

# LEGENDplex™ Assay Setup Protocol

## for Cytex® Aurora and Northern Lights Flow Cytometers

This protocol provides step-by-step instructions for the setup of Cytex® Aurora and Northern Lights flow cytometers for data acquisition of BioLegend's LEGENDplex™ multiplex immunoassays.

A pre-made **LEGENDplex™ Experiment Template** that can easily be imported into the SpectroFlo® software is available for download at [biolegend.com/en-us/legendplex](http://biolegend.com/en-us/legendplex) under the Instrument Setup tab.

If desired, users can also create their own Experiment Template by following the instructions found at the end of this protocol.

Unmixing of spectral data is not required. Please export Raw FCS files for downstream analysis. This is analogous to using uncompensated data on a conventional flow cytometer.

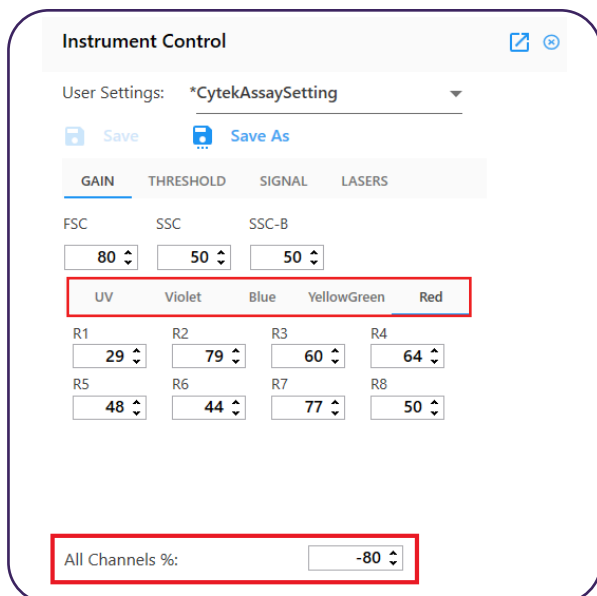
When analyzing LEGENDplex™ Assay FCS files generated on an Aurora or Northern Lights flow cytometer, the assay beads emission spectra should be analyzed using the following recommended channels:

- Without a yellow-green (YG) laser: Bead Classification = R4, Reporter = B4
- With a yellow-green (YG) laser: Bead Classification = R4, Reporter = YG1

## Protocol

### 1) Setting Up LEGENDplex™ for the First Time on Your Cytex® Instrument:

1. Perform instrument start up procedure and instrument QC per manufacturer's recommendations.
2. Import the **LEGENDplex™ Experiment Template** into the SpectroFlo® software, or follow the instructions at the end of this protocol to generate a new template.
3. Under Instrument Settings, start by selecting **CytekAssaySetting**. Lower gains for all channels by -80% by entering this into the **All Channels %** field. Please remember to do this for every laser line.
4. Vortex the vial of LEGENDplex™ Setup Beads 3: Raw Beads for 30 seconds to resuspend the beads.
5. Transfer 400 µL of the Raw Beads to a new FACS tube.
6. Load the tube of Raw Beads onto the cytometer.
7. Acquire data with the instrument flow rate set to LOW. The **LEGENDplex™ Experiment Template** is set to have stopping criteria of 4,000 singlet events.
8. Adjust the FSC and SSC gains such that the 2 bead populations are on scale and fall within the gates provided in the **LEGENDplex™ Experiment Template**. You can find typical FSC and SSC gain settings below.



