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).

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Instrument Setup - Beckman Coulter CytoFlex™

Step 1. Configure Filters.

Configure the CytoFlex™ for Violet Side Scatter (VSSC) detection. If using an instrument in a Shared Resource Lab (SRL or core facility) seek assistance from the staff. A detailed description of how to perform this operation can be found in the CytoFlex Operation Manual. Briefly, you will access the Violet laser optical filters inside the instrument, place the Violet 405nm filter in position 2, the Violet 450, 525, 610 and 660 filters in positions 3-6, and an unused filter in position 1.
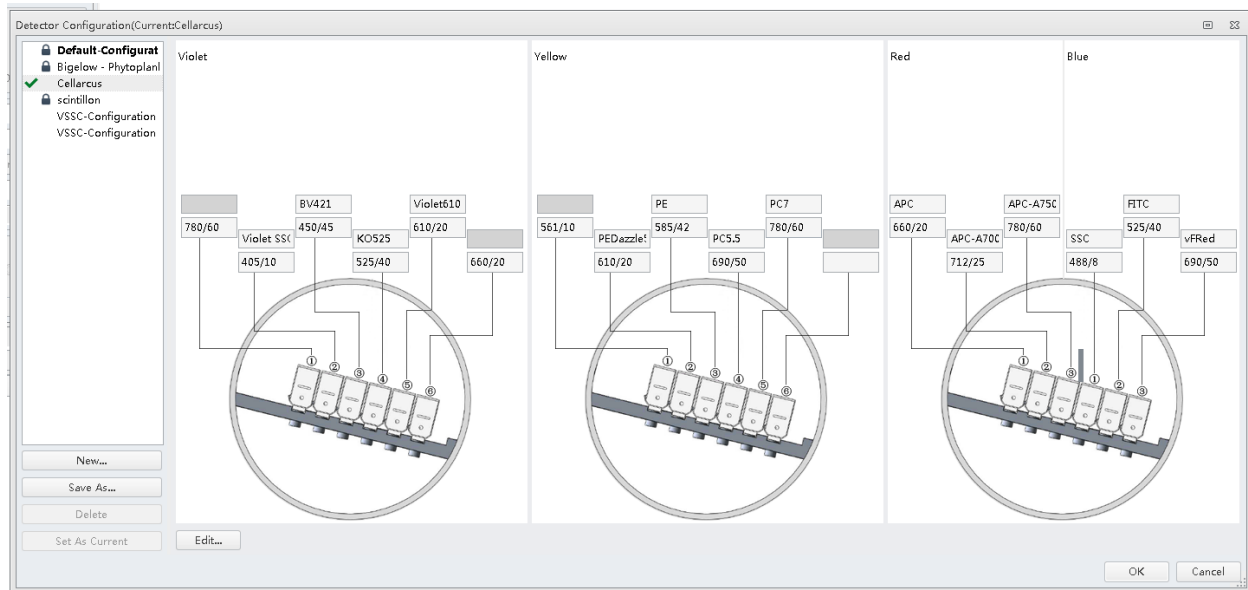
From the CytExpert software, create a new Detector Configuration (*Cytometer>Detector Configuration> New*) and Save as "vFC Assay". Populate the *Fluorophore* and *Filter* fields as indicated in the Filter Diagram. You will need to *Add* a Fluorophore named vFRed with center band of 690 nm to the Fluorophore list. Finally, right click on the 405nm channel and select *Set SSC*. *Save* the Template as "vFC Assay" and select *Set as Current*.

Step 2. Create a vCal™ Bead Acquisition Template for Instrument Calibration.

The CytoFlex acquisition settings are stored in a .xitm file, which contains data acquisition setting 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), compsation (Protocol 0.4),and spectral unmixing (Protocol 0.5). The Bead Acquisition Template It 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 (.xitm file), create a New Experiment (*File> New experiment*), then follow steps below.

