Instrument QC and Qualification

For the CytoFLEX

Instrument QC and Calibration with nanoRainbow Beads

Purpose

Assess fluorescence resolution and laser alignment and calibrate instrument flow rate using Cellarcus vCal™ nanoRainbow beads (nRBs). First-time users will need to perform Instrument Setup and create a vCal™ Bead Template for data as described in **Appendix A**.

Materials

Kit Component	Size		Store
vCal™ nanoRainbow beads (CBS6 – 2.5 mL)	2.5 mL		4°C
vCal™ Instrument QC and Qualification data analysis Layout			

Materials to be Provided by user

- Gloves
- Microwell plate (Sartstedt 82.1583.001)
- FCS Express or FCS Express Reader software (FCS Express Reader) –

  *Before first use, refer to Instrument-specific instructions in **Notes** (page 8).*

Procedure

1. Create a New Experiment by opening the vCal™ Bead Template created during **Instrument Setup** (see **Appendix A**).
2. Define save file name as “YYMMDD CytoFlex nanoRainbow”.
3. Vortex nanoRainbow beads well.
4. Place five drops (~250 uL) undiluted nanoRainbow beads in a well (or tube).
5. Load the plate (or tube), begin acquisition, and ensure the singlet bead population can be identified (see Appendix A. Step 2a. Figure).
6. Record the nanoRainbow bead sample for 30 seconds on the High sample flow rate.

Data Analysis

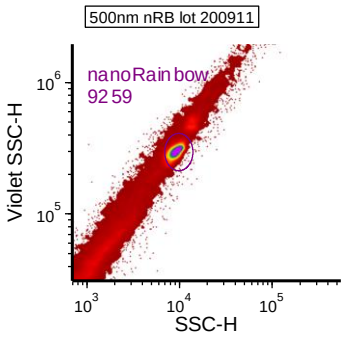
1. Open FCS Express.
   Before the first use of FCS Express, select the instrument-specific options as instructed in Notes, below.
2. Open the **Instrument QC and Qualification** data analysis Layout in FCS Express and load the nanoRainbow Bead data file.
3. On the **A1. nRB Report Tab**, inspect the time history and adjust the nanoRainbow gate on the population of single nanoRainbow Beads (**Figure 1A**).

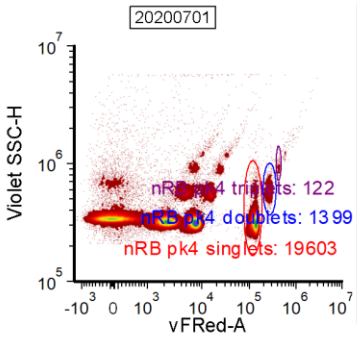
A. Laser Alignment and Fluorescence Resolution

1. Inspect the fluorescence histograms of the vFRed Plot to the left of the page (under the A1. Bead Peak Gating header). Adjust so that the appropriate markers include each of the 4 peaks (Figure 1B).
2. Laser alignment is assessed by inspecting the CV of the bright bead population, which should be <10%. If CVs are higher, or there is an apparent leftward shoulder to the peak, alignment on that laser may be sub-optimal.
3. Fluorescence resolution is assessed via the Separation Index (SI), which reflects the resolution of dim signals from background by comparing the difference between the blank and dimmest bead to the standard deviation of the background. This semi-quantitative metric is useful for an initial analysis of instrument performance, and the vFRed™ SI should be > 4.0 to ensure efficient EV detection.

