

LEGENDplex™ Data Analysis Software Version 8 User Guide

Introduction

Welcome to the user's guide for Version 8 of the LEGENDplex™ data analysis software for Windows® based computers¹. This tutorial will cover all the steps necessary to analyze a typical LEGENDplex™ data set, and assumes users followed the protocol included in their assay manual.

The software can be downloaded free of charge from under the software tab at our website: biolegend.com/legendplex.

If you run into any questions, please contact our Technical Services Team (tech@biolegend.com). We'll be happy to provide answers, and if desired, analyze your data for you.

Once installed, the software provides easy-to-use analysis of flow cytometry files generated from BioLegend's LEGENDplex™ bead-based immunoassays.

- To begin, insert the USB dongle which was included inside your LEGENDplex™ assay kit into a USB port on your PC. The dongle is used as the license key for the software.

Once it has been inserted please wait at least 15 seconds for your computer to recognize the dongle, after which you may launch the software.

After launching, the use of the LEGENDplex™ Data Analysis Software proceeds through 4 sequential modular steps, each of which will be covered in detail in this guide:

1. Loading FCS Files
2. Gating Bead Populations
3. Review of Analysis Results
4. Data Analysis Reports

¹The LEGENDplex™ Data Analysis Software Version 8 User Guide is an independent publication and is neither affiliated with, nor authorized, sponsored, or approved by, Microsoft Corporation.



Step 1: Loading FCS Files

Loading FCS Files:

The LEGENDplex™ data analysis software supports the following file types: FCS 2.0, 3.0, 3.1, and .LMD files.

Note:

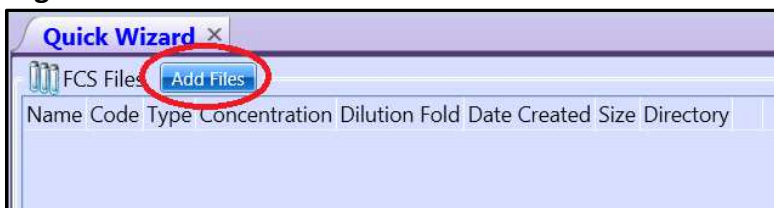
All FCS files from your assay need to have been acquired under exactly the same flow cytometer settings in order for the software to be able to analyze them together.

Be sure to include all files generated from your LEGENDplex™ assay. This includes both standard curve files as well as those from unknown samples.

Files can be added to the quick wizard window using one of the following methods:

1. Click the blue '**Add Files**' button. This will prompt a pop-up window which users can use to locate the folder containing their assay files. Once located, select all relevant files and drag them from the pop-up into the quick wizard window.

Figure 1. Add Files Button



Click this button to open a pop-up window which allows you to locate the folder containing your assay's FCS files.

2. Alternatively, open the folder containing their assay files independent of the software and then select and drop all assay files onto the quick wizard window.

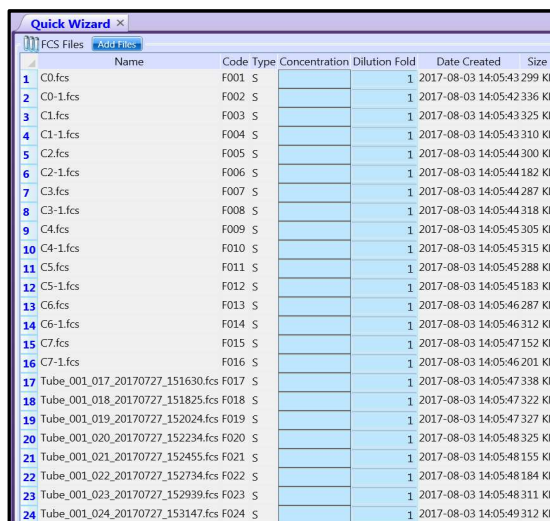
Step 1: Loading FCS Files

Ordering FCS Files:

Once all files have been loaded into the quick wizard window, the files should be arranged so that standard curve files are at the top of the list followed by all unknown sample replicates.

- Click on the name tab to sort the FCS files alphabetically. This option is usually sufficient to properly order the files, provided that you have followed the instructions in the assay manual, and named the files using a consecutive numbering scheme.
- If necessary, files can also be left click-and-dragged to their appropriate location on the list as an alternative method of ordering the data.
- When finished, a properly ordered file list will contain the standard curve files at the top of the list followed by all unknown samples, sequentially arranged by replicate (see image below).

Figure 2. Properly Ordered Files List



| | Name | Code | Type | Concentration | Dilution | Fold | Date Created | Size |
|----|----------------------------------|------|------|---------------|----------|------|---------------------|--------|
| 1 | C0.fcs | F001 | S | | | 1 | 2017-08-03 14:05:43 | 299 KB |
| 2 | C0-1.fcs | F002 | S | | | 1 | 2017-08-03 14:05:42 | 336 KB |
| 3 | C1.fcs | F003 | S | | | 1 | 2017-08-03 14:05:43 | 325 KB |
| 4 | C1-1.fcs | F004 | S | | | 1 | 2017-08-03 14:05:43 | 310 KB |
| 5 | C2.fcs | F005 | S | | | 1 | 2017-08-03 14:05:44 | 300 KB |
| 6 | C2-1.fcs | F006 | S | | | 1 | 2017-08-03 14:05:44 | 182 KB |
| 7 | C3.fcs | F007 | S | | | 1 | 2017-08-03 14:05:44 | 287 KB |
| 8 | C3-1.fcs | F008 | S | | | 1 | 2017-08-03 14:05:44 | 318 KB |
| 9 | C4.fcs | F009 | S | | | 1 | 2017-08-03 14:05:45 | 305 KB |
| 10 | C4-1.fcs | F010 | S | | | 1 | 2017-08-03 14:05:45 | 315 KB |
| 11 | C5.fcs | F011 | S | | | 1 | 2017-08-03 14:05:45 | 288 KB |
| 12 | C5-1.fcs | F012 | S | | | 1 | 2017-08-03 14:05:45 | 183 KB |
| 13 | C6.fcs | F013 | S | | | 1 | 2017-08-03 14:05:46 | 287 KB |
| 14 | C6-1.fcs | F014 | S | | | 1 | 2017-08-03 14:05:46 | 312 KB |
| 15 | C7.fcs | F015 | S | | | 1 | 2017-08-03 14:05:47 | 152 KB |
| 16 | C7-1.fcs | F016 | S | | | 1 | 2017-08-03 14:05:46 | 201 KB |
| 17 | Tube_001_017_20170727_151630.fcs | F017 | S | | | 1 | 2017-08-03 14:05:47 | 338 KB |
| 18 | Tube_001_018_20170727_151825.fcs | F018 | S | | | 1 | 2017-08-03 14:05:47 | 322 KB |
| 19 | Tube_001_019_20170727_152024.fcs | F019 | S | | | 1 | 2017-08-03 14:05:47 | 327 KB |
| 20 | Tube_001_020_20170727_152234.fcs | F020 | S | | | 1 | 2017-08-03 14:05:48 | 325 KB |
| 21 | Tube_001_021_20170727_152455.fcs | F021 | S | | | 1 | 2017-08-03 14:05:48 | 155 KB |
| 22 | Tube_001_022_20170727_152734.fcs | F022 | S | | | 1 | 2017-08-03 14:05:48 | 184 KB |
| 23 | Tube_001_023_20170727_152939.fcs | F023 | S | | | 1 | 2017-08-03 14:05:48 | 311 KB |
| 24 | Tube_001_024_20170727_153147.fcs | F024 | S | | | 1 | 2017-08-03 14:05:49 | 312 KB |

