LEGENDplex™ Data Analysis Software

Version 7.1

For Mac Computers

Contents

Welcome

Welcome to LEGENDplex™, the superior software solution for the analysis of bead-based assay data from flow cytometers with full automation, great accuracy, high throughput, and simple user interface.

LEGENDplex™ Data Analysis Software has the following main features:

- Easy-to-use user interface
- Simple wizard for repetitive tasks required for FCS data analysis generated from flow cytometers
- Automated curve-fitting and sample concentration calculations
- Robust curve-fitting algorithm
- Detection limit determination
- Integrated data visualization within original data, calculated data and curve mapping
- Standardized data reports
- Support both quantitative and qualitative analysis
- Normalization options for control analytes or control samples

Related topics:

- System Requirement
- Start LEGENDplex™
- Toolbar
- Installation
- Wizard Step 1

Getting Started

Overview

LEGENDplex™ data analysis software is designed for analysis of data of FCS file types generated from flow cytometers. Compatible FCS files that are in FCS 2.0, FCS 3.0, FCS 3.1 standard formats and some list mode files (.lmd).

System Requirements & Recommendations

Software:

- Mac OS X version 10.7 (Lion) and later.
- Mono 4.0 or higher

Related topics:

- Welcome
- Installation
- Start LEGEND*plex*™
- Toolbar
- Wizard Step 1

Installation

Download the software package from VigeneTech web site (http://www.vigenetech.com/LEP7register.asp) and save it to your computer.

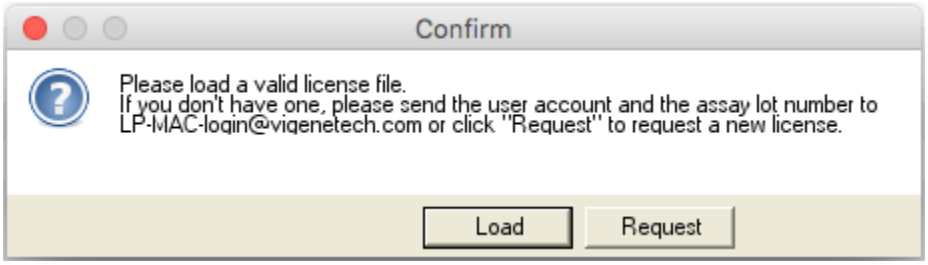
Check your computer for appropriate environment. Installation of Mono 4.0 or a higher is required prior to installation of LEGENDplex™ data analysis software.

To install,

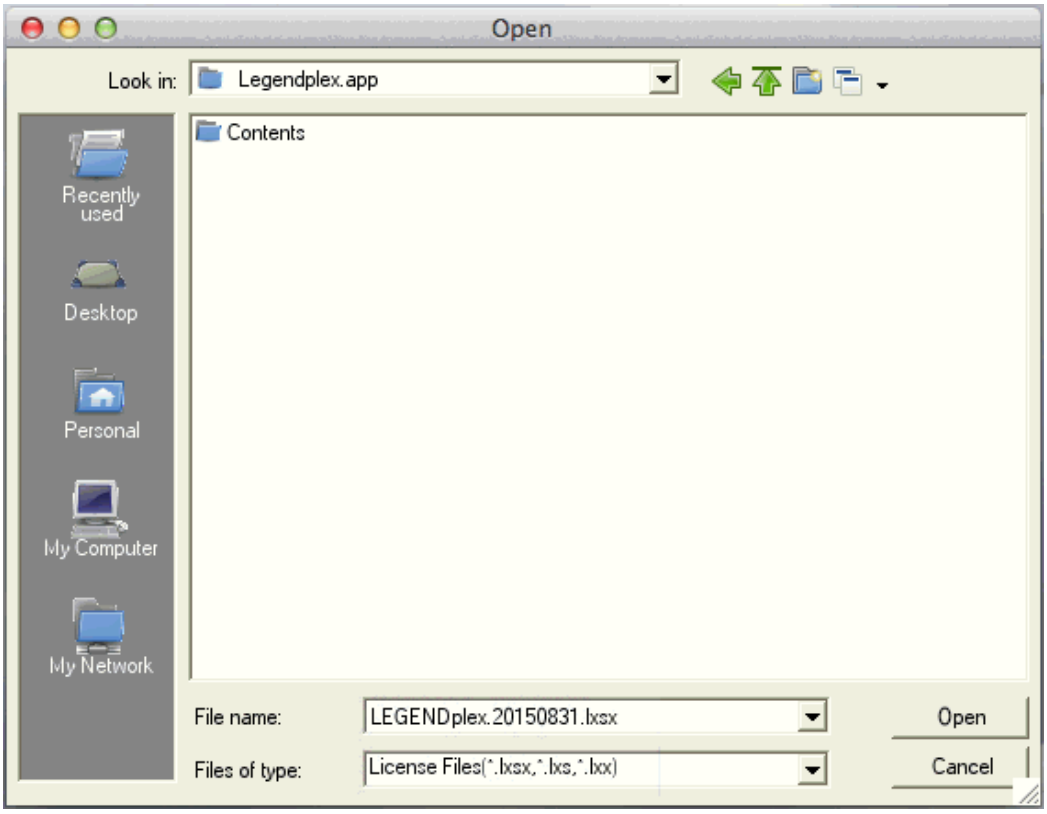
1. Double click the installation package in the folder where the software package is saved to unzip the software package, the icon of LEGENDplex™ will appear.
2. Click the icon of LEGENDplex™, a window will pop up asking for user information. Enter your contact information in the **Name**, **E-mail Address** and **Organization** fields. Click **OK**.

3. The **Trial License** dialog box will prompt. Click **Yes** to run the software on a trial version.

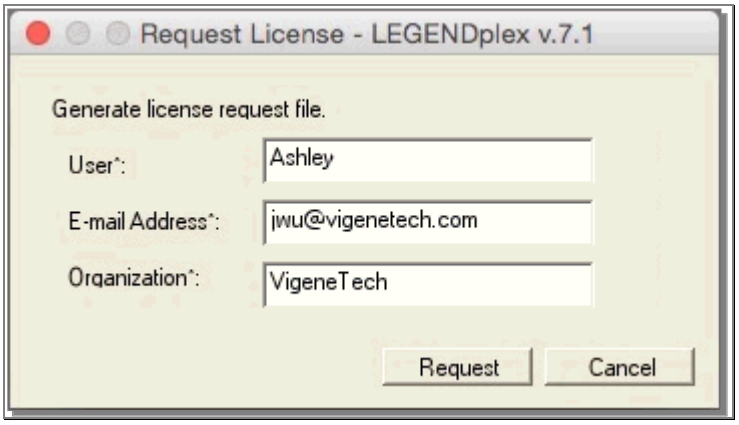
4. Click **No** to proceed to setting up software license.



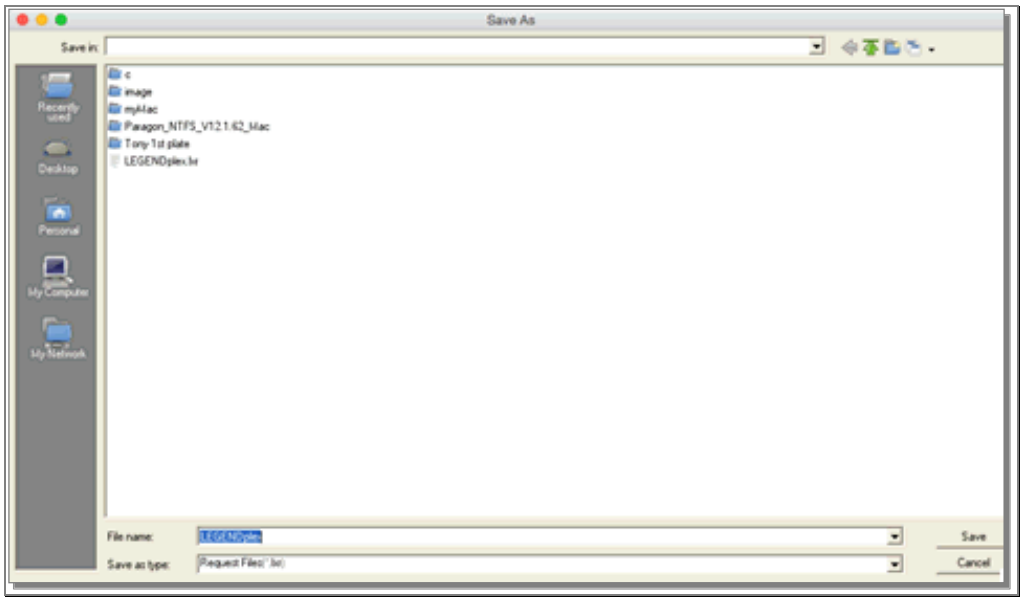
5. If you already have a file license, click **Load**, an **Open** dialog box pops up. This **Open** dialog box is for a user to enter a valid software license file you receive from VigeneTech. Change the directory, select a valid license file and click **Open** if you have a valid software license file.



6. If you do not have a valid license (most first time users do not have a license), click **Request** and confirm the user contact information.

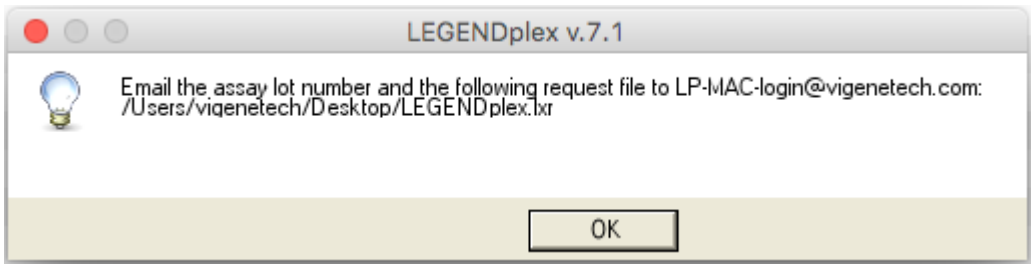


7. Click **Request.**

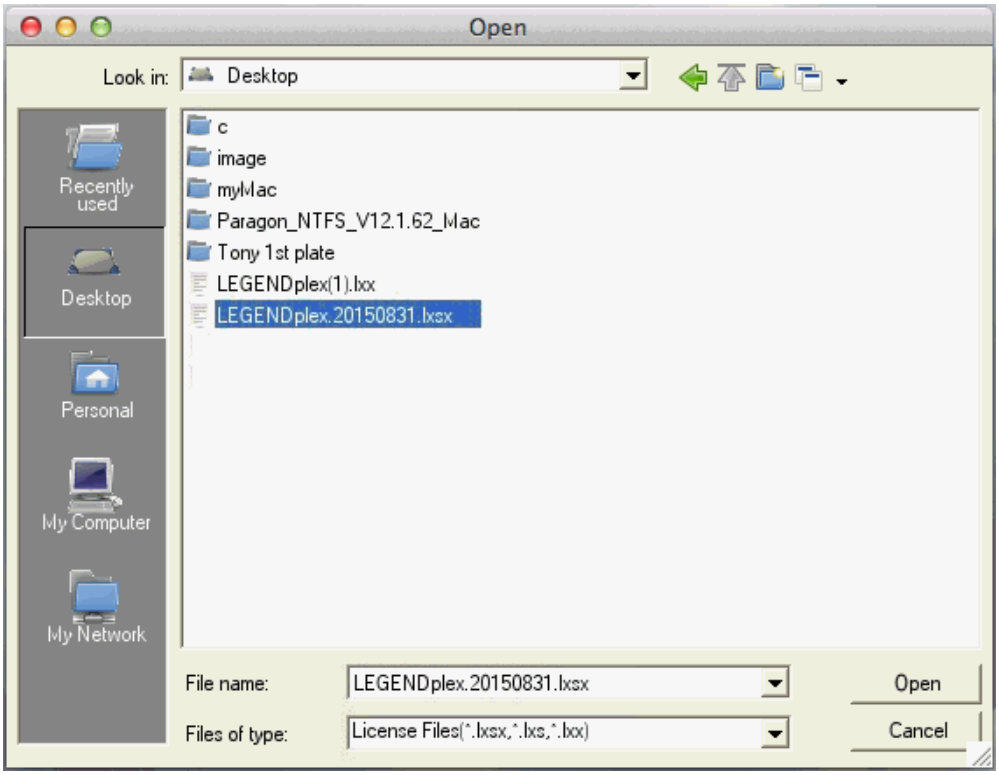


8. Click **Save** to save the license request file ***LEGENDplex.lxr*** to an appropriate directory on your computer.

   Send the LEGENDplex™ kit lot number and the license request file as an email attachment to the Email address shown below.



9. After you receive the valid license file (***LEGENDplex.lxsx or LEGENDplex.lxx)***, save the license file in an appropriate location on your computer.
10. Start LEGENDplex™ data analysis software and then the software will ask for a valid license again. Locate the software license file you receive from VigeneTech on your computer and click Open.



11. Software will launch.

Related topics:

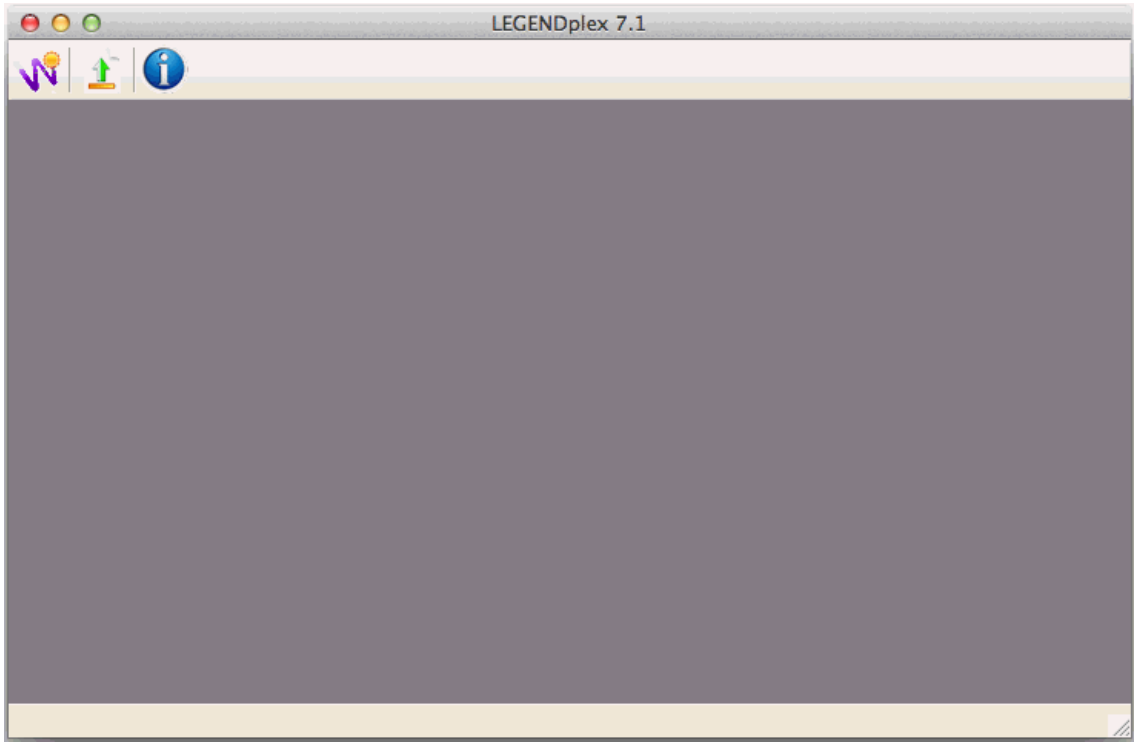
- System Requirement
- Start LEGENDplex™
- Toolbar

Start LEGENDplex™

Use the following method to launch LEGENDplex™ Data Analysis Software:

Double click the icon  on the desktop.

Then LEGENDplex™ is launched.



Click  to start a new wizard session. Alternatively, you may click  to open a status file from a previous saved analysis. A status file is a saved file set containing previously analyzed data and settings.

Related topics:

- System Requirement
- Toolbar

Toolbar

The toolbar in LEGENDplex™ data analysis software shows the functions related to the current operation step. For example, the toolbar below shows the buttons that are available for use with Wizard Step 3 operations.

Following table summarizes basic functions of each button.

Menu	Icon	Command
New wizard		Load a saved status file or start a new data analysis session.
Step 1		Step 1 (Open data file, add standard curve settings and define gating settings).
Step 2		Step 2 (Verify curve fitting Options and Standard Curve Options).
Step 3		Step 3 (View result data, display standard curves, data review and presentations, data report).
Open		Open a saved Status (.blpx) file.
Save		Click the icon: Save all data.
		Select from the dropdown list: Save, FCS-VG files, Status files or Result Data Files separately.
Save Result Data		Save Result Data only.
Option		Change standard curve-fitting options. Define outliers, and display options.
Flag		Flag selective samples or data points from calculation. The raw data remains intact.
UnFlag		Cancel the flag.

Auto Flag		Auto flag the data.
Clean All Flag		Clean all flag.
Run		Start or re-start data analysis process or start data analysis of selective samples.
Gate		Start gate settings.
Standard Curve		View individual Standard Curves on the screen.
Bar Chart		View Bar Chart. Selected data can be visualized in 2D bar chart format.
Heatmap		View heatmap of a selected data range.
Clustering		View clustering map of a selected data range.
About		Display the **software version** information.

Related topics:

- System Requirement
- Installation
- Start LEGENDplex™

Files

Status File

A status file contains the information about the status of your computer and file working environment including all the optional settings, files layout, manual mark-outs, completed or partially completed analysis of a data set. An analysis can be saved as a status (*.blpx) file, which can be opened later. Working environment will be reset when exiting the software, but a status file will memorize how that data were analyzed.

A status file (*.blpx) contains the .fcs files (or .lmd files), manual flag information, optional settings. This feature supports both Quantitative Analysis and Qualitative Analysis.

- **Save A Status File**

  Click the arrow  and select **Status File** from the drop down list to save a status file.

- **Load A Status File**

  Click  on the Toolbar to load a status file.

Related topics:

- FCS-VG File
- Start LEGENDplex™
- Installation
- Wizard Step 1

FCS-VG File

The FCS-VG files are generated once you generate a report or save any result data.
The FCS-VG files can be re-analyzed further without consuming additional dongle data points from a dongle license

See also Add Files for how to load FCS-VG files.

Related topics:

- Status File
- Start LEGENDplex™
- Installation
- Wizard Step 1
- FCS Files List
- Add Files

Data Analysis

Quantitative Analysis

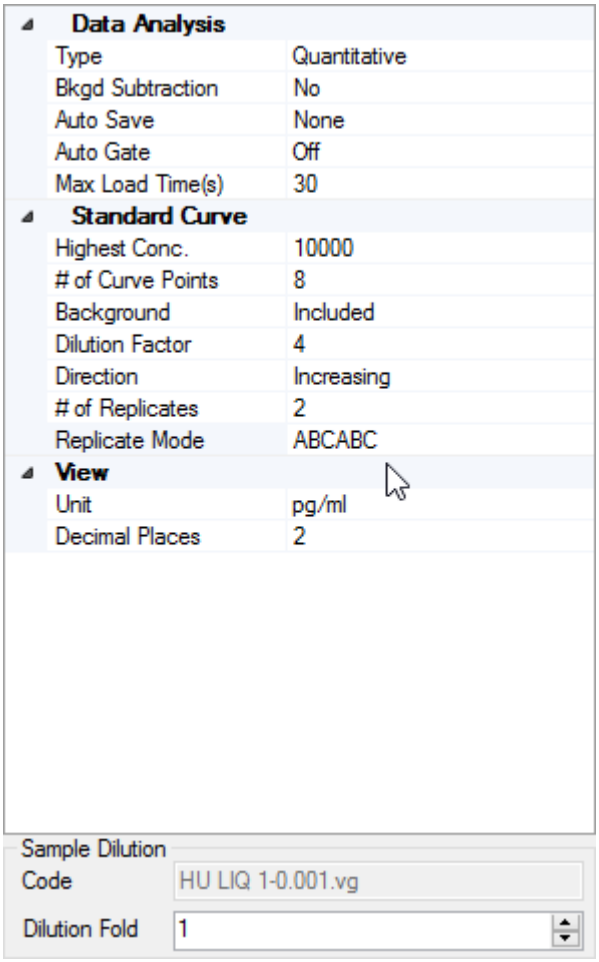
Quantitative Analysis Wizard

Quantitative Settings

Quantitative settings include information for sample IDs, file names, analyte names, standard concentrations, etc.

The quantitative settings are used to analyze raw data. It can be defined on the right panel after the files have been loaded at Wizard Step 1

Select **Quantitative** from the dropdown list under **Type**. The parameters for quantitative analysis are displayed as shown below:



Data Analysis

- **Quantitative ---** An analysis method to calculate sample signals and concentrations using standard curves.

- **Background ("Bkgd") subtraction ---** The background noise subtraction (signal intensities of the background non-specific binding noise) can be subtracted from raw data prior to data analysis. If zero concentration the Standard curve (C0) is present, the signal intensities of C0 will be subtracted from the signal intensities in all remaining files including remaining standard files and sample files. If the signal intensity of an analyte becomes negative after subtraction, the signal intensity will be mapped to a value between 0 and 1 in the same numerical order as the original data.

  Note: If the standard curve of an analyte is downward, background subtraction will be skipped and a message will pop up to show the analyte name.

- **Auto Save** --- Select Auto Save type (The default setting is **None**). Auto Save will automatically save results and status files and consume data points from a license dongle.
  - **None:** Not to automatically save anything after analysis is complete.
  - **Data:** Save the result data after analysis is complete.
  - **Report:** Save the default report in the same file folder as raw data after analysis is complete.
  - **Both:** Save result data and the default report in the same file folder as raw data after analysis is complete.
- **Auto Gate ---** When auto gating is **ON** it will always gate automatically with each file. When it's set at **OFF**, the first gating result will be used as a template and applied it to other files in the same analysis. When Auto Gate is ON, it will take longer time to perform the data calculations.
- **Max Load Time ---** The maximum time to load.

Standard Curve

- **Highest Conc. --- The highest concentration of the standard curves. If not all analytes share the same highest concentration, just use a concentration**

that works for most analytes. The highest concentrations for individual analytes can be adjusted in the Modify Standard Concentration before analysis.

- **# of Curve Points** --- The number of standard curve points in the standard curve that includes the background well, the number of standard points should be bigger than 5 in order to use the 5 or 4-parameter logistic curve-fitting methods.
- **Background (C 0)** --- Choose whether the background noise is included or excluded in the standard curve and sample calculations.
- **Dilution Factor** --- Serial dilution factor for standard dilutions.
- **Direction** --- Standard curve dilution direction: **Increasing** or **Decreasing**. Whether a curve is Increasing or Decreasing is relative to the sequential order of the standard concentrations increase or decrease in the FCS file selections. If the sequential order of the highlighted files is the same as the order from C0 to C7, the Direction should set as Increasing. If the sequential order of the highlighted files is from C7 to C0, the direction should be Decreasing.
- **# of Replicates** --- The number of replicates for each standard point. Sample replicates can be defined in the file list.
- **Replicate Mode** --- Select a replicate mode for standard curve.
  - **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 group size (e.g. sample 1, 2, 3, 4…; sample 1, 2, 3, 4…).