a. Define data acquisition display plots

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.
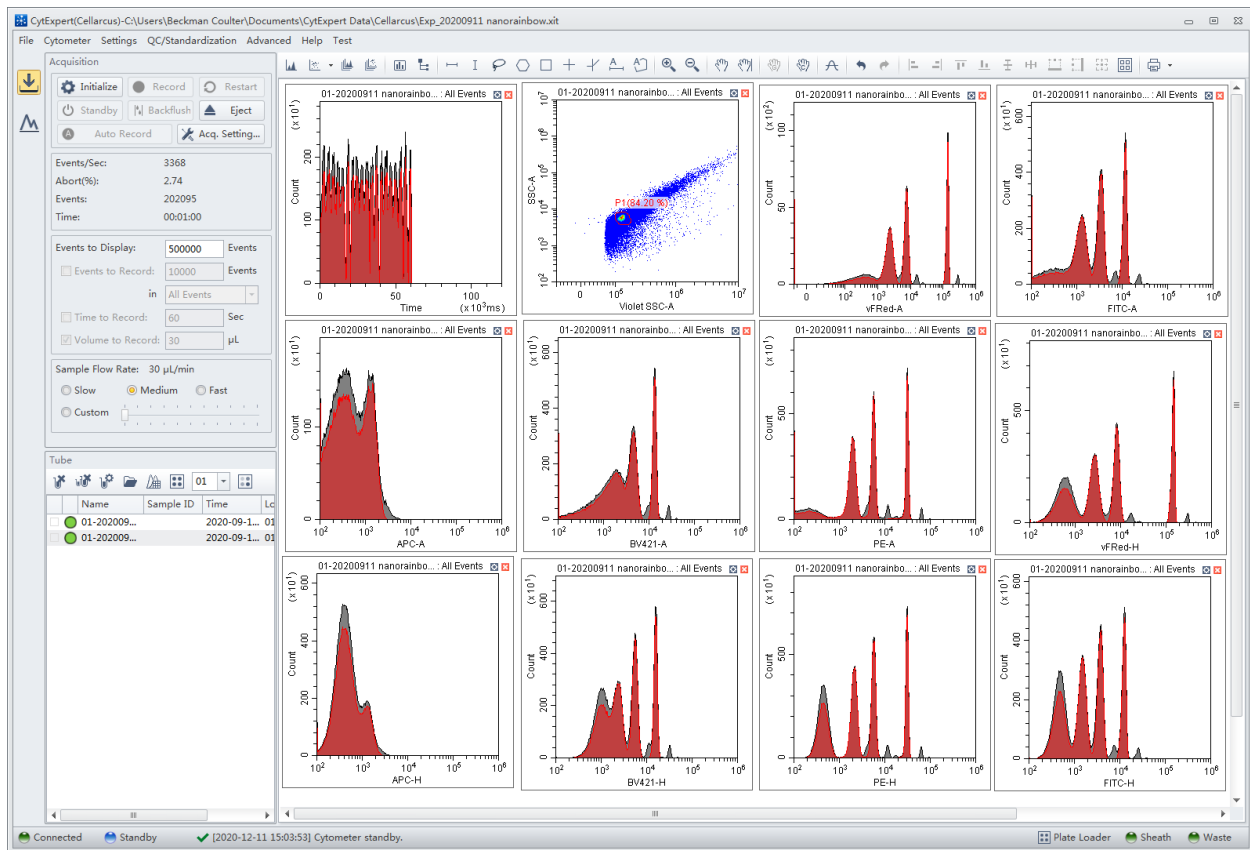

Table 1	
Plot/Gate: Description	**Purpose**
Plot 1: Time	Monitor fluidic stability
Plot 2: VSSC-A vs SSC-A	Gate singlet beads
Gate 1: Singlet gate	Select single beads and exclude doublets and background
Plots 3-n: FL1-FLn	Individual fluorescence channel intensity resolution

b. Define data acquisition settings

Set Detector Gains, Thresholds, and other Settings for the Template as defined in the Table below.

Note that the vCal™ Bead and vFC™ Assay Templates differ in their detection Threshold channel and level.

Dialogue	Setting	vCal™ beads	vFC™ assay
Acquisition Settings	Active Channels	All channels	All channels
	Scatter gain (VSSC, SSC and FSC)	100	500
	Fluorescence gain (all channels)	1000	1000
	Primary Threshold:	VSSC-H	vFRed-H
	Manual Threshold:	100,000	1900
	Width Parameter:	vFRed (690/50)	vFRed (690/50)
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)

On the *Acq.Setting* tab, set *Gains* for all fluorescence channels at 1000, 488 nm scatter (*FSC* and *SSC*) at 100, 405 nm scatter (*VSSC*) at 100. *Note: Gain 1000 has been found to be optimal for many CytoFlex instruments. Confirmation of optimal Gain for a specific instrument can be determined by performing a Gain sweep using nanoRainbow beads and examining the effect of Gain on the Separation Index between the blank and dim beads, as described in Protocol 0.5.*

Set the *Primary Threshold* to the vSSC channel and select the Height (-H) parameter and set the *Manual* threshold to 100,000. Set the *Width* channel to vFRed (690/50).