Example of a properly ordered files list with FCS files for the assay's standard curve followed by unknown experimental samples.

Step 1: Loading FCS Files

- Click the green 'NEXT' button on the bottom right of the quick wizard.

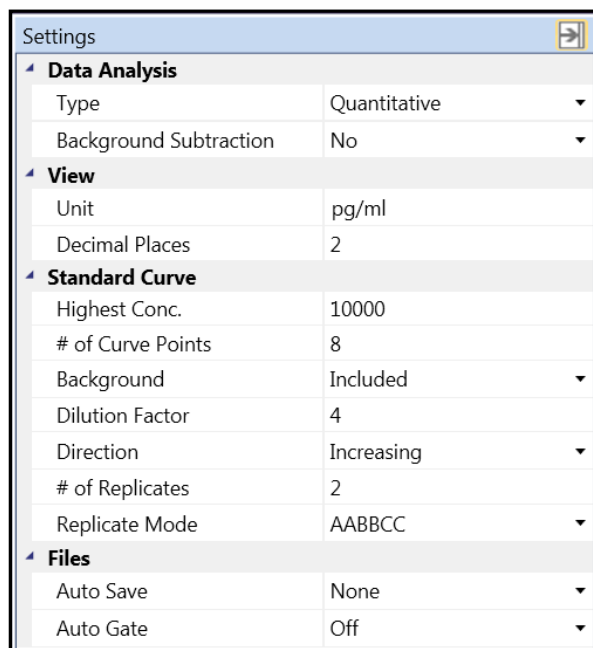
Figure 3. Next Button



The Settings Window:

After clicking next, the 'Settings' window will appear to the right of the file list. This window is used to define the parameters associated with the standard curve files from the assay.

Figure 4. Settings Window



| Settings | |
|------------------------|--------------|
| Data Analysis | |
| Type | Quantitative |
| Background Subtraction | No |
| View | |
| Unit | pg/ml |
| Decimal Places | 2 |
| Standard Curve | |
| Highest Conc. | 10000 |
| # of Curve Points | 8 |
| Background | Included |
| Dilution Factor | 4 |
| Direction | Increasing |
| # of Replicates | 2 |
| Replicate Mode | AABBCC |
| Files | |
| Auto Save | None |
| Auto Gate | Off |

Note:

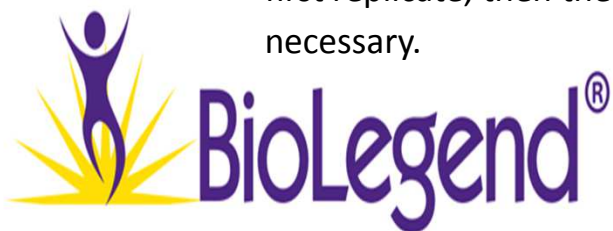
The LEGENDplex™ data analysis software has been designed so that many of the default settings in this window do not need to be adjusted if you have adhered to the protocol found in your assay manual.

Step 1: Loading FCS Files

There are several important setting options found in the window. These include the following:

Under the Standard Curve Section:

1. **Highest Concentration:** this value is set to a default 10,000 pg/mL. Certain LEGENDplex™ assays may contain alternative standard concentrations. Please consult the Certificate of Analysis included with your assay to determine the top standard concentrations for your specific assay. If your panel has top standard concentrations other than 10,000 pg/ml, these values will be adjusted later in the analysis. **Do not attempt to adjust the values here, leave the value set to 10,000 pg/mL.**
2. **Direction:** This parameter setting informs the software whether the FCS files for the standard curve are ordered on the quick wizard list from lowest to highest (and thus increasing) or from highest to lowest (i.e. decreasing). Select the appropriate setting now.
3. **Number of Replicates:** Ensure that this value matches the number of replicates you have for your assay's standard curve points. The number of replicates for each standard curve point recommended by the manual is 2. However, some researchers may elect to run the assay using a different number.
4. **Replicate Mode:** This parameter has 2 settings. AABBC or ABCABC. The AABBC mode is for standard curve files arranged with replicates adjacent to one another (i.e. both replicates of C7, followed by both replicates of C6, etc.). The ABCABC mode is used if the standard curve files are listed by the entire dilution series followed by the replicate series, i.e. C7-C0 files in the first replicate, then the C7-C0 files of the second replicate. Adjust as necessary.



Step 1: Loading FCS Files

Under the Files Section:

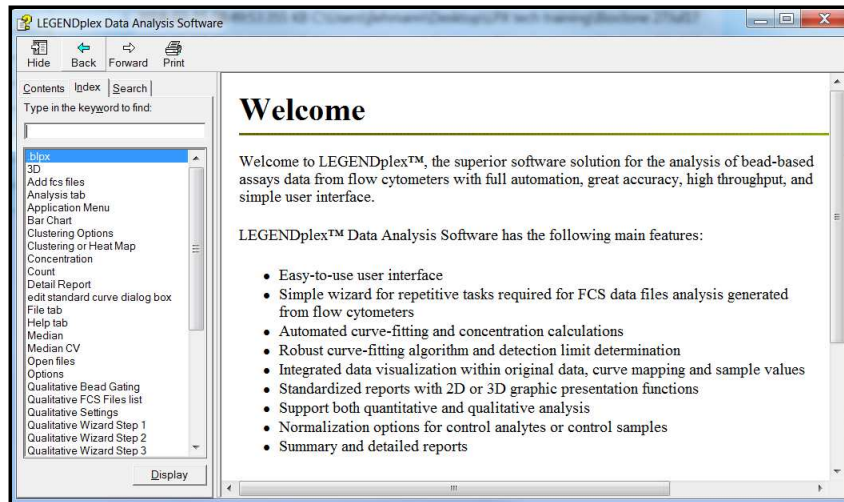
1. **Auto Save:** This feature can be adjusted to have the software automatically save an output report, a data status file, both file types, or none.