View

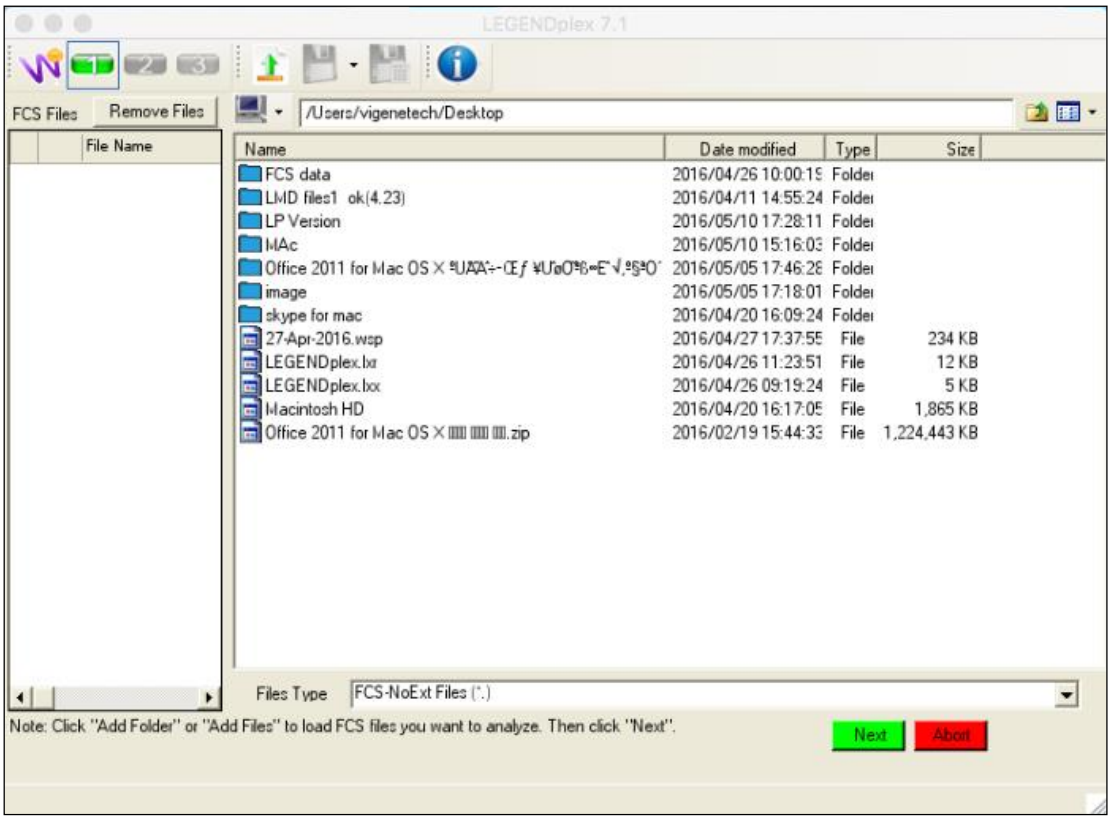
- **Decimal Places** --- The decimal places to keep for all the calculated numbers in the program
- **Unit** --- The default unit for calculated concentrations for all analytes. If different analytes have different units, they can be defined in Gate settings.

Related topics:

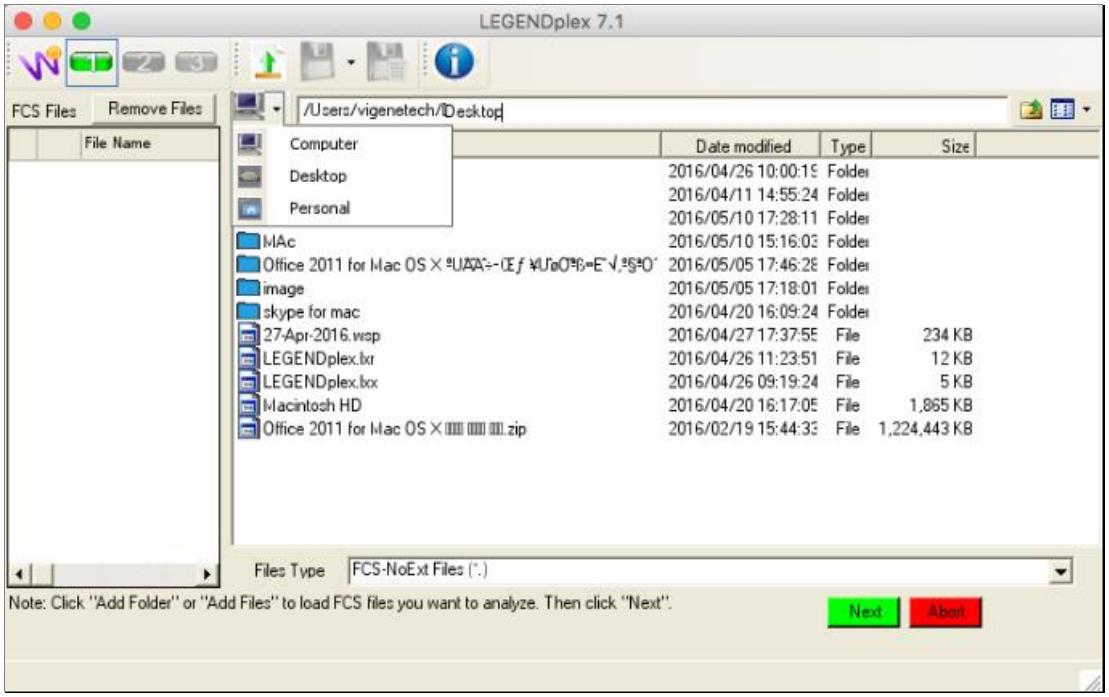
- Wizard Step 1
- Wizard Step 2
- Wizard Step 3
- Start LEGENDplex™
- Edit Standard Curve

Wizard Step 1

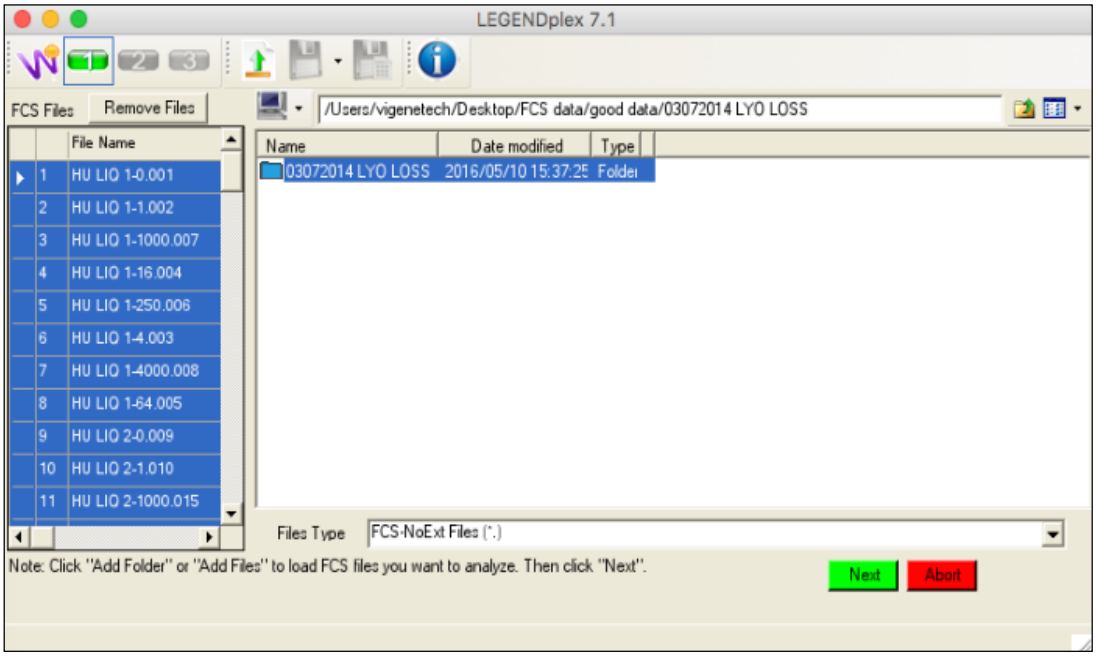
1. Click  to start a new wizard session.



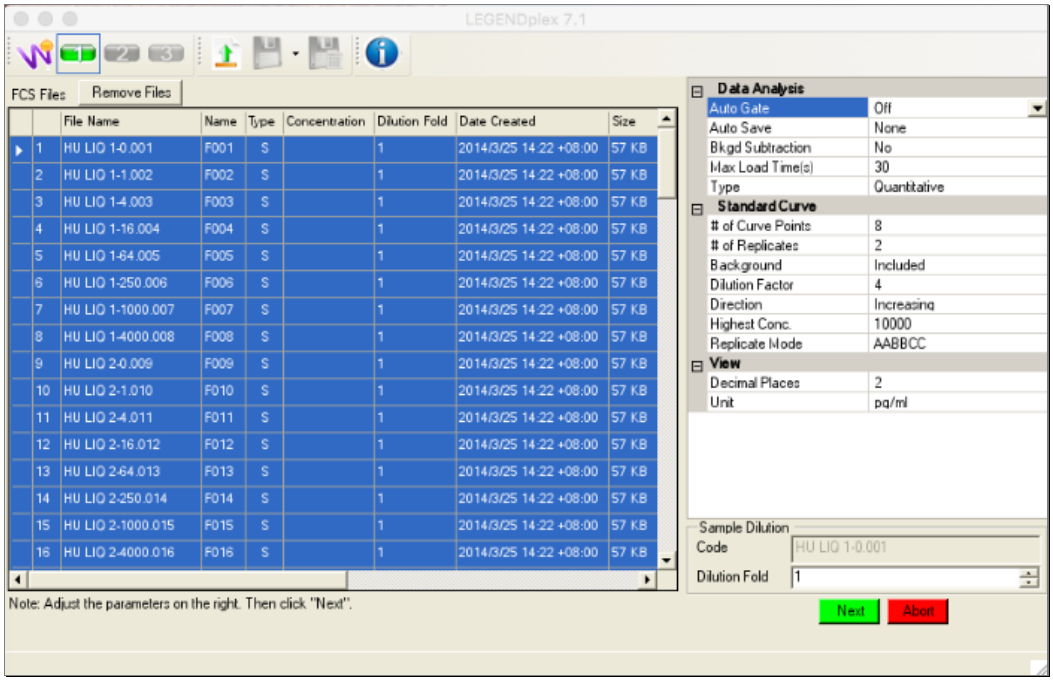
2. Click the dropdown list of my computer  .



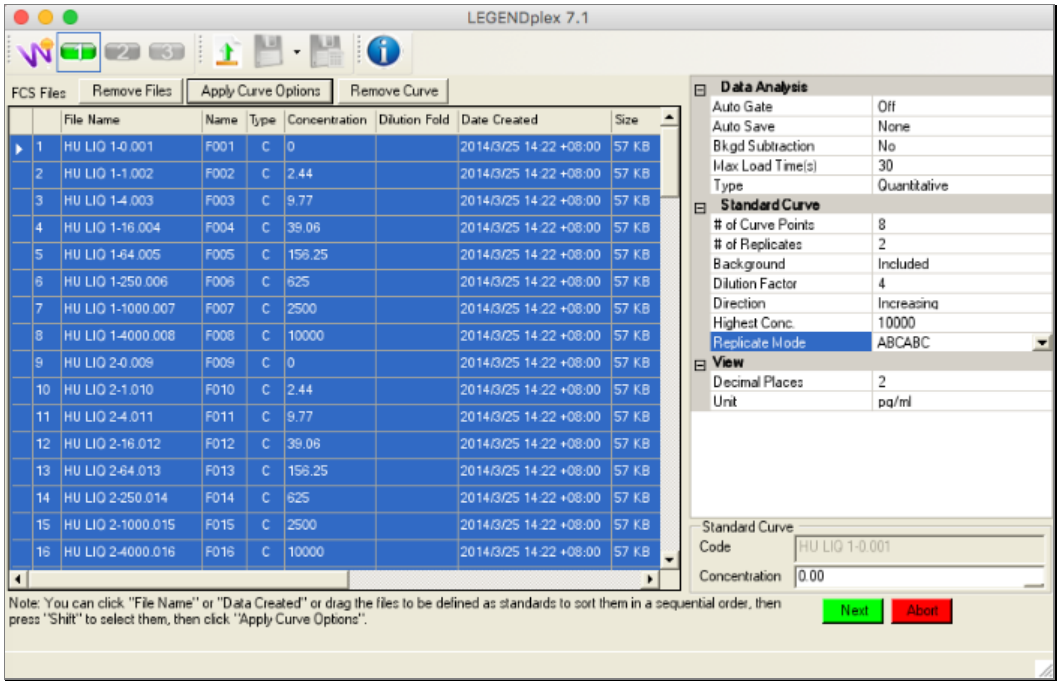
3. Select a file folder containing the FCS files to be analyzed and drag the folder to the left side of the screen. The files will be automatically loaded onto the blank space in the program. The folder can be opened and selective files can be dragged in the same manner.



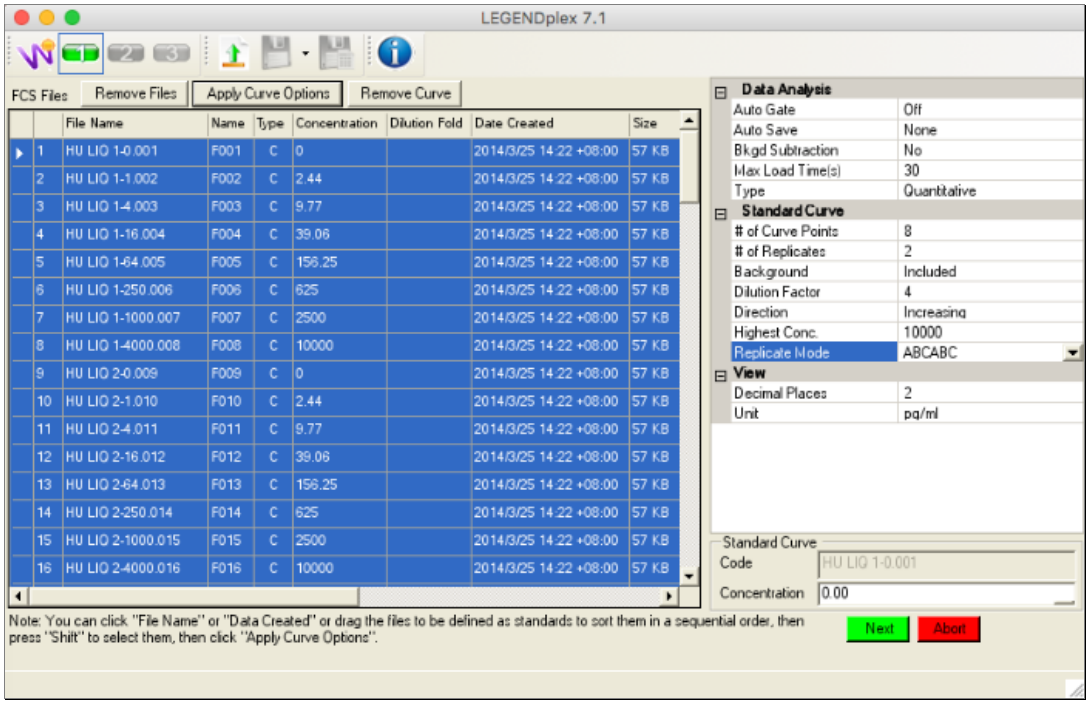
4. Click **Next**. On the right side of the window, a dialog setting box will appear. Adjust the parameters in Data Analysis settings according to the assay protocols and file arrangement.



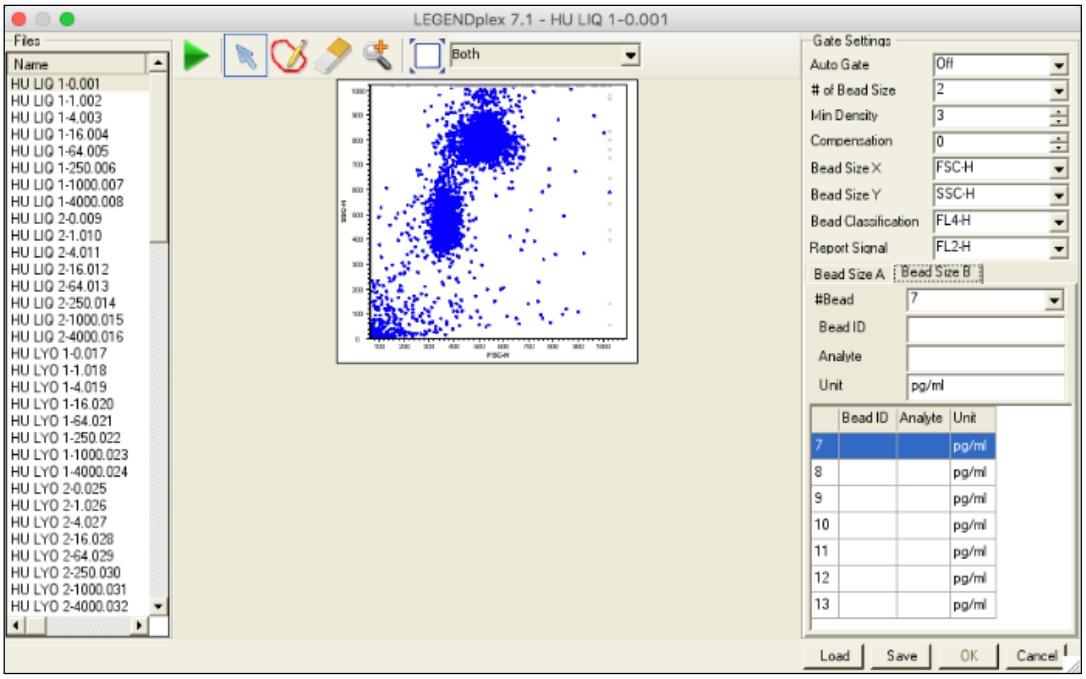
5. Click **Next.** Click "File Name" or "Data Created" or drag the files to sort them in a sequential order, then press "Shift" to select them, then click **Apply Curve Options**.



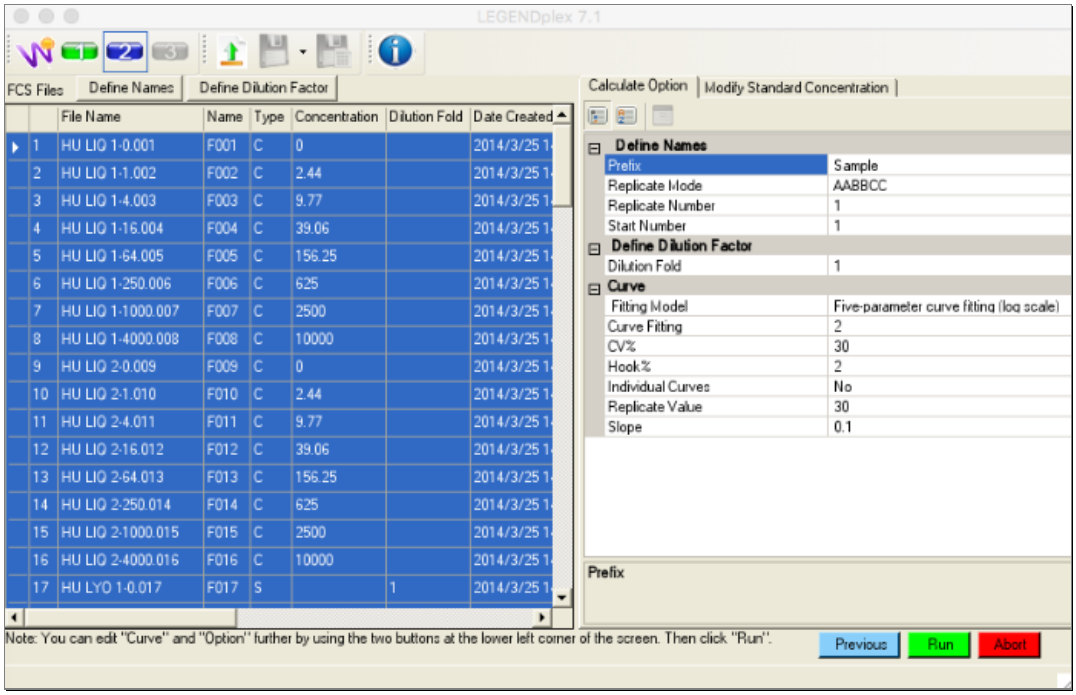
6. Standard concentrations will be added to the corresponding files. Check to make sure that the concentrations are applied correctly. If necessary, the added values can be removed by using **Remove Curve** button.



7. Click **Next**, the Bead Gating window will pop up automatically. Load Gate Protocol files or change Gate Setting if necessary. Click  , do Gating.



8. Click **OK** after gating is finished.



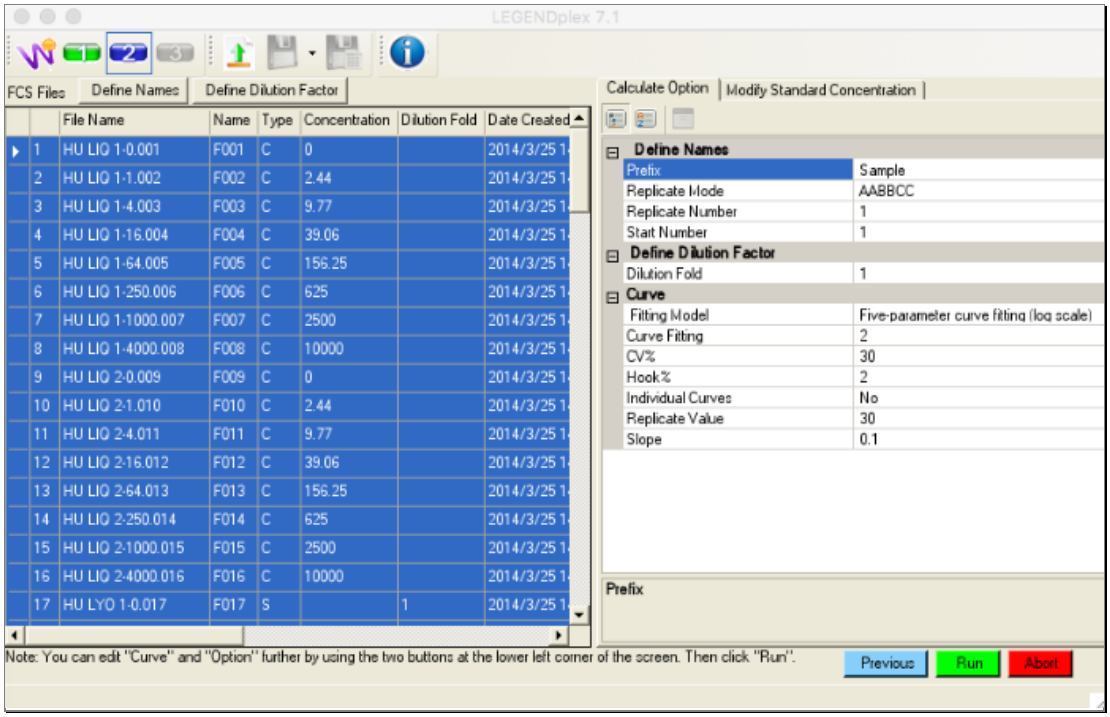
9. You can edit curve-fitting options" and/or "Standard Curve" further by using the two tabs "**Calculate Option"** and "**Modify Standard Concentration"** at the right side of the window. Then click **Run**.