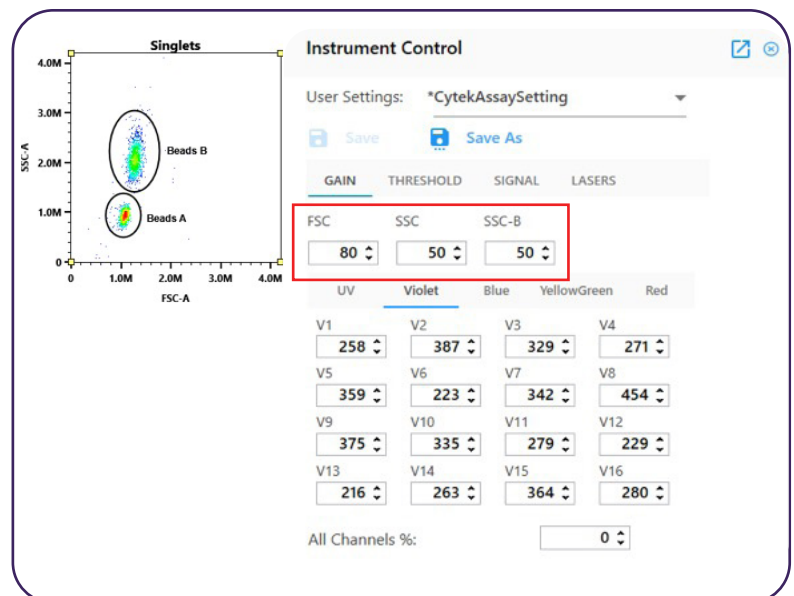
**Instrument Control**

User Settings: \*CytekAssaySetting

Save Save As

GAIN	THRESHOLD	SIGNAL	LASERS
FSC	SSC	SSC-B	
80	50	50	
UV	Violet	Blue	YellowGreen
R1: 29	R2: 79	R3: 60	R4: 64
R5: 48	R6: 44	R7: 77	R8: 50

All Channels %: -80



**Instrument Control**

User Settings: \*CytekAssaySetting

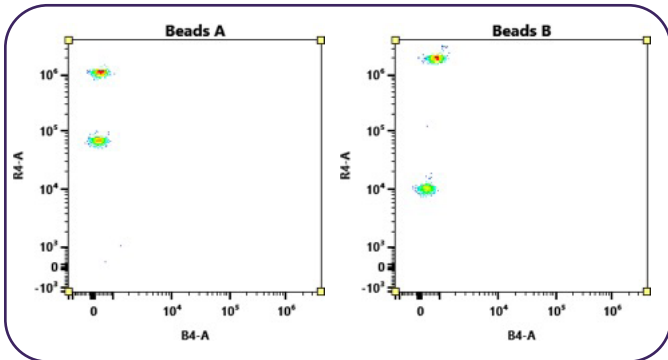
Save Save As

GAIN	THRESHOLD	SIGNAL	LASERS
FSC	SSC	SSC-B	
80	50	50	
UV	Violet	Blue	YellowGreen
V1: 258	V2: 387	V3: 329	V4: 271
V5: 359	V6: 223	V7: 342	V8: 454
V9: 375	V10: 335	V11: 279	V12: 229
V13: 216	V14: 263	V15: 364	V16: 280

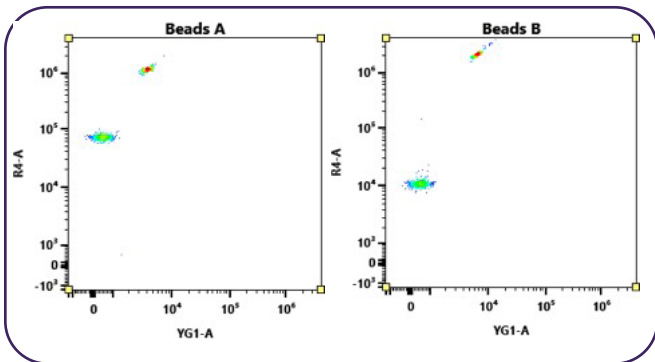
All Channels %: 0

9. Verify that the 2 bead populations within the beads A and B gates are on scale on the R4 axis in the R4 vs. YG1/B4 plots found in the downloaded LEGENDplex™ Experiment Template.

a. If your Cytex® instrument does not have a YG laser:



b. If your Cytex® instrument has a YG laser:

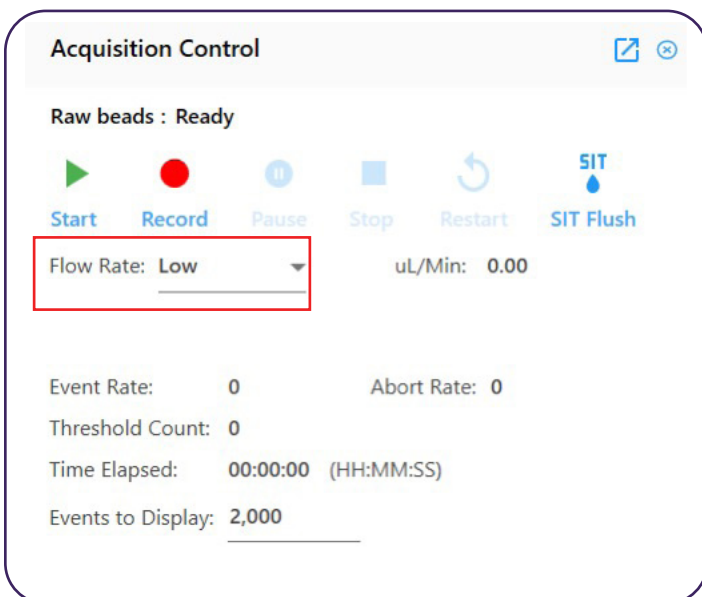


10. Vortex the vial of LEGENDplex™ Setup Beads 2: PE Beads for 30 seconds to resuspend the beads.

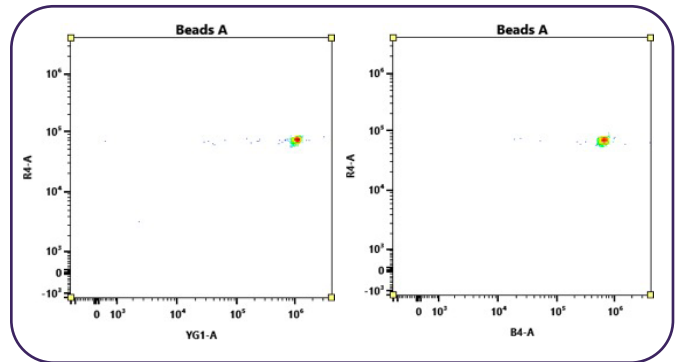
11. Transfer 400 µL of the PE Beads to a new FACS tube.

12. Load the tube of PE Beads onto the cytometer.

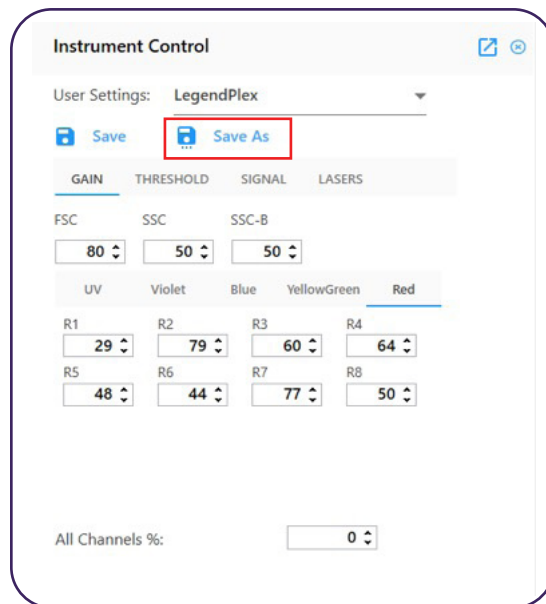
13. Acquire data with the instrument flow rate set to LOW.



14. Verify that for Beads A, the bead population is on scale on the YG1 or B4 axis in the R4 vs. YG1/B4 plots found in the downloaded LEGENDplex™ Experiment Template.