Remaining Parameters:

There are other features that may be adjusted within the settings window; however, the instances that would require changing the default settings are limited.

For more information about these settings please press the F1 key to open the software's help window, and then search for the term '**quantitative settings**'.

Figure 5. LEGENDplex™ Data Analysis Software Help Window



Press F1 to open the help window anywhere in the software. Search the index for further details on accessory software functions not covered in this guide.

Step 1: Loading FCS Files

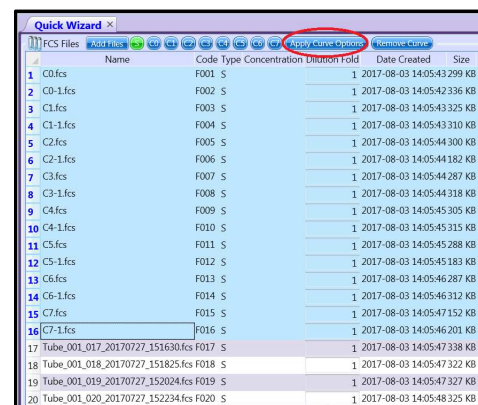
Defining the Standard Curve:

Once you have adjusted all the necessary parameters in the settings window, you will need to define which FCS files belong to the assay's standard curve using the following steps:

1. Select all the standard curve files in the list, and then click the **'Apply Curve Options'** button at the top of the screen.

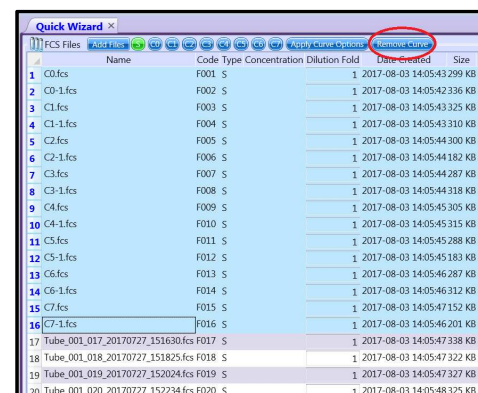
This will assign a standard curve point (C0 -C7) to all highlighted files based on the adjustments just made in the settings window.

Figure 6. Apply Curve Options Button



2. Click the **'Remove Curve'** button to undo the assignment if you notice any errors.
3. Make any necessary parameter adjustments in the settings window.
4. Click the **'Apply Curve Options'** again to apply the new settings.

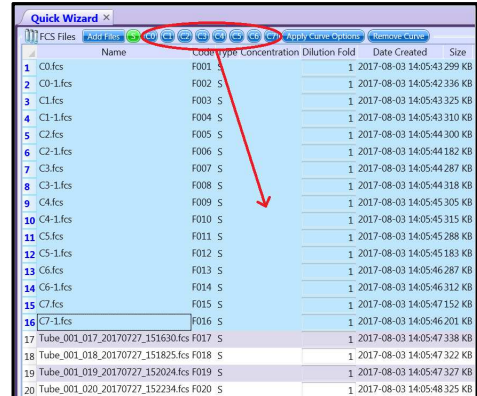
Figure 7. Remove Curve Button



Step 1: Loading FCS Files

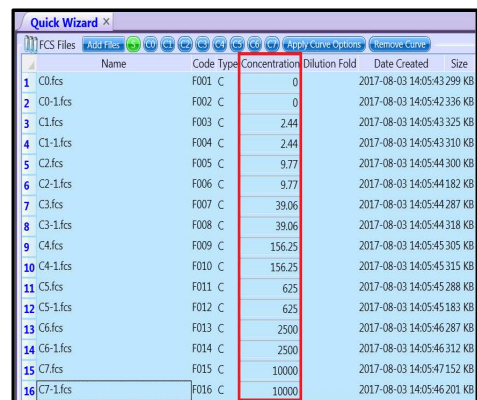
- Alternatively, you may also left click and drag the circular blue standard curve point icons to their respective FCS files to assign a designation.
- Releasing the dragged standard curve point icon over a file name will fill in the concentration column with the corresponding value.

Figure 8. Standard Curve Icons



- When you are finished each standard curve FCS file should have been assigned a corresponding concentration value.
- Verify this is correct by reviewing the information in the **Concentration** column.

Figure 9. Concentration Column



| | Name | Code Type | Concentration | Dilution Fold | Date Created | Size |
|----|----------|-----------|---------------|---------------|---------------------|--------|
| 1 | C0.fcs | F001 C | 0 | | 2017-08-03 14:05:43 | 299 KB |
| 2 | C0-1.fcs | F002 C | 0 | | 2017-08-03 14:05:42 | 336 KB |
| 3 | C1.fcs | F003 C | 2.44 | | 2017-08-03 14:05:43 | 325 KB |
| 4 | C1-1.fcs | F004 C | 2.44 | | 2017-08-03 14:05:43 | 310 KB |
| 5 | C2.fcs | F005 C | 9.77 | | 2017-08-03 14:05:44 | 300 KB |
| 6 | C2-1.fcs | F006 C | 9.77 | | 2017-08-03 14:05:44 | 182 KB |
| 7 | C3.fcs | F007 C | 39.06 | | 2017-08-03 14:05:44 | 287 KB |
| 8 | C3-1.fcs | F008 C | 39.06 | | 2017-08-03 14:05:44 | 318 KB |
| 9 | C4.fcs | F009 C | 156.25 | | 2017-08-03 14:05:45 | 305 KB |
| 10 | C4-1.fcs | F010 C | 156.25 | | 2017-08-03 14:05:45 | 315 KB |
| 11 | C5.fcs | F011 C | 625 | | 2017-08-03 14:05:45 | 288 KB |
| 12 | C5-1.fcs | F012 C | 625 | | 2017-08-03 14:05:45 | 183 KB |
| 13 | C6.fcs | F013 C | 2500 | | 2017-08-03 14:05:46 | 287 KB |
| 14 | C6-1.fcs | F014 C | 2500 | | 2017-08-03 14:05:46 | 312 KB |
| 15 | C7.fcs | F015 C | 10000 | | 2017-08-03 14:05:47 | 152 KB |
| 16 | C7-1.fcs | F016 C | 10000 | | 2017-08-03 14:05:46 | 201 KB |

Defining Replicates in Unknown Samples:

After defining the Standard Curve Files, you will need to define any replicate schemes in your unknown samples.