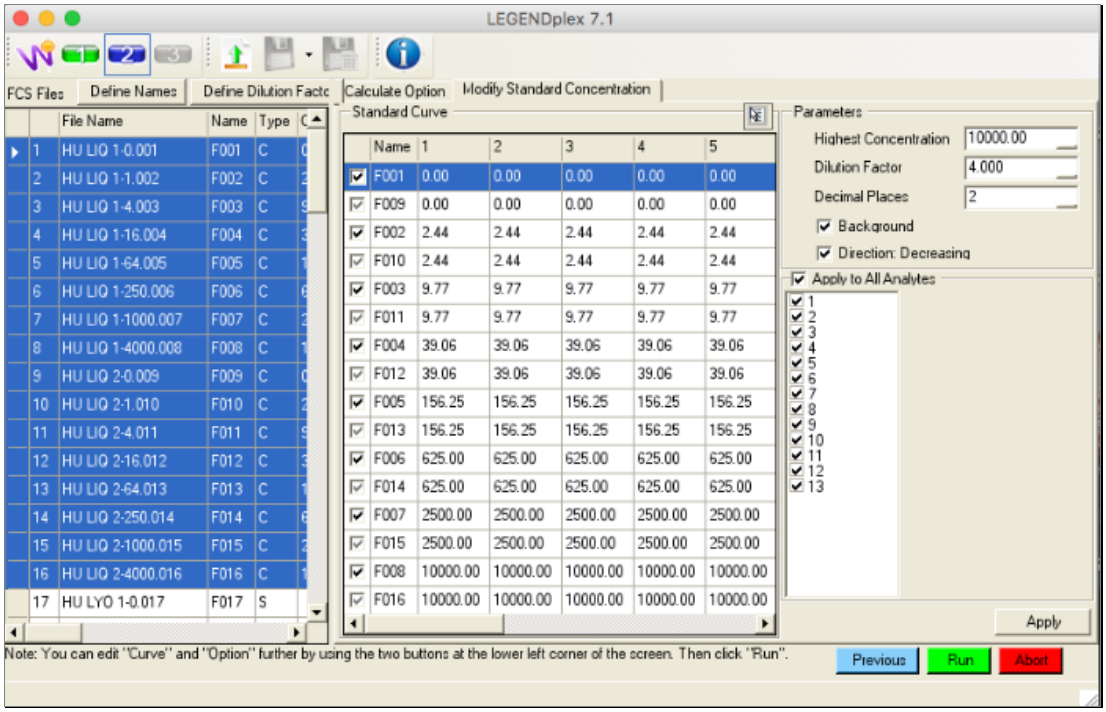
Related topics:

- Wizard Step 2
- Wizard Step 3
- Quantitative Settings

Wizard Step 2

1. In this step, you can edit "Option" and "Standard Curve" further by using the two buttons **Calculate Option** and **Modify Standard Concentration** at the right side of the window.
   - Edit Standard Curve
   - Edit Options
   - Flag Data





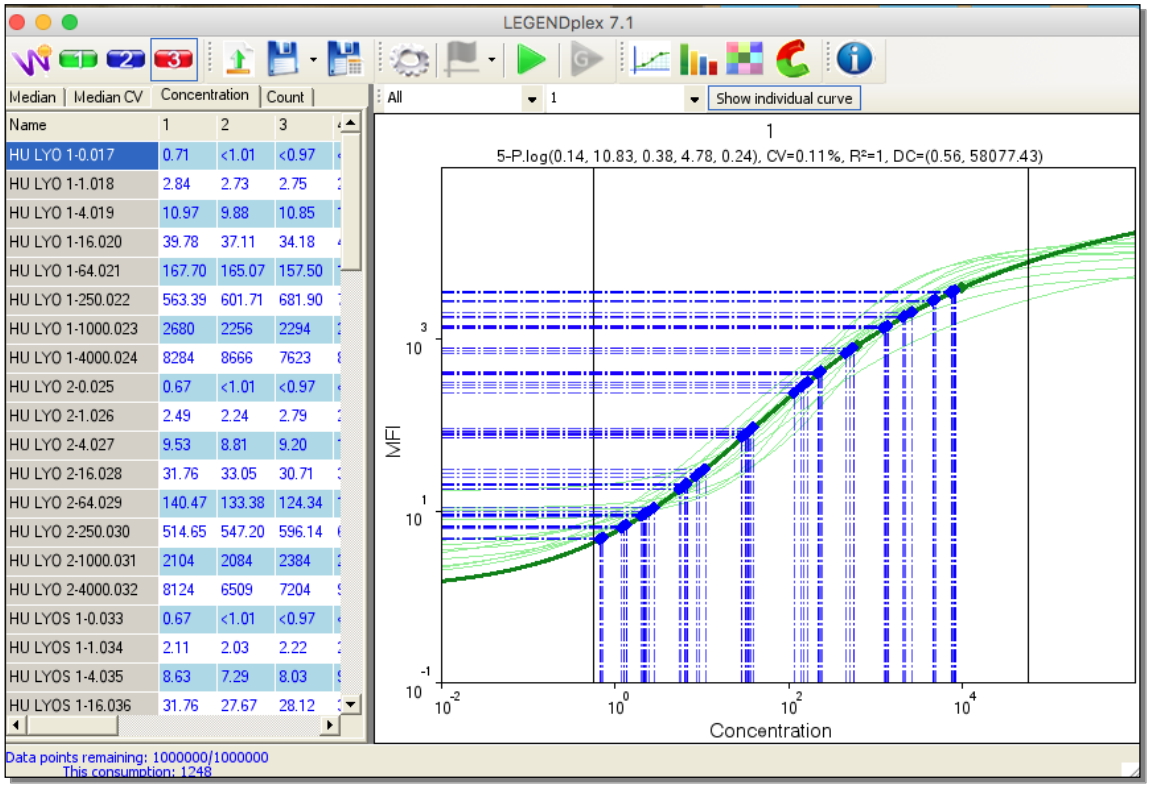
2. Click **Run**.

Related topics:

- Quantitative Settings
- Wizard Step1
- Wizard Step 3

Wizard Step 3

The result will be displayed as below.



If you know that there is gate shift for selected files, you can highlight selective files and perform gating again to obtain better result for the selected files.

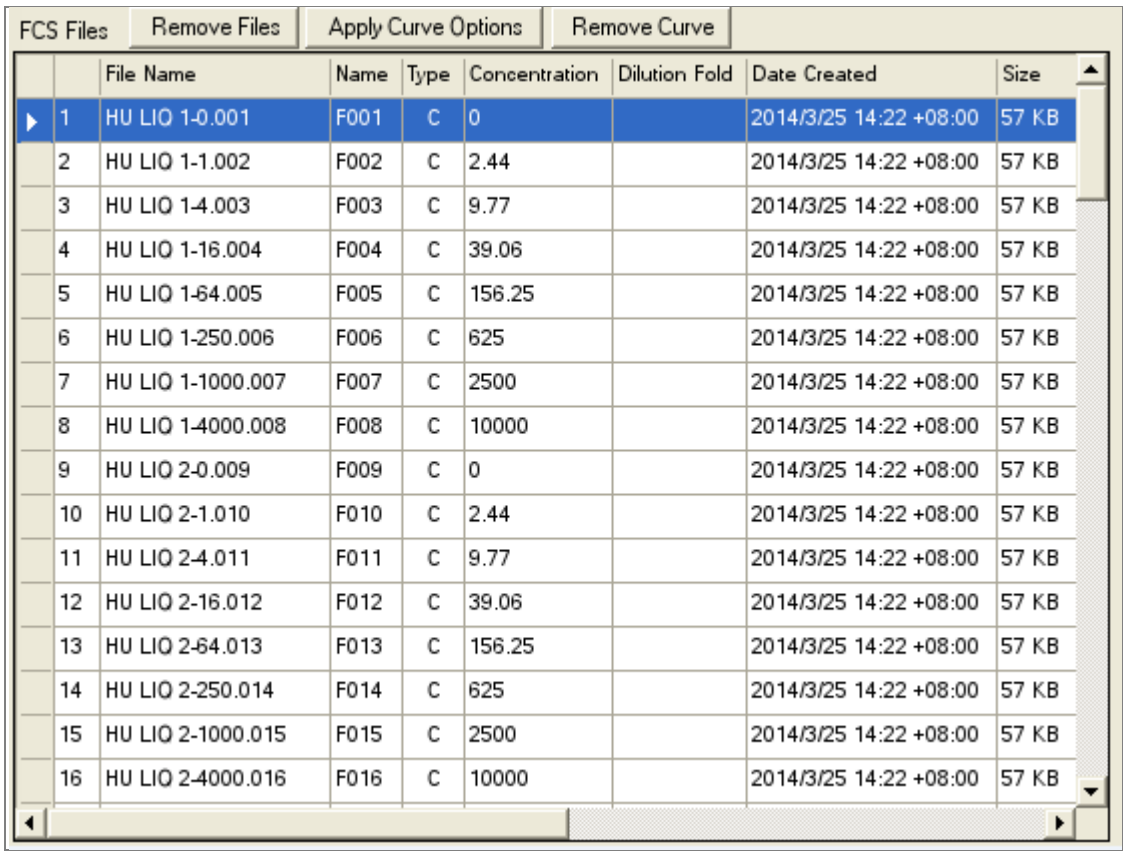
Related topics:

- Wizard Step1
- Wizard Step 2
- Quantitative Settings

Quantitative Operation Items

Quantitative FCS Files List

The Quantitative **FCS Files** list is a place where standard and sample files are displayed and edited. It lists the FCS files information including File **Name**, **Name**, **Type**, **Concentration**, **Dilution Fold**, **Data Created Size,** and **File Directory**.

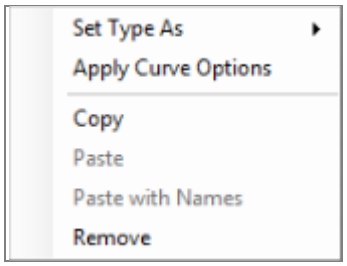


	File Name	Name	Type	Concentration	Dilution Fold	Date Created	Size
1	HU LIQ 1-0.001	F001	C	0		2014/3/25 14:22 +08:00	57 KB
2	HU LIQ 1-1.002	F002	C	2.44		2014/3/25 14:22 +08:00	57 KB
3	HU LIQ 1-4.003	F003	C	9.77		2014/3/25 14:22 +08:00	57 KB
4	HU LIQ 1-16.004	F004	C	39.06		2014/3/25 14:22 +08:00	57 KB
5	HU LIQ 1-64.005	F005	C	156.25		2014/3/25 14:22 +08:00	57 KB
6	HU LIQ 1-250.006	F006	C	625		2014/3/25 14:22 +08:00	57 KB
7	HU LIQ 1-1000.007	F007	C	2500		2014/3/25 14:22 +08:00	57 KB
8	HU LIQ 1-4000.008	F008	C	10000		2014/3/25 14:22 +08:00	57 KB
9	HU LIQ 2-0.009	F009	C	0		2014/3/25 14:22 +08:00	57 KB
10	HU LIQ 2-1.010	F010	C	2.44		2014/3/25 14:22 +08:00	57 KB
11	HU LIQ 2-4.011	F011	C	9.77		2014/3/25 14:22 +08:00	57 KB
12	HU LIQ 2-16.012	F012	C	39.06		2014/3/25 14:22 +08:00	57 KB
13	HU LIQ 2-64.013	F013	C	156.25		2014/3/25 14:22 +08:00	57 KB
14	HU LIQ 2-250.014	F014	C	625		2014/3/25 14:22 +08:00	57 KB
15	HU LIQ 2-1000.015	F015	C	2500		2014/3/25 14:22 +08:00	57 KB
16	HU LIQ 2-4000.016	F016	C	10000		2014/3/25 14:22 +08:00	57 KB

On top of the list, there're some small icons which provide functions to manipulate the files.

- Remove Files
- Define Names
- Define Dilution Factor
- Remove Curve

Right click the mouse in the list to display popup menu as below:

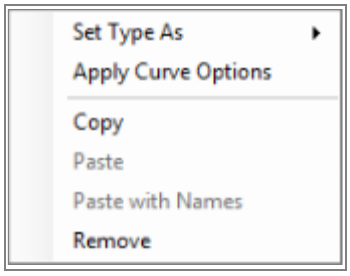


- **Set Type As**: Choose a type (S, Background, C0, C1, C2...) for current file.
- Apply Curve Options
- **Copy**: Copy the selected data.
- **Paste:** Paste the type and concentration.
- **Paste with Names**: Paste the type, concentration and code.
- **Remove**: Remove the file.

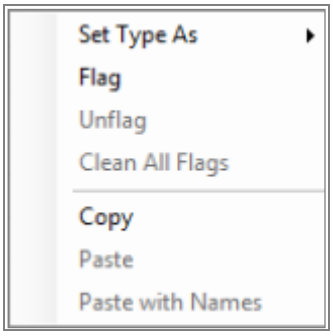
Standard Curve Files

Standard Curve files need to be predefined in Quantitative Settings before the quantitative data analysis and they're marked with **"C" type**.

Right click on the standard curve files to display the popup menu.



Step 1



Step 2

- **Set Type As**: Choose a type (Sample, Background, C0, C1, C2...) for current file.
- Flag, Unflag, Clean All Flags
- **Copy**: Copy the selected data.
- **Paste:** Paste the type and concentration.
- **Paste with Names**: Paste the type, concentration and code.

Related topics:

- Wizard Step 1
- Edit Sample ID

- Set Standard Curve
- Edit Standard Curve

Sample Files

Right after loading FCS files to the FCS Files list, all files are shown by default "S" in the **Type** column, indicating that they are samples. For the files corresponding to standard curves, the file type can be changed by defining Standard curves and Define Dilution Factor in Settings.

The files with S in the **Type** column indicate samples and with C in the **Type** column indicating a Standard.

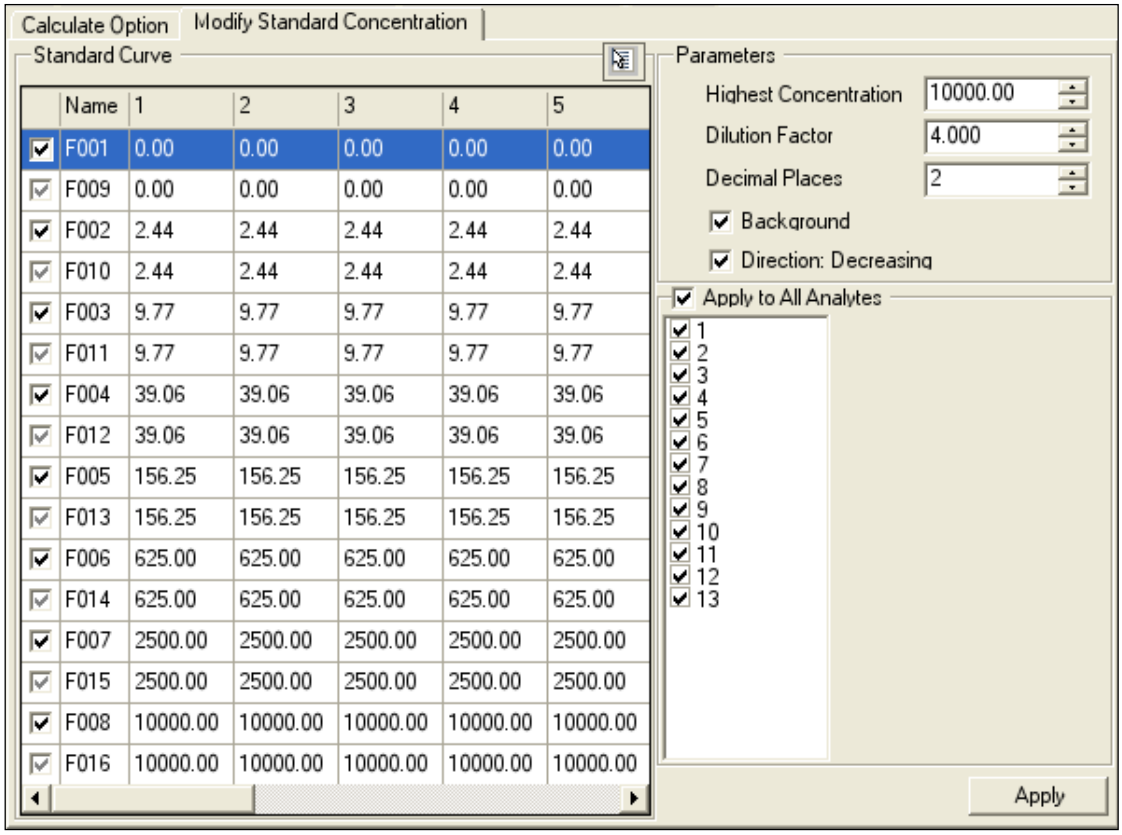
Change the following if necessary:

- **Name** --- Define the samples' name with series by Define Names.
- **Dilution Fold** --- Define Dilution Factor if the sample is diluted.

Modify Standard Concentration Dialog box

At Wizard Step 2, click Curve Tab to display the **Modify Standard Concentration** setting dialog box as below:

It contains three setting sections: Standard Curve List, Parameters, and Analyte Lists.



	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

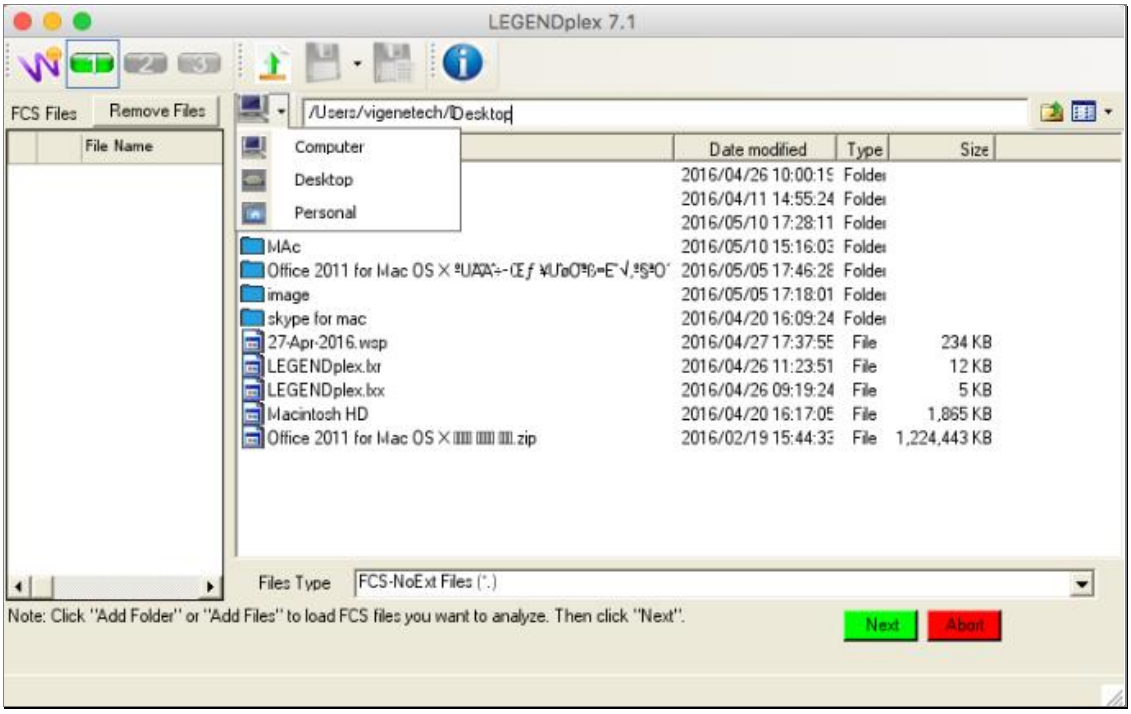
Related topics:

- Remove Standard Curve
- Set Standard Curve
- Edit Standard Curve

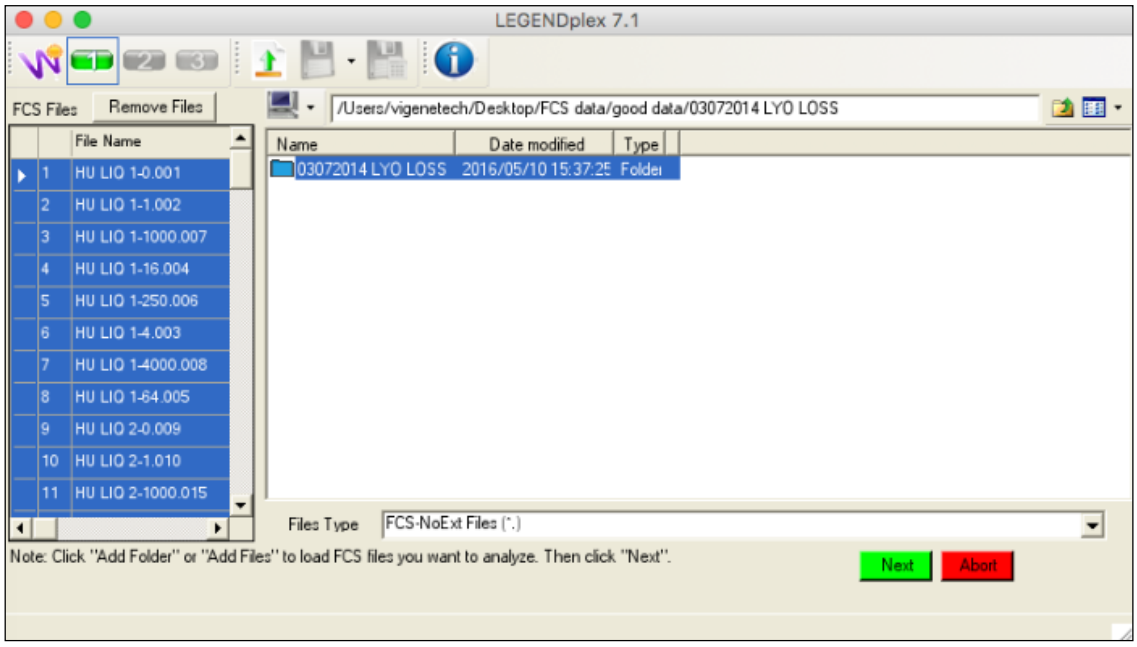
Add FCS Files

Click  to find the folder you need. Select the .fcs files and drag them to the **FCS Files** list. Select **FCS-VG Files (*.vg)** from the **File Type** drop down list to load the FCS-VG files.

Alternatively, you can drag a file folder containing the .fcs files to be analyzed to the left, LEGENDplex™ will load all the .fcs files in this file folder and automatically filters non .fcs files.

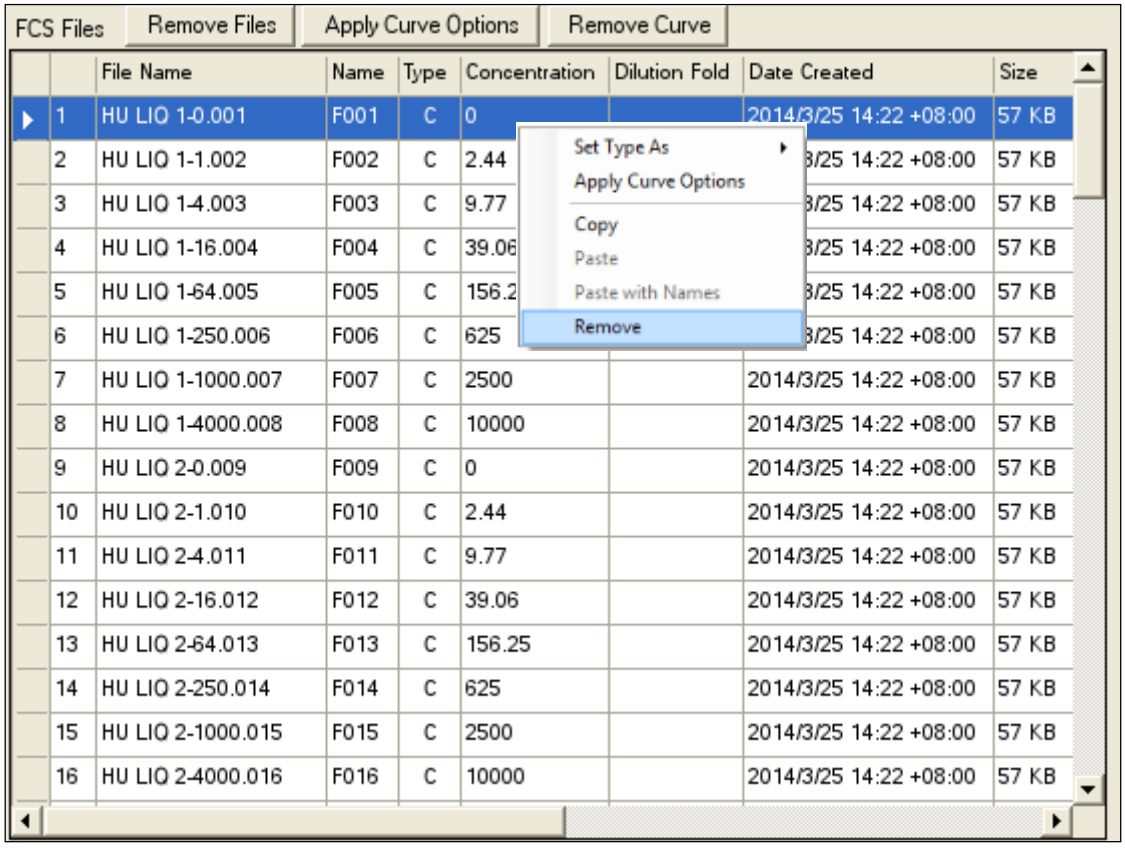


Drag the folder with all the files you need or select needed files to the left.



