquisition plots

Once Acquisition settings are set correctly as described above, add informative plots to acquisition screen. Add the following 1- and 2-parameter plots and gates to view data:

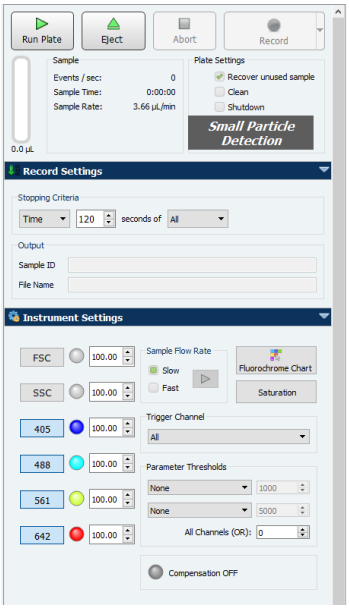


Note that Plots will be populated with data while samples are being acquired in Run mode, but not when in Record mode. Gating and data analysis do not take place during acquisition, but will be performed post-acquisition using the vFC™ Analysis layout in FCS Express or FCS Express Reader.

Save Settings file

Save the Settings file as “vCal Bead Settings YYMMDD”.

Define vFC™ Assay Settings



In **Record Settings:**

Set Stopping Criteria: Time, 120 seconds in All gates.

In **Instrument Settings**:

Set the FSC and SSC lasers to Off (icon will be grey)

Set all other enabled lasers to 100%.

Set Parameter Thresholds to None.

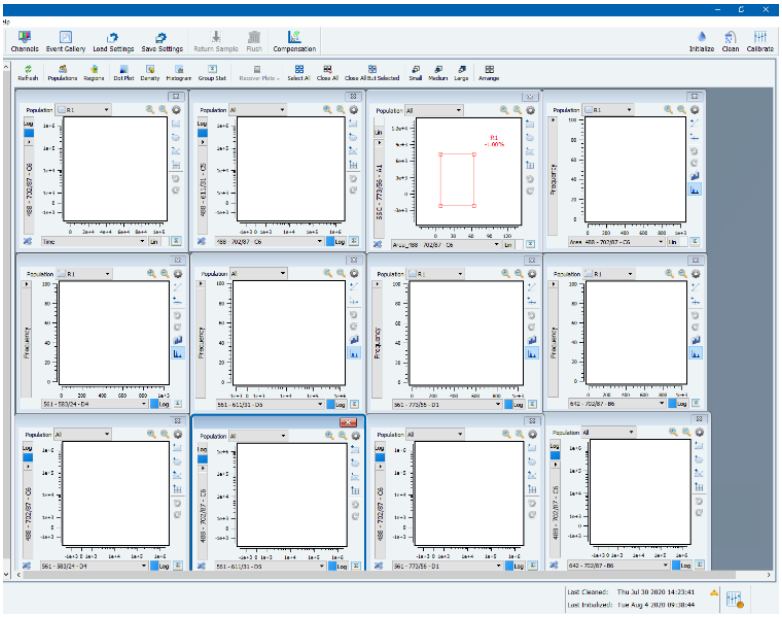
Set the Trigger Channel to “All.”

Set the All Channels (OR) threshold to 0.

Set the Sample Flow Rate to Slow (3.66uL/min).

Define acquisition plots

Once Acquisition settings are set correctly as described above, add informative plots to acquisition screen. Add the following 1- and 2-parameter plots and gates to view data:



Note that Plots will be populated with data while samples are being acquired in Run mode, but not when in Record mode. Gating and data analysis do not take place during acquisition, but will be performed post-acquisition using the vFC™ Analysis layout in FCS Express or FCS Express Reader.