- Select all FCS files associated with a particular replicate.

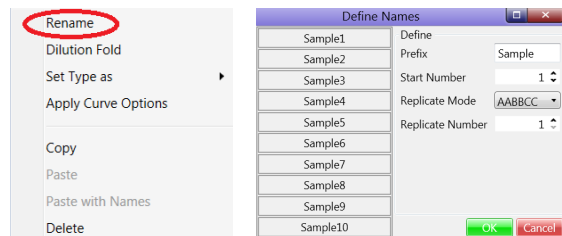
Note:

These files should be adjacent to one another in the file list, as previously described.

Step 1: Loading FCS Files

- Right click on any of the highlighted files and select '**Rename**' from the list of options that appears to open the '**Define Names**' pop-up window.

Figure 10. Defining Sample Names



From the **Define Names** window, the following parameters should be adjusted:

- Prefix:** Enter the name you would like the software to call the replicate in the data output reports. The default name is '**Sample**' but this can be changed by typing into the text box.
- Start Number:** The software requires that all replicate names include a number as well. The default start number is '1' but can be adjusted to any positive integer.
- Replicate Mode:** This setting's description is the same as it was for the standard curve files. Adjust as required.
- Replicate Number:** Adjust this value to reflect the number of files in a given replicate, i.e. adjust this value to '2' if describing an unknown sample assayed in duplicate.

Note:

Multiple groups of unknown replicates can be defined in a batch.

For example, if you were to select 14 FCS files, and then apply the following settings: Prefix = sample, Start # = 1, replicate = 2; the software would then define 7 replicates (named sample 1-7) each comprised of 2 FCS files starting with the first 2 and then moving down through the selected file list.

Step 1: Loading FCS Files

- Click the green OK button at the bottom of the window to close the window.

The replicate names that have been assigned to your FCS files will now appear in the **'Code'** column of the quick wizard file list, to the immediate right of the file name.

Figure 11. Code Column

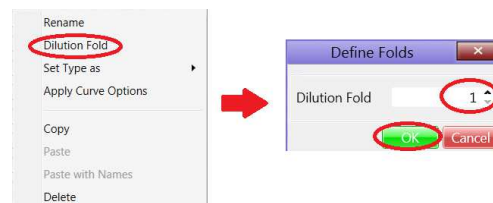
| | | | | |
|----|----------------------------------|-----------|--|---|
| 17 | Tube_001_017_20170727_151630.fcs | Sample1 S | | 1 |
| 18 | Tube_001_018_20170727_151825.fcs | Sample1 S | | 1 |

Defining Dilutions in Unknown Samples:

Next, define any dilutions made to unknown samples using one of the following methods:

- Manually define the dilution factor for a particular FCS file by typing the value into the **'Dilution Fold'** column of the quick wizard file list.
- Alternatively, if multiple unknown samples share the same dilution factor, select all the relevant FCS files and then right click and select **'Dilution Fold'**.
- Enter the dilution factor in the window and click **'Ok'** to apply this value to all selected files.

Figure 12. Defining Sample Dilutions



- Click the green **'Next'** button at the bottom right of the screen to proceed to the gating portion of the LEGENDplex™ data analysis software when you finish defining sample dilutions.

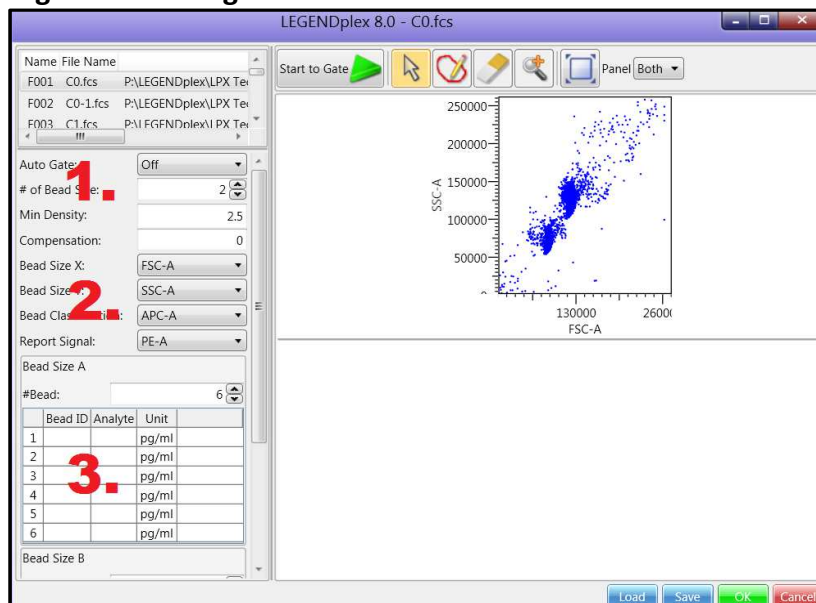
Step 2: Gating Bead Populations

The Gating Window:

The gating window will pop-up after clicking the '**Next**' button. This window is used to define the parameters used during acquisition on your flow cytometer, the analyte targets in your assay, and a global gating strategy for your samples.

- We recommend maximizing this window in order to have the best view of all settings and options.
- In the top left corner of the gating window you will find a list of all files that were loaded into the quick wizard.
- To find a specific file, use the scroll bar to the immediate right of the list.
- Below the file list are a number of other important parameters that you will need to define prior to drawing gates for the bead populations:

Figure 13. Gating Window



The Gating Window is used to define cytometer channels, analyte bead IDs, and gates for assay bead populations.