15. Save settings as LEGENDplex™.



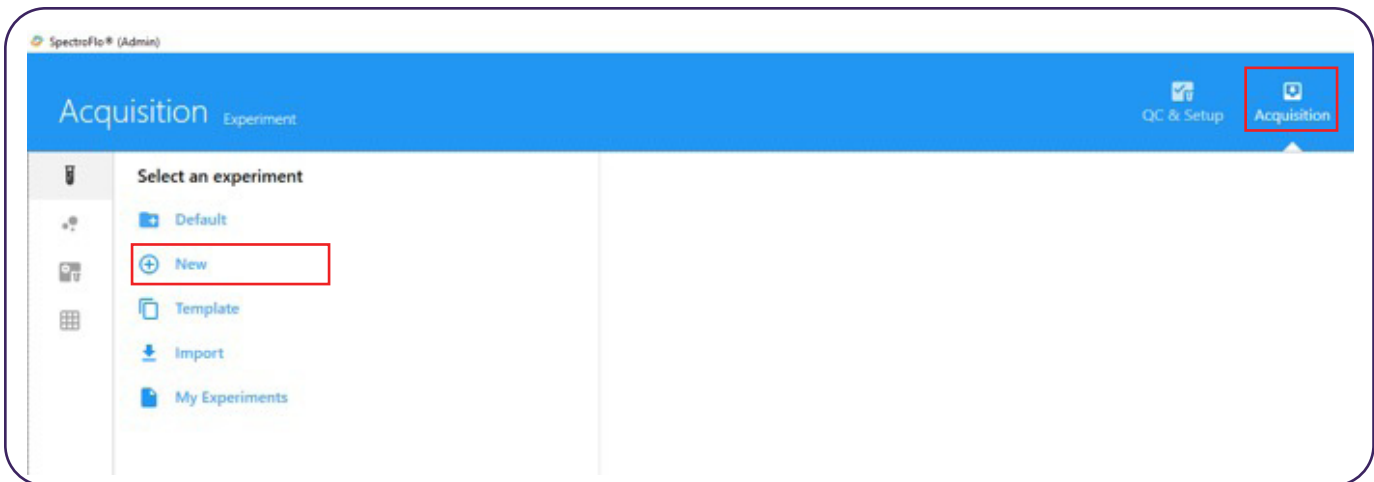
16. Your Cytex® flow cytometer is now ready for sample acquisition.

