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 Qualification and Calibration 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 – Cytek Aurora™

Step 1. Create a vCal™ Bead Acquisition Template for Instrument Calibration.

The Cytek acquisition settings are stored in a .exptfile, which contains data acquisition settings as well as the 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.

This Template will be used to measure the vCal™ nanoRainbow beads as part of instrument characterization and calibration (Protocol 0.1). It will also be used to measure vCal™ Antibody Capture Beads used for fluorescence calibration (Protocol 0.3), and spectral unmixing (Protocol 0.5). The Bead Acquisition Template uses light scatter triggering to measure the bead fluorescence intensity under the same instrument conditions (flow rate, laser power, detector gain) as will be used for vFC™.

To configure the initial vCal™ Bead Acquisition Template (.expt file), create a New Experiment (*Acquisition > Select an experiment > New*), then follow steps below.

a. Fluorescent Tags Tab – Add Fluorescence Tags

At the top of this first tab you can set a name and description for the experiment. Add vFC fluorescence tags to the Spectral Library. Click next.

Detector	Parameter Name ($PnN)	Laser	EX-EM/BP	Stain Name ($PnS)
1	B06	Blue	B6	vFRed
2	B02	Blue	B2-525/17	AlexaFluor488
3	YG01	YellowGreen	YG1-577/20	PE
4	YG03	YellowGreen	YG3-615/20	PEDazzle
6	YG09	YellowGreen	YG9-780/30	PECy7
7	V01	Violet	V1-428/15	BV421
8	V07	Violet	V7-542/17	BV510
9	V10	Violet	V10-615/20	BV605
11	R01	Red	R1-660/17	APC
12	R02	Red	R2-678/18	AlexaFluor647

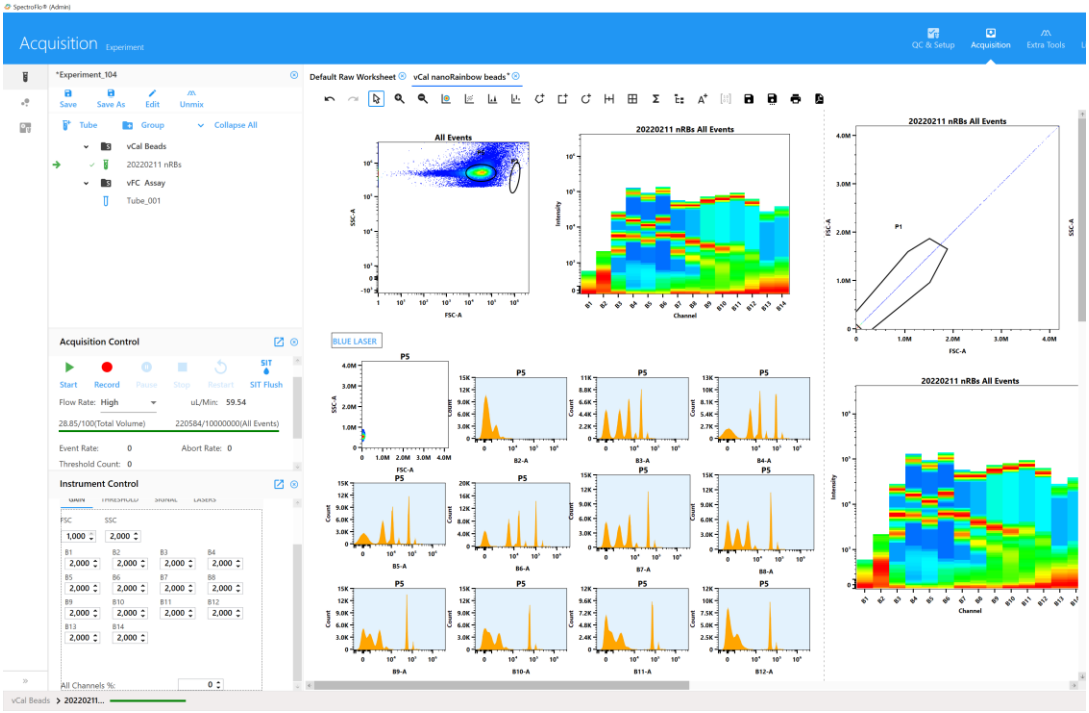
b. Groups Tab – Create Reference Group

Right click on the experiment group then select Add Reference Group and new window will open. The first unstained control will automatically be created. Ensure control type is “Beads.”