Step 2: Gating Bead Populations

1. # of Bead Size:

- The default setting for this parameter is 2. The majority of LEGENDplex™ assays contain 2 bead sizes, the smaller A beads and slightly larger B beads.
- If your particular assay contains only one size of beads, please adjust this value to 1.

2. Scatter Plot Parameters:

Parameters are imported directly from your FCS files, and may vary with the flow cytometer and data acquisition software used during your assay.

- For **Bead Size X**, select the forward scatter parameter from your cytometer.
- For **Bead Size Y**, select the side scatter parameter.
- For **Bead Classification** select the channel used to detect APC.
- For **Report Signal** select channel used to detect PE.

3. Bead IDs:

- 13-plex assays will have 6 A beads and 7 B beads. Adjust these values, as required by your assay, using the **#bead** fields.
- Enter the **bead IDs** and **analyte names** for all targets in your assay in both the A and B bead tables. Please refer to your kit manual for this information.
- Enter the lowest numbered bead ID at the top of the list first and fill in the rest of the list sequentially downward.

Figure 14. Bead ID Fields

| Bead Size A | | | |
|-------------|---------|---------|-------|
| #Bead: 6 | | | |
| | Bead ID | Analyte | Unit |
| 1 | A4 | IL-5 | pg/ml |
| 2 | A5 | IL-13 | pg/ml |
| 3 | A6 | IL-2 | pg/ml |
| 4 | A7 | IL-6 | pg/ml |
| 5 | A8 | IL-9 | pg/ml |
| 6 | A10 | IL-10 | pg/ml |

| Bead Size B | | | |
|-------------|---------|-----------|-------|
| #Bead: 7 | | | |
| | Bead ID | Analyte | Unit |
| 7 | B2 | IFN-gamma | pg/ml |
| 8 | B3 | TNF-alpha | pg/ml |
| 9 | B4 | IL-17A | pg/ml |
| 10 | B5 | IL-17F | pg/ml |
| 11 | B6 | IL-4 | pg/ml |
| 12 | B7 | IL-21 | pg/ml |
| 13 | B9 | IL-22 | pg/ml |

Enter the number of bead regions, bead IDs and analyte identities for your specific LEGENDplex™ Assay.

Step 2: Gating Bead Populations

Note:

If you plan on running the same LEGENDplex™ assay in the future, save a gate protocol file. This file can be loaded during future analyses and will automatically load all the assay parameters and bead IDs you have just defined.

- Click the blue '**Save**' button located at the bottom right of the window to save a gate protocol file. For future analyses, when you first enter the gating window click the '**Load**' button, find your protocol file, and then click open.

Figure 15. Saving Gate Protocol Files



Drawing Gates:

After all assay parameters have been defined it's time to move on to drawing gates.

The objective for this step in the analysis is to use one FCS file to draw a set of gates that correctly identifies all relevant bead populations from your assay.

These gates will then automatically be applied to all other FCS files, and can be thought of as a global set of parameters that can later be adjusted for individual files if necessary. **At this point in the analysis you cannot adjust the gates for only a singular FCS file**, as any changes will be applied to the rest your other files as well.

We will discuss the proper procedure for adjusting the gates for individual FCS files later in this tutorial.

To begin, you will need to create two gates on the forward versus side-scatter plot. One which identifies the A beads and a second that identifies the B beads.

Step 2: Gating Bead Populations

- Select the drawing tool icon at the top of the window.
- Move your mouse cursor to the FSC vs. SSC plot, near the A beads population.
- Click-and-hold the left mouse button to draw a gate around the A beads population (**Fig. 17**, green gate). Release mouse button when finished.
- Repeat this process for the B beads (**Fig. 17**, yellow gate).
- If bead populations are difficult to see, select the magnification icon and click-and-hold to define an area of the plot to zoom into.
- Release the mouse button to complete this action. This process can be repeated up to 3 times.
- Click the '**Reset Range**' button to return to the default view.

Figure 16. Manual Gating Tool



Figure 17. A & B Bead Sizing Gates

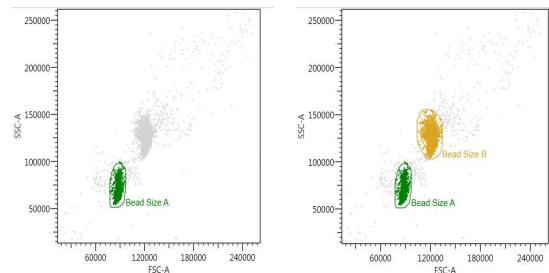


Figure 18. Magnification Tool



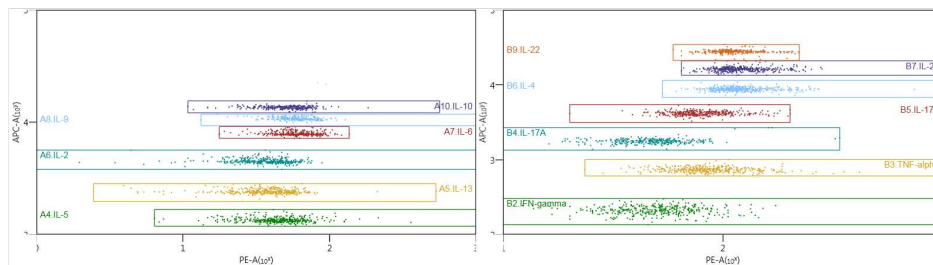
Figure 19. Reset Range Tool



Step 2: Gating Bead Populations

After you draw the A and B bead gates, the software will automatically attempt to gate the bead IDs in the APC versus PE scatter plots located below the sizing plot.

Figure 20. Bead ID Gates from PE vs. APC Fluorescence Plots



- If you make a mistake or would like to adjust the fluorescence scatter plot gates, select the eraser icon from the toolbar (**Fig. 21**, circled in red). Left click on any gate you wish to erase.

Figure 21. Eraser Tool



Note:

If you click on an area of the plot that does not contain a gate all existing gates in that plot will be erased.

- Select the drawing tool and click-and-hold on a scatter plot to draw a new gate.
- Once you are satisfied with the global gates, review how the software has applied them to your remaining FCS files by clicking through the list found in the upper left of the gating window.
- Make a note of any FCS files whose gates will need to be adjusted.
- Remember, any necessary gating adjustments for individual files will be made later in the analysis.

Step 2: Gating Bead Populations

Provided your flow cytometer was properly setup during acquisition there should be good resolution between all bead populations on the scatter plots.