On the *Stopping Rules* tab, check *Time to Record* and enter 30 seconds. Uncheck *Events to Record*. At High Sample Speed (~60 uL/min) on the CytoFlex, this will collect data from 30 ul of sample.

c. Save the data acquisition template

Save Template as *vCal Bead Template.xitm*.

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Step 3. Create a Data Acquisition Template for vFC^{TM} Assays

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

To configure the initial vFC^{TM} Acquisition Template (.xitm file), create a New Experiment (*File> New experiment*), then follow steps below.

a. Define data acquisition display plots

Add the following 1- and 2-parameter plots and 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^{TM} Report layout.
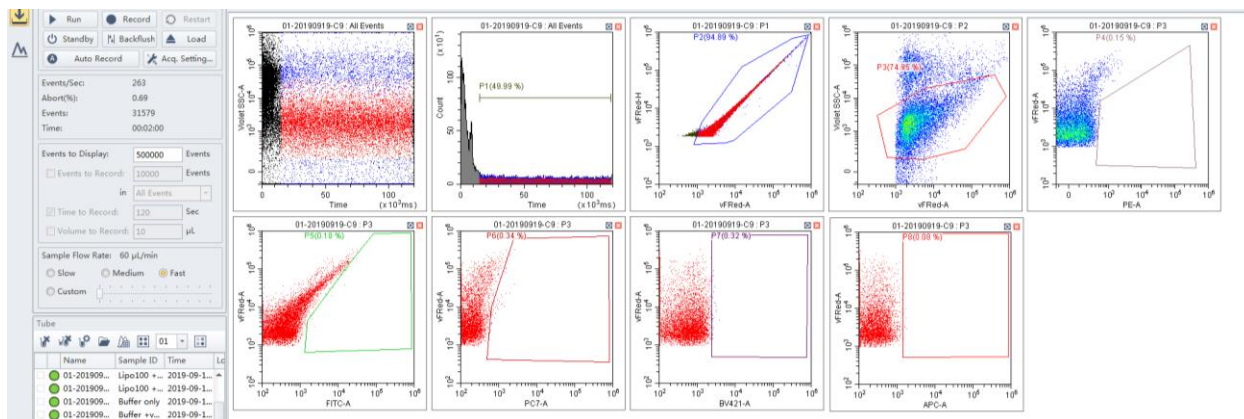

Plot/Gate: Description	Purpose
Plot 1: VSSC-A vs Time	Monitor fluidic stability
Plot 2: Time	Monitor fluidic stability
Gate 1: Time gate	Exclude the first 20 sec and collect the final 100 sec.
Plot 3: vFRed-A vs vFRed-H	Monitor event pulse characteristics
Gate 2: Pulse width gate	Exclude short pulse 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

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b. 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 set as *Sample Wells*.