Check “Define Additional Negative Control(s) for Spillover Calculation” and a new section will appear. Click “Add” to create a new negative control. Name the new negative control something like “Unstained EV” and set the control type to “Cells.”

Under Fluorescent Tags section ensure that the positive controls have the appropriate control type and negative control (vFRed should be Cells and Unstained EV and the rest should use the Unstained Bead control). Enter appropriate Labels for each positive control. Click Save.

Click Next (Skipping Markers tab). Click Next (Skipping Keywords tab).



c. Acquisition Tab – Set Acquisition Settings

Set Stopping Time to 30 seconds for all beads (in Step 2 its 120 seconds for vFC).

Set Events to Record to 5000 for all beads.

Click Save and Open.

d. Loader Settings Tab – Set Run Order

Click on Loader Settings on the right side of the screen.

Specify the Run Order to be by Columns ↓↓↓.

e. Setup Worksheet – Define Acquisition Plots

Start with the default raw worksheet. Create the histograms and gates to assess the sample during data acquisition. During data acquisition, the Singlet beads (Gate 1) will be adjusted to facilitate visual inspection during acquisition. Analysis of the data will take place in the appropriate vCal™ Bead Report layout.

Table 1	
Plot/Gate: Description	**Purpose**
Plot 1: Time	Monitor fluidic stability
Plot 2: SSC-A vs FSC-A	Gate singlet beads
Gate 1: Singlet gate	Select single beads and exclude doublets and background
Plots 3-n: FL1-FLn	Individual fluorescence channel intensity resolution

f. Instrument Control – Set Instrument Settings

Click on Instrument Control below the Plate Window under Acquisition.

Under Instrument Control – Voltage: Set the gain for all scatters to 5000 and set the gain for all fluorescence channels to 2000.

Under Instrument Control – Threshold: Set the channel to SSC-B-A and set the threshold to 100,000.

Under Instrument Control – Signal: Make sure at least B6 (vFRed) is chosen for the width values.

g. Save As – Save the data acquisition template

Save Template as ***vCal Bead Template.expt***.

Step 2. Create a Data Acquisition Template for vFC™ Assays

This Template will be used to measure the vCal™ Lipo100™ Vesicle Standard (Protocol 0.2) as part of instrument Calibration and for most vFC™ assays, including measuring EV concentration and size (Protocol 1) and measuring EV surface markers (Protocol 2).

To configure the initial vFC™ Acquisition Template (.expt file), create a New Experiment (*Acquisition > Select an experiment > New*), then follow steps below.

a. Fluorescent Tags – Add Fluorescent Tags Tab

Add vFC fluorescence tags to the Spectral Library.

Detector	Parameter Name ($PnN)	EX-EM/BP	Stain Name ($PnS)
1	B06	B6	vFRed
2	B02	B2-525/17	FITC
3	YG01	YG1-577/20	PE
4	YG03	YG3-615/20	PEDazzle
6	YG09	YG9-780/30	PECy7
7	V01	V1-428/15	BV421
8	V07	V7-542/17	BV510
9	V10	V10-615/20	BV605
11	R01	R1-660/17	APC
12	R02	R2-678/18	AlexaFluor647

Click next at the bottom of the screen or go to the Groups section.

