Diaclone DIAplex

DIAplex Pro 1.0 Software

Instructions for use

For research use only

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1. Introduction

DIAplex is a sensitive multiplex fluorescent bead-based immunoassay for the simultaneous quantification of multiple analytes from a single sample by Flow Cytometry.

Utilising bead populations with distinct fluorescence intensities and unique antibody specificities DIAplex can accurately measure multiple analytes in a single sample with a significantly reduced assay time and sample volume requirement when compared to traditional ELISA techniques.

Antibodies highly specific to the analytes to be detected are coupled to fluorescently labelled detection beads. Each bead is analogous to an individually coated well in an ELISA providing a capture surface for a specific analyte. One (simplex) or more (multiplex) populations of the beads are combined in suspension with the sample or standard under test. Any analytes present in the sample will bind to the specific antibodies linked to the fluorescent beads. A biotin-conjugated antibody mixture is then added which binds to any analytes captured by the first antibodies. Streptavidin-Phycoerythrin (PE) is added, which binds to the biotin conjugates and emits a yellow fluorescent signal. The unique size and fluorescent signature of each bead is differentiated by Flow Cytometry.

Following sample data acquisition using a Flow Cytometer, analyte concentration in the test sample is calculated simply by using the DIAplex Pro 1.0 analysis software.

2. DIAplex Pro 1.0 installation

2.1. System Requirements

The DIAplex Pro 1.0 software has been validated for use on PC computers running Windows XP with 256 MB RAM.

The software is compatible with the following data acquisition files; .FCS 2.0
.FCS 3.0
.LMD

2.2. DIAplex Pro 1.0 License

You are granted a non-exclusive, non-transferable limited license without license fees to use this software to analyze and evaluate DIAplex experiments using Diaclone products.

The redistribution of this software using any kind of support is forbidden.

2.3. Software Installation

Put the CD-rom in the CD drive of your computer. If your computer supports autostart, the installation procedure will follow automatically. If not, select your CD drive and start the installation manually by double-clicking on the file “setup_DIAplex_Pro.exe”.

Or

Go to the website www.gen-probe.com to download and execute the “setup_DIAplex_Pro.exe”.

Select the directory where you want to install the software, the name in the start menu and if you want icons on the desktop or in the quick launch zone. After installation is complete, start the program by clicking on either the desktop icon, the quick launch icon in the start menu (by default “Start/Programs/DIAplex Pro/DIAplex Pro”) or in the install directory (by default “C:\Program files\DIAplex Pro\”) DIAplex.exe.

3. DIAplex analysis

3.1. Software Presentation

The DIAplex Pro 1.0 screen is divided into 3 sections (Figure 1.): the menu at the top of the screen, analysis steps 1 to 4 on the left hand side of the screen and the central section corresponding to the information related to the current step being performed.