Specify the Run Order to be by Columns ↓↓↓.
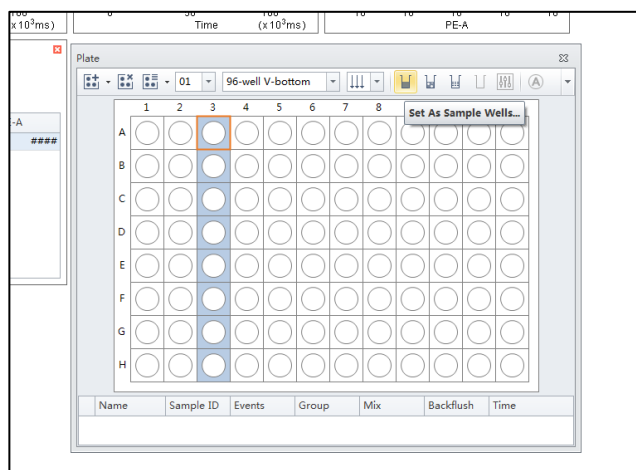
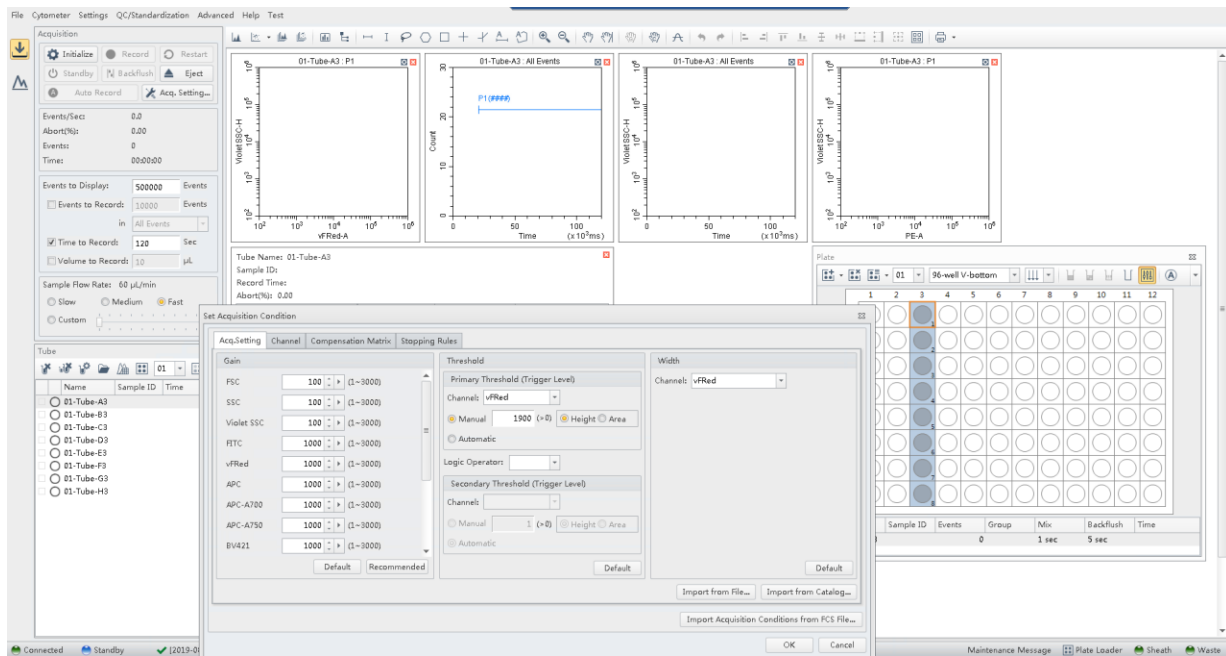### c. Define data acquisition settings

On the *Acq.Setting* tab, set *Gains* for all fluorescence channels at 1000, 488 nm scatter (*FSC* and *SSC*) at 100, 405 nm scatter (*VSSC*) at 500.

Set the *Primary Threshold* to the vFRed channel and select the Height (-H) parameter and set the *Manual* threshold to 1900. Set the *Width* channel to vFRed (690/50).

On the *Stopping Rules* tab, check *Time to Record* and enter 120 seconds. Uncheck *Events to Record*. At High Sample Speed (~60 uL/min) on the CytoFlex, this will collect data from 120 ul of sample.