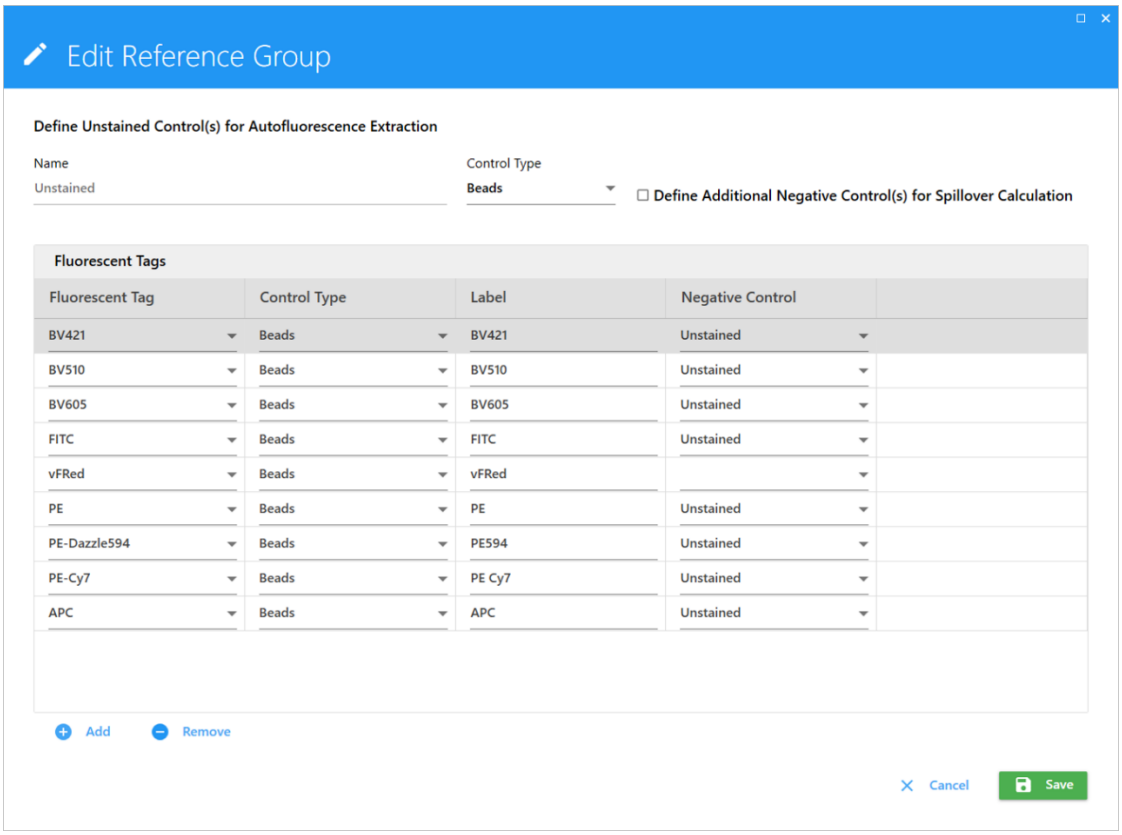
b. Groups Tab - Define plate and select sample wells to run

From the *Tube* dialogue, select the *Add Plate* icon and specify 96 well v-bottom plates.

Highlight the relevant rows identified in the Protocol Plate Map and click the add group icon (little folder on right with +).

c. Groups Tab – Create Reference Group

Click on Reference Group to open the edit reference group screen. Ensure all the control type for all the controls are bead. Fill in the labels if necessary.



At the top of the window should be an Unstained control. Ensure control type is bead.

Check “Define Additional Controls for Spillover Calculation” then add a negative control the liposome (either instrument background or lipo autofluorescence). Set control type to Cells.

Set vFRed (lipo) control type to “Cell.” Ensure that all the bead controls use the Unstained as the negative control (Setting top one should set others). Set the vFRed control to use appropriate negative control (either system background or unstained lipo).

Click Save. Click Next.

d. Markers Tab – Specify Markers (if needed)

If necessary specify markers for each sample.

Click Next. Click Next (skipping keywords tab).

e. Acquisition Tab – Set Acquisition Settings

Set Stopping Time to 120 seconds for vFC Samples.