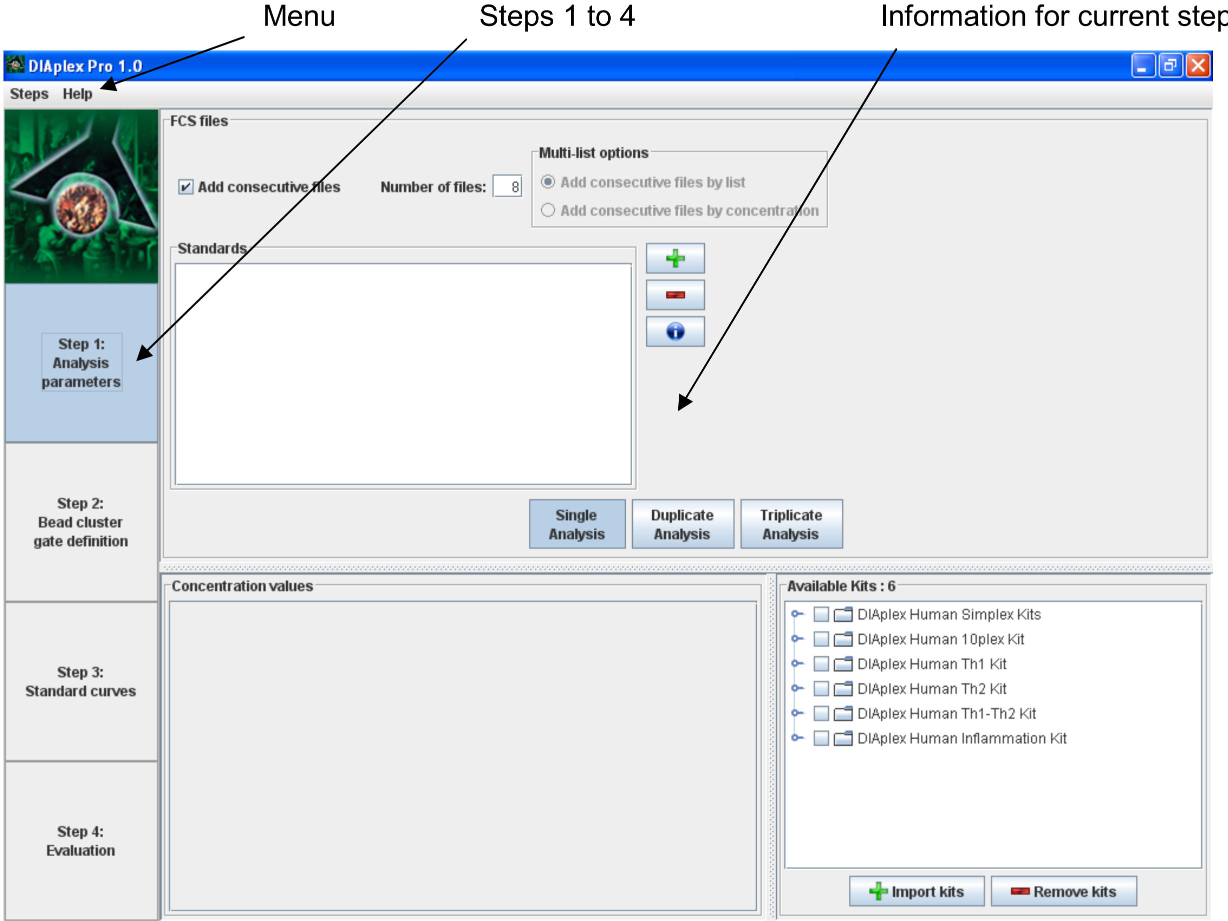


Figure 1: Software presentation

3.2. Analysis Steps

There are four steps in a DIAplex analysis, corresponding to the analysis progression:

Step 1: Analysis parameters
Step 2: Bead Cluster gate definition
Step 3: Standard curves
Step 4: Evaluation

These four steps correspond to the buttons shown in figure 2. There is always a selected button which corresponds to the current step in the analysis. The button corresponding to the next step in the analysis sequence can be selected if the prior step is complete, otherwise the button is deactivated.

