De Novo Software
Turning Cytometry Data Into Results

Universal Antibody Titration Template with Integrated Stain Index Calculations for FCS Express 7

Overview

The instructions below will guide users of the Universal Antibody Titration Template(s) in FCS Express 7. The template is designed to allow loading and analysis of data from any instrument for use with any parameter, including raw data spectral detector parameters from Cytek Aurora systems. When opening the layout, users will be presented with a "start-up script wizard" to select data files and parameters to use in the analysis which may also be dismissed for a manual set up by pressing cancel. The steps below outline manual setup and other details on layout usage. Please make sure to read the additional information in the Titration Layout Information page provided in the layout.

With the manual set up and instructions provided in this document, users will be instructed to load data into the data list, organize the data by titration points, change parameters on plots to adjust for parameter order difference, and replace gates to match gates to the correct parameters.

With both wizard and manual methods, stain index metrics for both classical stain index and the 84th percentile methods will automatically by graphed and regressed while providing the antibody amount to use at 90% binding. Data can be exported with "1 click" via the Batch actions.

The instructions below make use of a 7 point titration data set. 5 point, 6 point and 7 point titration templates can be accessed on the Staining Index Page of the De Novo Software website.

Please contact support@denovosoftware.com with any questions or for assistance using the template.

**Note – if you are using Raw mixed data files from the Cytek Auora, please make sure to update your FCS Express instrument specific settings to allow loading of the Raw detector channels. See the end of the document for information on updating Cytek Instrument Specific settings.*

De Novo Software
Turning Cytometry Data Into Results

Manual Setup Instructions:

1.) Launch FCS Express and open the template .fey file from the **File** tab→**Open**.

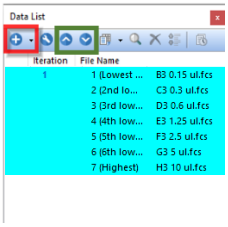

Figure 1 - Order the data list with the lowest titration at the top and the highest at the bottom.

Skip to step 2 if you already selected a data file and parameters in the start-up script

2.) Add data files to the data list by clicking on the blue plus icon (*Figure 1 – red box*) or by dragging the data files into the data list.

3.) Ensure data files in the data list are ordered from the lowest to highest titration concentrations.

**Note - If data files need to be reordered in the data list the up and down arrows can be used (Figure 1 - green box) or files can be dragged to the appropriate place in the list.*

Skip to step 7 if you already selected a data file and parameters in the start-up script

4.) On the **Analysis Plots** page(s), multiple select all plots in the column of plots at the left by drawing a selection area around the plots (much like selecting multiple objects in PowerPoint) (*Figure 2– Green Border*).
(Note: Steps 4 and 5 only required if correct parameters are not displayed on the plot)

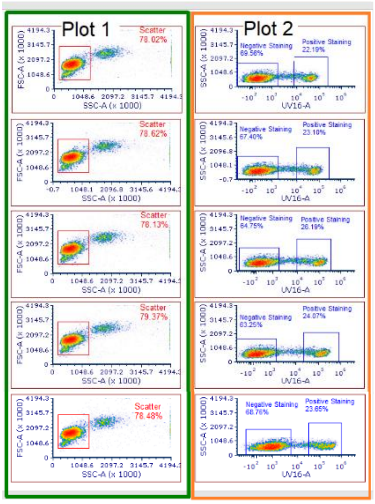

Figure 2 - Completed Titration Layout. Green border: plots used to choose scatter parameters, orange border: plots used to choose negative and positive staining populations

5.) Click on the **Format** tab→**Overlays** icon. In the **Overlays** format window change the **X** and **Y parameter**s to desired parameters to display and gate for the "Scatter" population of interest (*Figure 3*).
**Notes – typing while the dropdown menu is open will facilitate selecting parameters.*

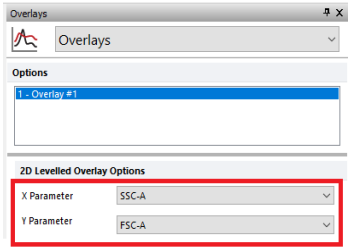

Figure 3 - Formatting parameters for Scatter Plots in Overlays dialog.

6.) On the Overlay page, change the parameter on the Histogram X axis if it is not already the desired fluorescence parameter.