Make sure that Events to Record is blank for vFC samples so that it doesn't stop based on number of events.

Click Next.

f. Loader Settings Tab – Set Run Order

Specify the Run Order to be by Columns ↓↓↓.

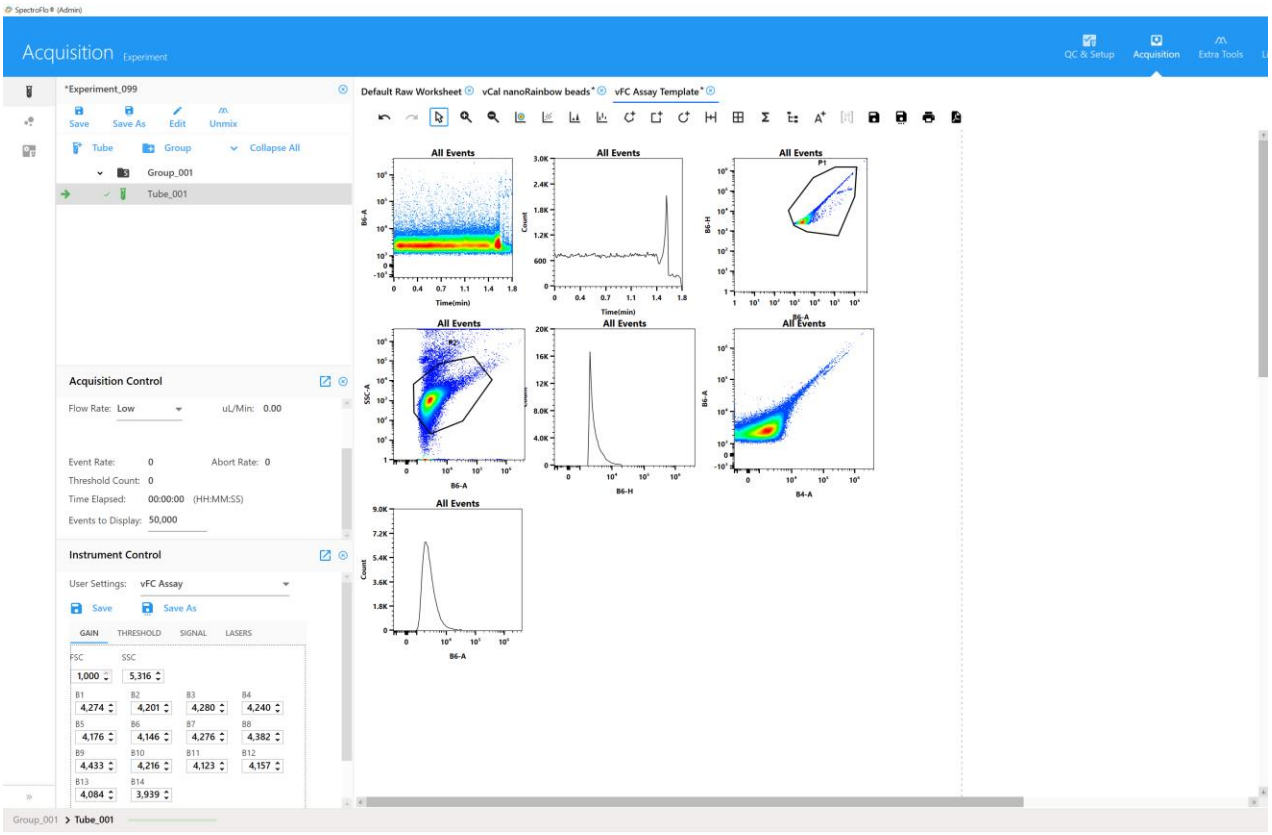
Click Save and Open.

g. Import FCS File – Load Controls into Reference Group

To load a control right click on the sample name in the reference group (back in Acquisition) then click "Import FCS File" from the dropdown menu. Navigate to folder with you controls then load the corresponding control. Do this for each control. (If you haven't yet run the controls you may do this later but need controls to do vFC).

g. Setup Worksheet – Define data acquisition display plots and gates.