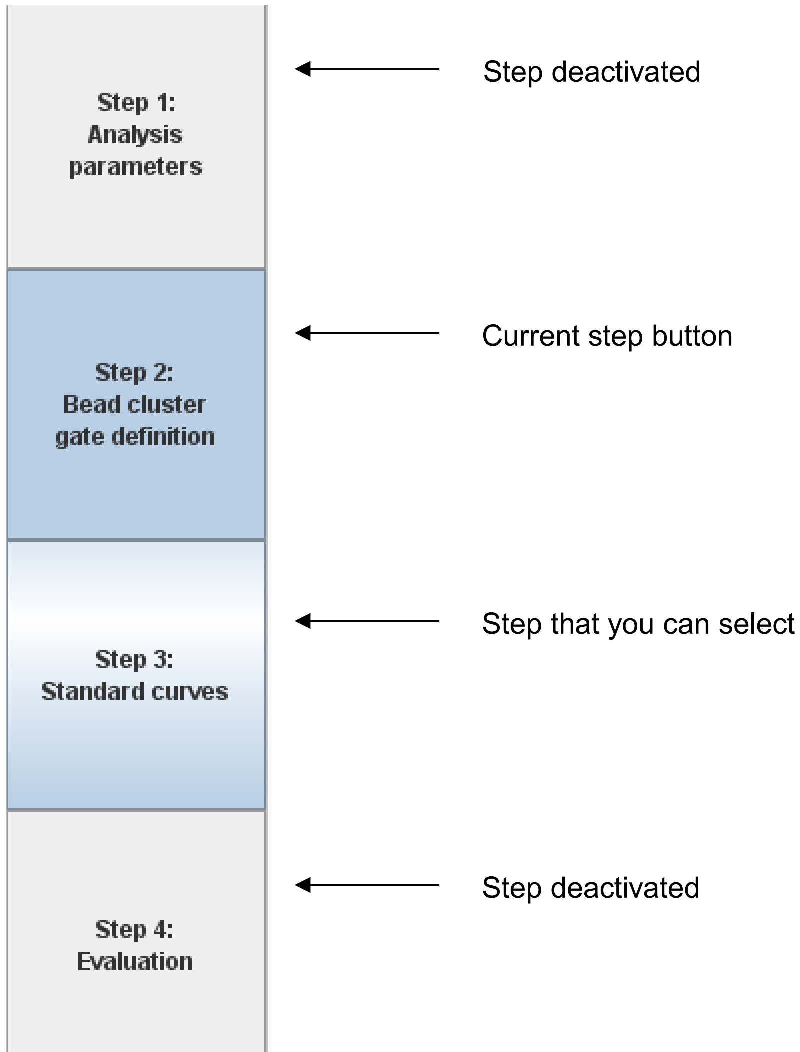


Figure 2: Analysis Steps

3.3. Menu

The menu is composed of the ‘steps’ menu and the ‘help’ menu.

Under step menu, you can start a ‘new analysis’. This will delete everything you have already entered. Once data is entered and steps have been completed under the ‘step menu’ you have the option to return to the preceding step in the analysis sequence. This will result in a loss of the information entered at the step that you leave. i.e. going from Step 3 back to Step 2 will result in data loss from Step 3. Under the step menu you can also ‘exit’ from the software.

In the help menu, you can display a help frame which provides further guidance on each step of the software analysis. When opened, the help frame will display the help of the current step in process.

4. Step 1: Analysis parameters

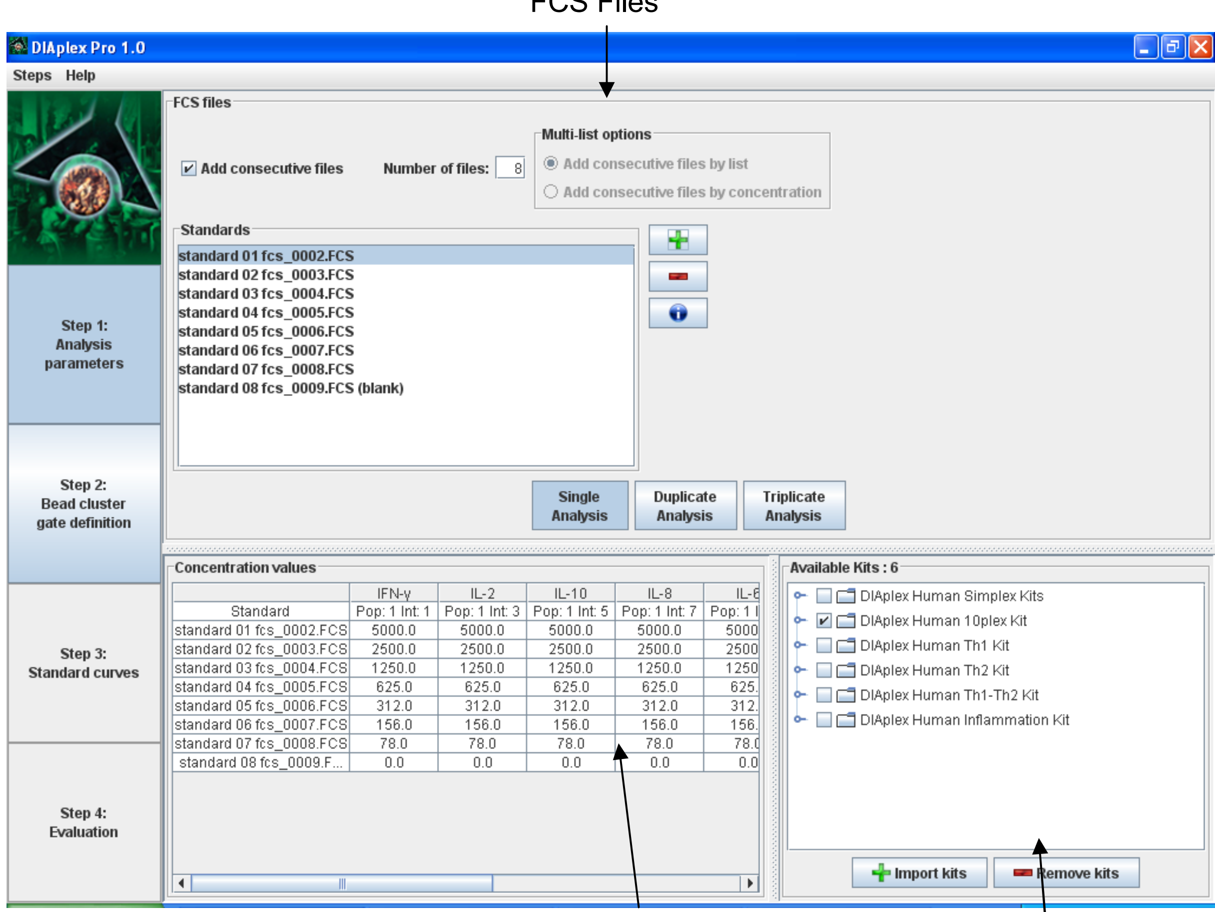
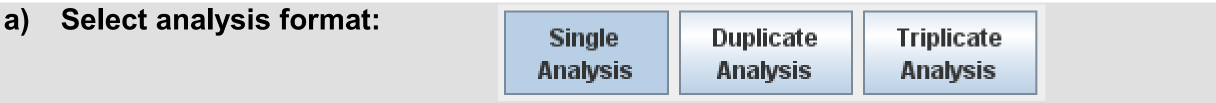