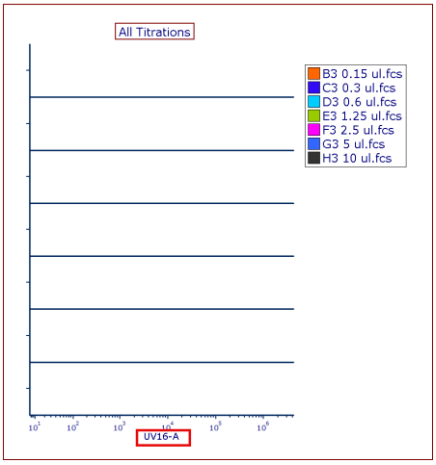

Figure 4 – The stacked histogram overlays

7.) Enter titration information on the Analysis Plots page of the layout
   a. Enter the antibody name under Titration Antibody. This name will be used as the name of output files created during batch processing.
   b. Optionally, enter the antibody Lot number.
   c. Below **Antibody concentration or amount**, enter the units used to measure the value (*Figure 5- blue box*).
   d. Enter the value for each tube (Lowest, 2nd Lowest, 3rd Lowest, 4th Lowest, 5th Lowest, 6th Lowest and Highest) (*Figure 5-red box*).
      i. A value greater than zero must be entered for all edit boxes except Lowest.
      ii. Enter zero in Lowest edit box if including an unstained sample as a titration point.
      iii. The values must be integer or decimal. Fractions or ratios need to first be converted to decimals.

	Antibody concentration or amount
Unit of measure*	uL
Lowest	0.15
2nd Lowest	0.3
3rd Lowest	0.6
4th Lowest	1.25
5th Lowest	2.5
6th Lowest	5
Highest	10

Figure 5 – Entering the Antibody concentrations

8.) Depending on your data files parameter order and range of data, the default Scatter gate may or may not appear in the column of plots on the left.
   a. If a Scatter gate <u>is not shown</u> on the plot:
      i. Select the **Gating** tab→choose the desired gate type→draw a new Scatter gate on the upper left plot (*Figure 2 – Plot 1*).
      ii. In the **Create New Gate** window select **Replace existing gate**→choose "**Scatter**" from the dropdown list→click **OK** (*Figure 6*).
   b. If a Scatter gate <u>is shown</u> on the plot continue to the next step.

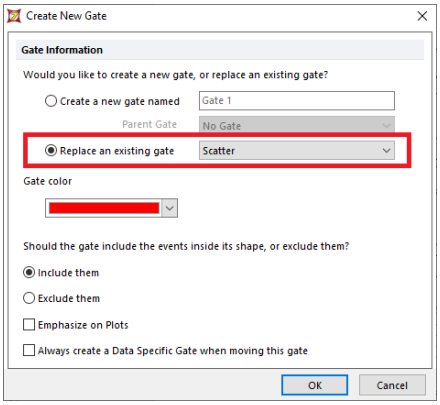

Figure 6 - Replacing the Scatter gate.

9.) Adjust "Scatter" gate as needed.
**Note* – Holding down the shift key while moving the gate will allow you to create Data Specific Gates for each data file if needed.

Skip to step 12 if you already selected parameters in the start-up script

10.) Multiple select all plots in the right column of plots (Figure 2 – right column with orange border).

11.) In the **Overlays** format window (see step 5) change the **X parameter** to the channel for titration measurements to define negative and positive populations and change the **Y parameter** to the Side Scatter parameter used in step 5 (*example shown in Figure 7*).

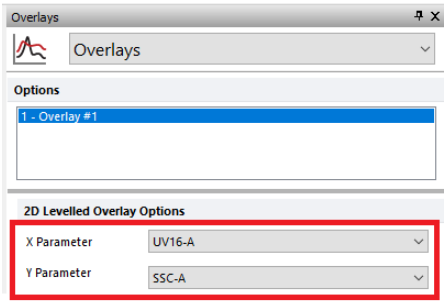

Figure 7 - Formatting parameters for Staining Plots in Overlays dialog.

11.) Repeat step 8 to view, replace, and adjust the "**Negative Staining**" and "**Positive Staining**" gates that are set up by default in the layout using the top plot from the middle column in the layout (*Figure 2 - Plot 2 and Figure 8*). Do not use the right column of plots which is reserved for 84th percentile calculations.

