1. What files can be analyzed by the LEGENDplex™ Data Analysis Software?

The LEGENDplex™ Data Analysis Software is compatible with FCS files in FCS 2.0, FCS 3.0, and FCS 3.1 standard formats. The software is also compatible with list mode file format (.lmd) generated from flow cytometers from some vendors. Occasionally, data acquisition software on a flow cytometer may generate FCS or LMD files in modified or non-standard format, which may lead to data incompatibility with LEGENDplex™ data analysis software. In this case, users are requested to send sample files(s) to supports@vigenetech.com for a quick solution. Vigenetech is committed to continuously improving product quality and customer satisfaction.

2. How can I get help?

LEGENDplex™ data analysis software instructions are available online **(http://www.vigenetech.com/LEP7register.asp; www.biolegend.com/legendplex).**

For specific issues, you may also email us at supports@vigenetech.com. Please provide us your contact information (email, phone) and describe details of issues or questions.

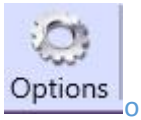
For LEGENDplex™ kit use and performance questions, please address questions to **techserv@biolegend.com**.

3. How do I use the Compensation function in the gating step?

Occasionally, when working with a single laser flow cytometer or a flow cytometer requiring compensations, significant spill-over of the reporting signal (e.g. PE) into the classification channel (e.g. , APC) may occur, resulting in upward or downward drifting of the bead sub-populations. In this case, the parameter for compensation function in bead gating settings may be adjusted to re-align the bead sub-populations horizontally. The function works only with slightly mis-compensated datasets. Best approach for avoid data compensation issue is proper instrument setup.

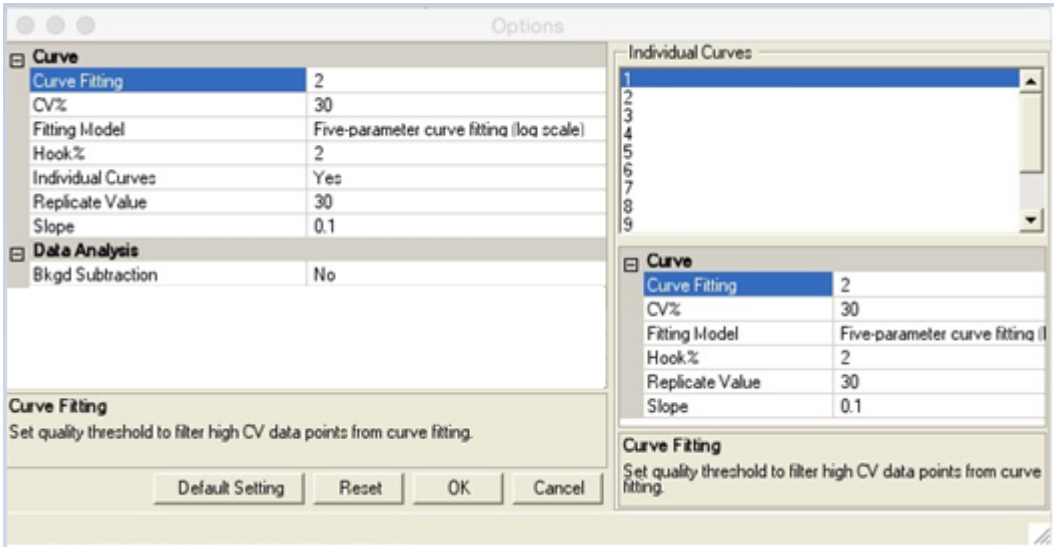
4. I did not get calculated concentrations for my standard curves and samples for selected analytes. What should I do?

When the analysis failed to perform calculations using default settings, it usually indicates that the curve-fitting model selected did not quite fit the actual data and a different curve-fitting algorithm needs to be selected for better data-fitting. To change a curve-fitting method,

a. Click 

on the Analysis ribbon tab to open **Options** dialog box.
b. Change the **Individual Curves** to “Yes”. Select the analyte(s) you want to change curve-fitting method.

c. Change the Curve Fitting method to a different model. Typically from the default 5-parameter curve-fitting method to 4-Parameter curve-fitting. Method.
d. Click OK. The data will be automatically recalculated.