## II) For Subsequent Use of LEGENDplex™ on Your Cytex® Instrument

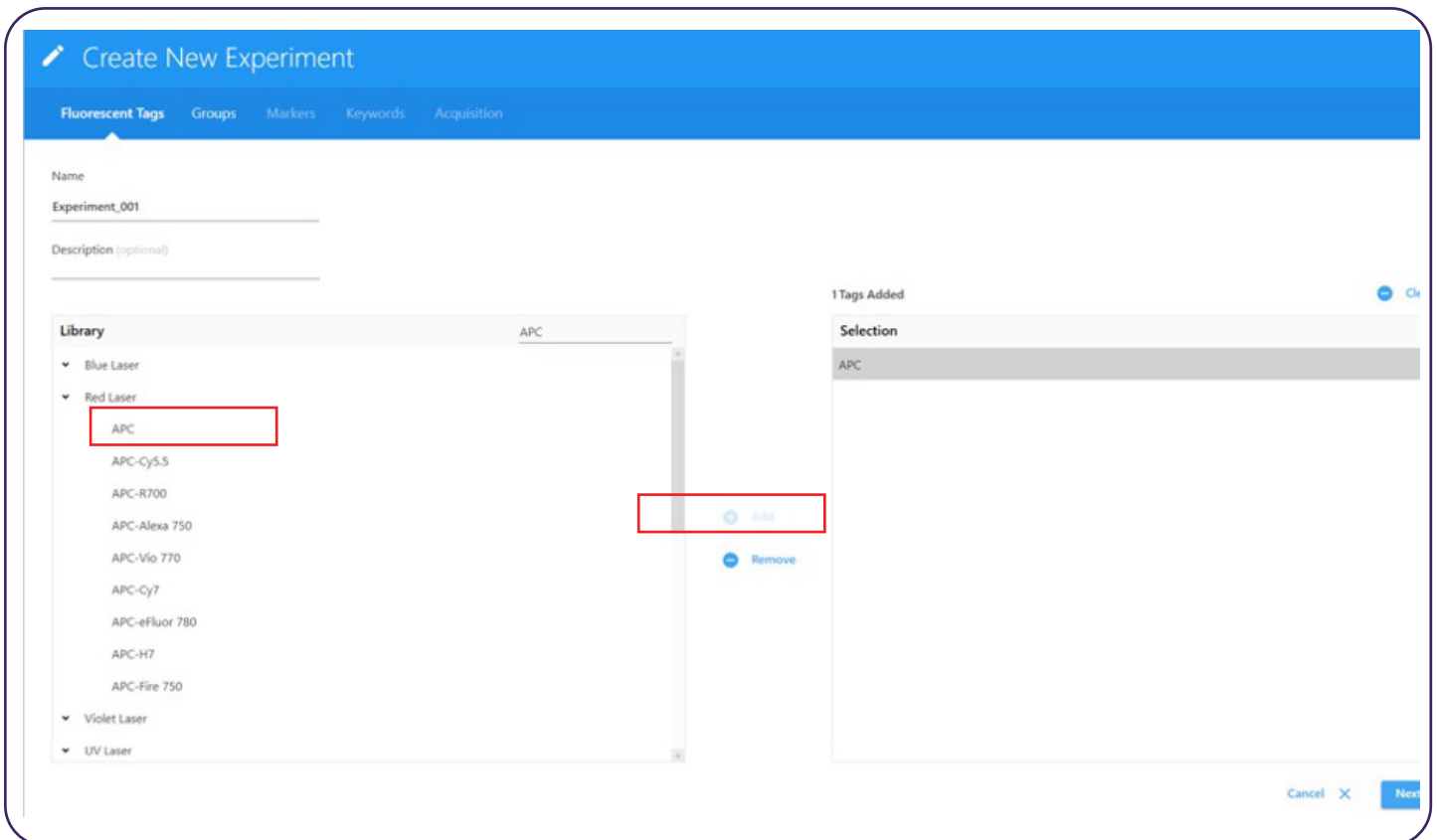
1. Perform instrument start-up procedure and instrument QC per manufacturer's recommendations.
2. Complete one of the following options:
  - a. Option A Under the "Acquisition" module, click on "Template" and open the existing LEGENDplex™ Experiment Template.
  - b. Option B Under the "Acquisition" module, click on "My Experiments." Find the previous LEGENDplex™ experiment that you ran, right click, and "Duplicate without Data". This will create a new experiment with the same tube layout and acquisition criteria as your previous experiment. Open the newly created duplicated experiment.
3. Under Instrument Settings, select your saved LEGENDplex™ settings.
4. Your Cytex® flow cytometer is now ready for sample acquisition.