Remove Files

At Wizard Step 1, select one or more files in the FCS Files list, right click press **Remove** on the dropdown list to remove them.



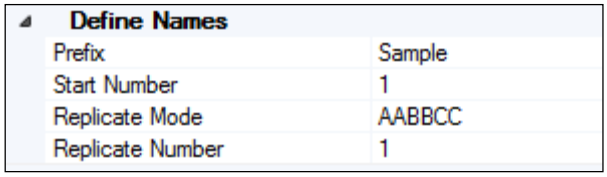
	File Name	Name	Type	Concentration	Dilution Fold	Date Created	Size
1	HU LIO 1-0.001	F001	C	0		2014/3/25 14:22 +08:00	57 KB
2	HU LIO 1-1.002	F002	C	2.44		2014/3/25 14:22 +08:00	57 KB
3	HU LIO 1-4.003	F003	C	9.77		2014/3/25 14:22 +08:00	57 KB
4	HU LIO 1-16.004	F004	C	39.06		2014/3/25 14:22 +08:00	57 KB
5	HU LIO 1-64.005	F005	C	156.2		2014/3/25 14:22 +08:00	57 KB
6	HU LIO 1-250.006	F006	C	625		2014/3/25 14:22 +08:00	57 KB
7	HU LIO 1-1000.007	F007	C	2500		2014/3/25 14:22 +08:00	57 KB
8	HU LIO 1-4000.008	F008	C	10000		2014/3/25 14:22 +08:00	57 KB
9	HU LIO 2-0.009	F009	C	0		2014/3/25 14:22 +08:00	57 KB
10	HU LIO 2-1.010	F010	C	2.44		2014/3/25 14:22 +08:00	57 KB
11	HU LIO 2-4.011	F011	C	9.77		2014/3/25 14:22 +08:00	57 KB
12	HU LIO 2-16.012	F012	C	39.06		2014/3/25 14:22 +08:00	57 KB
13	HU LIO 2-64.013	F013	C	156.25		2014/3/25 14:22 +08:00	57 KB
14	HU LIO 2-250.014	F014	C	625		2014/3/25 14:22 +08:00	57 KB
15	HU LIO 2-1000.015	F015	C	2500		2014/3/25 14:22 +08:00	57 KB
16	HU LIO 2-4000.016	F016	C	10000		2014/3/25 14:22 +08:00	57 KB

Related topics:

- Sample Files
- Remove Standard Curve
- Edit Standard Curve

Define Names

The default sample name is the original file name by default. However, default sample names can be modified.
At Wizard Step 2, highlight a file or multiple files in the FCS Files List then in the Calculate Option, enter the Prefix, Replicate Mode, Replicate Number and Start Number in the Define Number section, and click **Define Names** button on top of the File List table.



- **Prefix** --- Enter a **Prefix** of sample name series (The default is Sample).
- **Start Number** --- Enter a starting number of sample name Series.
- **Replicate Mode** --- Select a **Replicate Mode**: **AABBCC** or **ABCABC**. The **AABBCC** mode will replicate samples in consecutive manner when number of replicates is defined. The **ABCABC** mode will replicate samples in repeating groups with defined size.
- **Replicate Number** --- Enter number of replicate for the selected samples.

Related topics:

- Sample Files
- FCS Files List
- Wizard Step 2
- Wizard Step 1

Define Dilution Factor

Select a file or multiple files in the Quantitative FCS Files List or Qualitative FCS Files List, set the **Dilution Fold** in Calculate Option. Then click **Define Dilution Factor** button.

- **Dilution Fold:** Input the dilution factor value.

Related topics:

- Sample Files
- FCS Files List
- Wizard Step 2
- Wizard Step 1

Flag Data

Flagging a file can be achieved in two methods: Flag and Auto Flag.

- **Flag**: FCS files corresponding to selected samples can be flagged. The flagged files will not be analyzed (regardless whether it has been determined to be an outlier from the algorithm during analysis). The data outliers can be removed by using a manual flagging function.
- **Auto Flag:** Data will be flagged as an outlier by the algorithm parameters set in Curve Option during the analysis process.

The flagged data will only have **Median** value displayed in results.

Flagging data:

- Select one or a group of samples, right click the data and select **Flag** from the popup menu, or

UnFlag Data:

- Select one or a group of flagged samples, right click the data and select **UnFlag** from the popup menu, or

Reset the data back to Auto Flag state:

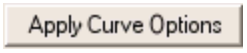
Select one or a group of samples, right click the data and select **Auto Flag** from the popup menu.

Data Sorting

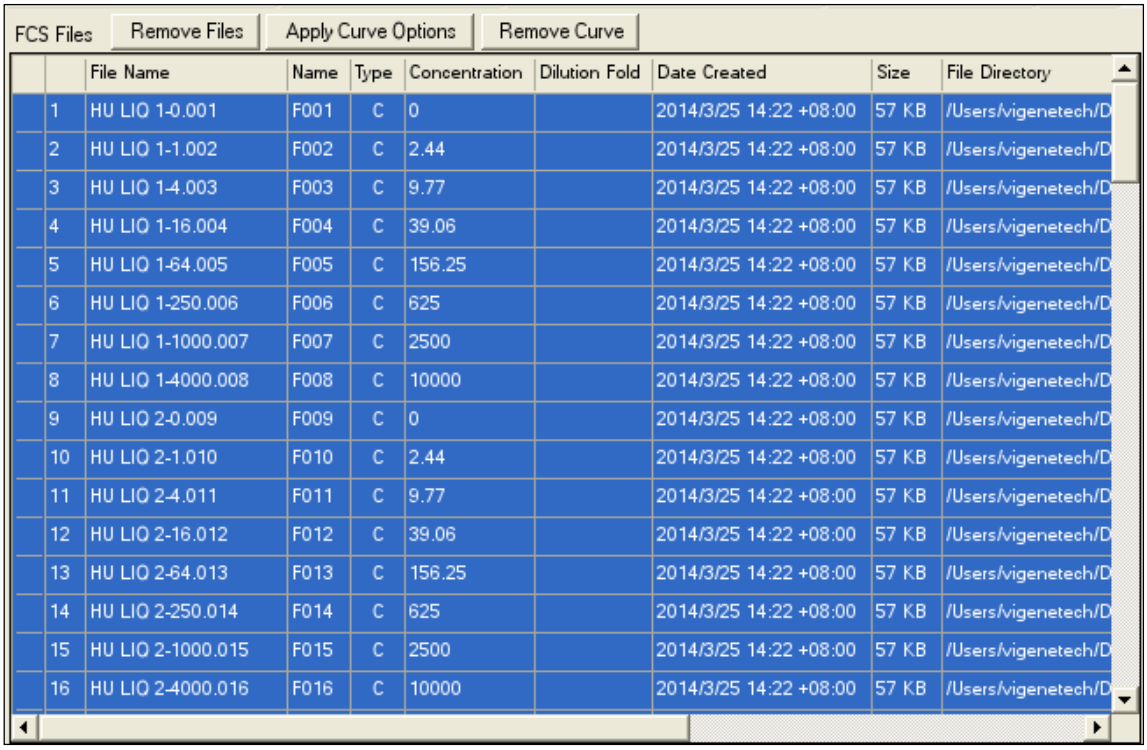
In the **FCS File** list, the column title such as **File Name**, **Data Created** and **Size** can be used to sort the files in ascending/descending order. Common use of the sorting function is to arrange the order of files for standard curves to match with experimental layout so that data can be calculated correctly.

Alternatively, files or a file can be dragged and moved to a different location within the file list by using a mouse.

Set Standard Curve

If the FCS files for the standard curve are located consecutively and the related parameters are correctly set in Settings, drag the mouse to select all the files that you want to include, click 

or right click and select **Apply Curve Options** to apply the standard curve parameters according to **Settings**.



	File Name	Name	Type	Concentration	Dilution Fold	Date Created	Size	File Directory
1	HU LIO 1-0.001	F001	C	0		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D
2	HU LIO 1-1.002	F002	C	2.44		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D
3	HU LIO 1-4.003	F003	C	9.77		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D
4	HU LIO 1-16.004	F004	C	39.06		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D
5	HU LIO 1-64.005	F005	C	156.25		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D
6	HU LIO 1-250.006	F006	C	625		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D
7	HU LIO 1-1000.007	F007	C	2500		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D
8	HU LIO 1-4000.008	F008	C	10000		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D
9	HU LIO 2-0.009	F009	C	0		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D
10	HU LIO 2-1.010	F010	C	2.44		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D
11	HU LIO 2-4.011	F011	C	9.77		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D
12	HU LIO 2-16.012	F012	C	39.06		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D
13	HU LIO 2-64.013	F013	C	156.25		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D
14	HU LIO 2-250.014	F014	C	625		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D
15	HU LIO 2-1000.015	F015	C	2500		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D
16	HU LIO 2-4000.016	F016	C	10000		2014/3/25 14:22 +08:00	57 KB	/Users/vigenetech/D

Click "File Name" or "Data Created" or drag the files to sort them in a sequential order, then press "Shift" to select them, and then click "**Apply Curve Options**". Alternatively, you may set type as C0, C1, C2, …C7 to appropriate files corresponding to the standard curve point.

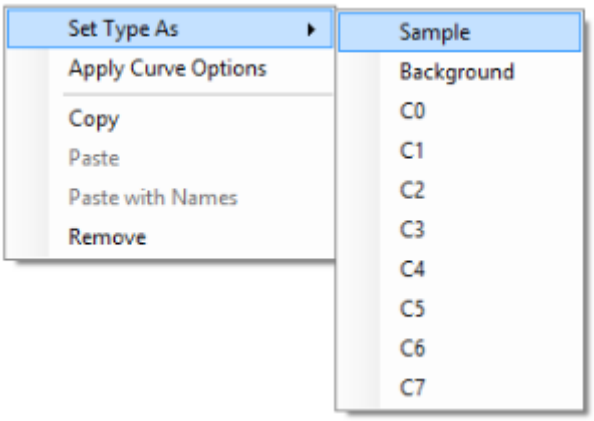
Related topics:

- Standard Curve Files
- FCS Files List
- Modify Standard Concentration Dialog Box
- Remove Standard Curve
- Set Standard Curve Manually

Set Standard Curve Manually

If not all the FCS files for the standard curve are consecutively located in the file list, select individual file or multiple files (if consecutive) and right click to set the type as C0, C1, C2…. You can change the concentration in the setting tab.

.



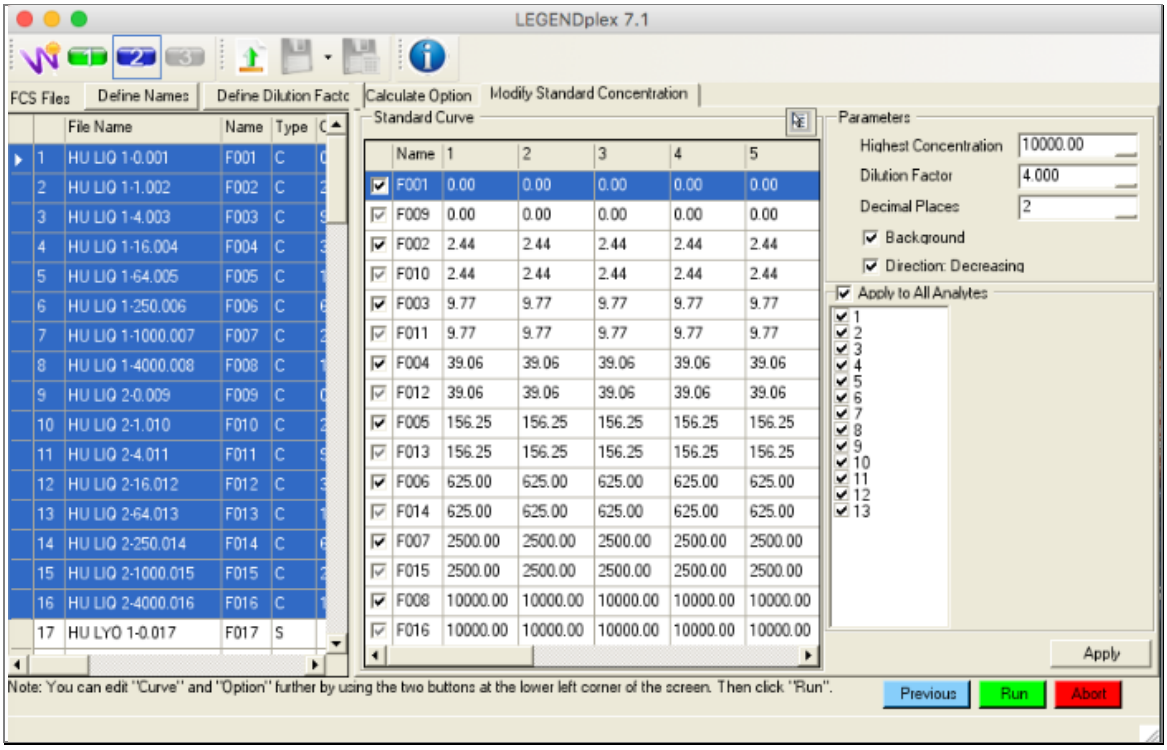
Or select the file whose type is C and input the concentration you need in the setting tab.

Remove Standard Curve

Click Remove Curve on top of the **FCS Files** list to remove the standard curve for setting a new standard curve.

Edit Standard Curve

From the Modify Standard Concentration, you can define the standard curve further, group the files by dilution level, sort and list the standard curve files in dilution series, and set up starting concentration.



Step1: Open **Edit Standard Curve** tab.

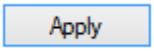
- Click the Modify Standard Concentration button on the right side of the screen to open the **Edit Standard Curve** tab.

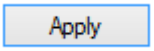
Step2:

- Verify or redefine the standard curve well list in the left panel (Standard Curve).
- Select the check boxes in front of file IDs. If the box is grey, it is the replicate of the file immediately above it.

Step3:

- Enter a value for highest concentration, dilution factor for the standard curve and how many decimal digits for the calibrated sample concentrations.

- Check **Background (C0)** check box to indicate the standard curve files include background files. Uncheck it to indicate the standard curve files do not include background files.
- Check **Direction: Increasing** to define standards curve files have a serial dilutions from low to high (upward curve shape); or Check Decreasing for standard concentrations from high to low (downward curve shape). Increasing is used most often with typical sandwich assays involving S shaped curves. **Decreasing** is often used for competition assay curves.
- If all analytes have the same starting concentrations, select **Apply To All Analytes** check box (default). Click 

button to continue.
- If not all analytes have the same starting concentrations, uncheck the box for **Apply To All Analytes**, select the analytes which share the same concentrations, enter the concentration in the **Highest Concentration** box and then click 

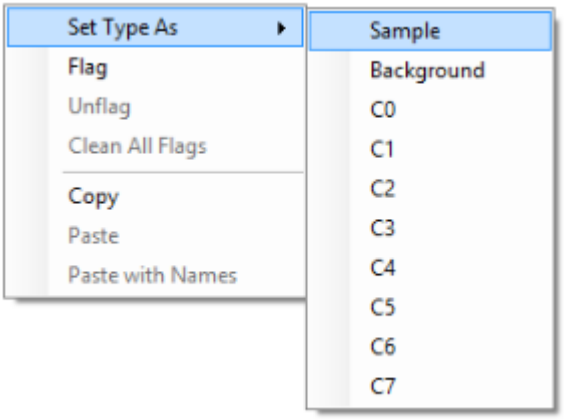
.Repeat the same operations for other targets which have different highest concentrations. When all analytes are defined, click **RUN** button to continue.
- If not all Analytes have the same starting concentration, uncheck the box labeled as Apply to All Analytes. Then enter a different concentration for the analyte(s) that differ from others and click Apply. Check the dilution table to make sure concentrations are correct.

Related topics:

- Set Standard Curve
- Set Standard Curve Manually
- Modify Standard Concentration Dialog Box

Convert To Sample

Select standard curve files, right click to show popup menu and select **Set Type as -> S** to convert the selected files to sample files.

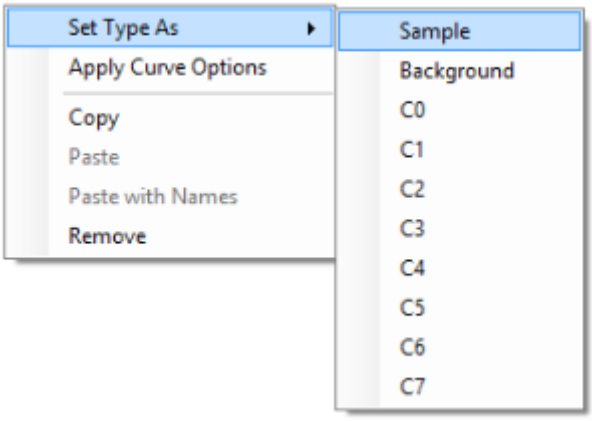


Related topics:

- Calibrator Files
- Sample Files
- FCS Files List

Convert To Standard Curve

Select some sample files, right click to show the popup menu and select **Set Type as** and select one of **C0, C1, ..., C7** to convert the selected files to standard curve files.
Note: The number of **C0, C1, ..., C7** is determined by **# of Curve Points**.



Related topics:

- Calibrator Files
- Sample Files
- FCS Files List

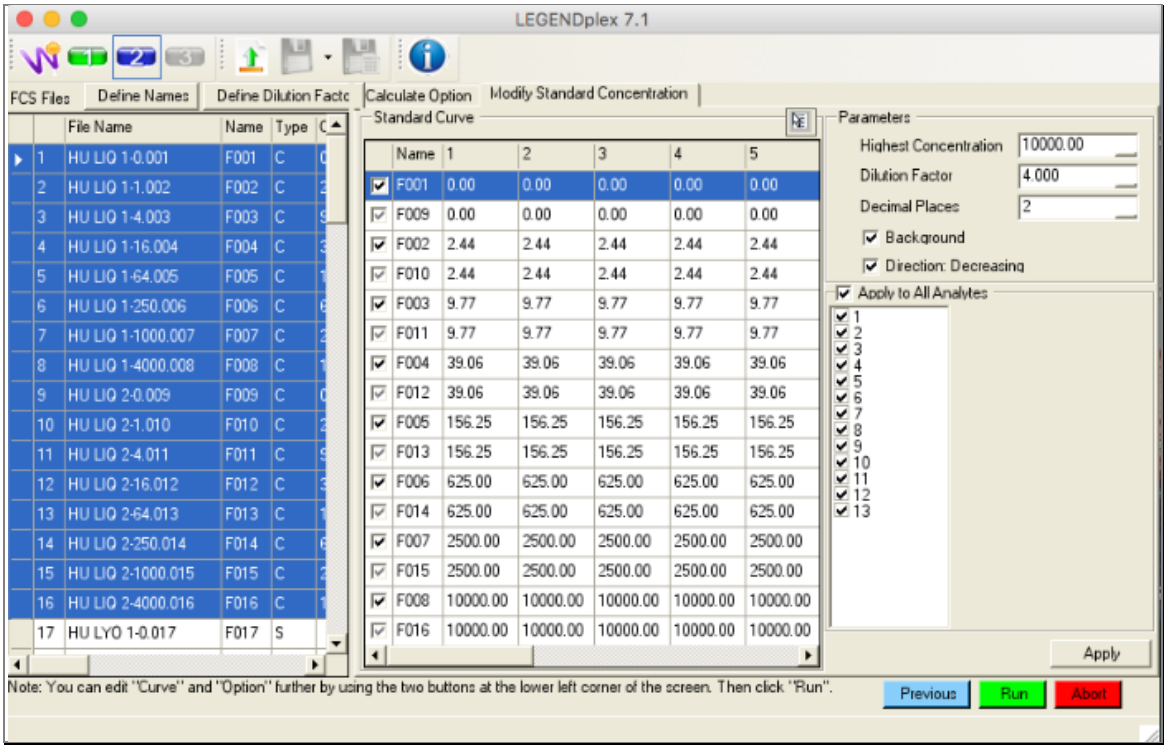
Replicates

Sample replicates can be defined by:

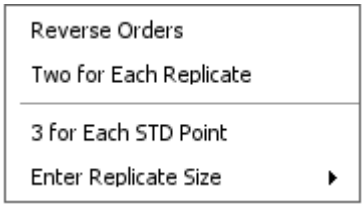
Highlight samples, click on the **Define Names**. Define the **Replicate Mode** and **Replicate Number**.

Standard Curve-Set Standard Well Dilution Order

Click Modify Standard Concentration Dialog button on the middle of the screen to open the Modify Standard Concentration Dialog box. The **Standard Curve** section is shown as below:



Click  on the standard curve, a popup menu will be displayed:



- **Reverse Orders** --- Reverse the standard curve file dilution order.
- **Two for Each STD Point** --- Set the standard files as two replicates for each dilution level.
- **3 for Each STD Point** --- Set the standard files as 3 replicates for each dilution level. It will be changed according to the replicate size used in **Enter Replicate Count** as below.
- **Enter Replicate Count** --- Define the number of replicate files at each dilution level and click the "# for Each Replicate" above to implement.

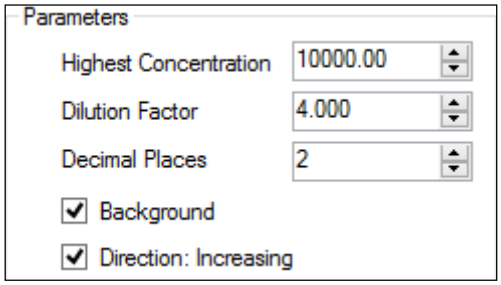
Related topics:

- Edit Standard Curve
- Calibrator Files
- Wizard Step 2

- Replicates

Parameters-Set for the Highest Standard Concentration

Open the Edit Standard Curve dialog box. The **Parameters** section is shown as below:



- **Highest Concentration** --- Enter the highest concentration value of the standard curve.
- **Dilution Factor** --- Enter the dilution factor of the standard curve.
- **Decimal Places** --- Enter the number of the decimal places up to 6 for concentration value for quantitative analysis results output.
- **Background (C0)** --- Check if there are zero concentration wells in the Standard Curve.
- **Direction: Decreasing** --- Check to set the curve dilution series from high to low (upward curve). Uncheck to set the curve dilution series from low to high (downward curve).

Note: No decimal place is set for concentrations with values > 100.