Figure 3: Step 1: Analysis parameters Concentration values Available Kits

4.1. FCS files

a) Select analysis format:



Activate the button corresponding to the required analysis format: a standard analysis (with one list of standard files), a duplicate analysis (two lists: Standard and Control 1) or a triplicate analysis (three lists: Standard, Control 1 and Control 2).

b) Add standard curve data files:

Click on the + button to add the standard curve data files. Go to your data files directory and select the file corresponding to the highest standard point first.

There are two options to import the data file list for the full standard curve:

i) Select the **Add consecutive files** by checking the box and insert the **Number of files** for your standard curve (usually 8). If the selected file's name ends with numeric characters, the software will automatically fill the list in the order in which they are named. E.g. select the Standard_01.FCS in the first table row and the following rows will select Standard_02.FCS…Standard_08.FCS, creating a complete standard curve from 7 standard files and 1 blank file (Figure 4.).

ii) Unselect **Add consecutive files** if your standard files do not correspond to consecutive order and open them one by one.

Note: Standard curve data files need to be imported from the highest standard down to lowest with the blank file last. When naming files during FACS acquisition thought should be given to naming with numerical characters as the last digits to enable consecutive file addition (Figure 4.)

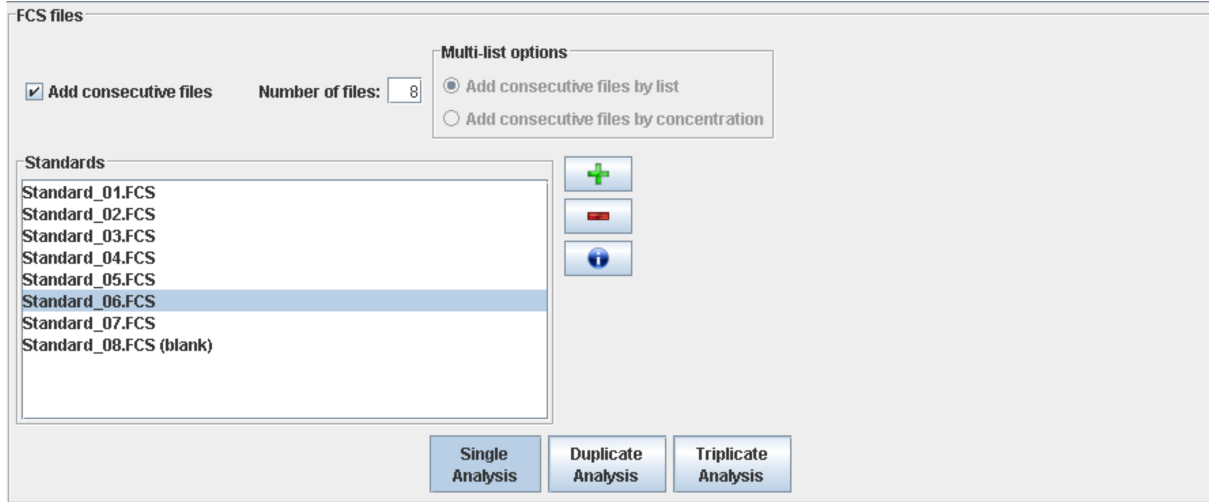


Figure 4: Add consecutive files selection

For a duplicate or triplicate analysis, select the appropriate standard files naming scheme in the multi-list options section:

Select **Add consecutive files by lists:** The software will start from file Standard_01.FCS to Standard_08.FCS as first standard curve (if **Number of files**: 8) and continue with Standard_09.FCS to Standard_16.FCS as second standard curve (control 1)).