5. Save Settings file

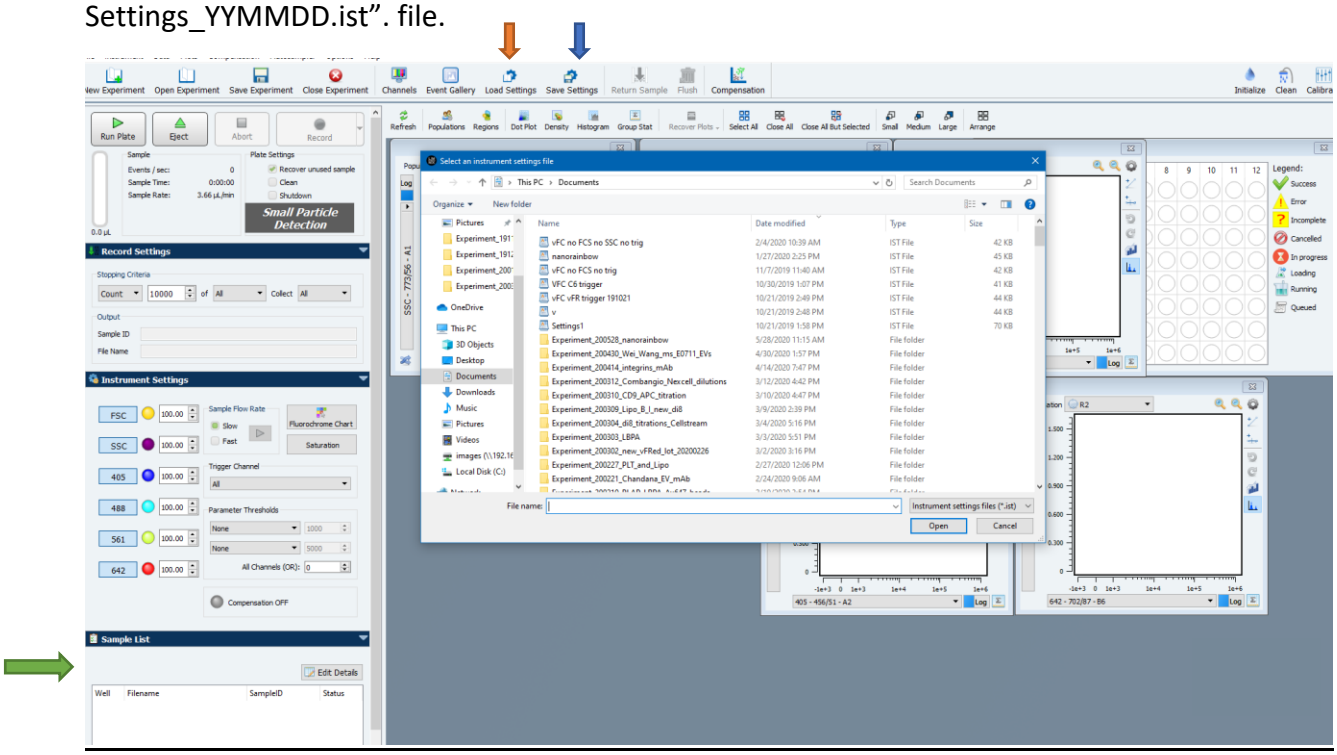
Save current settings as: “vFC Assay Settings_YYMMDD.ist”.

Running a vFC™ Assay

Once the instrument and assay performance has been characterized and validated, standard protocols for EV counting and sizing and cargo measurement can be performed. These assays generally include a dilution series to determine the concentration and optimal dilutions for unknown samples (Protocol 1), staining for markers of surface or internal cargo and detergent treatment to demonstrate sensitivity of the measured particles (Protocol 2). The general procedure is outlined below. See the relevant detailed vFC™ Assay Protocols for assay-specific instructions.