If you feel confident in your cytometer setup, you can use the software's auto-gating function to apply gates instead of manually drawing them yourself.

- This is accomplished by clicking the green '**Start to Gate**' icon found at the top of the window when you are ready to start gating.

Figure 22. Auto-gating Button



Click this button to have the software attempt to automatically gate all bead populations. Only use if there is good resolution between populations.

The software will then apply gates, which will be displayed on the scatterplots.

Note:

You can still use the manual gating tools after the auto-gating function draws your initial global gates should you wish to make any adjustments.

- When you are finished, click the green '**OK**' button, located at the bottom right of the screen, to close the gating window and proceed to the next step in the analysis.

Step 2: Gating Bead Populations

Adjusting Top Standard Concentrations:

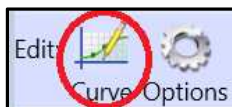
In the initial steps of the analysis, the top concentration for all standard curves is left at a default setting of 10,000 pg/mL.

A large portion of LEGENDplex™ assays do indeed have standards set to this concentration, but for some assays it is necessary to change these values.

Please consult the certificate of analysis included with your assay to determine the appropriate top standard concentrations.

If you need to adjust the concentration for a particular analyte target, this can be accomplished by accessing the **'Curve'** button that is now visible in the lower left corner of the screen.

Figure 23. Curve Button



- Click the **'Curve'** button now to open the **Edit Standard Curve** window.

Figure 24. Edit Standard Curve Window

| Name | Concentration |
|------|---------------|
| F001 | 0 |
| F002 | 0 |
| F003 | 2.44 |
| F004 | 2.44 |
| F005 | 9.77 |
| F006 | 9.77 |
| F007 | 39.06 |
| F008 | 39.06 |
| F009 | 156.25 |
| F010 | 156.25 |
| F011 | 625 |
| F012 | 625 |
| F013 | 2500 |
| F014 | 2500 |
| F015 | 10000 |

Parameters

Highest Concentration: 10000
Dilution Factor: 4
Decimal Places: 2
☒ Background
☒ Direction: Increasing
☒ Apply to All Analytes
A4.IL-5
A5.IL-13
A6.IL-2
A7.IL-6
A8.IL-9
A10.IL-10
B2.IFN-gamma
B3.TNF-alpha

Apply OK Cancel

This window is used to adjust the top standard concentrations of the recombinant proteins used in your LEGENDplex™ assay's standard curve.

Step 2: Gating Bead Populations

- Begin by unchecking the “**Apply to All Analytes**” box found in the middle portion of the right side of the window.
- Next, check only the box for the particular analyte that you wish to change.
- In the highest concentration box enter the value provided by your assay’s certificate of analysis or manual.
- If you ordered your standard curve FCS files in decreasing order on the files list (i.e. from C7 to C0) please uncheck the Direction: Increasing box.
- Click the blue ‘**Apply**’ button at the bottom to assign the new value.
- Uncheck the box for that analyte.
- Repeat these steps for each analyte standard concentration that needs to be adjusted.
- When finished, click the green ‘**OK**’ button to close the pop-up window.
- Click the green ‘**Run**’ button to run the analysis and proceed to the output report tabs.

Figure 25. Run Button



Step 3: Review of Analysis Results

Viewing Analysis Results:

After the curve fitting algorithm finishes calculating concentrations for samples, the results will be displayed in step 3 of the analysis software.

Standard Curves:

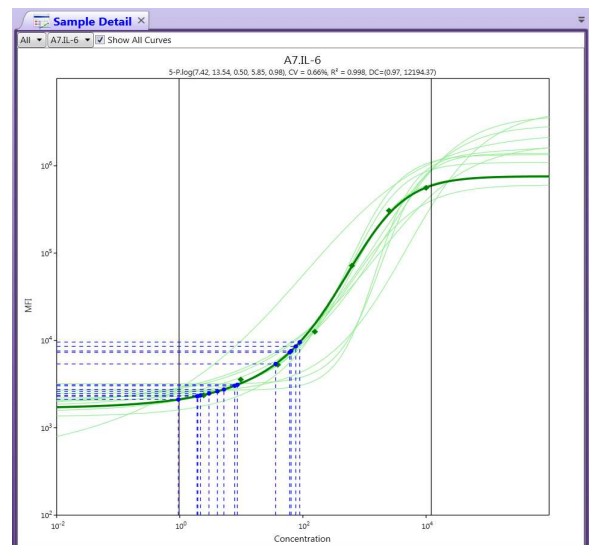
On the right of your screen you will see the standard curve graphs for your LEGENDplex™ assay:

In the upper left corner of the graph you will find 2 pull down tabs.

The pull down tab on the right can be used to select a standard curve for a particular analyte. The selected curve will be highlighted in bolded dark green in the graph, with the other curves being displayed in a lighter green.

- Unselect the '**Show All Curves**' box to view the standard curve for the currently selected target only.

Figure 26. Standard Curve Graphs



These graphs display the standard curves generated by the LEGENDplex™ Data Analysis Software's curve fitting algorithm.

The pull-down tab on the left can be toggled to show where unknown samples fall onto the standard curves in the assay.

The default setting is set to display all unknown samples, but you may view just a single FCS file by selecting it from the pull-down list.

Step 3: Review of Analysis Results

In the graph header you will see the name of the analyte. Below the name you can also find the following information:

- The coefficients for all variables used in the 5-parameter logistic curve fitting.
- The CV % and R^2 value of the curve fitting.
- The theoretical upper and lower limits of quantification for the curve which are found in the DC range bracket. For the curve currently selected, these limits are also depicted visually by the black vertical lines on the graph.

Result Tabs:

Four result tabs are displayed on the left of the screen. The tabs are as follows:

Figure 27. Assay Result Tabs



1. The Concentration Tab:

This tab displays the calculated analyte concentrations for all unknown sample files.

If you defined multiple files as being replicates earlier in the analysis, then the values displayed on this tab represent the average calculated concentrations of all replicates.

Unknown sample with concentrations that fall of the bounds of the standard curve will be reported as inequalities (i.e. < lower limit of quantification or > upper limit of quantification).

Step 3: Review of Analysis Results

2. The Median CV Tab:

This tab displays the %CV values between any FCS files defined as replicates.

In general, values should be less than 30%. Variation between replicates higher than this is usually indicate a need for troubleshooting.