Start with the default worksheet since it includes all of the parameters. Ensure the following 1- and 2-parameter plots are present and draw gates to view data during acquisition. These plots are only for visual inspection during acquisition. Analysis of the data will take place in the appropriate vFC™ Report layout.



Plot/Gate: Description	Purpose
Plot 1: VSSC-A vs Time	Monitor fluidic stability
Plot 2: Time	Monitor fluidic stability
Gate 1: Time gate	Exclude the first 20 sec and collect the final 100 sec.
Plot 3: vFRed-A vs vFRed-H	Monitor event pulse characteristics
Gate 2: Pulse width gate	Exclude short pulse width background events
Plot 4: vFRed-A vs VSSC-H	Monitor event vFRed and VSSC intensities
Gate 3: Vesicle gate	Select events characteristic of single vesicles, exclude high- and low-VSSC events.
Plot 5: PE-A vs vFRed-A	Monitor PE immunofluorescence
Gate 4: PE positive	Estimate number of “PE positive” events
Additional Plots and Gate	For additional immunofluorescence channels as needed/desired

h. Instrument Control – Set Instrument Settings

Click on Acquisition Control below the Plate Window under Acquisition.

Under Instrument Control – Voltage set the gain for all of the scatters to 5000 and set the gain for all of the fluorescence channels to 2000.

Under Instrument Control – Threshold set the channel to the vFRed Parameter (B6-A) and set the threshold to 1900.

Under Instrument Control – Signal make sure at least B6 (vFRed) is chosen for the width values.

Note: Typically, data from the first 20 ul (20 seconds) are discarded, due to high background that frequently occurs at the beginning of a well, so that data from 100 uL is analyzed for each sample. This gating will happen automatically using the vFC™ Data Analysis Layout.

Dialogue	Setting	vCal™ beads	vFC™ assay
Acquisition Settings	Active Channels	All channels	All channels
	Scatter gain (VSSC, SSC and FSC)	5000	5000
	Fluorescence gain (all channels)	2000	2000
	Primary Threshold:	VSSC-H	B6-H
	Manual Threshold:	100,000	1700
	Width Parameter:	B6-H	B6-H
Stopping Rules	Time to Record	Checked: 30 sec	Checked: 120 sec
	Events to Record	Unchecked	Unchecked
Acquisition	Events to Display	500,000	500,000
	Sample Flow Rate	Fast (60 uL/min)	Fast (60 uL/min)

In the *Acquisition* dialogue, set *Events to Display* to 500,000 to ensure that all data are displayed.

i. Save As – Save the data acquisition template

Save Template as *vFC Assay Template.expt*.