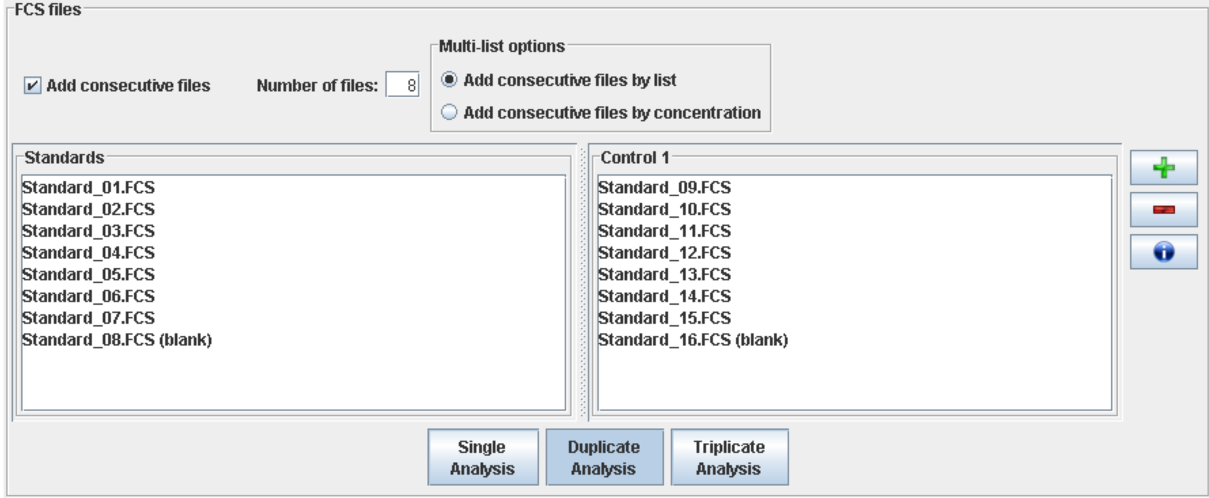


Figure 5: Add consecutive files by list in duplicate analysis

Select **Add consecutive files by concentration**: The software will sort the files by concentration values. E.g. Standard_01.FCS, Standard_03.FCS… Standard_15.FCS for the first standard curve and Standard_02.FCS, Standard_04.FCS… Standard_16.FCS for the second curve.

FCS files

Add consecutive files Number of files: 8

Multi-list options

Add consecutive files by list

Add consecutive files by concentration

Standards	Control 1
Standard_01.FCS	Standard_02.FCS
Standard_03.FCS	Standard_04.FCS
Standard_05.FCS	Standard_06.FCS
Standard_07.FCS	Standard_08.FCS
Standard_09.FCS	Standard_10.FCS
Standard_11.FCS	Standard_12.FCS
Standard_13.FCS	Standard_14.FCS
Standard_15.FCS (blank)	Standard_16.FCS (blank)

Single Analysis Duplicate Analysis Triplicate Analysis

Figure 6: Add consecutive files by concentration in duplicate analysis

c) Removing standard curve data files:

To remove files, select them and click on the button.

d) File information:

The button provides the information on the file's name, the file's version, the parameters number and the events number.

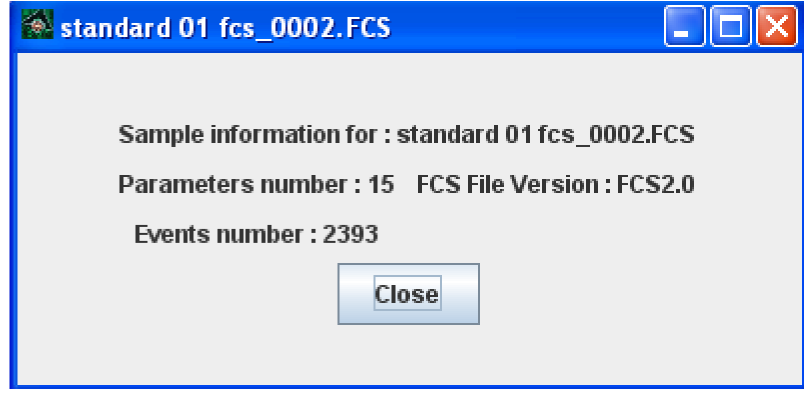


Figure 7: FCS file information

4.2. Available kits

Select the square check box next to the DIAplex analysis Kit required. Select the analytes to be included in analysis.