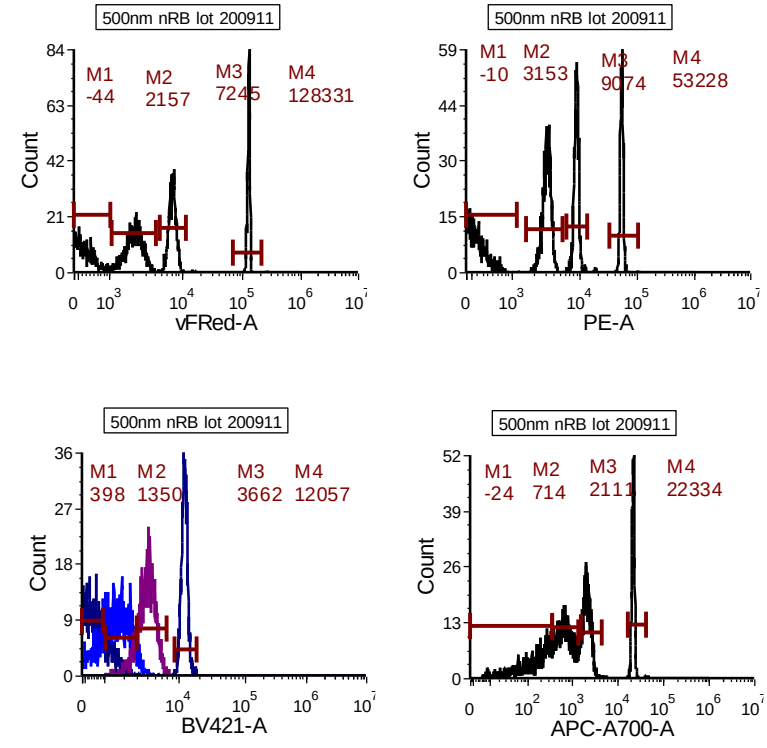
B. Calibrate Sample Flow Rate

1. On the **A2. Flow Rate Tab**, adjust the gates selecting the Peak 4 singlets, doublets and triplets (Figure 1C). The Layout will calculate the sample flow rate using the known concentration of the nanoRainbow beads (1e7/mL).





	A	B	C	D
1	Pk 4 conc	1.0E+03	/uL	
2	Pk4 singlets	19603		
3	Pk4 doublets	1399		
4	Pk4 triplets	122		
5	Gated	22767		
6	**Flow rate**	**0.76**	**uL/sec**	
7				
8	Volume	30000	nL	
9	Conc	7.6E+02	/uL	



	A	B	C	D	E
1	**vFRed CV**	**4.58**	**%**		
2	pos	2157			
3	neg	-44			
4	st dev	361			
5	**vFRed SI**	**3.05**			
6					
7	**PE CV**	**6.33**	**%**		
8	PE pos	3153			
9	neg	-10			
10	st dev	269			
11	**PE SI**	**5.88**			
12					
13	**BV421 CV**	**9.84**	**%**		
14	pos	1350			
15	neg	398			
16	st dev	261			
17	**BV421 SI**	**1.82**			
18					
19	**APC CV**	**6.93**	**%**		
20	pos	714			
21	neg	-24			
22	st dev	263			
23	**APC SI**	**1.40**			
24					

Figure 1. Instrument QC and Qualification. A. Single nanoRainbow beads are gated by light scatter. **B.** The laser and fluidic alignment is assessed by the Peak 4 (Bright) CV, while the fluorescence resolution is assessed busing the Separation Index, which reflects the resolution between Peak 2 (Dim) and Peak 1 (Blank). **C.** The sample flow rate is estimated from the number of Peak 4 (Bright) beads, including doublets and triplets, measured for a fixed time.

Notes

CytoFlex-specific Instructions:

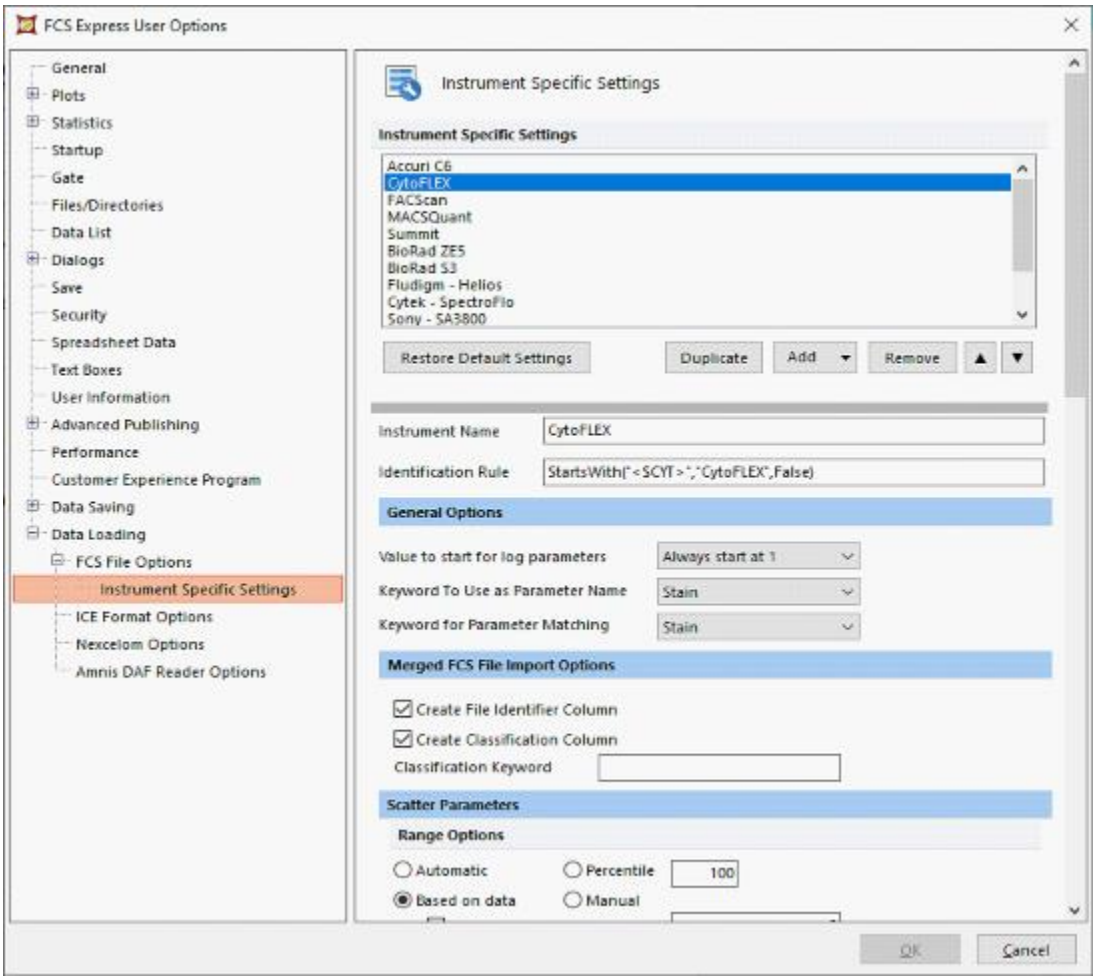
Before loading data files

Configure FCS Express Options to recognize CytoFlex data file parameters by name:

File>Options>Data Loading> Instrument Specific Settings> CytoFLEX>General Options>

Keyword to Use as Parameter Name: **Stain**
Keyword for Parameter Matching: **Stain**

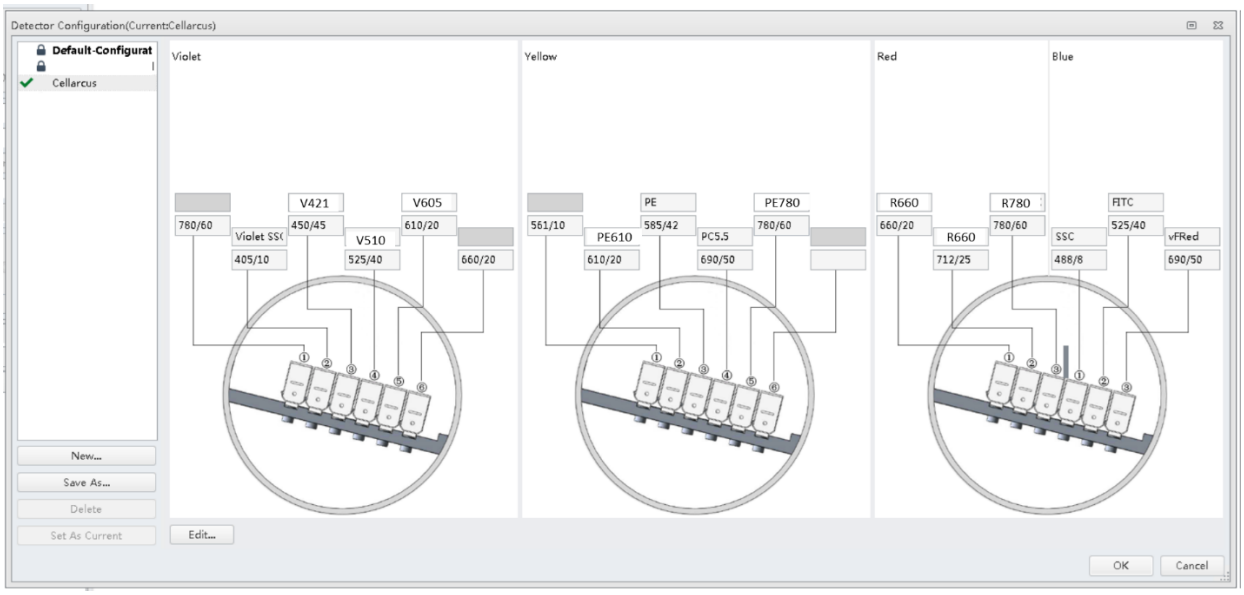
Click: **OK**



Appendix A. 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 QC and Qualification (Protocol A). It will also be used to measure vCal™ Antibody Capture Beads used for Fluorescence Calibration and Compensation (Protocol B). The Bead Acquisition Template uses light scatter triggering to detect beads and measures 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.