**Note – Holding down the shift key while moving the gate will allow you to create Data Specific Gates for each data file if needed.*

Data, plot, parameter, and gate setup is now complete (*As seen in Figure 2*). The statistic spreadsheets and regression plots located in the **Stain Index** page tab will automatically update as changes to plots/gates are made in the template.

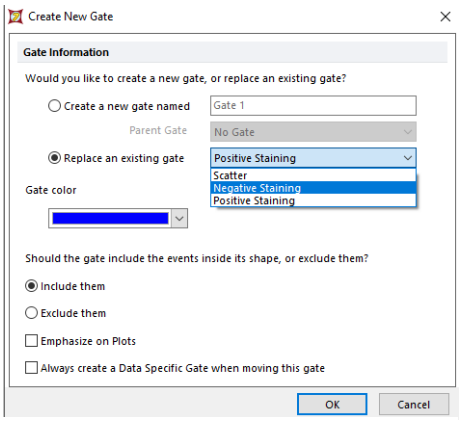

Figure 8 – Replacing Negative and Positive Staining gates.

Optional step: Displaying all tubes on a density plot.

a) Click **Data** tab > **Panels**
b) Click **Edit**
c) Increase the Number of Tubes by 1 (e.g. if the value is 5, change it to 6).
d) For the name for the new tube, enter "Merged" (*Figure 9*)
e) Click **OK**
f) Click **OK**
g) Click the **+** button in the Data list
h) Highlight all titration data files (use Shift key or Ctrl key to multiple select) (*Figure 10*)
i) From the dropdown menu, select **Merge FCS Files**
j) Click **Open File**
k) A message will appear saying *"An active panel with X tubes is currently applied to this layout… Would you like to automatically add the next X data files?"*
    a. Click **No**
l) Go to the **Overlay** page of the layout.
m) Drag and drop the Merged file from the Data List onto the white space of the Overlay page.
n) Select **Density**. Click **OK.**
o) Change the X axis parameter to the fluorescence parameter for the antibody. (*Figure 11*)
p) Change the Y axis parameter to Classification identifier. (*Figure 11*)
q) Click the Gating tab. From the Current Gate dropdown menu, select **Scatter**.
r) Make any desired plot formatting changes.

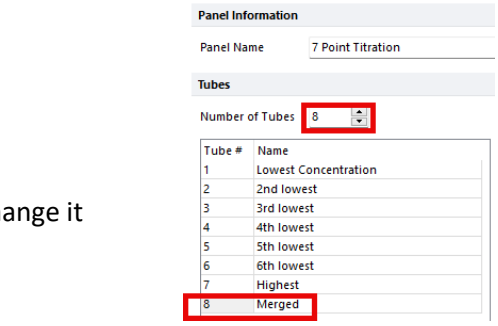

Figure 9 – Expanding the panel

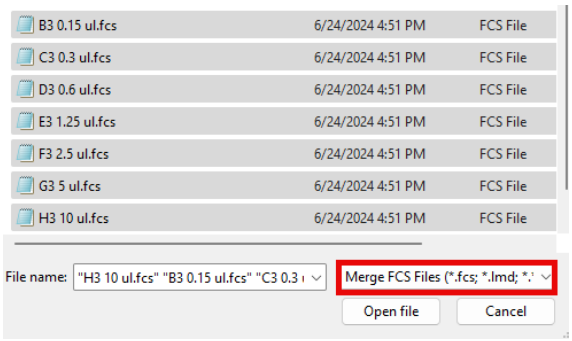

Figure 10 – Adding a Merged file

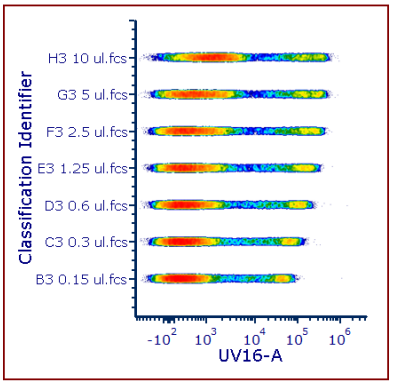

Figure 11 – Merged file on a density plot

To quickly export results:

12.) Click on the **Batch & Export** tab→ **Actions & Reports** (or **Batch** tab→**Batch Actions** in versions earlier than 7.22.0006). Double click on each action and confirm the location for exports to be saved for any actions selected is correct (*Figure 12*).

a. By Default, the files will be saved to the Desktop in a new folder labeled with the current date.