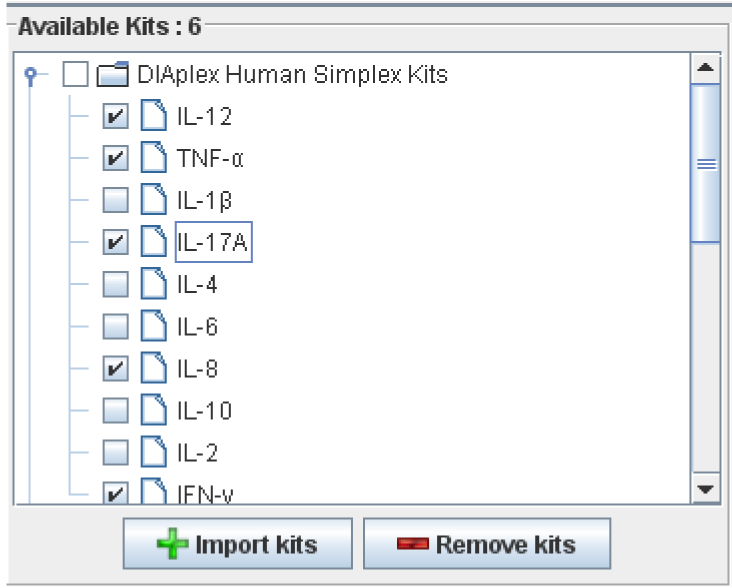


Figure 8: Available kits

Import kits button and **Remove kits** button allows you to import or delete available kits from the tree.

4.3. Concentration values

The software provides automatically a table with the recommended standard range for each selected analyte. The blank file always has a concentration of zero.

Concentration values

	IFN-γ	IL-8	IL-17A	TNF-α	IL-12
Standard	Pop: 1 Int: 1	Pop: 1 Int: 7	Pop: 2 Int: 4	Pop: 2 Int: 8	Pop: 2 Int: 10
Standard_01.FCS	5000.0	5000.0	5000.0	5000.0	5000.0
Standard_02.FCS	2500.0	2500.0	2500.0	2500.0	2500.0
Standard_03.FCS	1250.0	1250.0	1250.0	1250.0	1250.0
Standard_04.FCS	625.0	625.0	625.0	625.0	625.0
Standard_05.FCS	312.0	312.0	312.0	312.0	312.0
Standard_06.FCS	156.0	156.0	156.0	156.0	156.0
Standard_07.FCS	78.0	78.0	78.0	78.0	78.0
Standard_08.FCS (blank)	0.0	0.0	0.0	0.0	0.0

Figure 9: Concentration values

Note: If the default standard concentrations have not been used the standard concentration values can be changed by double-clicking on a concentration. If you change the first concentration of an analyte, the subsequent concentrations can be automatically amended with the dilution factor of ½. The values can also be changed individually by manual input.

Once the analysis parameters are determined, go to step 2 by selecting **Step 2: Bead cluster gate definition.**

5. Step 2: Bead cluster gate definition

Once selected this step will automatically process the standard curve data files imported to produce a 2D-clustering plot which is used to distinguish the two bead populations by size. Each of the two beads populations are then further differentiated by analysis of their fluorescence characteristics using the fluorescence clustering plots 1 (blue, 5μm beads) and 2 (red, 4μm beads). Each of these plots can be selected for manipulation using the tabs at the top of the screen.

5.1. 2D-Clustering

This page displays a scatter-plot of the events of the standard file selected in the files list above the chart. You can switch through the standard files just by clicking on ← or →. Alternatively, you can choose another file by clicking on the black arrow at the right of the file's name and choosing a file in the list displayed.

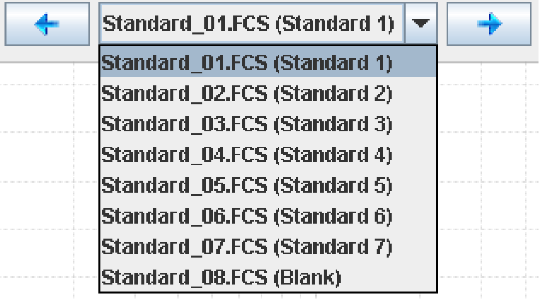


Figure 10: 2D-Clustering File Selection

Choose the required axis parameter (units) by clicking on the relevant axis. For example select forward scatter for the x-axis and Sideward scatter on the Y-axis for size discrimination (the parameter name will depend on your instrument e.g. FSC-H and SSC-H in a BD Flow Cytometer , Guava Technologies or FSLin and SSLin in a Beckman Coulter).

