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)

Procedure

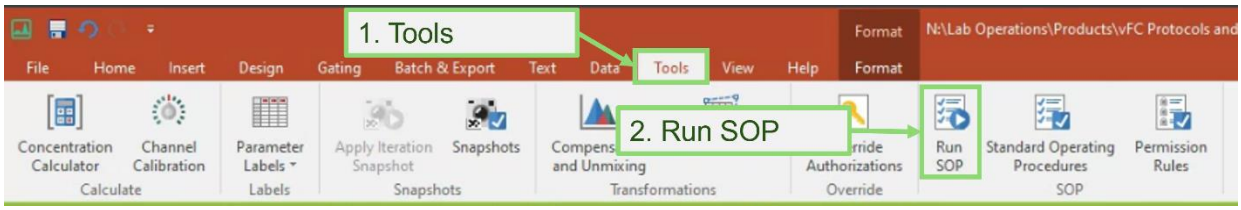
1. Create a New Experiment by opening the vCal™ Bead Template created during **Instrument Setup** (see **Appendix A**).
2. Define save file name as “YYMMDD CytoFlex nanoRainbow”.
3. Vortex nanoRainbow beads well.
4. Place five drops (~250 uL) undiluted nanoRainbow beads in a well (or tube).
5. Record the nanoRainbow bead sample for 30 seconds.

Data Analysis

Run SOP

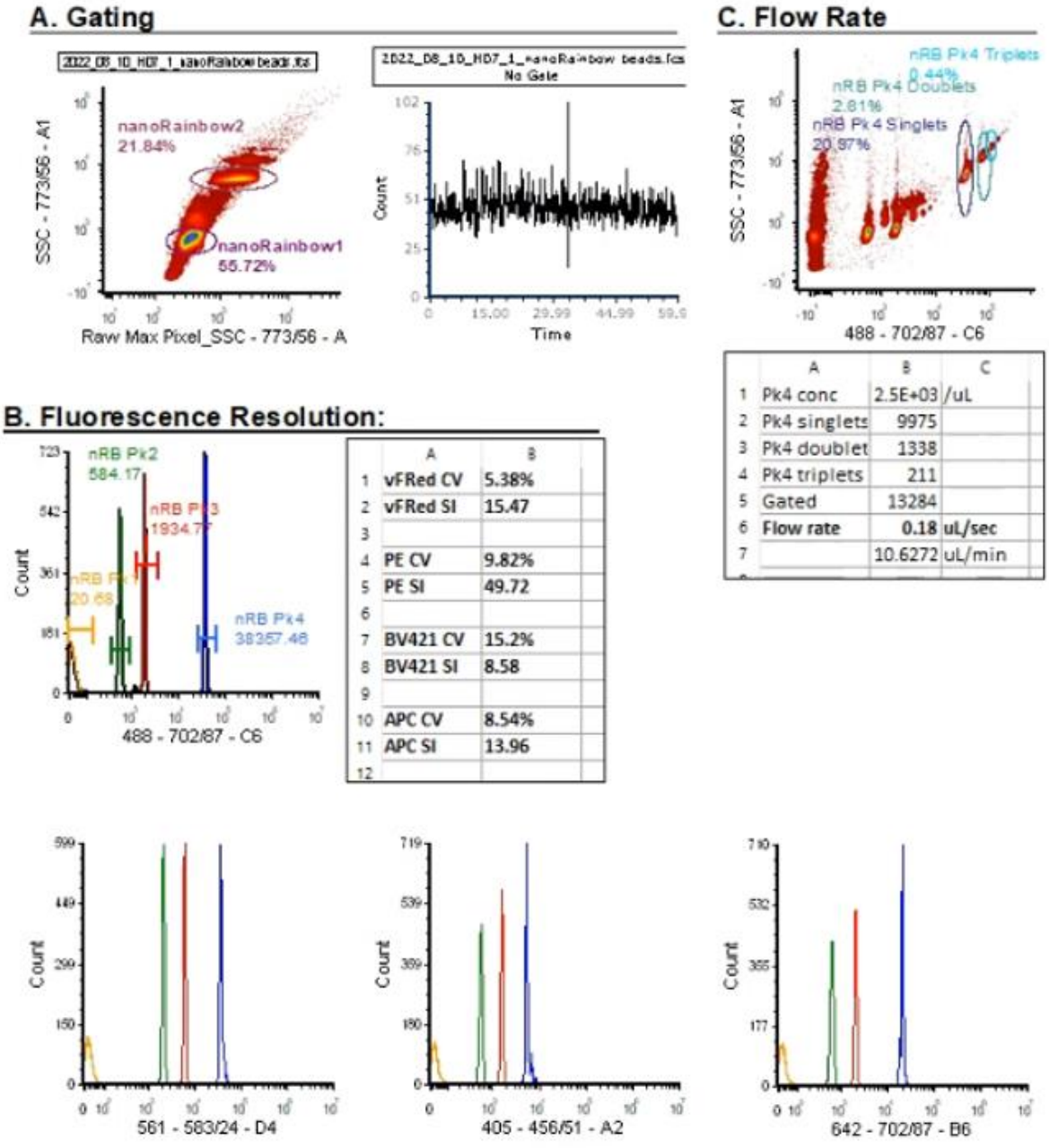
Open the Aurora (including Northern Lights) **Instrument QC and Qualification** data analysis Layout in FCS Express and run the SOP by going to Tools>Run SOP.