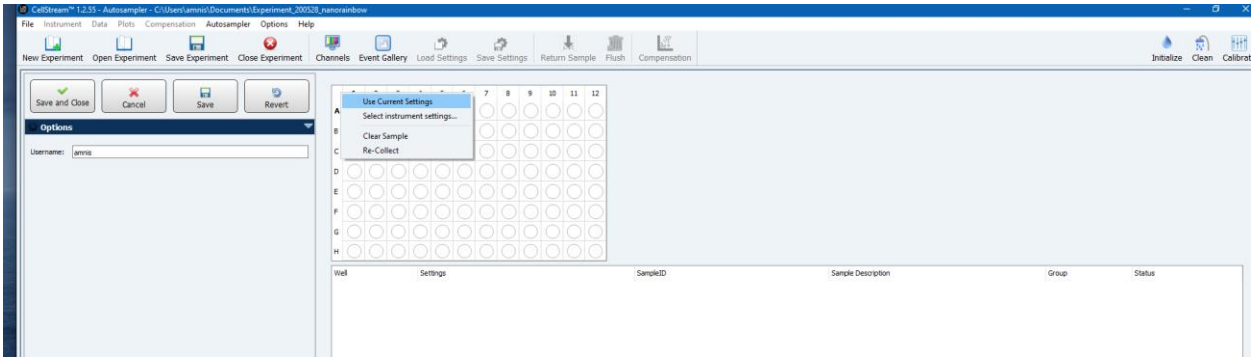
1. Open a new vFC™ Experiment and Load Settings

Open a New Experiment and choose “Load Settings” from top ribbon menu and load “vFC Assay Settings_YYMMDD.ist”. file.



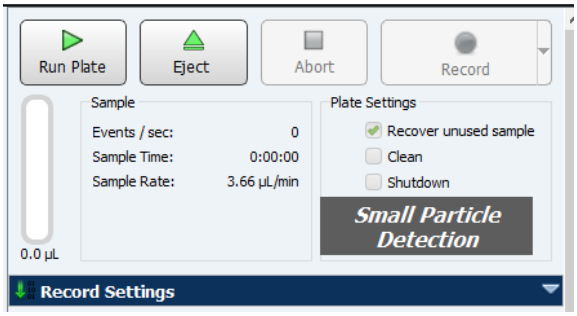
2. Create Sample List

Under Sample list choose “Edit Details”. This will bring up a new window for naming samples. Highlight plate wells to be run and choose “Use current Settings” for data collection. Files will be named yyyy_mm_dd_WellID_plate#_”text entered in sample ID field”.fcs Enter Sample IDs as described in the specific vFC™ Assay Protocol. Once sample IDs have been entered choose “Save and Close” to return to acquisition screen.



3. Acquire Data

Under Plate Settings deselect “recover unused sample” and check “Clean”. If you are not running another plate also check “Shutdown”. Then click the “Run Plate” button to begin recording data.



Cellstream: Parameter tables for Compensation

When using the compensation protocol the Cellstream parameter design will interfere with FCS Express ability to assign each bead to the right channel. As a result you will likely need to assign each single-stained control to its corresponding parameter manually. See chart below.

Detector	Parameter Name ($PnN)	EX-EM/BP	Stain Name ($PnS)
A1	SSC - 773/56 - A1	SSC - 773/56	
A2	405 - 456/51 - A2	405 - 456/51	V450
A3	405 - 528/46 - A3	405 - 528/46	V525
A4	405 - 583/24 - A4	405 - 583/24	
A5	405 - 611/31 - A5	405 - 611/31	V610
A6	405 - 702/87 - A6	405 - 702/87	
B1	642 - 773/56 - B1	642 - 773/56	APC780
B3	642 - 528/46 - B3	642 - 528/46	
B4	642 - 583/24 - B4	642 - 583/24	
B5	642 - 611/31 - B5	642 - 611/31	
B6	642 - 702/87 - B6	642 - 702/87	APC
C1	488 - 773/56 - C1	488 - 773/56	
C2	no laser - 456/51 - C2	no laser - 456/51	
C3	488 - 528/46 - C3	488 - 528/46	FITC
C4	488 - 583/24 - C4	488 - 583/24	
C5	488 - 611/31 - C5	488 - 611/31	
C6	488 - 702/87 - C6	488 - 702/87	vFRed
D1	561 - 773/56 - D1	561 - 773/56	PE780
D2	FSC - 456/51 - D2	FSC - 456/51	
D3	no laser - 528/46 - D3	no laser - 528/46	
D4	561 - 583/24 - D4	561 - 583/24	PE
D5	561 - 611/31 - D5	561 - 611/31	PE594
D6	561 - 702/87 - D6	561 - 702/87	PE780

Notes

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