5. If files corresponding to the standard curves are not all together in the file list and are instead scattered in the FCS file list, what options do I have to define my standard curves?

There are four methods you may use individually or in combinations.

a. Drag files according to the sequential order you need and move them together;
b. Sort file list by clicking File Name and Date Modified;
c. Highlight a file, right click your mouse and select **Set Type as** C0, C1…C7;
d. Select the file whose type is C and type the concentration you need in concentration box of the setting tab.

6. How do I define sample dilutions and sample replicates?

In Step 2 of Analysis tab, highlight selective sample files, enter the dilution factors in the settings on the right side of the screen, then click on the Define Dilution Factor to define sample dilution factors.

Similarly in Step 2 of Analysis tab, highlight selective samples, click on the Define Names to rename sample IDs.

7. What does Replicate Mode mean?

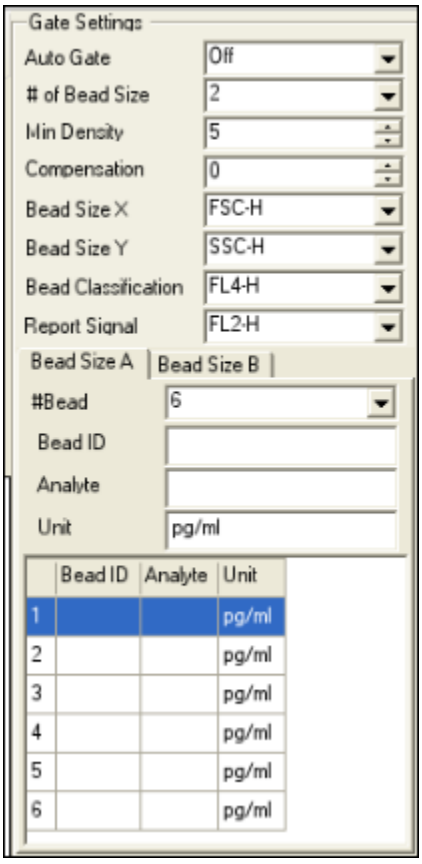
Replicate Mode defines modes of sample replicates.

- **AABBCC**: The AABBCC mode will replicate samples in consecutive manner when the number of replicates is defined (e.g. sample 1, sample 1; sample 2, sample 2 …).

  ABCABC. The ABCABC mode will replicate samples in repeating groups with defined size (e.g. sample 1, 2, 3, 4…; sample 1, 2, 3, 4…).

8. Where and how do I enter Bead ID and analyte names?

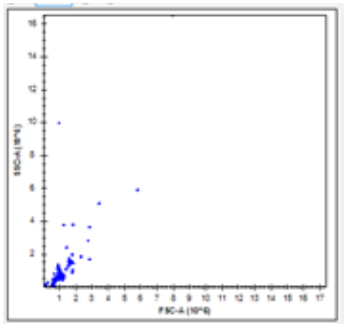
In Wizard Step 1, Click Gate. On the right side of the screen, type in Bead ID and Analyte names in the Bead Gating window in the middle section of the Gate Settings for each highlighted selection of Bead/Analyte in the lower section of the Gate Settings. Each bead size has a separate tab. The alphabetical order of "Bead ID" should match the numerical order of the index number. For example, enter A4, A5, A6…for "Index" 1, 2, 3.