### III) Instructions for Experiment Template Creation

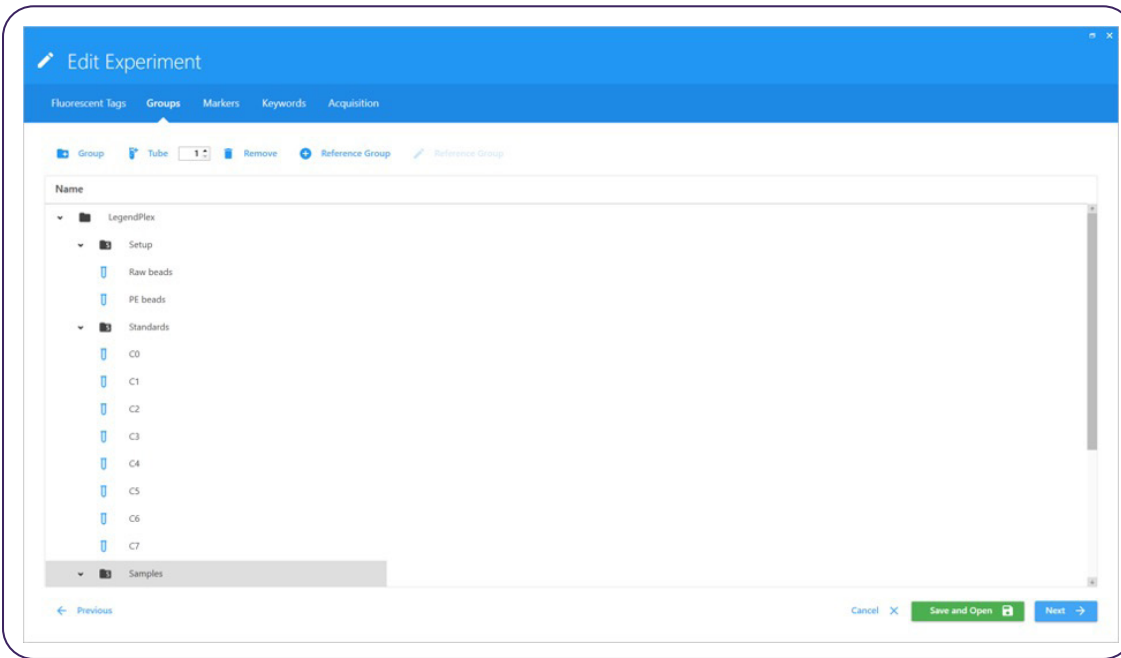
1. Under the **Acquisition** module, create a **New** experiment. A pop-up wizard will appear stepping you through how to create your experiment.



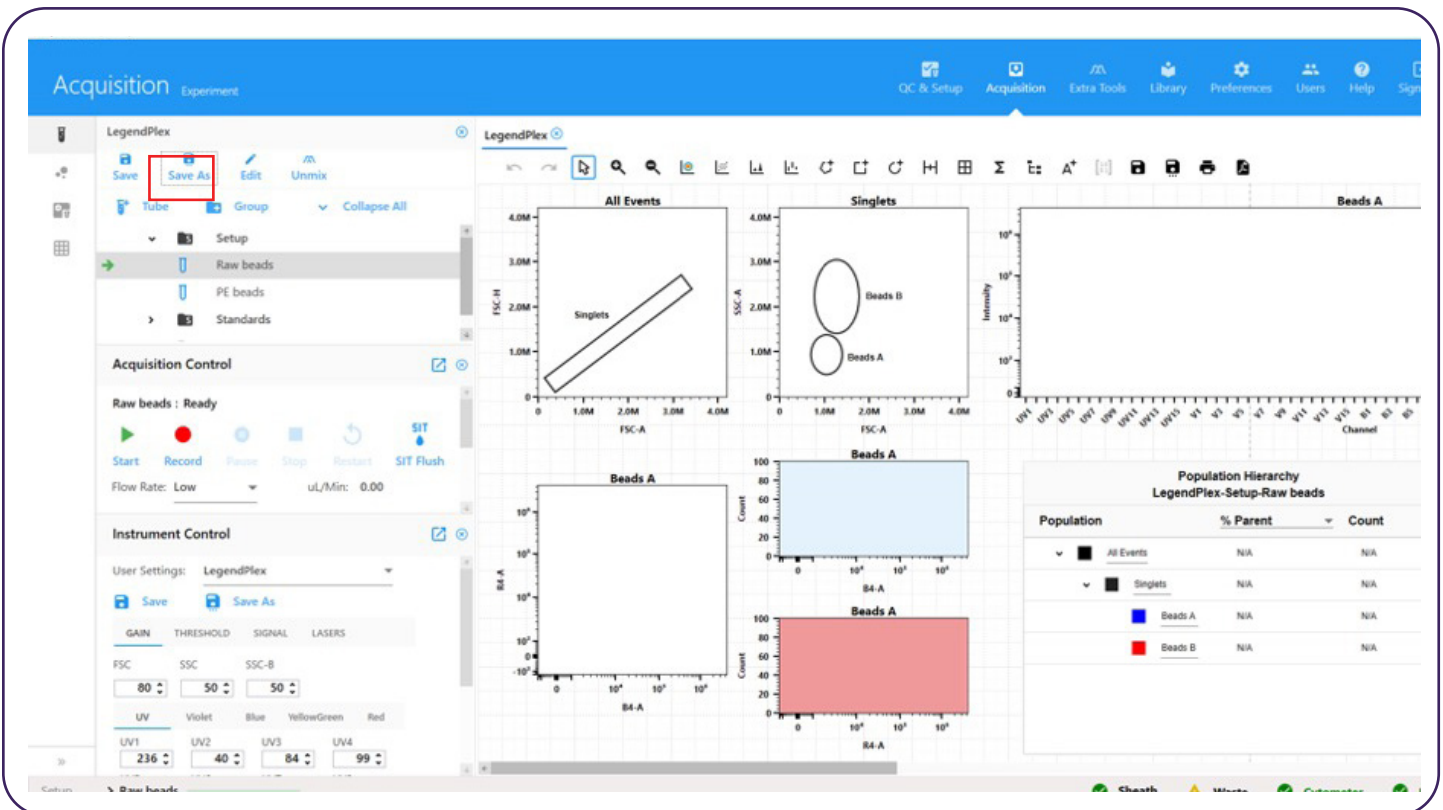
2. First, enter the desired name and description (optional) for your experiment.
3. You will need to add two or more fluorescent tags to continue. In this case, we will add PE and APC. Then click the Next button in the bottom right.