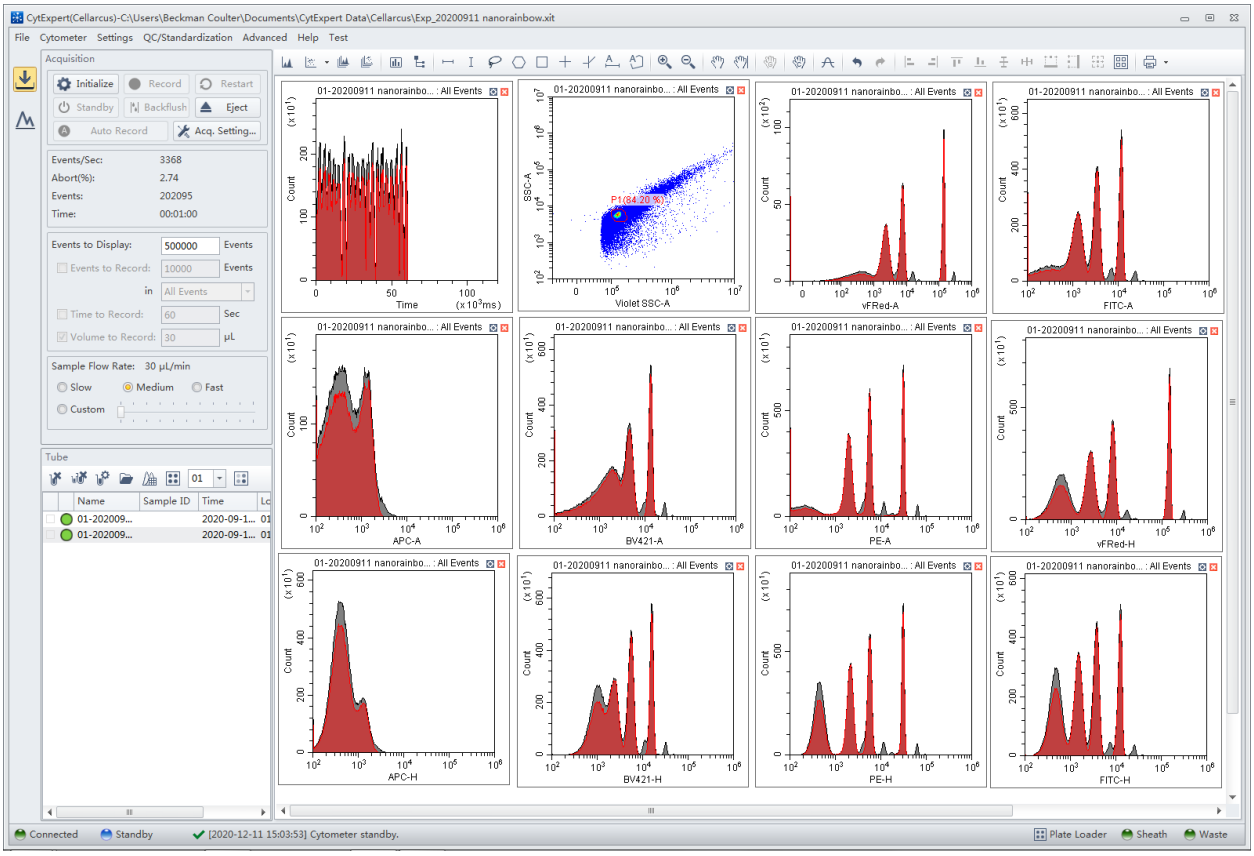


Table 1	
Plot/Gate: Description	**Purpose**
Plot 1: Time	Monitor fluidic stability
Plot 2: VSSC-A vs SSC-A	Gate singlet beads
Gate 1: Singlet gate	Select single beads and exclude doublets and background
Plots 3-n: FL1-FLn	Individual fluorescence channel intensity resolution

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 (500 for buffer control)	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 YYMMDD.xitm*.

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