9. How do I do Gating?

LEGENDplex™ Data Analysis Software supports three methods of gating:

a. Gating with Auto Gate OFF: This method uses gates from one sample and then applies the same gates to rest of the files in the same analysis. When data is clean with minimum junk signals and/or aggregated signals, proper gating can be performed by directly clicking  icon after Gate Settings (channels, number of bead size, bead sub-populations, etc.) are selected. Some files may show bead clusters too close to the corner of the dot map (see below,left panel), zoom function () may be used to zoom out the selected region before clicking on  . Zooming may need to be repeated for best result.



b. Manual gating: Find the bead sub-populations based on your manual drawing on bead size clusters After proper selection of gate settings for number of bead sizes, number of sub-populations for each bead size, analyte names, bead IDs, channel names for axes, the drawing tool () may be used to directly draw a circle around the bead populations (upper panel) or sub-populations (bands in lower panels). The drawing function () can be used in combination with the Eraser function () and the zooming function ().

c. Full Auto gating: Full auto gating function requires that the Auto Gate at ON position. After proper selection of parameters for gate settings, clicking on  will make the software automatically checking for required gates in each individual files independently. This method takes significantly longer time because of extra time required for the calculations.

10. What does Min Density in the gate settings mean?

The gating function is based on the setting on the density of a cluster of events. The density parameter defines the size of an area for threshold signal detection. The larger the value, the smaller the gated cluster area will be found.

11. If I have samples that show gate drifting or I have files from more than one experiments with different gates, Can I still analyze the datasets together?

Combined files to be analyzed in one analysis run have to be generated from the same instruments with the same selected channels and settings. In general, one may try one or combinations of the following four strategies to handle the files with different or drifted gates.

- Use manual gating function to draw bigger gates to cover all samples.
- Change the value of Min Density and then perform  ;
- Use of Full Auto gating function to gate individual samples independently.

The 3 methods above work only with samples with same settings (size and subpopulations)

- Use of "Re-gating" function
    a. At regular gating step, check individual files for appropriate gating and identify those files with inappropriate gates.
    b. Continue the gating and analysis process.
    c. At Step 3 after the analysis is finished, select the Median tab. Highlight those files that showed inappropriate gates, click on  , perform gating on those samples and then Click on OK. Click Run. The highlighted samples will be automatically re-analyzed with the new gates and the data will be combined in the original dataset with rest of the data.

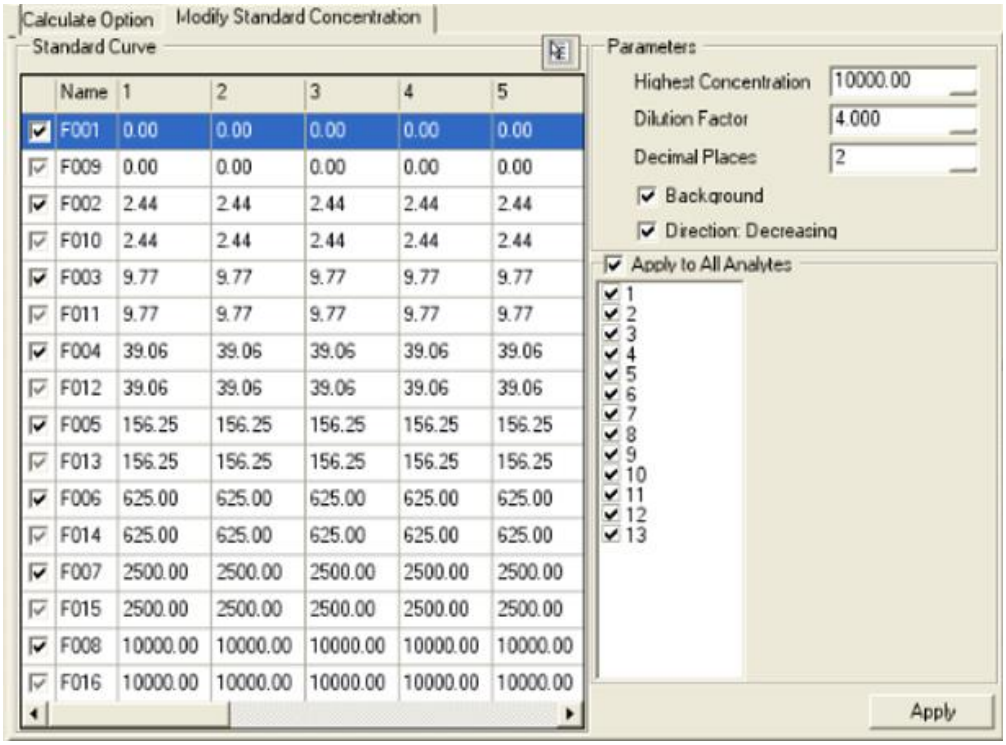
12. At Gating step, how do I select appropriate X-axis and Y-axis labels?