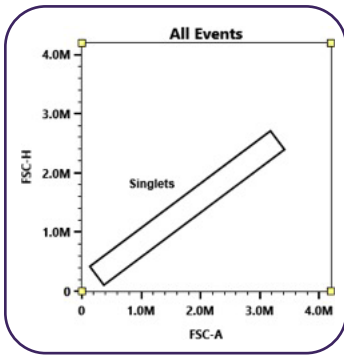
- You will now add the necessary Groups and Tubes. For the LEGENDplex™ assay, you will need to next create a Setup group, a Standards group, and a Samples group. See below for naming of your Setup and Standards beads. Then click Next to go to the next tab in the Create New Experiment wizard.



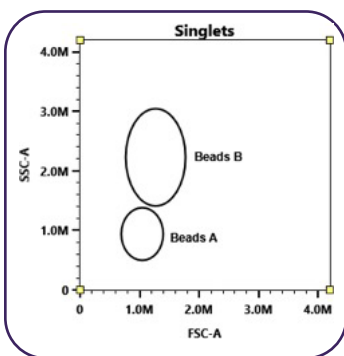
- You can skip the following tabs going over Markers and Keywords and skip straight to the Acquisition tab.
- Under the Analysis tab, you can select any pre-designed analysis worksheet, as well as stopping criteria. When starting to acquire your tubes, please ensure you collect no more than 5,000 events per tube.
- Once done with the Create New Experiment wizard, click "Save As" to create your experiment template.



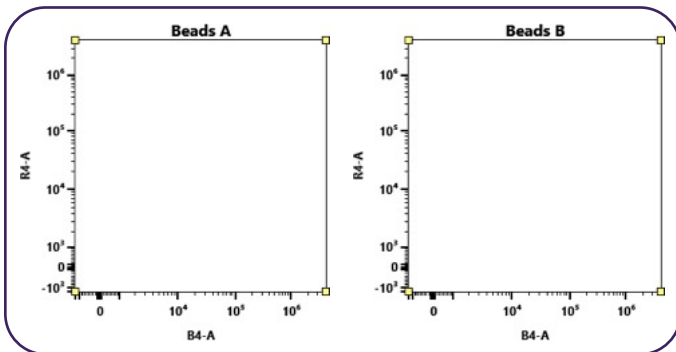
#### IV) Instructions for Setting Up Your Worksheet Template



1. For your analysis worksheet, first gate around your bead singlets.



2. Next, you will see a Beads A and Beads B population when gating off FSC-A and SSC-A.



3. For both Beads A and Beads B, make sure you can see all events when looking at the R4 and B4/YG1 channels.