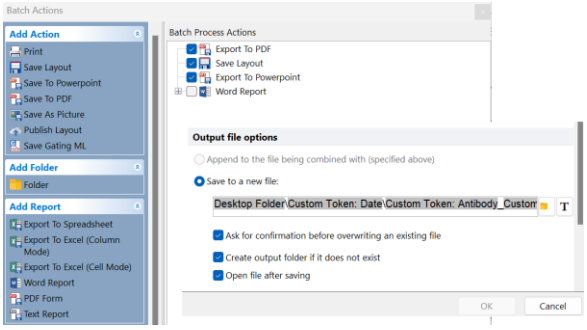

Figure 12 - Batch Process Actions window and partial view of action window to display output file options.

Optional step: Exporting a pre-designed Microsoft Word Report

a) By default, the Word Report batch action is unchecked. Checkmark the action if you wish to export a pre-designed Word Report (*Figure 13*).

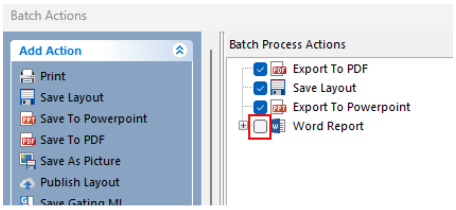

Figure 13 – Exporting the Microsoft Word Report

13.) Click on the **Batch & Export** tab→**Run** (or **Batch** tab→**Run** in versions earlier than 7.22.0006) to proceed with **Batch processing** which will export the results to PDF, PPT, optionally Microsoft Word, and save as a new layout.

14.) Navigate to the "Stain Index" page. Select the spreadsheet below the layout page (reporting "Filename" in cell A1) Choose the **Layout** tab now available at the top of the workspace (*Figure 14*).

15.) Click on the **Export** icon→define the location to be saved to/file name→click **Save**. This action will export the results of the FCS Express spreadsheet to Excel.

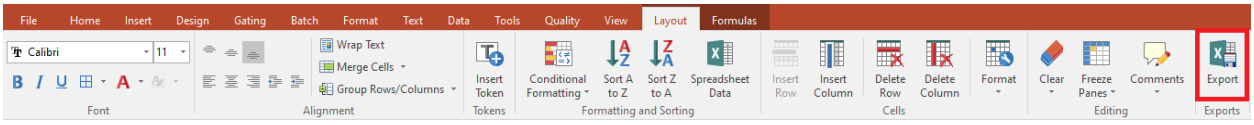

Figure 14 - Layout tab and export icon to export spreadsheet from Stain Index page

Setting Up Cytek Instrument Specific Settings

If you are using Raw mixed data files from the Cytek Auora, please make sure to update your FCS Express instrument specific settings (*Figure 15*) to allow loading of the Raw detector channels as follows:

1. Click on the **File** tab→**Options** to open the FCS Express User Options.
2. Expand **Data Loading→FCS File Options→Instrument Specific Settings**.
3. Select **Cytek – SpectroFlo** from the listed instruments under **Instrument Specific Settings**.
4. Confirm that the checkbox next to **Delete fluorescence parameters after spectra merge is unchecked**. In version 7.24.0024 and later, this setting is called **Hide individual detector parameters from raw data**.

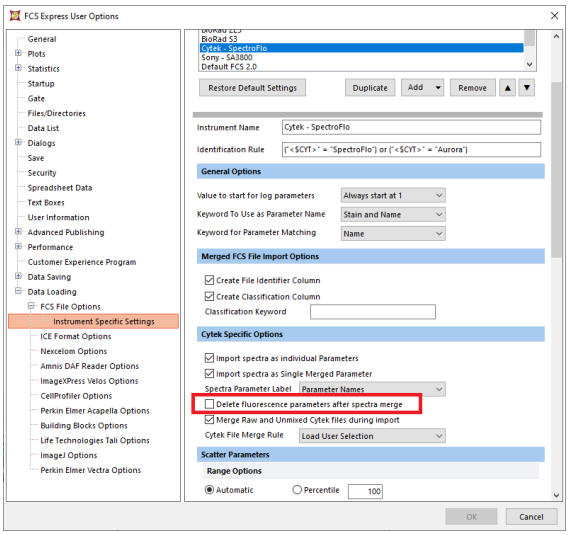

Figure 15 - Adjusting Instrument Specific Settings for Cytek data files to allow loading of raw paramaters.

Please reach out to support@denovosoftware.com with any questions.