Proper selection of X-Axis and Y-axis parameters is critical for appropriate analysis of data from a flow cytometer. The parameters in FCS or LMD files are usually pre-defined by the instrument setup and data acquisition software on the flow cytometer. LEGENDplex™ data analysis software uses some commonly used parameters in the default settings, end users are required to check the gate settings and adjust the parameters as necessary for appropriate data analysis. The top bead size panel always use FSC-A (or FSC-H) for X-axis and SSC-A (or SSC-H) for Y-axis. In the lower band panels, the X-axis is the Report Signal (e.g. PE-A or FL2 or equivalent depending on instrument channels) used for the result reporting and calculations; the Y-axis is the classification signal which distinguishes the bead sub-population bands (e.g. FL4 or APC-A, PE-Cy5-A, etc. depending on flow cytometer channels).

13. If I have different standard concentrations for different analytes, where and how do I enter the information?

At Wizard Step 2, on the right side of the screen, click Modifying Standard Concentration Tab to display the Modify Standard Concentration setting dialog box. It contains three sections: Standard Curve List, Parameters and Analytes Lists.

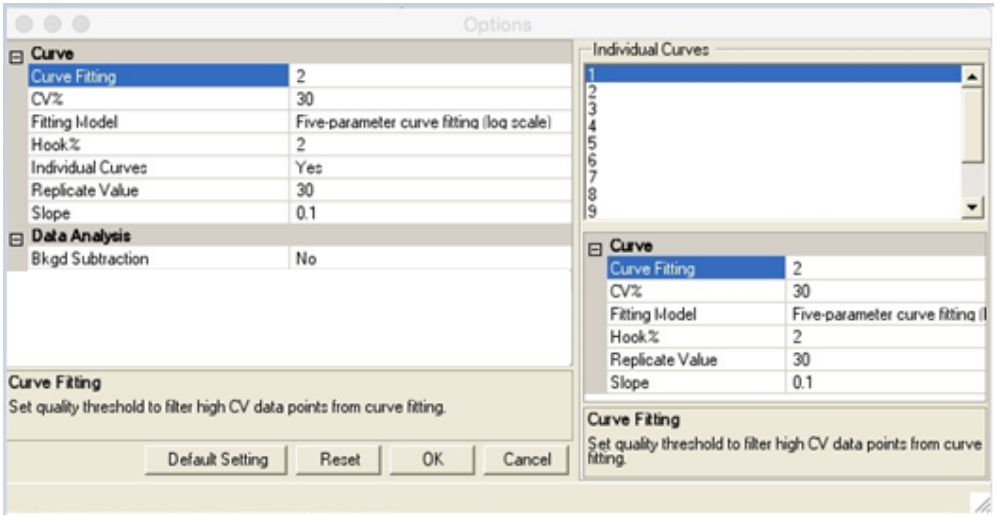
To change the starting concentration of a standard for particular analyte(s), uncheck the Apply to All Analytes. Select the analyte names that have different starting concentrations and enter the concentration in the box labeled as "Highest concentration", click on "Apply", the concentration table on the left side will refresh to display the correct concentrations of the diluted standards. After checking for errors, click "Run" and the data will be recalculated.