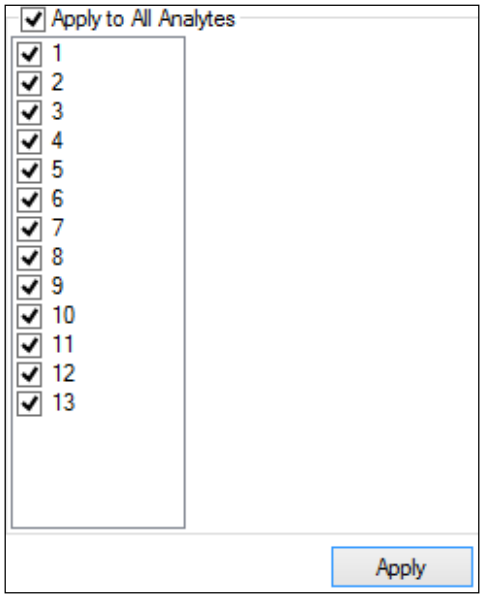
Related topics:

- Edit Standard Curve
- Calibrator Files
- Wizard Step 2
- Replicates
- Standard Curve-Set Standard Well Dilution Order
- Analyte List-Apply Settings To Analytes

Analyte List-Apply Settings To Analytes

Open the Edit Standard Curve dialog box.

All analytes are listed at the bottom of the box:



Apply to All Analytes

- Check the check box to apply the settings in this section of the Standard Curve List and Parameters to all analytes in the list, and then click Apply.
- Un-check the **Apply to All Analytes** check box, and individually select each analyte. Click Apply below to apply the settings for only selected analytes in the list.

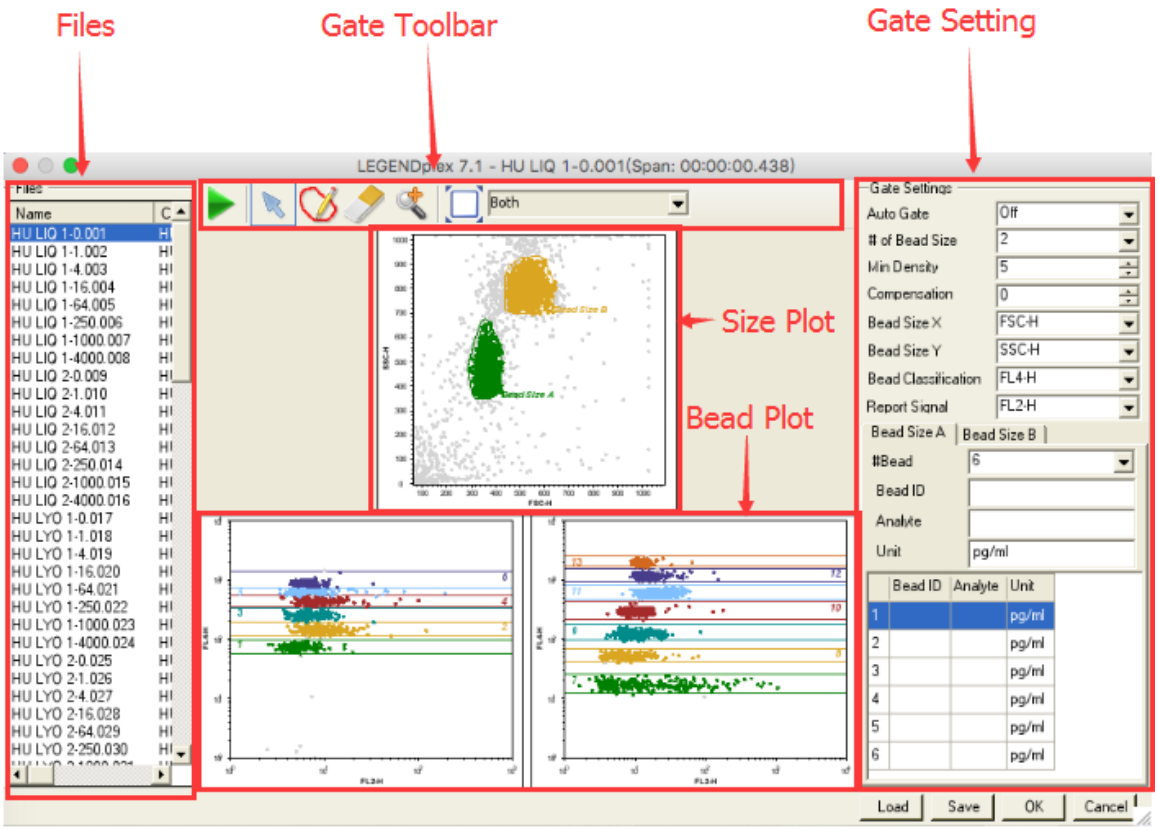
Related topics:

- Edit Standard Curve
- Calibrator Files
- Wizard Step 2
- Replicates
- Parameters-Set Standard Highest Concentration
- Standard Curve-Set Standard Well Dilution Order

Gating

Bead Gating Window

Select any sample and click  on the left bottom corner of your screen at Step 1 or the same icon on the Toolbar at Wizard step 3 when the data display is Median.



Gating Toolbar provides a series of tools to finish gating process.
Gate Settings is to define the gating parameters.
Size Plot and **Bead Plot** display the gating results.

Related topics:

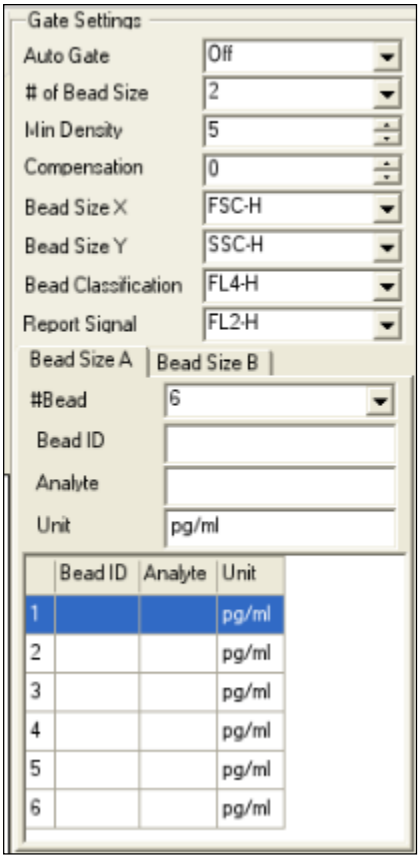
- Wizard Step 1
- Calibrator (Standard Curve) Files
- FCS Files List
- Gating Procedure
- Gate Settings

Gate Settings

On the Right of the **Bead Gating** window is **Gate Settings**.

On the right is the information about the file. After the gating process is finished, you can click each file name to view the corresponding gating result and check whether the result is correct.

The image below shows the parameters for gating.



a. Auto gate: Normally, set the Auto gate at OFF position. The On position is used for mixed samples with the same instrument settings and all different gates. The Auto gating process at ON position takes longer to process data.
b. **# of Bead Size**: the number of different Bead Sizes used in a multiplex assay panel.
c. **Min Density**: The region to find Bead Size, the value is lower, the region is bigger. It is recommended that set the value as 5. When the value is 0, there's no any process.
d. **Compensation**: Compensation, it's not recommended to use.
e. **Bead Size X**: Horizontal axis of Bead Size(e.g. FSC-A)
f. **Bead Size Y**: Vertical axis of Bead Size (e.g. SSC-A)

g. **Bead Classification**: Separate the horizontal axis of bead (e.g. APC-A etc.)
h. **Report Signal**: Select a signal channel (e.g. FL2, PE-A, etc.).
i. **# Bead**: The number of bead subpopulations for a given bead size.
j. **Bead ID**: Right click and enter the Bead ID in the dialog box. The alphabetical order of "Bead ID" should match the numerical order of the index number. For example, enter A4, A5, A6 …B3, B4, B5... for "Index" 1, 2, 3... 7, 8, 9... (Please check the kit instruction manual for specific bead region for a given analyte.)
k. **Analyte**: Right click and input the target analyte name (e.g. TNF-α, IL-6, etc.) in the dialog box according to the kit instructions manual.

Related topics:

- Gate Toolbar
- Gating Procedure
- Bead Gating Window

Gating Toolbar

Above the Bead Gating window shows the following gating toolbars.


Start a gating operation automatically.


The selection tool.


Used for manual gating. Click and then manually draw a circle around a cluster of beads on the Size panel (top) or Band panels (bottom).


An Eraser for erasing a gate. Click the Eraser first and then click a gated cluster of beads to erase the gate. This is usually used when auto gating fails to identify a particular bead population or subpopulation.


Zoom in a selected bead region for better gating. This is usually used to focus the gating on a selected area of the scatter plot (FSC vs. SSC plot).


Reset the zoomed range.

Select which panel (bead plot or size plot) to display for a zoom-in view for selected panel.

Gating Procedure

1. Click 

to open the **Bead Gating** window. Adjust # of Bead Size, Report Signal, # of Beads for Size A, # of Beads for Size B, etc. and select the axes for the dot plots. Type in **Bead ID** and **Analyte**, if so desired. If nothing is entered, the default setting will display the beads regions from small to highest in bead size and from low to high in bead internal signal intensity.
2. Depending on data acquisition software on your flow cytometers, FCS versions may be different. You will need to select appropriate X-axis and Y-axis labels (channels) on the Bead Size and Band plots. The default settings are for FCS files from common Research laboratory flow cytometers.
3. Then click  on the top of the **Bead Gating** window, LEGENDplex™ Data Analysis Software will find bead regions automatically. This works well with clean data. When data is dirty, manual gating is preferred.
4. For LEGENDplex™ beads-based assays, the auto-gating in general works well if you follow the kit's protocol, the flow cytometer used for sample reading is properly set up and compensated, and FCS files are exported with gated events only. If FCS file(s) you use for gating fail to properly separate the bead sub-populations, please use the following options for a proper solution:

    a. Change Min Density

    b. Use  to zoom out the selected region, maybe you need to repeat this operation many times to get the best effect, then click  .

    c. Use manual gating: Click  and then draw a polygonal or circular gate around the bead size population(s). The new gating will automatically apply. If you find that a Band sub-population is not acceptable, you may use the  to erase the rectangular gate for a particular sub-population and re-draw a new gate using  .

However, if you click on  , it will remove all manual gating.

d. Choose a different sample to gate. This is usually the most practical method.

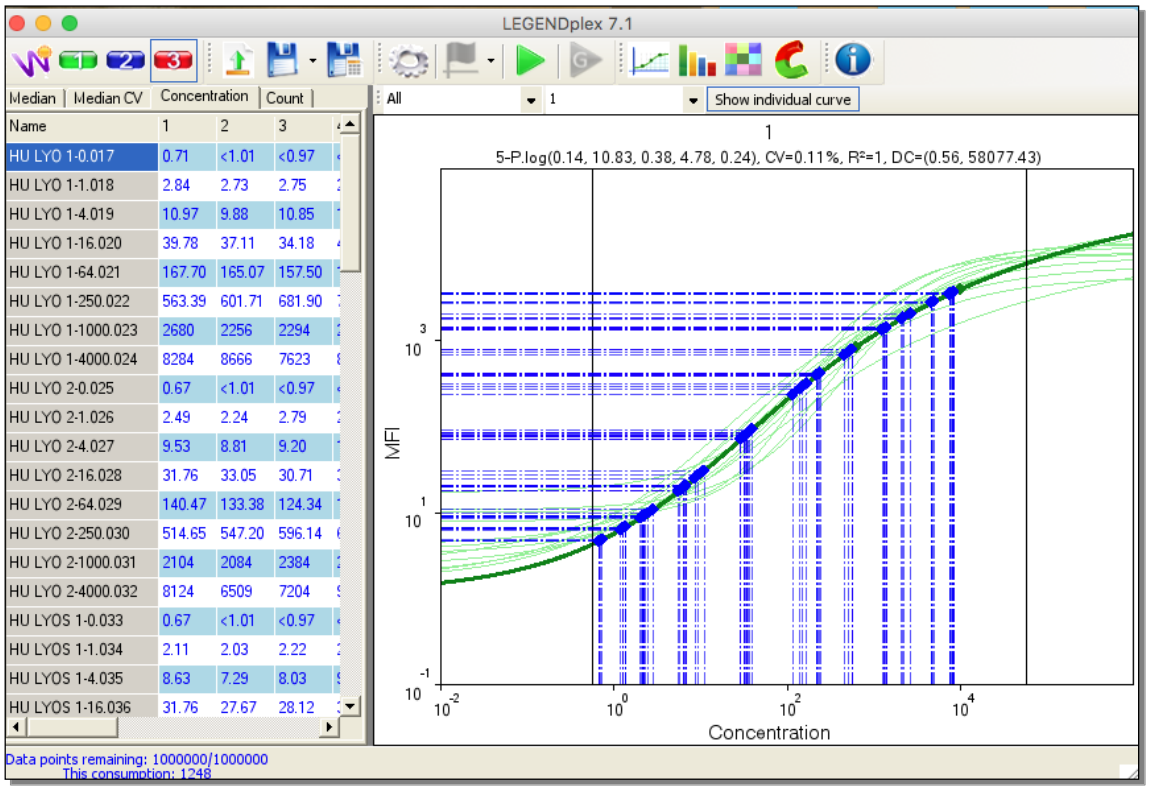
5. Click  to finish gating.

Related topics:

- Wizard Step 1
- Calibrator(Standard Curve) Files
- FCS Files List
- Gating Procedure
- Gate Settings
- Bead Gating Window

Quantitative Result

After data analysis, the sample and standard information displayed as shown below:



The left panel shows data for: Median, Median CV, Concentration and Count.

The right panel shows the graphic view: standard curve and Sample Detail. Click **Show individual curve** to view the individual curve information.

The Sample signal and concentrations are mapped in the standard curve(s). All sample concentrations are mapped to a standard curve by default.

Definitions of curve fitting:

- **R^2 ---** see R^2 for more information.
- **CV** --- The Coefficient of Variation of sample replicates. (CV% = 100 x Std Dev / Mean)

LEGENDplex™ offers other views: Bar Chart, Standard Curve, Clustering, Heatmap. Click the tabs to switch the results views. You can right click on the graphs to save them in the same folder of the image.

Related topics:

- Wizard Step 3
- Options
- Toolbar
- Bar Chart
- Standard Curve
-

Concentration

The Concentration tab displays the calculated sample concentrations generated from the standard curves.

Median | Median CV | Concentration | Count

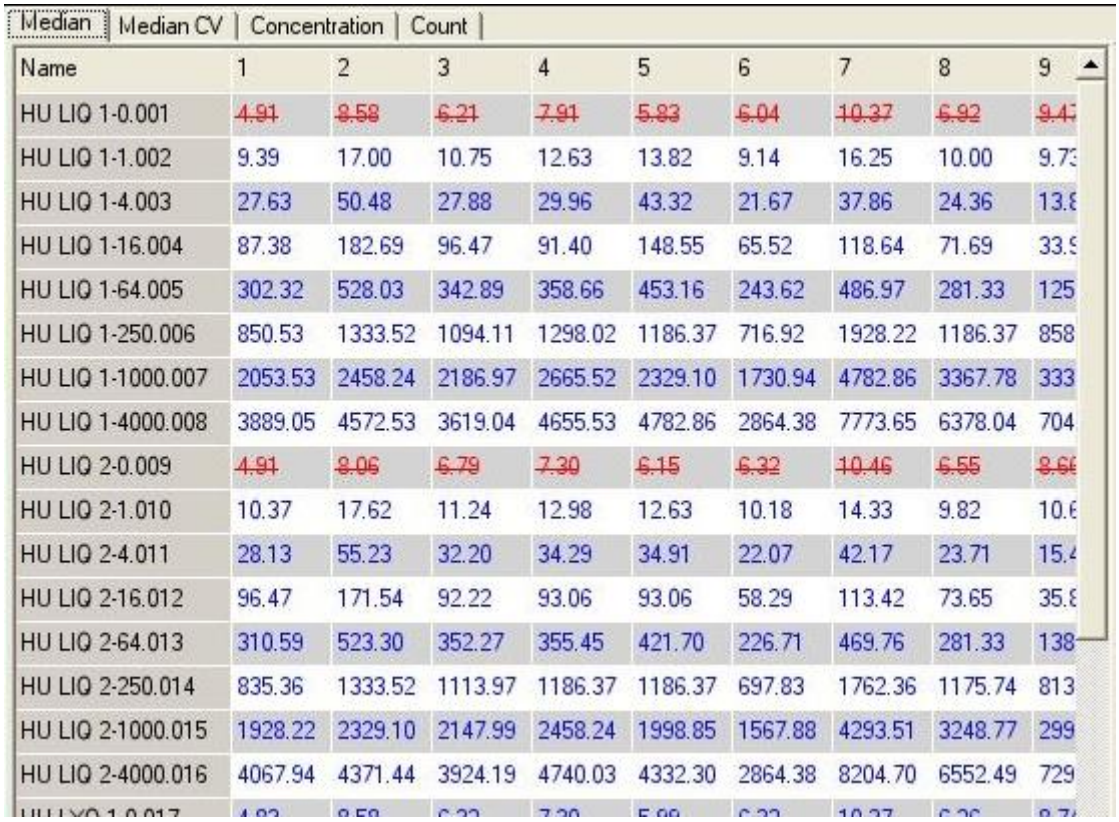
Name	1	2	3	4	5	6	7	8	9	10
HU LYO 1-0.017	0.71	<1.02	<0.93	<0.90	<1.02	0.76	1.07	<0.88	<0.95	1.41
HU LYO 1-1.018	2.85	2.74	2.72	2.80	2.32	2.42	2.34	2.67	1.81	3.06
HU LYO 1-4.019	10.97	9.96	10.96	13.10	6.77	9.94	8.09	9.25	11.10	10.90
HU LYO 1-16.020	39.76	37.01	35.05	41.40	22.12	39.93	27.47	33.41	41.12	34.14
HU LYO 1-64.021	167.52	164.96	157.66	166.43	89.32	162.00	113.66	137.72	138.42	168.54
HU LYO 1-250.022	562.95	602.32	674.15	737.83	420.69	541.03	484.30	544.02	565.68	533.21
HU LYO 1-1000.023	2684	2259	2304	2421	1568	2590	1877	2101	2459	1853
HU LYO 1-4000.024	8144	8661	7632	8825	8792	7770	6075	8515	9178	5808
HU LYO 2-0.025	0.67	<1.02	<0.93	<0.90	<1.02	<0.69	<0.90	<0.88	<0.95	1.34
HU LYO 2-1.026	2.49	2.26	2.80	2.61	2.26	2.32	2.42	2.67	2.84	2.52
HU LYO 2-4.027	9.53	8.70	9.29	10.51	6.70	7.72	6.77	8.68	7.83	7.65
HU LYO 2-16.028	31.74	32.95	30.90	35.75	17.50	30.87	23.40	30.54	35.57	32.10
HU LYO 2-250.030	514.22	547.70	597.31	667.09	303.54	515.76	441.72	538.75	549.15	445.52
HU LYO 2-1000.031	2106	2086	2394	2421	1132	2244	1794	2017	2224	1534

Related topics:

- Median
- Median CV
- Count
- Bar Chart
- Standard Curve

Median

The Median tab displays median fluorescence intensity of the reporting signal for a given bead sub-population.



Median | Median CV | Concentration | Count

Name	1	2	3	4	5	6	7	8	9
HU LIQ 1-0.001	4.91	8.58	6.21	7.91	5.83	6.04	10.37	6.92	9.4
HU LIQ 1-1.002	9.39	17.00	10.75	12.63	13.82	9.14	16.25	10.00	9.73
HU LIQ 1-4.003	27.63	50.48	27.88	29.96	43.32	21.67	37.86	24.36	13.8
HU LIQ 1-16.004	87.38	182.69	96.47	91.40	148.55	65.52	118.64	71.69	33.9
HU LIQ 1-64.005	302.32	528.03	342.89	358.66	453.16	243.62	486.97	281.33	125
HU LIQ 1-250.006	850.53	1333.52	1094.11	1298.02	1186.37	716.92	1928.22	1186.37	858
HU LIQ 1-1000.007	2053.53	2458.24	2186.97	2665.52	2329.10	1730.94	4782.86	3367.78	333
HU LIQ 1-4000.008	3889.05	4572.53	3619.04	4655.53	4782.86	2864.38	7773.65	6378.04	704
HU LIQ 2-0.009	4.91	8.06	6.79	7.30	6.15	6.32	10.46	6.55	8.66
HU LIQ 2-1.010	10.37	17.62	11.24	12.98	12.63	10.18	14.33	9.82	10.6
HU LIQ 2-4.011	28.13	55.23	32.20	34.29	34.91	22.07	42.17	23.71	15.4
HU LIQ 2-16.012	96.47	171.54	92.22	93.06	93.06	58.29	113.42	73.65	35.8
HU LIQ 2-64.013	310.59	523.30	352.27	355.45	421.70	226.71	469.76	281.33	138
HU LIQ 2-250.014	835.36	1333.52	1113.97	1186.37	1186.37	697.83	1762.36	1175.74	813
HU LIQ 2-1000.015	1928.22	2329.10	2147.99	2458.24	1998.85	1567.88	4293.51	3248.77	299
HU LIQ 2-4000.016	4067.94	4371.44	3924.19	4740.03	4332.30	2864.38	8204.70	6552.49	729

Data in red indicates auto-flagged or flagged files. The C0 files are always flagged.

Median CV

The Median CV tab displays the variance of the replicate samples.

Median | Median CV | Concentration | Count

Name	1	2	3	4	5	6	7	8	9	10	11	12	13
HU LIQ 1-0.001	0.00	3.15	4.49	4.05	2.70	2.25	0.45	2.70	4.49	2.70	5.39	1.35	0.00
HU LIQ 1-1.002	4.94	1.80	2.25	1.35	4.49	5.39	6.29	0.90	4.49	2.25	0.90	2.25	1.80
HU LIQ 1-4.003	0.90	4.49	7.18	6.74	10.75	0.90	5.39	1.35	5.39	9.86	2.70	3.15	10.31
HU LIQ 1-16.004	4.94	3.15	2.25	0.90	22.97	5.84	2.25	1.35	2.70	3.15	3.60	8.52	13.41
HU LIQ 1-64.005	1.35	0.45	1.35	0.45	3.60	3.60	1.80	0.00	4.94	1.80	0.00	4.49	1.35
HU LIQ 1-250.006	0.90	0.00	0.90	4.49	0.00	1.35	4.49	0.45	2.70	4.05	2.25	5.84	2.70
HU LIQ 1-1000.007	3.15	2.70	0.90	4.05	7.63	4.94	5.39	1.80	5.39	6.74	4.05	6.29	5.39
HU LIQ 1-4000.008	2.25	2.25	4.05	0.90	4.94	0.00	2.70	1.35	1.80	6.29	0.45	0.00	2.70
HU LYO 1-0.017	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HU LYO 1-1.018	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HU LYO 1-4.019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HU LYO 1-16.020	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HU LYO 1-64.021	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HU LYO 1-250.022	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HU LYO 1-1000.023	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HU LYO 1-4000.024	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HU LYO 2-0.025	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HU LYO 2-1.026	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HU LYO 2-4.027	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Count

The Count tab displays the count of events found in the defined gated area for a particular analyte. This is equivalent to the number of beads within the gated area.