Before the first use of FCS Express, select the instrument-specific options as instructed as specified in "READ ME FIRST" tab.



Analysis

1. Go to Data List (right side of page) and click the Add Data File icon to navigate to nanoRainbow data (.fcs) file.
2. In the "Data List" window click on the nanoRainbow (.fcs) file then open the "Change File" menu and select "Change Data On All Plots" from the dropdown menu.
3. Adjust the “nanoRainbows1” and the “nanoRainbows2” gates to include single beads (excluding background noise, doublets, etc).
4. Inspect the time history for any fluidics issues.
5. Inspect the fluorescence histograms of a representative channel from each laser and adjust the markers to select each of the four bead populations.
6. Laser alignment is assessed by inspecting the CV of the bright bead population, which should be <15%. If CVs are higher, or there is an apparent leftward shoulder to the peak, alignment on that laser may be sub-optimal.
7. 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 >3.0 to ensure efficient EV detection.
8. Set the "nRB Pk4 Singlets", "nRB Pk4 Doublets", and "nRB Pk4 Triplets" on the appropriate populations. The Layout will calculate the sample flow rate using the known concentration of the nanoRainbow beads (2e6/mL).
9. Go to File>Save As to save the layout with a descriptive name.



	A	B	C
1	Pk4 conc	2.5E+03	/uL
2	Pk4 singlets	9975	
3	Pk4 doublet	1338	
4	Pk4 triplets	211	
5	Gated	13284	
6	**Flow rate**	**0.18**	**uL/sec**
7		10.6272	uL/min

	A	B
1	vFRed CV	5.38%
2	vFRed SI	15.47
3		
4	PE CV	9.82%
5	PE SI	49.72
6		
7	BV421 CV	15.2%
8	BV421 SI	8.58
9		
10	APC CV	8.54%
11	APC SI	13.96
12		

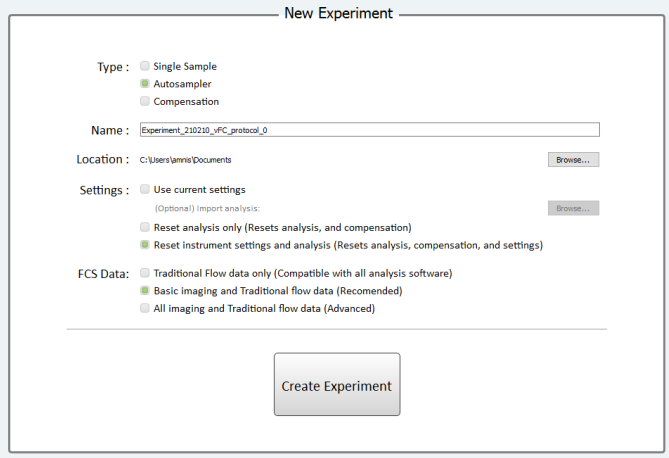
Figure 1. Instrument QC and Qualification. A. Single nanoRainbow beads are gated by light scatter. **B.** The laser and fluidic alignment is assessed by the Peak 4 (Bright) CV, while the fluorescence resolution is assessed using the Separation Index, which reflects the resolution between Peak 2 (Dim) 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.

Appendix A. 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

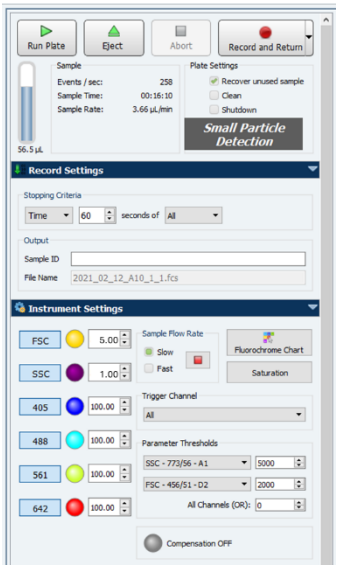
vFC™ assays using the CellStream are performed with all fluorescence excitation lasers at maximum power (100%), and the FSC and SSC lasers at 5% and 1%, all thresholds set to “none” (Table 1). If your instrument is equipped with 375 nm and/or 532 nm lasers, set these to 0% power.