Data Acquisition

a. Load Assay Template and Settings

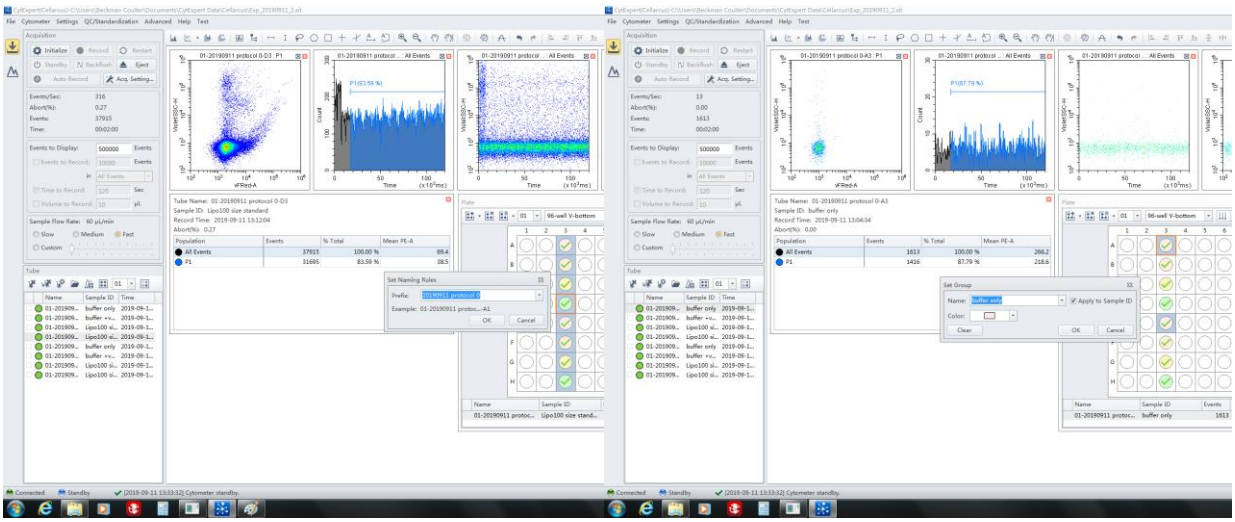
Set up your instrument with the appropriate template and settings files required to acquire vFC data outlined in the Instrument Setup Section above.

b. Adjust Trigger Channel Threshold.

Load a well of vFC™ Staining Buffer and allow system to run for 15 seconds on high flow rate (60ul/min). Set the instrument to trigger from the blue laser-excited, B6-H (vFRed) fluorescence channel at a threshold of 1700, then adjust the manual threshold until you get 10-15 events/second in a Buffer Only sample. Apply this threshold to all wells and save the Template file (.expt) for future use. In general, once optimized the threshold does not need to be changed from day to day.

c. Set File and Group names.

Highlight wells and define Names as YYYYMMDD (eg. 20190704) and Set Group names as defined in “staining wells” on the relevant Protocol Plate Map.



d. Auto Record data.

You are now ready to run samples. Click Auto Record to run the selected Sample wells.

Unmixing with Spectroflo

a. Load Experiment then Start Unmixing

Make sure the experiment to be unmixed is loaded. If you have just acquired data on current worksheet you probably don't need to load anything. Otherwise load experiment to be unmixed.

Click on the Unmix button (under acquisition tab).

b. Select Controls – Set Control Type and Assign Negative Controls

If you created the worksheet properly then you should see 2 negative controls. There should be a control for Beads and for Cells (EVs). Make sure the positive vFRed control has the control for EV's assigned to it. The rest of the positive controls should use the unstained bead control.

Check the "Autofluorescence as a Fluorescent Tag" checkbox.

Click Next.

c. Identify Positive/Negative Populations – Adjust gates for each control

Adjust the bead scatter gates to include only singlets. If you hold down shift while adjusting the scatter gates for one bead it will apply that scatter gate to all beads.

Adjust the Cells (EV) scatter gate to include the main population. It helps to check the checkbox for log for these controls to switch to loIg scaling. This makes it easier to adjust the gates for dimmer particles.

Adjust the positive and negative gates. If possible, it is better to adjust positive gates to include only the brighter half of the positive peak. Similarly, it is better to adjust negative gates to

include only the dimmer half of the negative peak. This improves Spectroflo's unmixing algorithm.

Click Next.

Click "Create New Unmixing Experiment." Note: You do NOT want to "Live Unmix" because this will replace the original data files with the unmixed data files causing you to lose the original data.

Other Spectroflo Help

a. Export an experiment along with data files (any experiment)

Saved experiments are stored inside spectroflo and if you want to send the whole experiment to someone or save it in a folder somewhere then you will need to export it first.

Click Save in upper left corner of the screen. Click the little x next to the experiment name to go back to the Select an experiment screen.

Go to "My Experiments" then select the saved experiment that you wish to export then click Export to choose a save location and name.

This will save an experiment as a compressed folder that contains the data files as well as the Spectroflo files (such as .xitm).

Notes

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