Median | Median CV | Concentration | Count

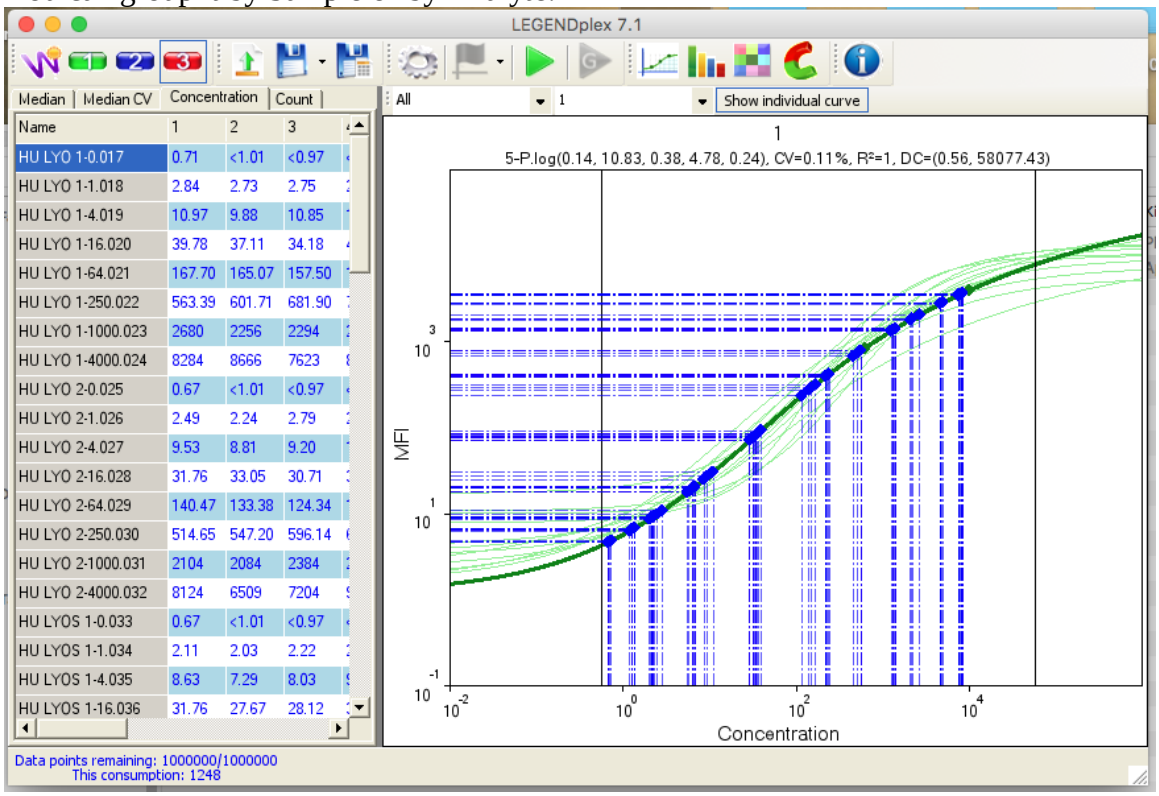
Name	1	2	3	4	5	6	7	8	9	10	11	12	13
HU LIQ 1-0.001	~~253~~	~~237~~	~~182~~	~~172~~	~~184~~	~~211~~	~~254~~	~~242~~	~~282~~	~~243~~	~~281~~	~~216~~	~~239~~
HU LIQ 1-1.002	253	229	203	222	170	161	277	287	276	291	284	235	233
HU LIQ 1-4.003	256	231	187	235	212	141	264	287	308	290	302	246	238
HU LIQ 1-16.004	253	215	196	218	209	152	256	269	273	303	328	240	214
HU LIQ 1-64.005	244	199	187	243	220	126	280	290	241	318	298	258	218
HU LIQ 1-250.006	252	234	181	281	228	104	267	268	257	279	287	240	211
HU LIQ 1-1000.007	245	234	204	239	203	117	283	294	275	315	253	228	207
HU LIQ 1-4000.008	250	250	214	252	211	132	277	283	270	286	278	227	239
HU LIQ 2-0.009	~~284~~	~~225~~	~~191~~	~~232~~	~~196~~	~~109~~	~~257~~	~~263~~	~~301~~	~~286~~	~~269~~	~~221~~	~~224~~
HU LIQ 2-1.010	246	206	218	285	199	98	268	246	286	270	271	217	240
HU LIQ 2-4.011	248	218	209	255	200	123	264	265	264	269	277	236	238
HU LIQ 2-16.012	245	224	189	261	176	107	268	240	270	272	235	218	206
HU LIQ 2-64.013	274	228	213	260	231	96	256	270	280	285	238	204	210

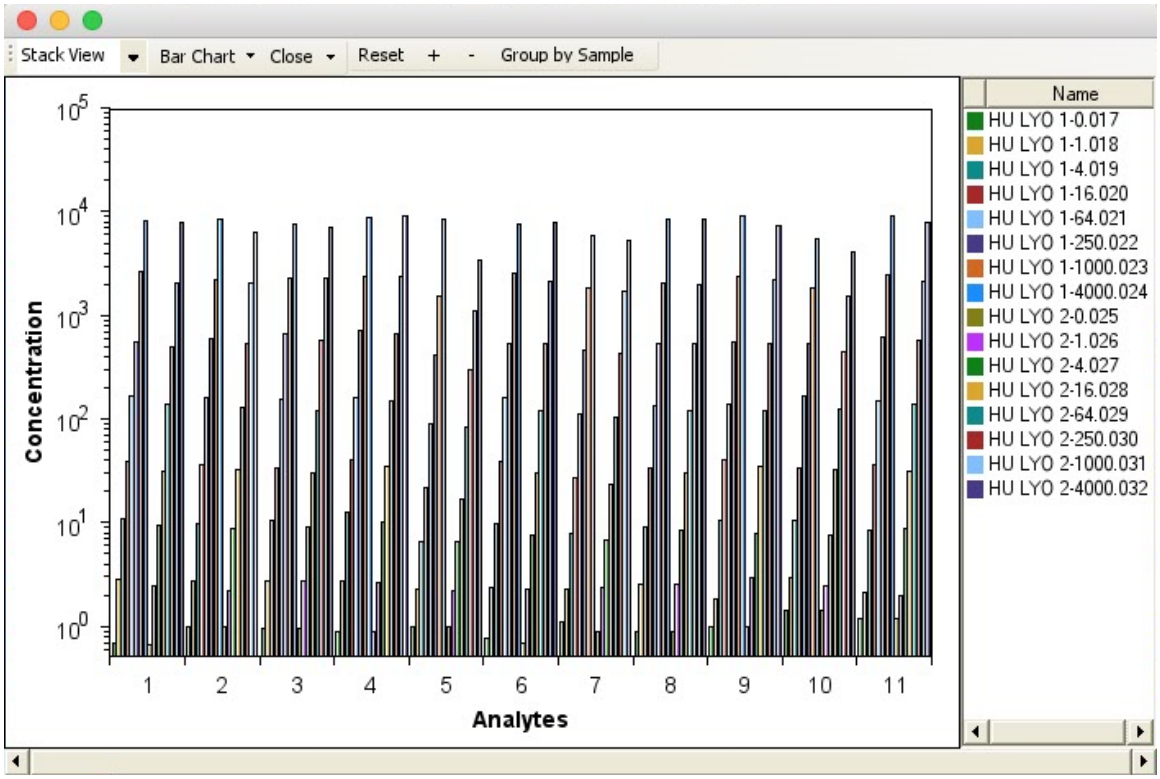
Bar Chart

Highlight the desired Concentration and Median data and click  on the Toolbar, the Bar Chart will be displayed as below. The image of a bar chart can be taken as snapping shot by using using “Command key + Shift key + 4 Key” simultaneously on a Mac computer.

If the highlighted number of data points is too many, the bar chart may not have enough room to display the chart properly. You can enlarge the display window to properly display the chart by click "+" on the chat toolbar. And click "-" to reduce them or reset it.

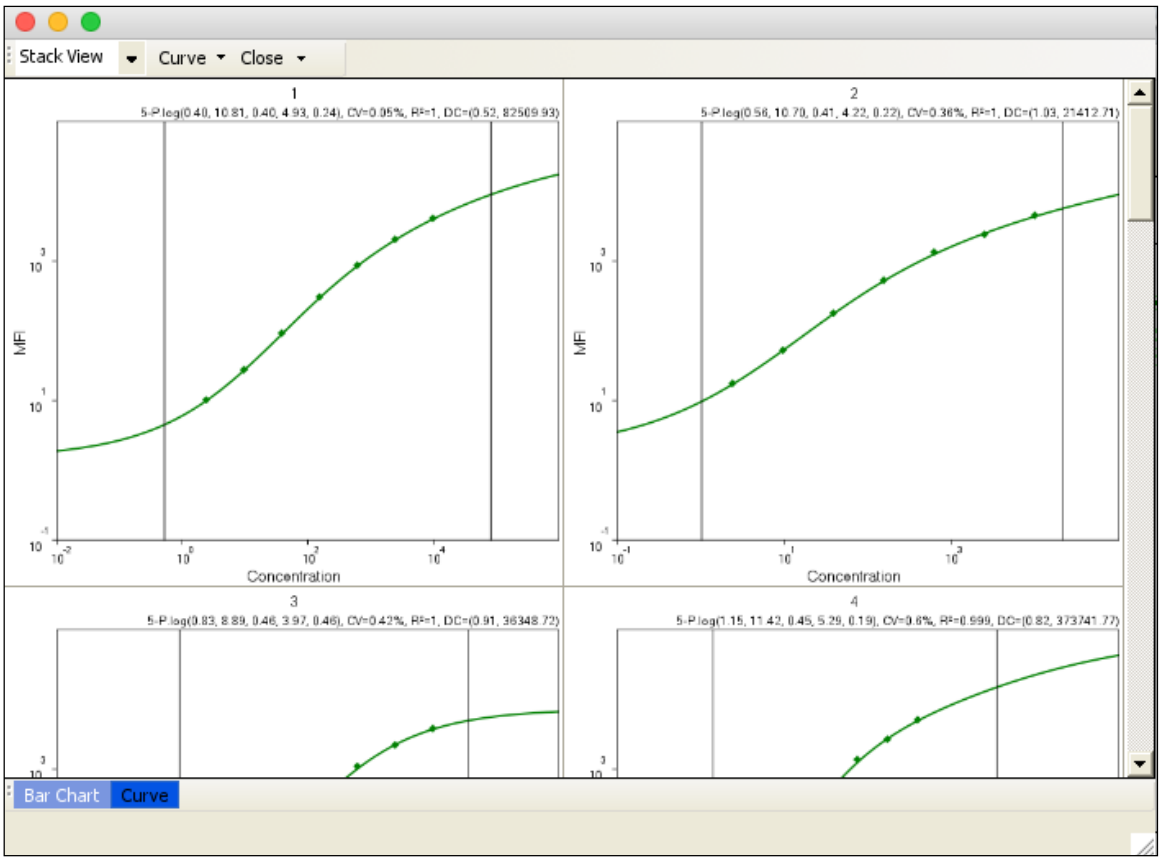
You can group it by Sample or by Analyte.





Standard Curve

In the **Concentration** tab, click  on the Toolbar, all the standard curve of the beads will be displayed individually. The curve-fitting parameters including curve coefficients, assay minimum and maximum detectable concentrations (DC) are displayed in an area just above each standard curve.



Scroll down the bar to view the curves.

Definitions of curve fitting:

- **CV** --- The Coefficient of Variation of one curve (CV = 100% x Chi/ Ymean)
- **R^2** --- The coefficient of determination is a statistical measure to assess the goodness of fit of a model. (R^2 = 1.0, indicates that the regression line perfectly fits the data.) For 4 parameter logistic fitting, y = a + (b – a)/[1 + exp(d(x – c))], we first convert x to v using v = 1/[1 + exp(d(x – c))], so y = a + (b – a)*v. We then apply linear regression to calculate R-squared as below:

$$R^2 = 1 - \frac{\sum_{i=1}^{N}(y_i - \hat{y}_i)^2}{\sum_{i=1}^{N}(y_i - \bar{y})^2}$$

Where N is the number of observations in the model, y is the dependent variable, y-bar is the mean of the y values, and y-hat is the value predicted by the model. Similar approaches are used for 5-parameter and other curve fitting methods. The approach might be over simplified in determining pseudo R-squared for non-linear regression. Since R squared is only meaningful to ordinary (or linear) least square regression, it is not beneficial to determine maximum R squared.

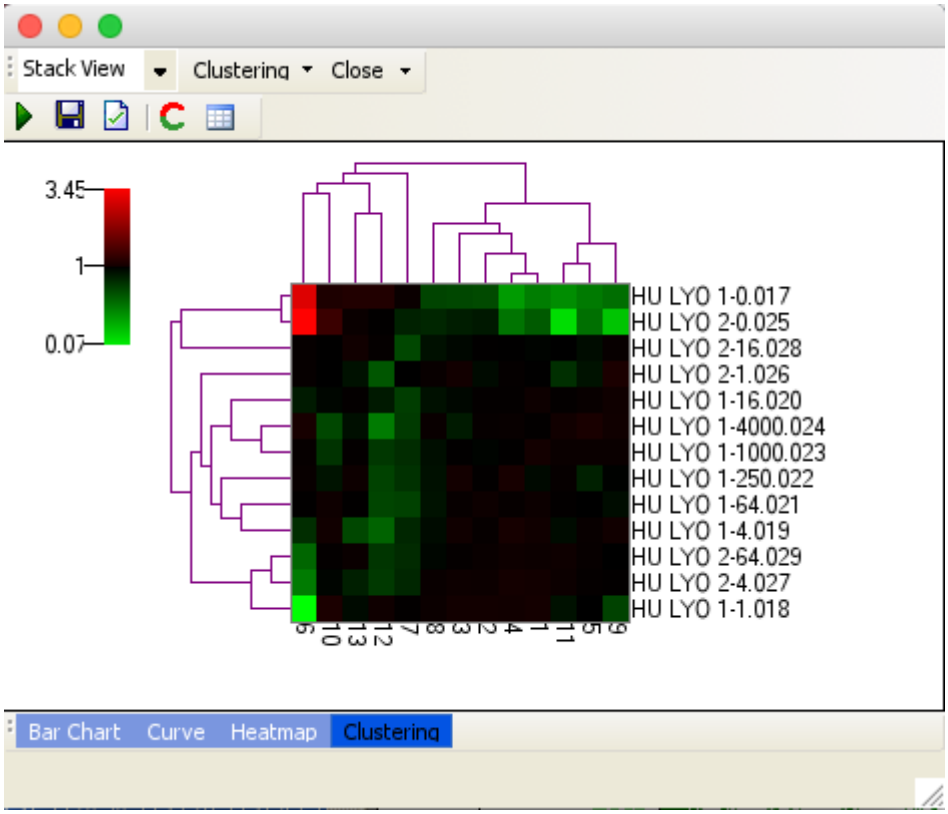
Related topics:

- Median CV
- Count
- Bar Chart
- Wizard Step 3
- Toolbar
- Sample Detail

Clustering

Clustering or Heat Map

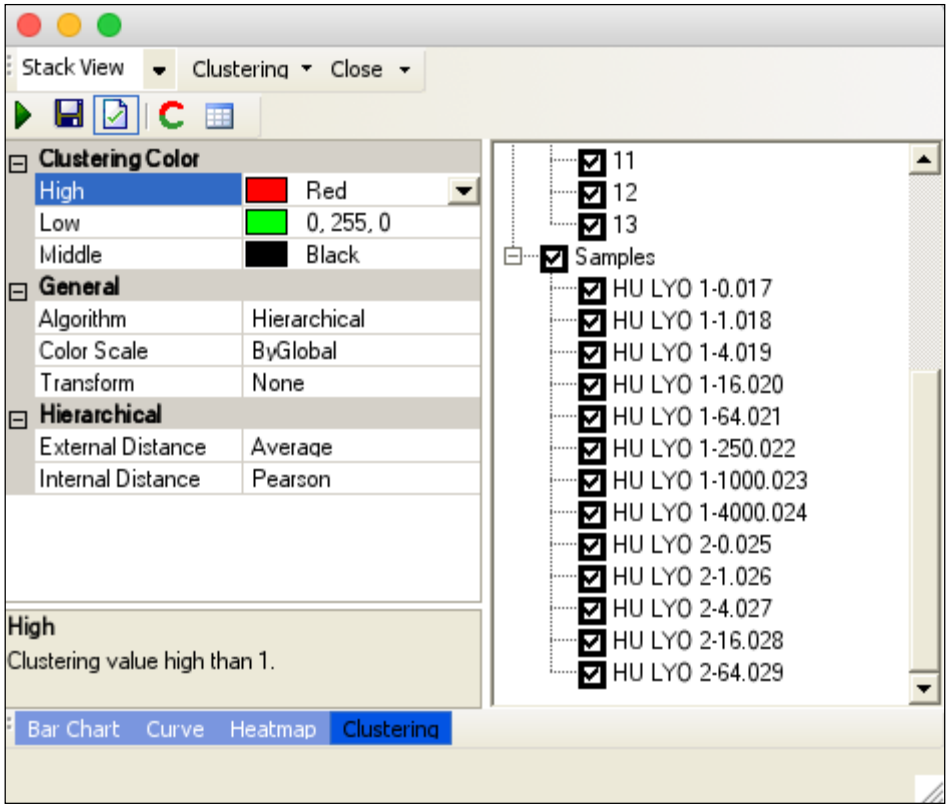
Highlight one or more data, click  on the **Toolbar**. The heatmap and clustering view will be displayed:



On the left is the Analytes/Samples list, check the check box to select the viewed data and click to apply the selection.

Clustering Options

Click on the top of Clustering dialog box to open the **Clustering Options** dialog box.



Clustering Color: Set the colors of clustering.

Show Category: Choose the mode of **Show Category**.

- **No**: Show nothing.
- **Value Only**: Only show the category value next to the sample, like {value}.
- **All**: Show the name and the value of category next to the sample, like {name: value}.

Algorithm: Select Clustering analysis algorithm model.

- **Hierarchical:** Hierarchical clustering procedure produces a series of partitions of the data, P_n, P_{n-1}... P_1. The first P_n consists of a single object "clusters", the last P_1, consists of single group containing all n cases.

  Note: The method refers to agglomerative hierarchical method.
- **K-means:** K-means is an algorithm to classify or to group your objects based on attributes/features into K groups. K is positive integer number. The grouping is done by minimizing the sum of squares of distances between data and the corresponding cluster centroid.

  Note: When you select K-means algorithm, you should also select a type of K-means distance.

Transform: Use linear or log transformed intensity or concentration data to do clustering analysis.

- **None**: Use the raw data for clustering analysis.

- **Log2**: Transform the raw data using Log2.
- **Ln**: Transform the raw data using Ln.

Color Scale: Clustering color can be scaled by whole data set (global) or analytes (biomarker).

- **Scale by global:** The heatmap colors are based on the overall (complete set) min and max values.
- **Scale by biomarker:** The heatmap colors are based on the biomarker min and max values.

Color Map: Choose a color tone to present the intensity or concentration data value.

Auto Scale: Based on the data set, highest and lowest values of data define the whole color range.
Min Scale: The lowest value of data sets for the top of color range.
Max Scale: The highest value of data sets for the top of color range.

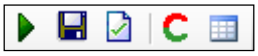
Internal Distance: Algorithm to group similar data to one class.

- **Euclidean:** The distance is defined as Euclidean distance.
- **Pearson correlation:** The distance is defined as 1-r.
- **Pearson squared:** The distance is defined as $1-r^2$.

External Distance: Algorithm to separate different data to two or more classes.

- **Average:** The average of distances between all pairs of objects.
- **Average group:** Form the groups by their mean values for each protein, that is their mean sample, and cluster distance is now defined in terms of distance between two such mean samples.
- **Single:** The distance between the closest pair of objects.
- **Complete:** The distance between the most distant pair of objects.

Clustering Toolbar



Icon	Name	Function
	Run	Run to analyze.
	Save	Save the Clustering Summary in a .pdf file.
	Options	Display the Clustering Options.
	Clustering	Display Clustering box

	Clustering table	Display Clustering table

Related topics:

- Median CV
- Count
- Bar Chart
- Standard Curve
- Wizard Step 3
- Toolbar
- Clustering or Heat Map
- Clustering Toolbar

Output

There are 2 types of data reports: Detail and Summary. Click  on the Toolbar, it will save all result data. Click **OK**.

Detail: Curve Information, Curve Image, Final Sample Concentration, Analyte Detail, count and Files Information, in .xlsx format.

Summary: Curve Information, Curve Image, Final Sample Concentration, Standard Concentrations, MFI, Count and Files Information, in .xlsx format.

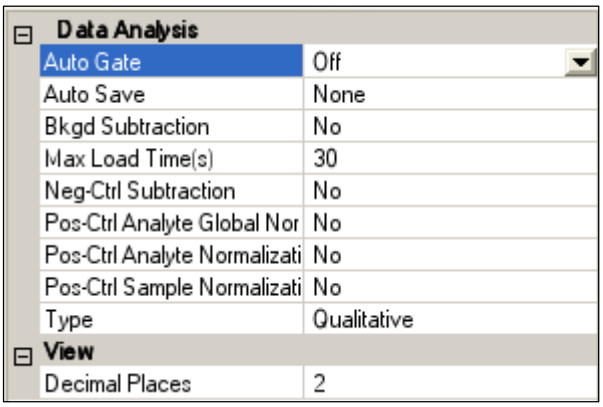
Qualitative Analysis

Qualitative Analysis Wizard

Qualitative Settings

Qualitative Settings includes sample name, analyte name, positive file and blank file. The qualitative settings are used to analyze raw data. The Settings can be predefined on the right panel in Wizard Step1.

Select **Qualitative** from the drop down list in **Data Analysis**, the parameters are shown below:



Data Analysis

- **Qualitative** --- Multiplex assay used to test samples against reference samples, or to test target analytes against control analytes with one or more levels of controls and/or normalization factors.
- **Background Subtraction** --- Background file (BKG) Subtraction: Each sample files will subtract background. For Qualitative assays, each analyte signal intensity MFI will be adjusted by subtracting the signal MFI for the corresponding analyte MFI in the background (BKG). Background can be defined **(**define Background).
- **Neg-Ctrl Subtraction** --- Negative Control Analyte Subtraction: In each file, each analyte signal intensity MFI will be adjusted by subtracting the negative control analyte signal MFI.

You should define some analyses' **Control Type** as **Negative Control**.

Note: Background Subtraction (sample) and **Negative Control Subtraction** (analyte) cannot be used at the same time in a single analysis process.

- **Pos-Ctrl Analyte Global Normalization** --- Positive Control Analyte Global Normalization: In each file, the MFI of the positive control analyte will be normalized. Each analyte intensity MFI value will be adjusted by multiplying the normalized positive control value. The normalized positive control value is derived by dividing the signal MFI of the highest intensity positive control analyte on the plate by the signal MFI of the positive control analyte.

  You should define some analyses' **Control Type** as **Positive Control**.
- **Pos-Ctrl Analyte Normalization** --- Positive Control Analyte Normalization: Each analyte signal intensity MFI in a file will be normalized to the intensity of the MFI of the positive control analyte in the same file.

  Note: Positive Control Analyte Global Normalization and **Positive Control (Analyte) Normalization** cannot be used at the same time in a single analysis process. You should define some analyses' **Control Type** as **Positive Control**.
- **Pos-Ctrl Sample Normalization** --- Each sample file can be normalized to the Pos-Ctrl Sample(s) (POS Well). The analyte signal intensities MFI of each sample will be normalized to the signal intensity MFI of the corresponding analyte in the positive control sample file.

  You should define some file type as **P** (Positive).
- **Auto Gate** --- It is recommended that the **Auto Gate** be set at **OFF** position.
- **Auto Save** --- Select Auto Save type: (The default setting is **None**)
  - **None:** Not to save anything after analysis is complete.
  - **Data:** Save the result data in the database after analysis is complete.
  - **Report:** Save the default report in the file folder as raw data after analysis is complete.
  - **Both:** Save result data and the default report in the file folder as raw data after analysis is complete.