Table 1. Acquisition settings

	vCal™ Calibration Beads		vFC™ Assay	
Laser	**Power (%)**	**Threshold**	**Power (%)**	**Threshold**
FSC	5	2000	5	none
SSC	1	none	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 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 5% and 1%, respectively

Set all other enabled lasers to 100%.

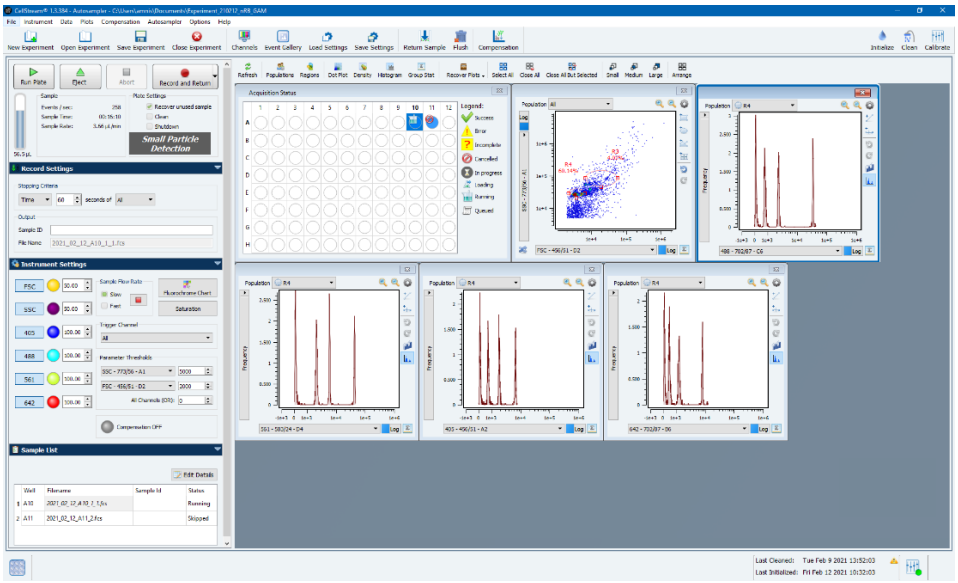
Set the Trigger Channel to All.

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 DATE”.

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 5% and 1%, respectively

Set all other enabled lasers to 100%.

Set Parameter Thresholds to None.

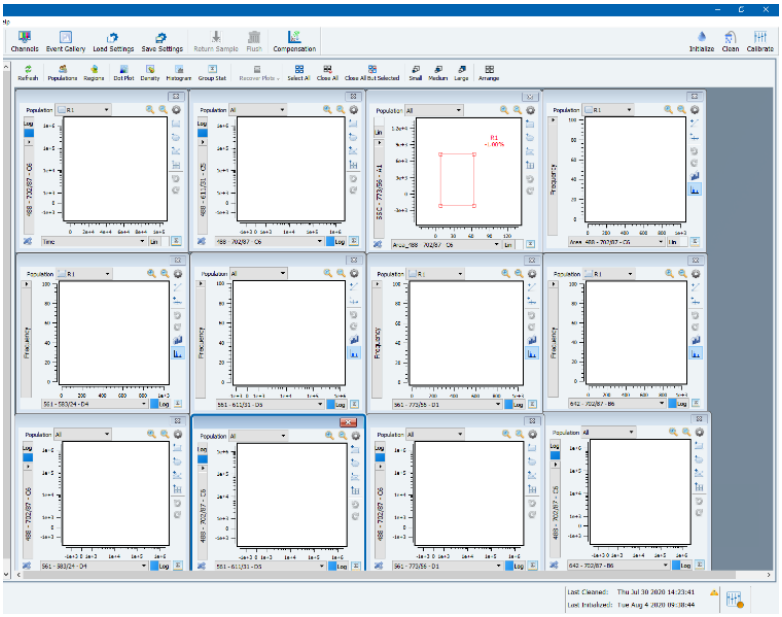
Set the Trigger Channel to “All.”