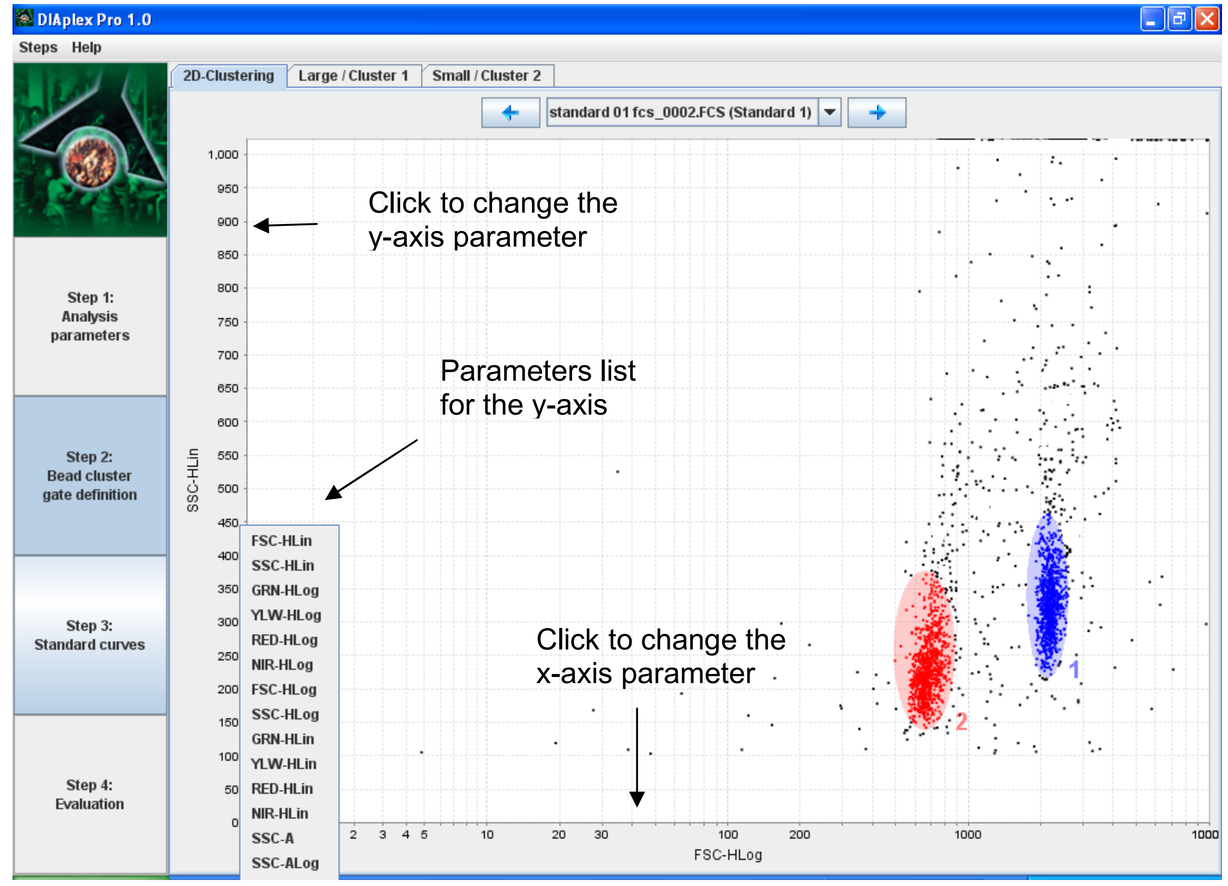


Figure 11: 2D-Clustering parameter axis change

The software will automatically determine and select the two different sized bead clusters (Blue 1 and Red 2), however this selection can be modified manually if you wish to exclude or include some data points. You can do so by clicking on the coloured ellipse, a black rectangle appears around the ellipse which can be re-sized.

Any changes made will automatically be adapted for the remainder of the analysis. Note that you can't overlay the two ellipses.

5.2. Fluorescence clusters 1 and 2

Choose the required axis parameter (units) by clicking on the relevant axis.

Select the fluorochrome for bead differentiation (far red, 685-690 nm fluorescence emission, the channel number depends on your instrument, e.g. 690 nm is FL3 in a BD Flow Cytometer but FL-4 in a Beckman Coulter) in a linear mode.

Select the fluorochrome for quantification (575 nm fluorescence emission, usually channel number FL2) in Log mode.

This chart shows the fluorescence intensities of the bead clusters either 1 (blue ellipse, 5µm beads) or 2 (red ellipse, 4µm beads).The coloured rectangles represent the different analytes. The software will automatically provide rectangles to separate bead clusters. You can adjust the borders by dragging the lines.