View

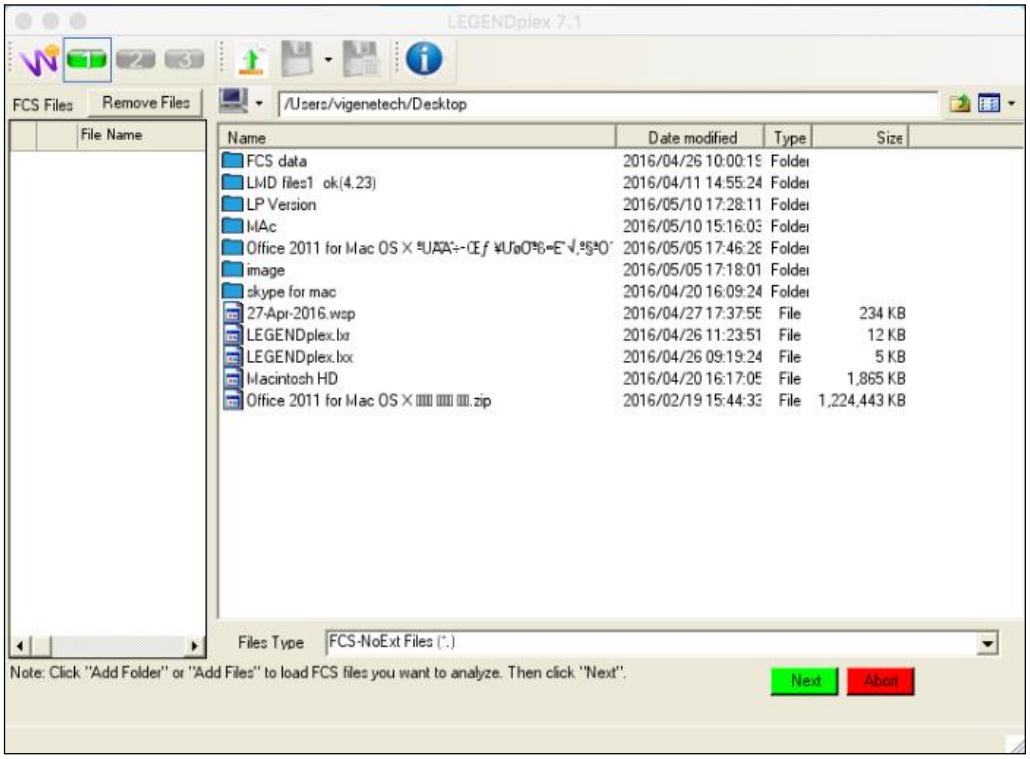
- **Decimal Places** --- The decimal places for all numbers in the program.

Related topics:

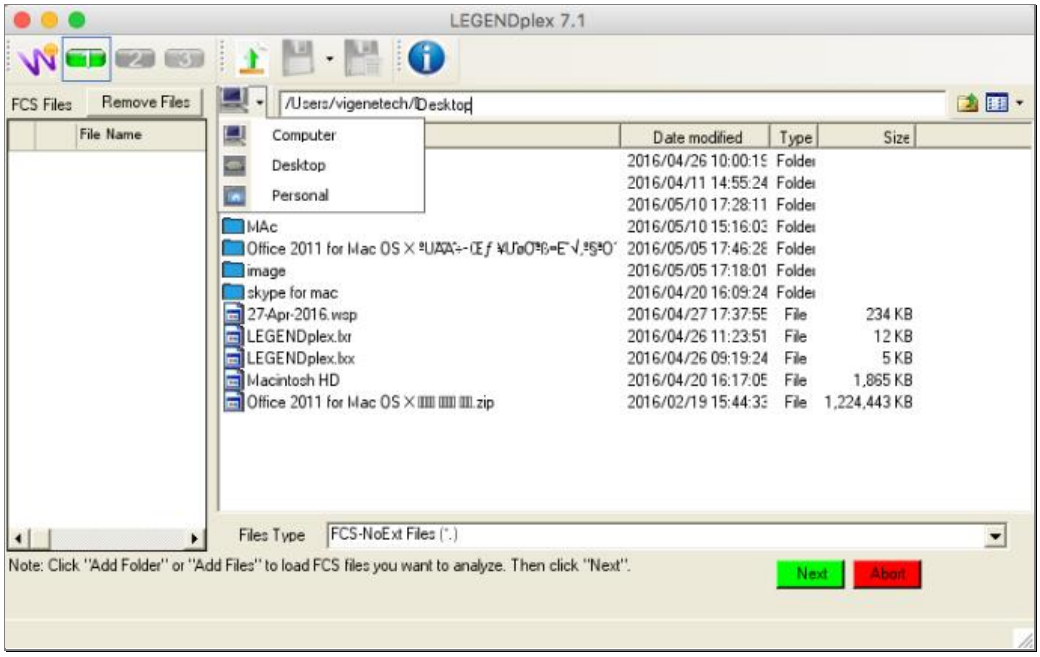
- Wizard Step 1
- Wizard Step 2
- Wizard Step 3
- Quantitative Settings

Wizard Step 1

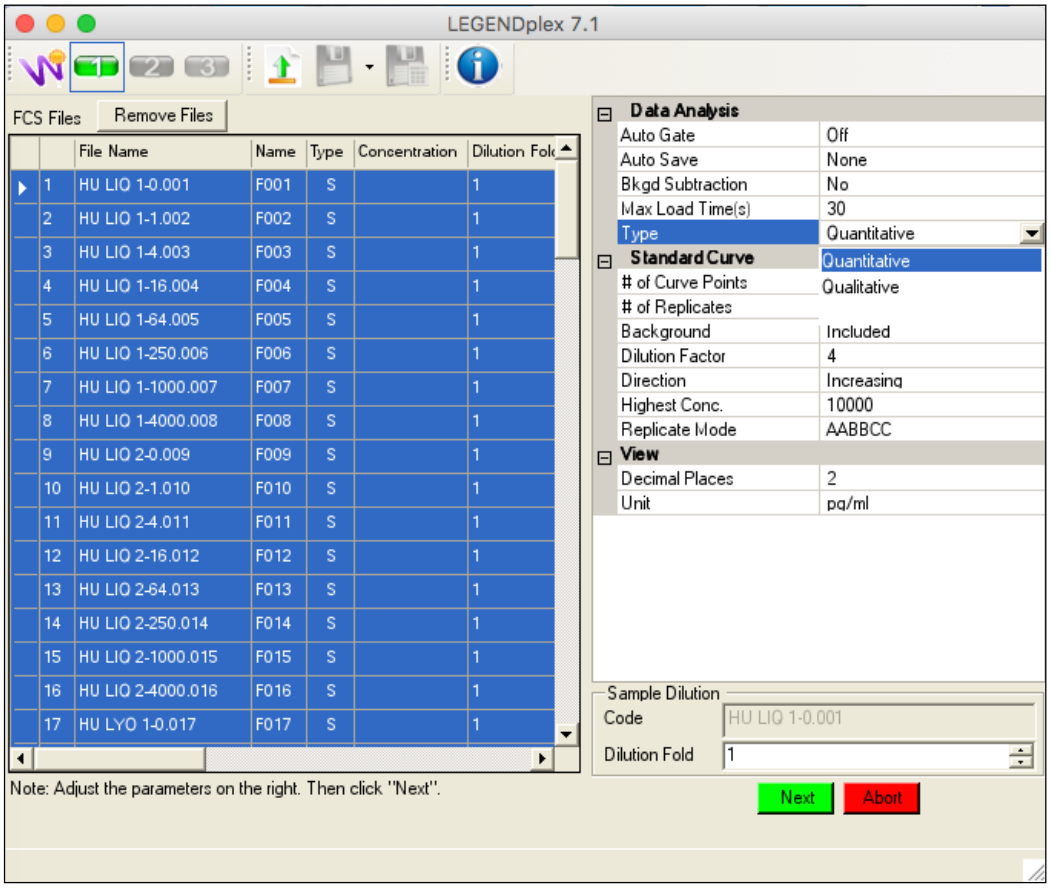
1. Click  to start a new wizard session.



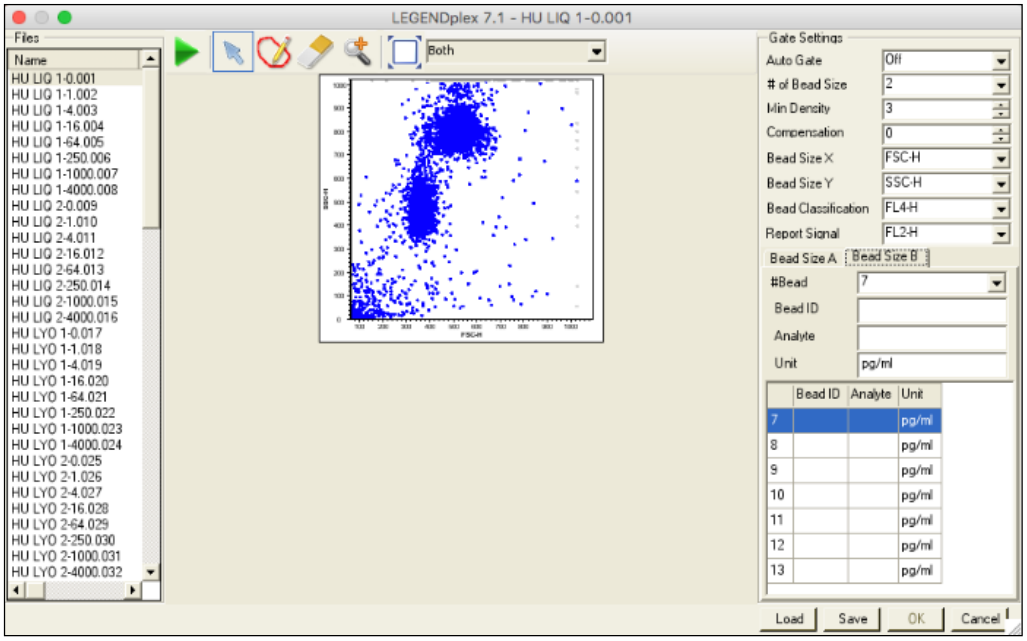
2. Click **Add File** to load FCS files to be analyzed a folder containing files to be analyzed, or click **Add Files** to load FCS files you want to analyze. Then click **Next**. Non-FCS files will be excluded from sample loading automatically.



3. Click **Next**, select **Qualitative** as **Type** in Settings.



4. Click **Next** on the right bottom of the window to start gating procedure.



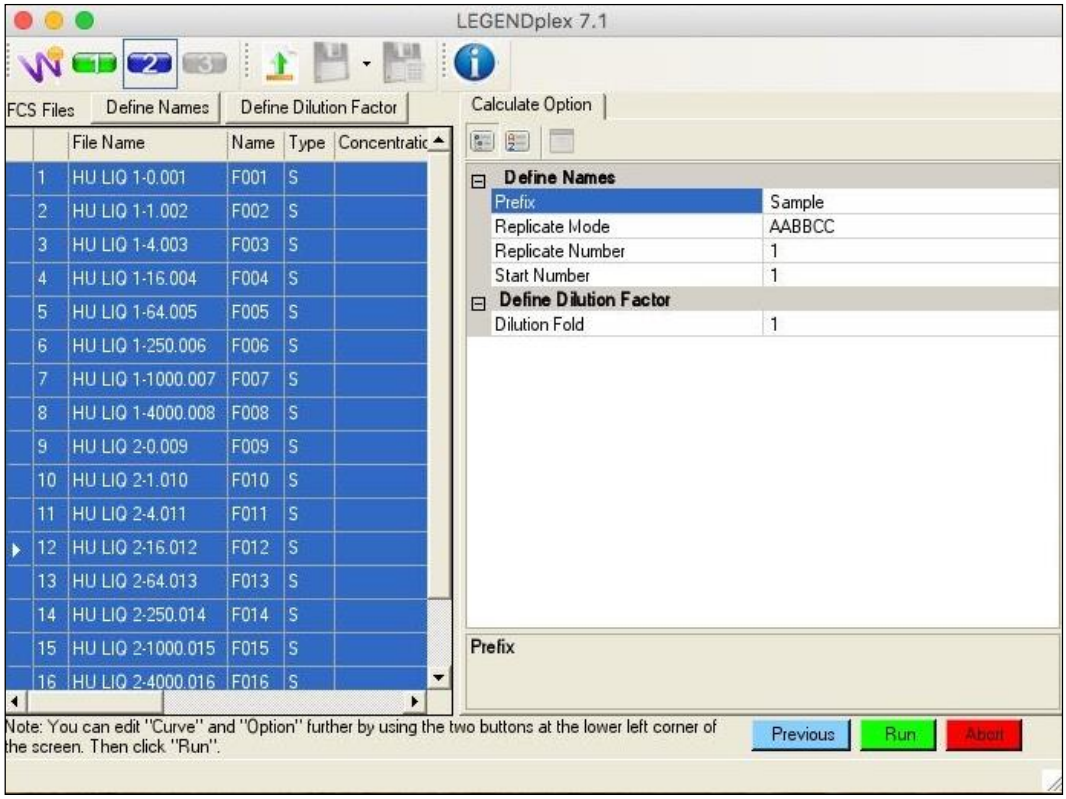
5. Click **OK** to go to the next step.

Related topics:

- Qualitative Settings
- Wizard Step 2
- Wizard Step 3

Wizard Step 2

1. Do the followings if necessary.
   - Define Name
   - Edit Calculate Options



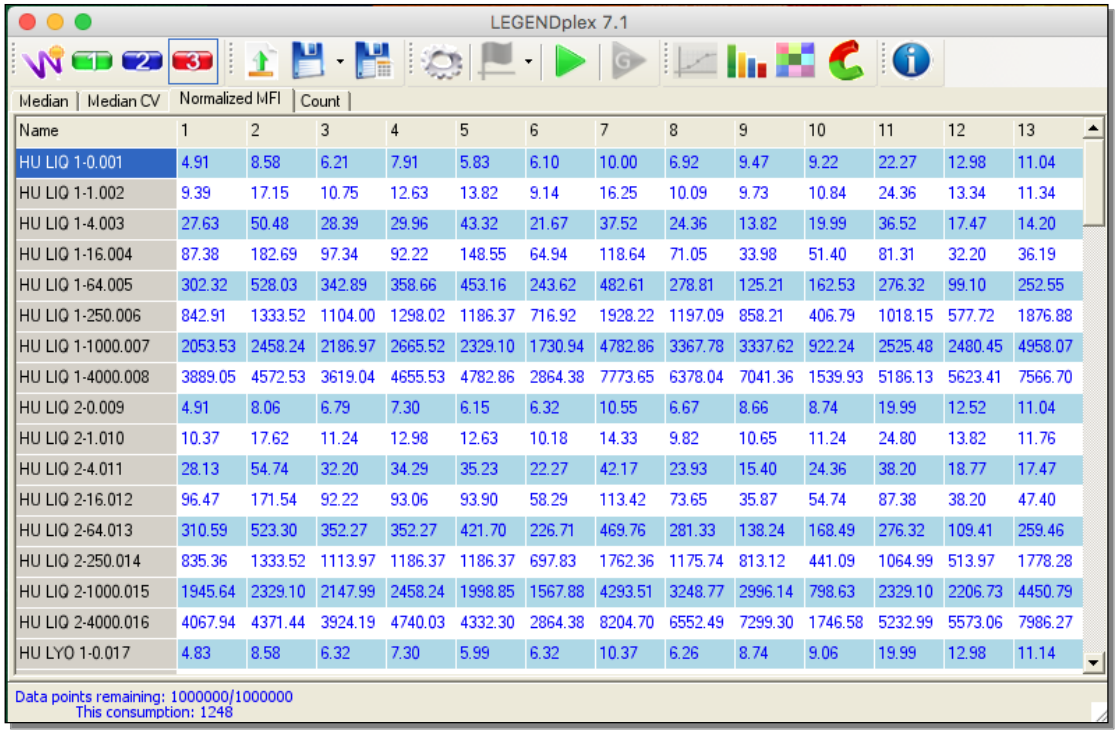
2. Click **Run**.

Related topics:

- Wizard Step3
- Qualitative Settings
- Wizard Step1

Wizard Step 3

The result will be displayed as below.



Name	1	2	3	4	5	6	7	8	9	10	11	12	13
HU LIQ 1-0.001	4.91	8.58	6.21	7.91	5.83	6.10	10.00	6.92	9.47	9.22	22.27	12.98	11.04
HU LIQ 1-1.002	9.39	17.15	10.75	12.63	13.82	9.14	16.25	10.09	9.73	10.84	24.36	13.34	11.34
HU LIQ 1-4.003	27.63	50.48	28.39	29.96	43.32	21.67	37.52	24.36	13.82	19.99	36.52	17.47	14.20
HU LIQ 1-16.004	87.38	182.69	97.34	92.22	148.55	64.94	118.64	71.05	33.98	51.40	81.31	32.20	36.19
HU LIQ 1-64.005	302.32	528.03	342.89	358.66	453.16	243.62	482.61	278.81	125.21	162.53	276.32	99.10	252.55
HU LIQ 1-250.006	842.91	1333.52	1104.00	1298.02	1186.37	716.92	1928.22	1197.09	858.21	406.79	1018.15	577.72	1876.88
HU LIQ 1-1000.007	2053.53	2458.24	2186.97	2665.52	2329.10	1730.94	4782.86	3367.78	3337.62	922.24	2525.48	2480.45	4958.07
HU LIQ 1-4000.008	3889.05	4572.53	3619.04	4655.53	4782.86	2864.38	7773.65	6378.04	7041.36	1539.93	5186.13	5623.41	7566.70
HU LIQ 2-0.009	4.91	8.06	6.79	7.30	6.15	6.32	10.55	6.67	8.66	8.74	19.99	12.52	11.04
HU LIQ 2-1.010	10.37	17.62	11.24	12.98	12.63	10.18	14.33	9.82	10.65	11.24	24.80	13.82	11.76
HU LIQ 2-4.011	28.13	54.74	32.20	34.29	35.23	22.27	42.17	23.93	15.40	24.36	38.20	18.77	17.47
HU LIQ 2-16.012	96.47	171.54	92.22	93.06	93.90	58.29	113.42	73.65	35.87	54.74	87.38	38.20	47.40
HU LIQ 2-64.013	310.59	523.30	352.27	352.27	421.70	226.71	469.76	281.33	138.24	168.49	276.32	109.41	259.46
HU LIQ 2-250.014	835.36	1333.52	1113.97	1186.37	1186.37	697.83	1762.36	1175.74	813.12	441.09	1064.99	513.97	1778.28
HU LIQ 2-1000.015	1945.64	2329.10	2147.99	2458.24	1998.85	1567.88	4293.51	3248.77	2996.14	798.63	2329.10	2206.73	4450.79
HU LIQ 2-4000.016	4067.94	4371.44	3924.19	4740.03	4332.30	2864.38	8204.70	6552.49	7299.30	1746.58	5232.99	5573.06	7986.27
HU LYO 1-0.017	4.83	8.58	6.32	7.30	5.99	6.32	10.37	6.26	8.74	9.06	19.99	12.98	11.14

See also Qualitative Result.

Related topics:

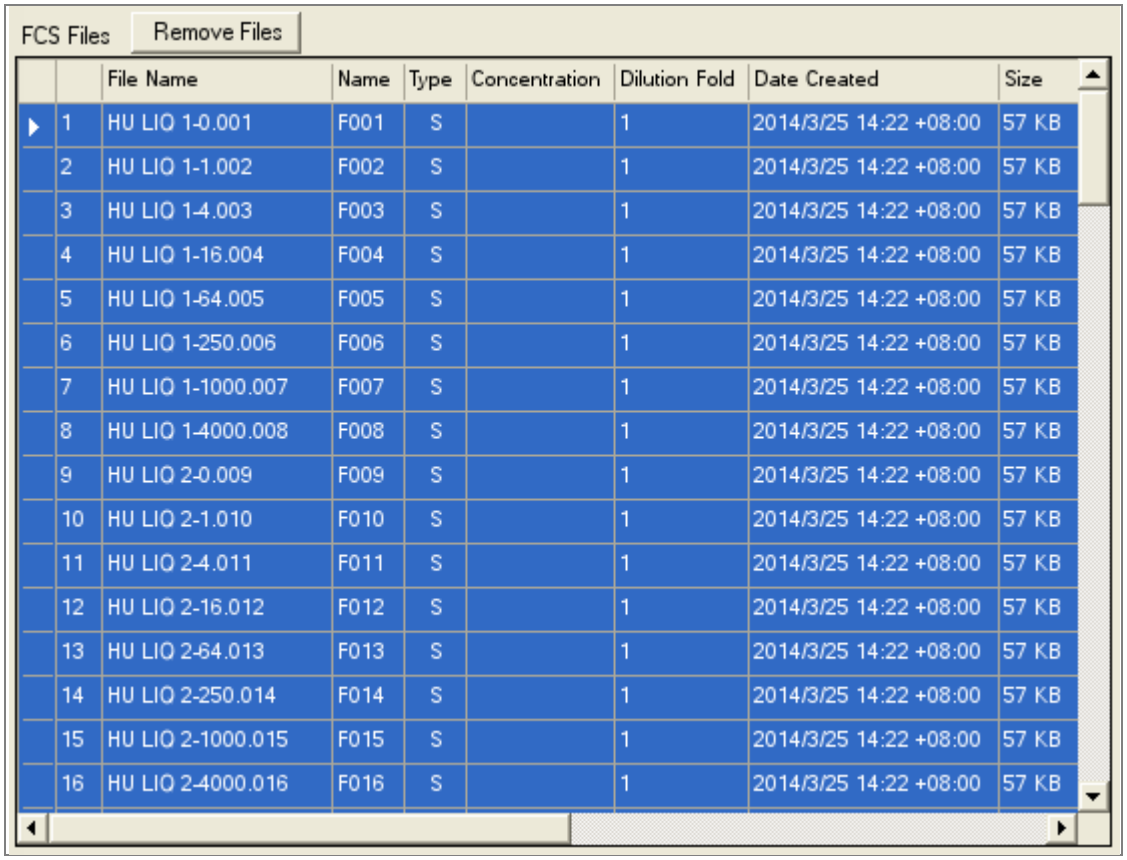
- Qualitative Settings
- Wizard Step 1
- Wizard Step 2

Qualitative Operation Items

Qualitative FCS Files List

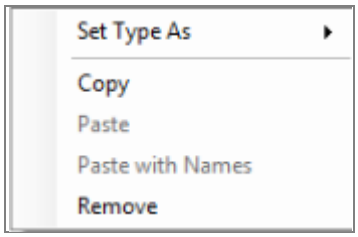
The Qualitative FCS Files list is a place to display and edit positive and sample files.

It lists the file information including **Name**, **Type**, **File Name**, **Data modified**, **Size** and **File Directory**.



	File Name	Name	Type	Concentration	Dilution Fold	Date Created	Size
1	HU LIO 1-0.001	F001	S		1	2014/3/25 14:22 +08:00	57 KB
2	HU LIO 1-1.002	F002	S		1	2014/3/25 14:22 +08:00	57 KB
3	HU LIO 1-4.003	F003	S		1	2014/3/25 14:22 +08:00	57 KB
4	HU LIO 1-16.004	F004	S		1	2014/3/25 14:22 +08:00	57 KB
5	HU LIO 1-64.005	F005	S		1	2014/3/25 14:22 +08:00	57 KB
6	HU LIO 1-250.006	F006	S		1	2014/3/25 14:22 +08:00	57 KB
7	HU LIO 1-1000.007	F007	S		1	2014/3/25 14:22 +08:00	57 KB
8	HU LIO 1-4000.008	F008	S		1	2014/3/25 14:22 +08:00	57 KB
9	HU LIO 2-0.009	F009	S		1	2014/3/25 14:22 +08:00	57 KB
10	HU LIO 2-1.010	F010	S		1	2014/3/25 14:22 +08:00	57 KB
11	HU LIO 2-4.011	F011	S		1	2014/3/25 14:22 +08:00	57 KB
12	HU LIO 2-16.012	F012	S		1	2014/3/25 14:22 +08:00	57 KB
13	HU LIO 2-64.013	F013	S		1	2014/3/25 14:22 +08:00	57 KB
14	HU LIO 2-250.014	F014	S		1	2014/3/25 14:22 +08:00	57 KB
15	HU LIO 2-1000.015	F015	S		1	2014/3/25 14:22 +08:00	57 KB
16	HU LIO 2-4000.016	F016	S		1	2014/3/25 14:22 +08:00	57 KB

At Wizard Step 1, right click mouse in the list to display popup menu as below:



- **Set Type As**: Choose a type (S, Background, Positive) for current file.
- **Copy**: Copy the selected data.
- **Paste:** Paste the type.
- **Paste with Code**: Paste the type and code.
- **Remove**: Remove the file.