	Name	1	2	3	4	5
☑	F001	0.00	0.00	0.00	0.00	0.00
☑	F009	0.00	0.00	0.00	0.00	0.00
☑	F002	2.44	2.44	2.44	2.44	2.44
☑	F010	2.44	2.44	2.44	2.44	2.44
☑	F003	9.77	9.77	9.77	9.77	9.77
☑	F011	9.77	9.77	9.77	9.77	9.77
☑	F004	39.06	39.06	39.06	39.06	39.06
☑	F012	39.06	39.06	39.06	39.06	39.06
☑	F005	156.25	156.25	156.25	156.25	156.25
☑	F013	156.25	156.25	156.25	156.25	156.25
☑	F006	625.00	625.00	625.00	625.00	625.00
☑	F014	625.00	625.00	625.00	625.00	625.00
☑	F007	2500.00	2500.00	2500.00	2500.00	2500.00
☑	F015	2500.00	2500.00	2500.00	2500.00	2500.00
☑	F008	10000.00	10000.00	10000.00	10000.00	10000.00
☑	F016	10000.00	10000.00	10000.00	10000.00	10000.00

14. How do I handle poor curve-fitting or lack of calculated data?

Sometimes, curve-fitting options are changed by the user for certain analysis and the settings were not restored to defaults. You may open **Option** Dialog box ( ), and click **Default Setting** on the bottom to restore the default settings.



One should also check the number of Count to see whether low bead count is caused by poor gating or gate shift.

After making sure that the default settings and low bead count are not the cause for lack of good curve fitting, one may change the curve-fitting algorithm for better curve fitting for individual analyte (e.g. after making Individual Curves to ON position, change the default 5-parameter curve-fitting to 4-parameter logistic curve-fitting for selected analytes that showed poor curve-fittings).

15. In the View tab, there is a Cluster Function. How do I interpret the clustering and heat map data?

Clustering: Display the hierarchical clustering data. Clustering is the task of assigning a set of objects into groups (called clusters) so that the objects in the same cluster are more similar (in some sense or another) to each other than to those in other clusters. Use this function with caution as it is much more complicated than a single click.

Heat Map Data: Display the intensity MFI data or analyte concentration data of selected samples with the color map to illustrate the relationship of the values for analytes of interest.

16. In the Standard Curve Plots, the curve-fitting coefficients and detectable limits are reported. How I export the data to a table format?

The values of the parameters are reported in table format after the results are saved in Detail and Summary files. You may use Excel or Numbers to open the report files.

17. There are 4 file types that can be saved. What are the differences among FCS.VG file, Result file, and Status files? For future use, which file should I keep？

FCS.VG file： The FCS-VG file is generated when a report is requested or when result data is saved. The .vg files can be used for future re-calculations using LEGENDplex™ data analysis software.

Result file： Save the summary and detailed result data including raw MFI, calculated concentrations, curve information and original file properties. The files can be opened in Excel or Numbers.

Status file： A status file is a memory of the analysis. The file contains the results, raw data and gating information. However the files can only be replayed by the LEGENDplex™ data analysis software.

Gate setting file (.gpd): This file can be generated from the Gating step. It serves as a template for future use for the same kit. It may be loaded from the gating step as well.

18. A PC dongle license does not work for my Mac computer. How can I use the software to analyze my LEGENDplex kit data?

After initial downloading of the software, it has a built-in trial license good for 30 days. During the use of the software, a user is reminded to request a formal license. Please follow the instruction on screen for

User License Requesting processes. A valid license will be issued after a user successfully provides requested information (the user account, LEGENDplex™ kit lot number, etc.). Users are requested to send a license request form to LP-MAC-login@vigenetech.com. A file license will be generated and sent to the email address provided in the request form. Upon receiving the license file, users will then need to import the license file into the program.

19. If I have bad samples or bad replicate that I need to exclude from analysis, how do I flag it without deleting the file(s)?

In Wizard Step 3 interface, select Median tab. Highlight sample file(s), then click 

the Toolbar or right click and select **Flag**.

Similarly, one can highlight a sample, unflag or clean all flags by right clicking mouse and choose appropriate functions.

20. My license is expired, how do I go get a new license?

To request a license, you will need to follow the instructions to generate a license request form after you start to launch the software. Send the request file as an email attachment to LP-MAC-login@vigenetech.com.

21. How to generate a report？

In Wizard Step 3 interface, click  to save the result data.