Common causes of variation are described in the troubleshooting section of any LEGENDplex™ assay manual.

3. The Median Tab:

This tab displays the raw median fluorescence intensity (MFI) values for each FCS file in the assay.

4. The Count Tab:

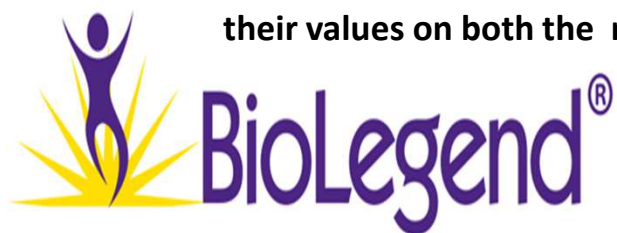
This tab displays the number of events acquired for each bead ID in a given file. The number of events collected should approximately equal 300 for each analyte if you properly followed the assay protocol.

Collecting at least 300 events is required to establish statistical confidence in the estimation of the MFI of the population, a value which is used for all downstream calculations by the software.

Individual FCS files with high variation between analyte bead counts should be reviewed for gating accuracy.

Note:

FCS files identified as blanks (*i.e.* C0) will display a red-dashed line through their values on both the median and count tabs.



Step 3: Review of Analysis Results

Reviewing and Adjusting Gates for Individual FCS Files:

In the first gating window, the LEGENDplex™ data analysis software simultaneously applied a global gating strategy to all FCS files.

In order to review and adjust the gates for individual FCS files please do the following:

1. Select either the Median or Count Tab.
2. Click on the small icon to the immediate left of the name of the first FCS file you wish to review. Unknown samples will have an 'S' icon and standards a 'C' icon. Once selected, the file will be highlighted blue.

Figure 28. Selecting Samples for Gating Adjustment

Concentration

Median CV

Median

Count

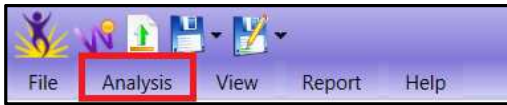
| Name | A4.IL-5 | A5.IL-13 | A6.IL-2 | A7.IL-6 | A8.IL-9 |
|------------------|-----------|-----------|-----------|-----------|-----------|
| C4.009-2018-01-3 | 3001.00 | 77947.00 | 9514.00 | 14248.00 | 11195.00 |
| C4.010-2018-01-3 | 3783.00 | 62325.00 | 13546.00 | 10948.00 | 9471.00 |
| C5.011-2018-01-3 | 8018.00 | 222476.00 | 40289.00 | 71879.00 | 71328.00 |
| C5.012-2018-01-3 | 7592.00 | 226010.00 | 35849.00 | 71895.00 | 70385.00 |
| C6.013-2018-01-3 | 23743.00 | 569979.00 | 143553.00 | 280283.00 | 300615.00 |
| C6.014-2018-01-3 | 96014.00 | 597344.00 | 291626.00 | 332430.00 | 310108.00 |
| C7.015-2018-01-3 | 246031.00 | 987803.00 | 686810.00 | 566519.00 | 592214.00 |
| C7.016-2018-01-3 | 281484.00 | 972756.00 | 742494.00 | 549688.00 | 587678.00 |
| Sample1 | 1984.00 | 6037.00 | 2830.00 | 2299.00 | 2490.00 |

3. Shift + Click to highlight multiple sequential files or Ctrl + Click individual files you wish to add to your selection. Newly selected files will also be highlighted blue.

Step 3: Review of Analysis Results

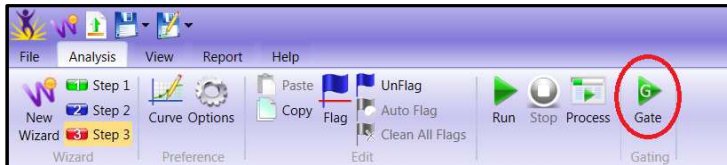
- Left click the analysis tab at the top of the window.

Figure 29. Analysis Tab



- Locate and left click the green 'Gate' triangle icon on the far right of the ribbon. This will re-open the gating window.

Figure 30. Gating Icon



Note:

The file list in the upper left now contains only the FCS files you just selected.

Any gating changes you make will now be applied to only the individual FCS file selected, as opposed to all files simultaneously like in the previous gating step.

- Select the first file you wish to adjust by clicking on the file's name from the list.
- Using the manual gating tools, adjust all necessary gates.

Once any changes are made, the FCS file's name will be changed from black to green text. This allows you to identify which files have already been re-gated.

- Repeat the above steps for all files on the list.

Step 3: Review of Analysis Results

- Click the green 'OK' button to close the gating window once you are finished.

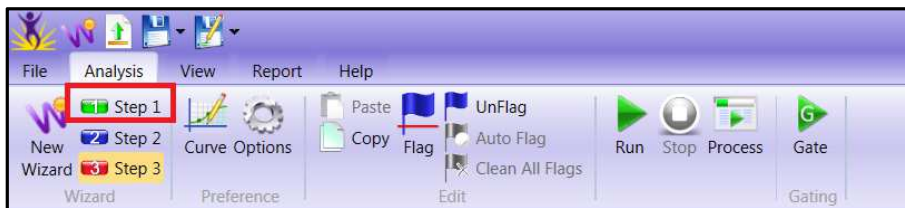
The analysis software will now re-calculate your results using the revised gating strategies.

Note:

Bead IDs and Analyte names cannot be adjusted here.

- To correct a mistake in one of these fields, please use the analysis tab to return to 'Step 1'.

Figure 31. Returning to Previous Analysis Steps



- Click on the green 'Gate' icon at the bottom left of the screen. This will reopen the original gating window where edits can be made to the bead IDs.

Flagging Files from Analysis:

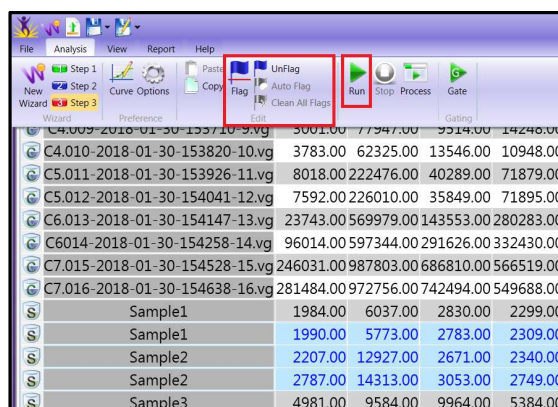
If necessary, the LEGENDplex™ data analysis software allows you to flag data points from the analysis.