Related topics:

- Add Files
- Quantitative FCS Files List

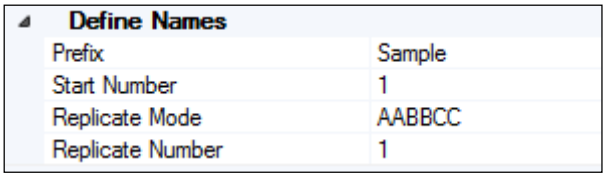
Files

Right after loading FCS files to the FCS Files list, all files are shown by default “S” in the **Type** column, indicating they are samples.

There're 4 file types in Qualitative assay: **C** (Control), **S** (Sample), **B** (Background), **P** (Positive). These can be converted to each other.

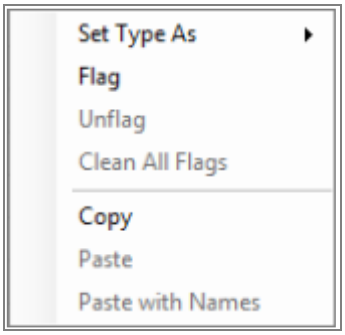
Do the following if necessary:

1. At Wizard Step 1, input a new sample name if necessary. Select a file or multiple files in the Qualitative FCS Files List, then click **Define Names** icons to define the samples' name with series.



2. At Wizard Step 1 and Wizard Step 2, change the sample file type by right clicking in the Qualitative FCS Files List and then choose a type from Set Type As on the dropdown list.

3. At Wizard Step 2, click on the files to display a similar popup menu as below:



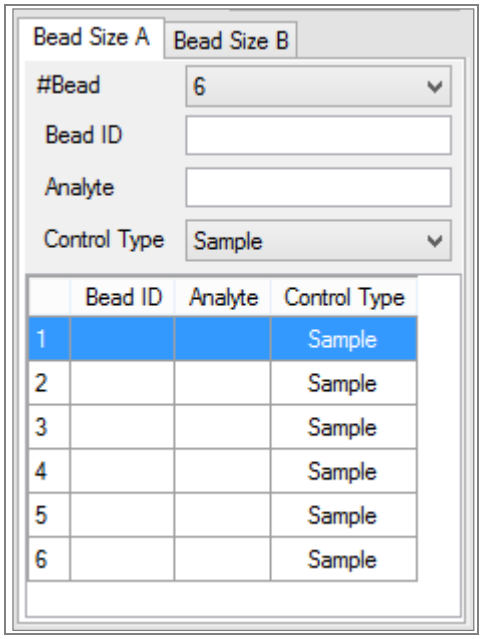
- **Set Type As**: Choose a type (Sample, Background,C0, C1, C2...) for current file.
- Flag, Unflag, Clean All Flags
- **Copy**: Copy the selected data.
- **Paste:** Paste the type and concentration.

Related topics:

- Convert To Samples
- Convert To Standard Curve
- Calibrator(Standard Curve) Files

Qualitative Gating

Qualitative gating is similar to Quantitative Gating Procedure. The only difference is the part in Gate Settings as shown below:



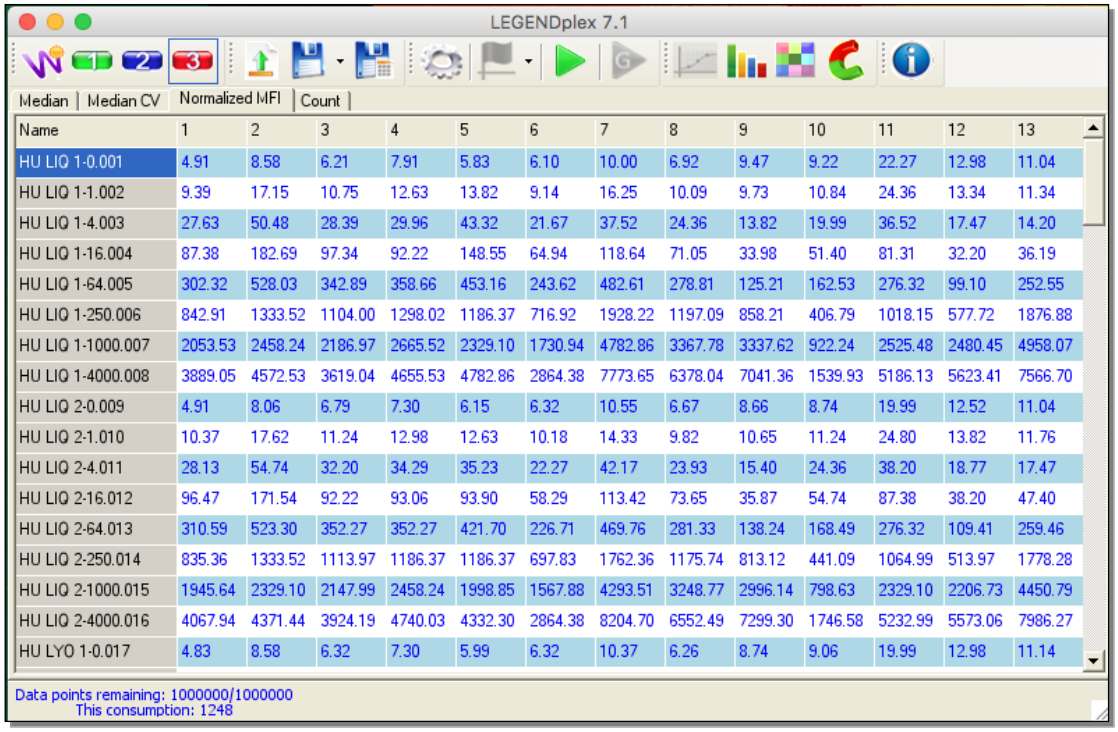
Click the drop down list of **Control Type** to select a control type: **Sample**, **Positive, Negative.**

Related topics:

- Bead Gating Window
- Gate Settings
- Gating Procedure
- Qualitative FCS Files List

Qualitative Result

After the data has been analyzed, the result data will be displayed as shown below:



Name	1	2	3	4	5	6	7	8	9	10	11	12	13
HU LIQ 1-0.001	4.91	8.58	6.21	7.91	5.83	6.10	10.00	6.92	9.47	9.22	22.27	12.98	11.04
HU LIQ 1-1.002	9.39	17.15	10.75	12.63	13.82	9.14	16.25	10.09	9.73	10.84	24.36	13.34	11.34
HU LIQ 1-4.003	27.63	50.48	28.39	29.96	43.32	21.67	37.52	24.36	13.82	19.99	36.52	17.47	14.20
HU LIQ 1-16.004	87.38	182.69	97.34	92.22	148.55	64.94	118.64	71.05	33.98	51.40	81.31	32.20	36.19
HU LIQ 1-64.005	302.32	528.03	342.89	358.66	453.16	243.62	482.61	278.81	125.21	162.53	276.32	99.10	252.55
HU LIQ 1-250.006	842.91	1333.52	1104.00	1298.02	1186.37	716.92	1928.22	1197.09	858.21	406.79	1018.15	577.72	1876.88
HU LIQ 1-1000.007	2053.53	2458.24	2186.97	2665.52	2329.10	1730.94	4782.86	3367.78	3337.62	922.24	2525.48	2480.45	4958.07
HU LIQ 1-4000.008	3889.05	4572.53	3619.04	4655.53	4782.86	2864.38	7773.65	6378.04	7041.36	1539.93	5186.13	5623.41	7566.70
HU LIQ 2-0.009	4.91	8.06	6.79	7.30	6.15	6.32	10.55	6.67	8.66	8.74	19.99	12.52	11.04
HU LIQ 2-1.010	10.37	17.62	11.24	12.98	12.63	10.18	14.33	9.82	10.65	11.24	24.80	13.82	11.76
HU LIQ 2-4.011	28.13	54.74	32.20	34.29	35.23	22.27	42.17	23.93	15.40	24.36	38.20	18.77	17.47
HU LIQ 2-16.012	96.47	171.54	92.22	93.06	93.90	58.29	113.42	73.65	35.87	54.74	87.38	38.20	47.40
HU LIQ 2-64.013	310.59	523.30	352.27	352.27	421.70	226.71	469.76	281.33	138.24	168.49	276.32	109.41	259.46
HU LIQ 2-250.014	835.36	1333.52	1113.97	1186.37	1186.37	697.83	1762.36	1175.74	813.12	441.09	1064.99	513.97	1778.28
HU LIQ 2-1000.015	1945.64	2329.10	2147.99	2458.24	1998.85	1567.88	4293.51	3248.77	2996.14	798.63	2329.10	2206.73	4450.79
HU LIQ 2-4000.016	4067.94	4371.44	3924.19	4740.03	4332.30	2864.38	8204.70	6552.49	7299.30	1746.58	5232.99	5573.06	7986.27
HU LYO 1-0.017	4.83	8.58	6.32	7.30	5.99	6.32	10.37	6.26	8.74	9.06	19.99	12.98	11.14

There are the Normalized MFI, Count, Median CV, and Median. Click tabs to switch between the views.

Normalized MFI and Median data could also be viewed in Bar Chart and Clustering or Heat Map view.

Related topics:

- Wizard Step 3
- Options

Output

There are 2 types of result reports: Summary and Detail.
Summary: Normalized MFI, MFI, Count, Files Information, in .xlsx format.
Detail: Normalized MFI, Detail MFI, Count, Files Information, in .xlsx format.

Options

Options dialog box is the user interface to define data analysis parameters and select options.

Click  on the Toolbar to open **Options** dialog box.

There are: Curve Option, Data Analysis Option and View Option sections.

After you apply the changes of the parameters in **Options**, LEGENDplex™ will reanalyze automatically.

LEGENDplex™ offers different parameters for Quantitative and Qualitative analysis.

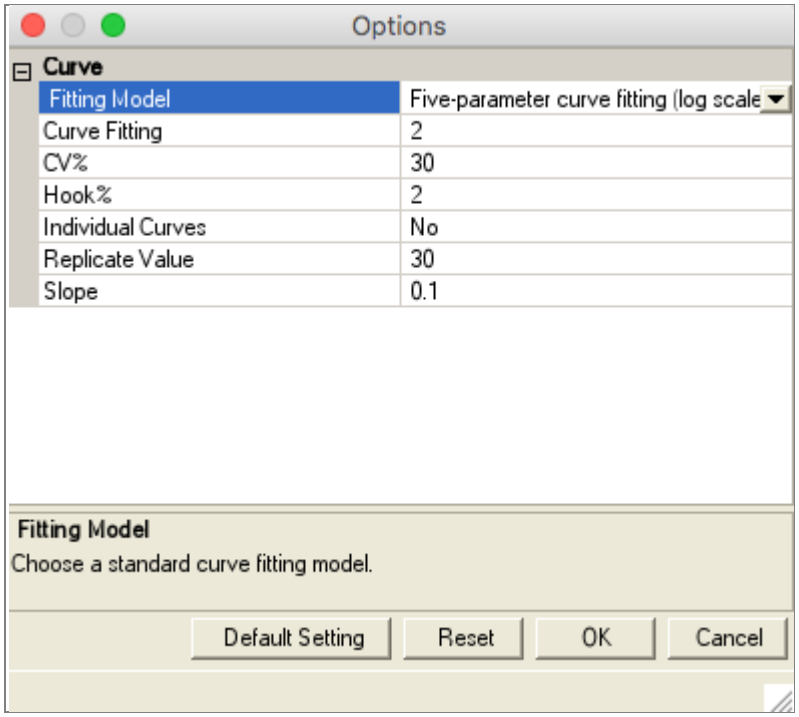


Figure 1. Quantitative Options

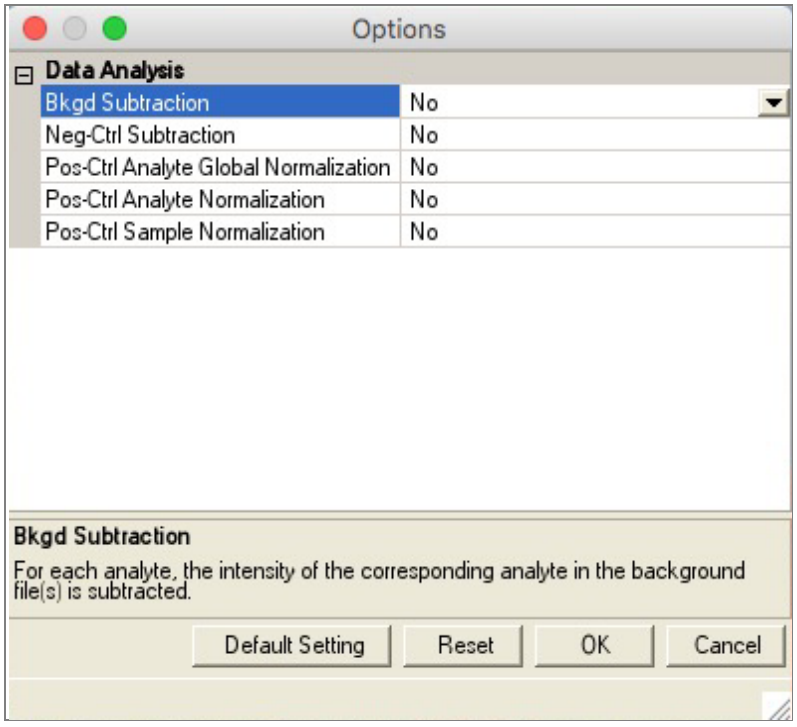


Figure 2. Qualitative Options

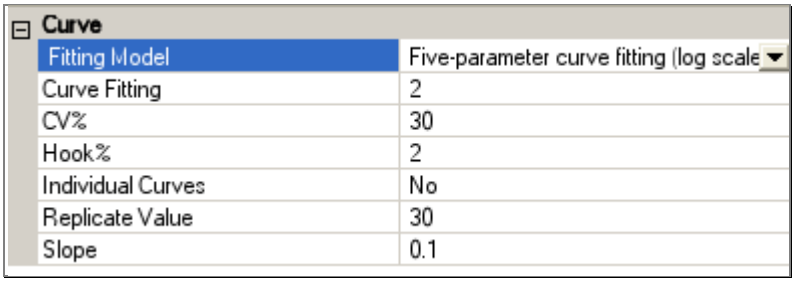
Click **Default Setting** to load the default value of the parameters.
Click **Reset** to load the value set last time.

Related topics:

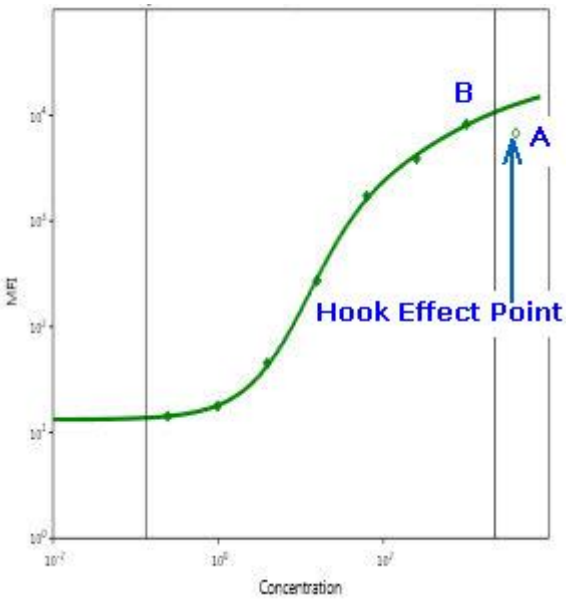
- Wizard Step 2
- Edit Standard Curve
- Curve Option
- Data Analysis Option
- View Option
- Standard Curve Fitting Model
- Toolbar

Curve Option

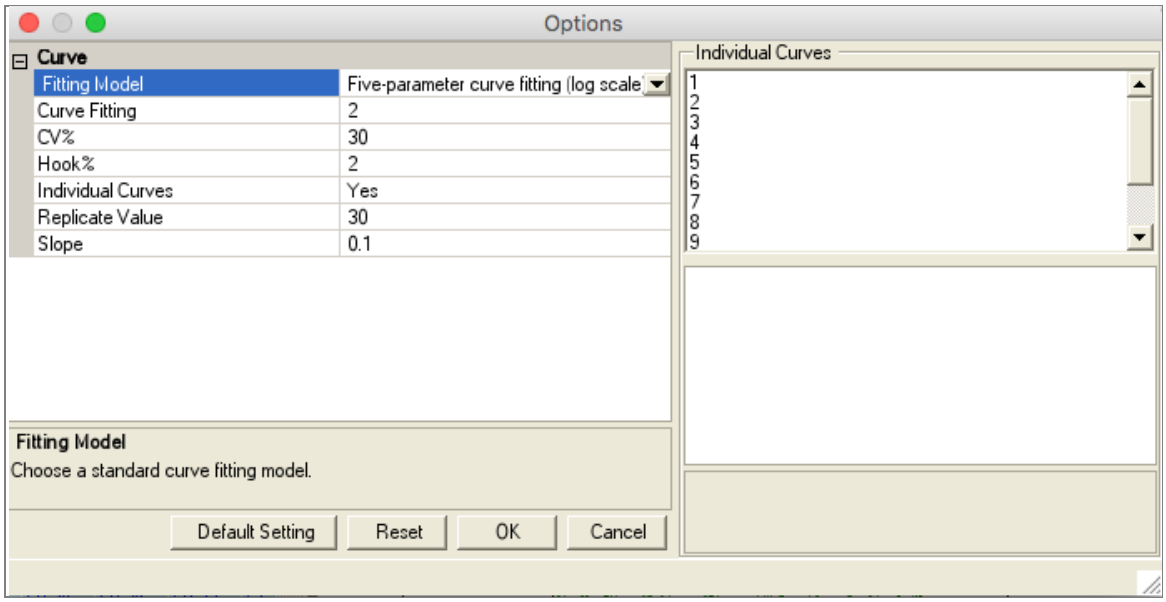
Open Options and see the **Curve** Option.



Parameter	Range	Means
Curve Fitting	**0.01-10**	Set quality threshold to filter high CV data points from curve fitting. Coefficient of variance of the curve fitting, curve outlier threshold is multiplied by this. Increase to flag out more curves. Set larger values for challenge curves. Default value is 2.
CV%	**0.1 - 100 (%)**	Set threshold of curve fitting CV in % to assess the data quality of curve fitting. Percentage of coefficient of variance of the fitting. Decrease to flag out more curves. • Status: Good, if **Curve CV < Threshold CV** • Status: Warning, if **Threshold CV < Curve CV < 2*Threshold CV** • Status: Poor, if **Curve CV > 2*Threshold CV** Default value is 30.
Fitting Model	/	Select one curve fitting model from the drop-down list Standard Curve Fitting Model.
Hook	**-100 - 100 (%)**	Set threshold of MFI value either jump or drop in % of whole curve Vertical rises to assess if it is hook effected standard curve. The hook threshold is the minimum difference between the highest (or lowest) point and the end point to qualify as a hook. Default value is 2. For example, from the last point (A) to the adjacent one (B), the difference in values between two points must exceed the specified percentage range of Y, |Ya-Yb|>Hook*(Ymax-Ymin). Otherwise, it won't be treated as a hook point.

		 If LEGENDplex™ finds a hook point at the last two points or the first two points and the hook threshold is >= 0, just remove the last point or the first point. Otherwise, check to see which point should be removed based on the chi square.
Replicate Value	**0.01 - 100**	Set CV threshold of replicate signal intensities average to filter out data point with large replicate CV. The average error threshold of replicate analytes. Increase to in upper the max CV value.(max CV = Threshold Replicate Data * Threshold CV) Default value is 30.
Slope	**0 - 1**	Set threshold of the slope at IC50 point to filter out very flat curves. Indicates the threshold slope at the inflection point. Increase to flag out more curves. • Status: Good, if **Curve Slope > Threshold Slope** • Status: Warning, if **Threshold Slope / 2 < Curve Slope < Threshold Slope** • Status: Poor, if **Curve Slope <Threshold Slope / 2** Default value is 0.1.

Individual Curves: Check the **Individual Curves** check box to recalculate a selected Individual Curve. Click each analyte to set the curve options respectively.

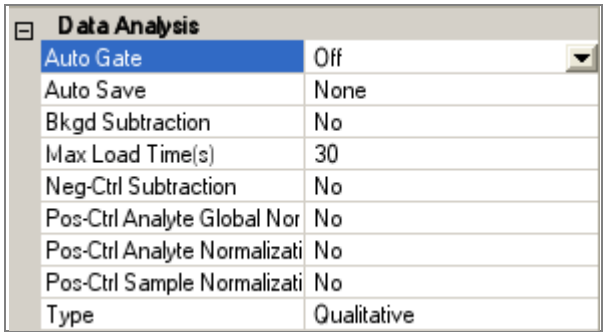


Related topics:

- Wizard Step 2
- Edit Standard Curve
- Data Analysis Option
- View Option
- Standard Curve Fitting Model
- Toolbar
- Flag Data

Data Analysis Option

Open Options and see the **Data Analysis** Option.

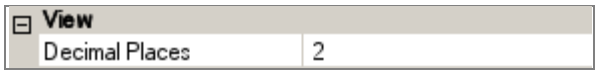


- Auto Gate
- Auto Save

- Background Subtraction
- Neg-Ctrl Subtraction
- Pos-Ctrl Analyte Global Normalization
- Pos-Ctrl Analyte Normalization
- Pos-Ctrl Sample Normalization

View Option

Open Options to see the **View** Option.



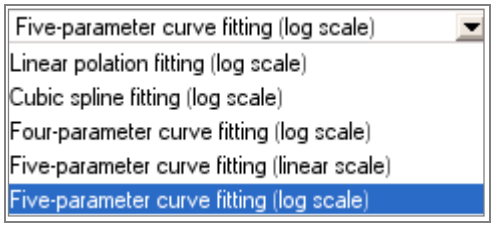
- **Decimal Places** --- Define the decimal places displayed in the program.

Related topics:

- Wizard Step 2
- Edit Standard Curve
- Curve Option
- Data Analysis Option
- Standard Curve Fitting Model
- Toolbar
- Median
- Quantitative Result

Standard Curve Fitting Model

Select the Standard Curve Fitting Model from the drop down list in Curve Option**.**



Select a following Standard Curve Fitting Model:

- **Linear curve fitting (log scale)** --- 2 parameters linear curve fitting model with log(x) and log (y) scale, the model is $Y = P1 + P2 \cdot x$.

- **Cubic spline fitting (log scale)** --- 3 parameters cubic spline curve fitting model with log(x) and log(y) scale.
- **Four-parameter curve fitting (log scale)** --- 4 parameters non logistic curve fitting model with log(x) and log (y) scale.
- **Five-parameter curve fitting (linear scale)** --- 5 parameters logistic curve fitting model with log(x) and y linear scale.
- **Five-parameter curve fitting (log scale)** --- 5 parameters logistic curve fitting model with log(x) and log (y) scale.

Related topics:

- Options
- Curve Option
- Edit Standard Curve Dialog Box
- Set Standard Curve
- Set Standard Curve Manually

Index