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

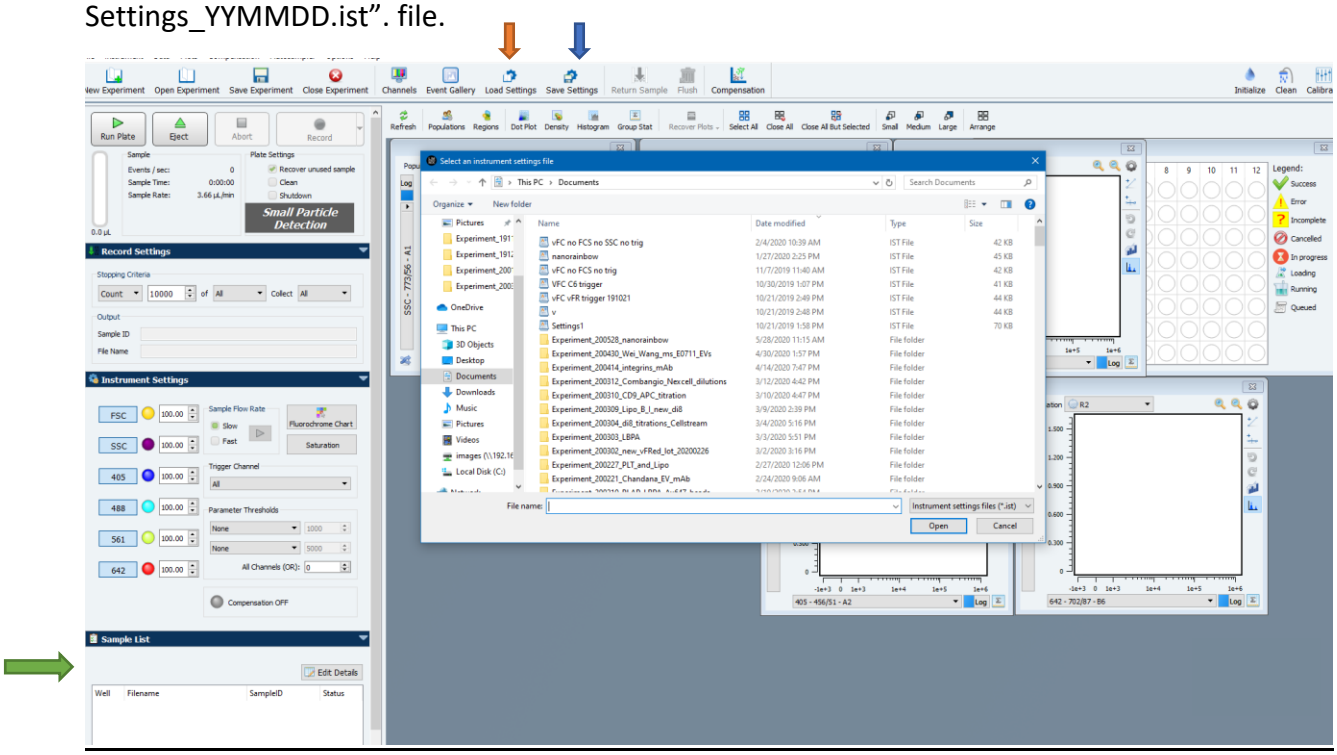
Save current settings as: “vFC Assay Settings_DATE”.

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.

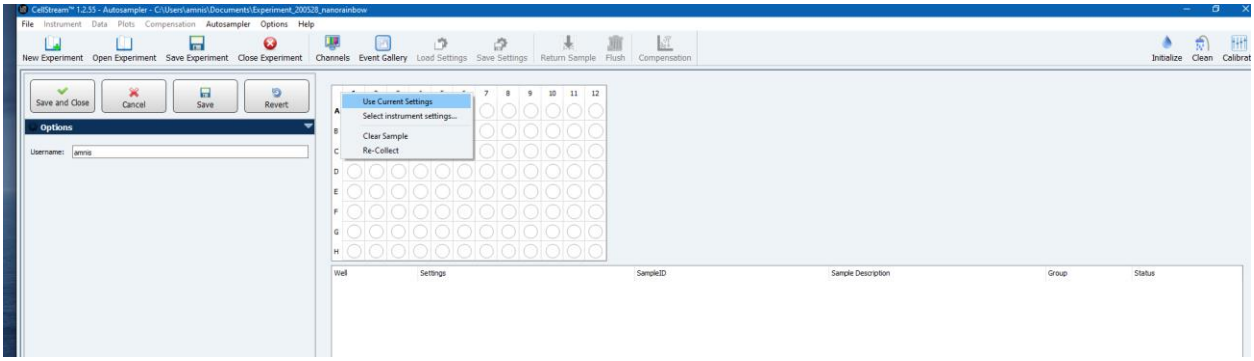
1. Open a new vFC™ Experiment and Load Settings

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



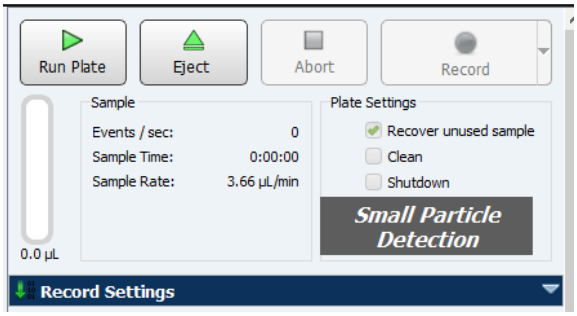
2. Create Sample List

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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