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)	100	500
	Fluorescence gain (all channels)	1000	1000
	Primary Threshold:	VSSC-H	vFRed-H
	Manual Threshold:	100,000	1900
	Width Parameter:	vFRed (690/50)	vFRed (690/50)
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 <u>500,000</u> to ensure that all data are displayed.
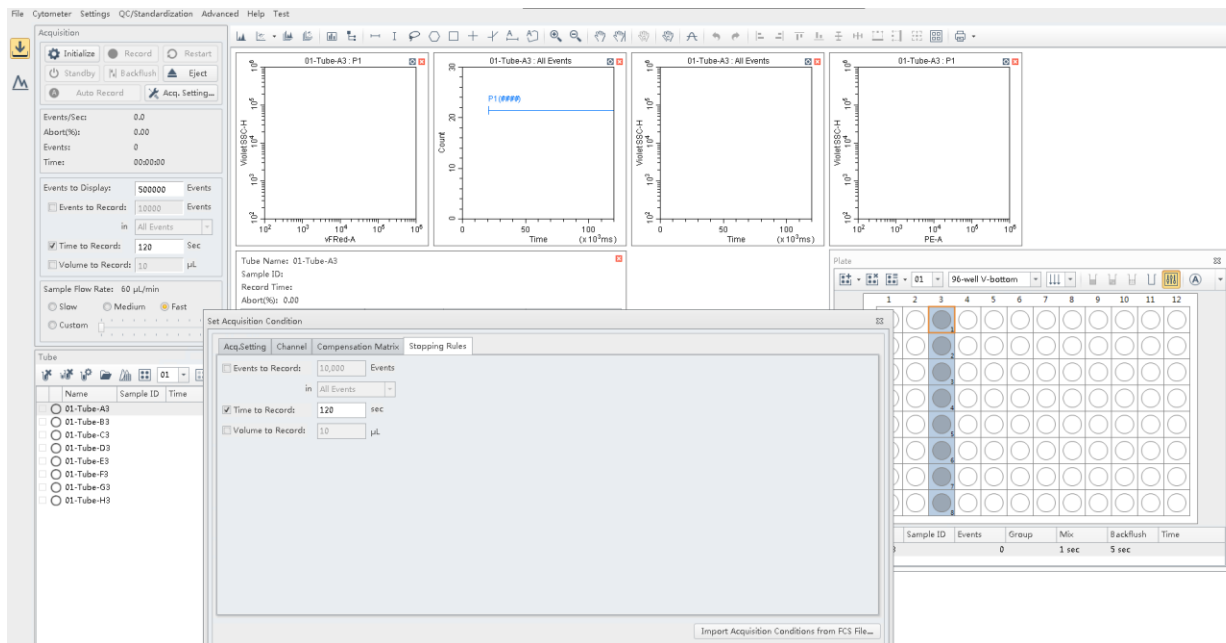
d. Save the data acquisition template

Save Template as *vFC Assay Template.xitm*.

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 Protocols

b. Adjust Trigger Channel Threshold.

Load a well of vFC^TM 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, 690/50-H (vFRed) fluorescence channel at 1900, 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 (.xitm) for future use. In general, once optimized the threshold does not need to be changed from day to day.

b. 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.
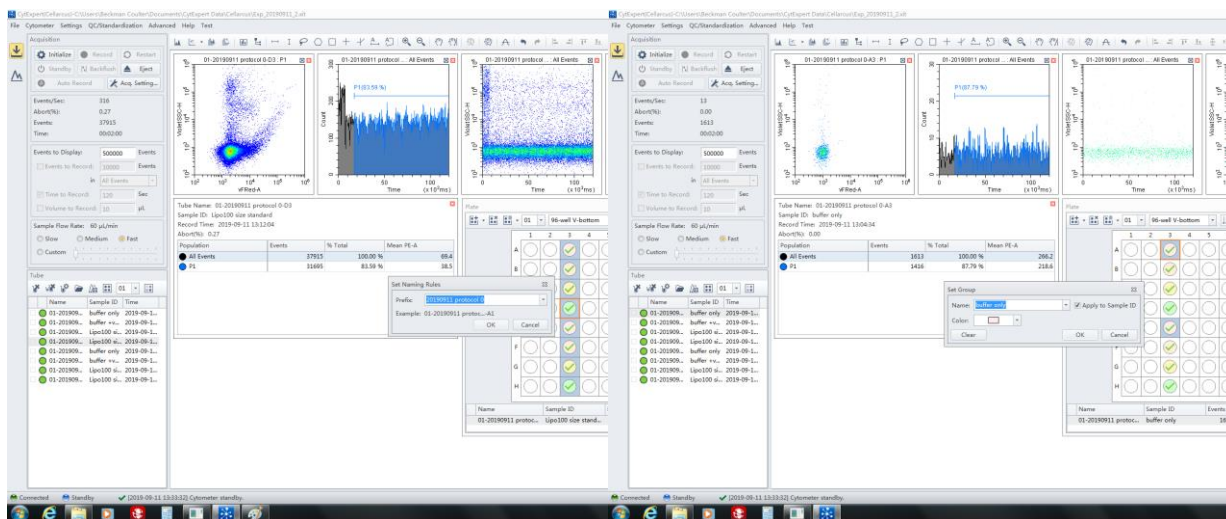
a.   Auto Record data.

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

Notes

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