You may exclude the data for just a single analyte all the way up to the entire FCS file. This is accomplished by using the flagging functions of the analysis software.

Step 3: Review of Analysis Results

- From either the '**Median**' or '**Count**' Tab, highlight the data points you wish to exclude from analysis by completing the following steps:
 - Click the well for the particular analyte you wish to exclude from analysis.
 - Ctrl+Click additional wells to exclude additional analytes.
 - Entire FCS files can be highlighted by clicking the small icon to the left of the file name.
- Next, click the analysis tab at the top of the window once you have identified all the data you wish to exclude.
- Click on the blue '**Flag**' icon from the list.
- This will add a green dotted line through all selected data wells.
- Return to the analysis tab and click the green '**Run**' icon. The software will now re-calculate your results.
- To clear flagged data, repeat this process using the '**Unflag**' icon instead.
- Alternatively, all flagged data points can be cleared simultaneously by selecting the '**Clean all flags**' icon.
- Remember to click the '**Run**' icon once you have finished unflagging any data.

Figure 32. Flagging Tools



Use these tools to highlight data points you wish to exclude from analysis.

Step 3: Review of Analysis Results

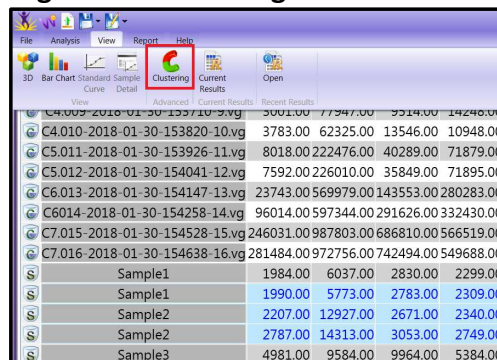
Heatmap and Clustering Analysis:

The LEGENDplex™ data analysis software can perform heatmap and clustering analysis on data selected from either the concentration or median tabs.

To perform this type of analysis, use the following steps:

1. Highlight the desired FCS files from either the concentration or the median tab.
2. Click on the '**View**' tab at the top of the window.
3. Click the clustering icon on the ribbon that is now visible.

Figure 33. Clustering Button

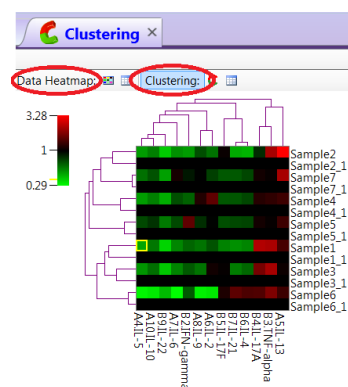


Use this button to access the heatmap & clustering functions of the LEGENDplex™ Data Analysis Software.

This will open a new tab that will now be visible on the right side of your screen.

- Toggle between the heatmap or clustering view by selecting either option at the top of the tab.
- The settings for these analyses can be adjusted by opening the '**clustering options**' icon.
- Right click on either of the maps and select '**Save as**' to save a copy of the image as one of the several graphic file types listed in the file type pull down menu.

Figure 34. Clustering Tab



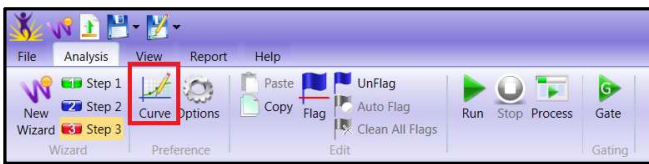
This tab found adjacent to the standard curve graphs.

Step 3: Review of Analysis Results

Edit Standard Curve Icon:

The edit standard curve icon that was previously used to adjust the top standard concentrations can also be found in the analysis tab should any other adjustments be necessary.

Figure 35. Curve Button Location



Returning to Previous Steps:

- Click on the analysis tab and then click on the analysis step you wish to return to (See Figure 31).

Note:

Due to the modular nature of the software, if you return to a previous analysis step all data flags will be removed and top standard concentrations reset to default. You will need to re-enter these values prior to saving any data analysis reports.

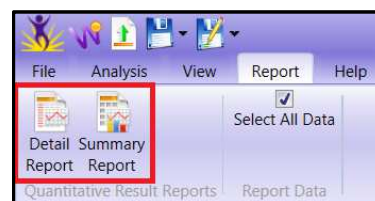
Step 4: Data Analysis Reports

Saving Data Analysis Reports:

After you have finished reviewing your analysis results you will want to export them out of the software platform.

- Click the **'Report'** tab at the top of the screen.

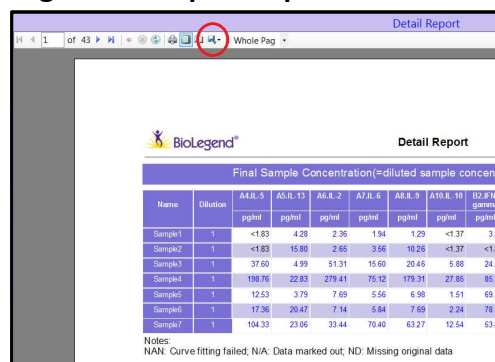
Figure 36. Report Tab



There are 2 types of output reports that the LEGENDplex™ data analysis software can generate. These are called the **Detail Report** and the **Summary Report**. The process for saving either report type is the same:

- Select the icon for the report you wish to save from the **'Report'** tab.
- Click the **'export'** icon at the top of the new window and select the file format you wish to save your data report as.
- Enter a file name, then click save.
- Close the pop-up window.

Figure 37. Report Export Button



Note:

We have found that Excel is often the most compatible with further downstream analyses, but you may select whichever format you prefer.

Step 4: Data Analysis Reports

You will also likely want to save a **Status File** of your analysis for experimental documentation purposes.

- Click the file tab and select '**Save As**' and then '**Status Files**'.

Note:

The status files have the extension '**.blpx**' and can be opened by the software in the future to return the report tabs without having to complete an entirely new analysis.

To load a status file from a prior analysis please do the following:

1. Launch a new session of the software.
2. Click on the '**File**' tab at the top of the screen.
3. Select '**Load Status File**'.
4. Load the file.

Figure 38. Loading Status Files