If events are selected in a rectangle, the MFI (Mean Fluorescence Intensity) appears as a vertical line in the rectangle. It corresponds to the median of the selected points.

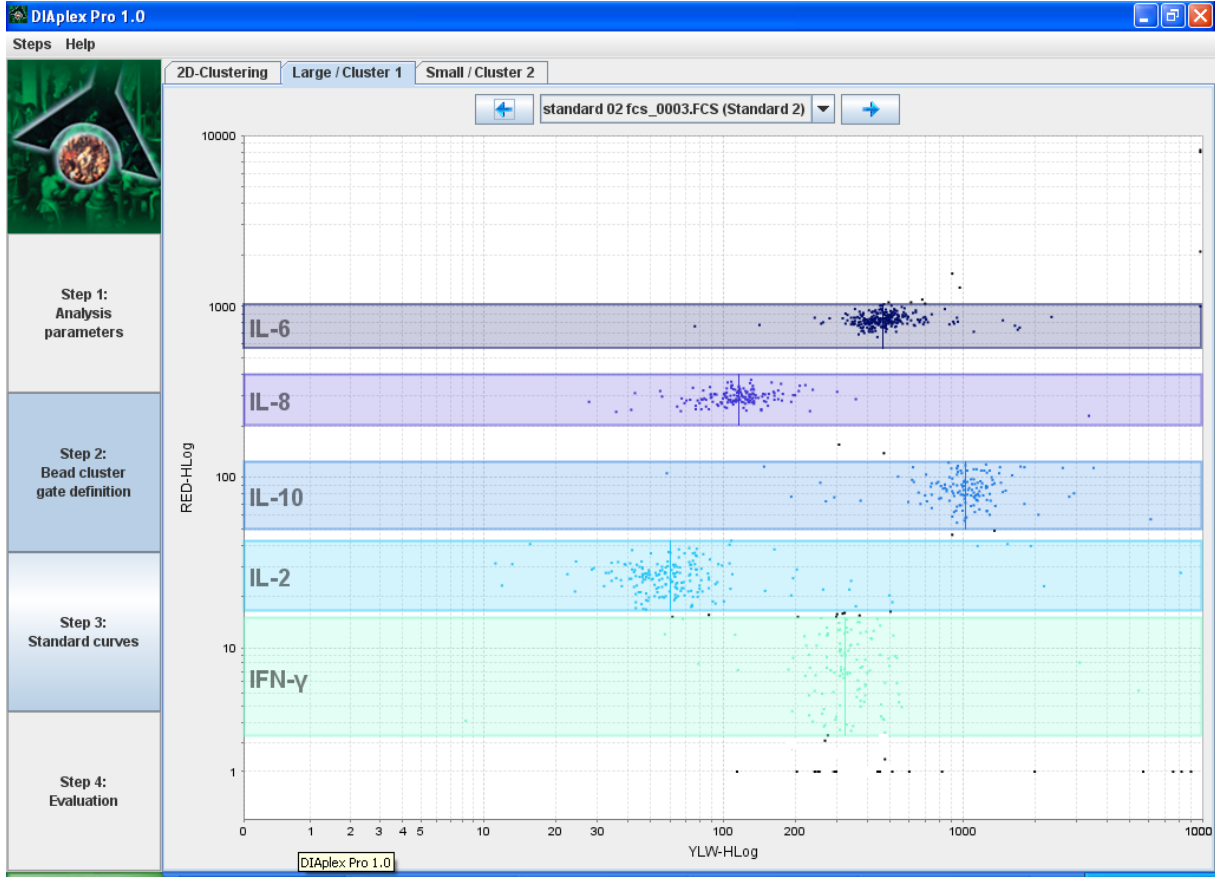


Figure 12: Fluorescence cluster 1

You can switch through the sample files just by clicking on ← or →. Alternatively, you can choose another file by clicking on the black arrow at the right of the file's name and choosing a file in the list.

If you want to adjust these regions you can do so by moving and/or resizing the rectangles. Once you are satisfied with the adjustments made to the 2D-clustering plot and fluorescence clustering plots 1 and 2, go to Step 3 by selecting **Step 3: Standard curves**.

6. Step 3: Standard curves

Once selected this step will automatically process the standard curve data files imported to produce a standard curve for each analyte under test. Standard curves can be reviewed and amended if necessary by omitting data points before the importation of the experimental sample data.

6.1. Standard curves

This panel displays the standard curves that have been created during steps 1-2. You can view the individual standard curves for each analyte by selecting from the analyte buttons above the curve.

An example standard curve is shown in Figure 13.

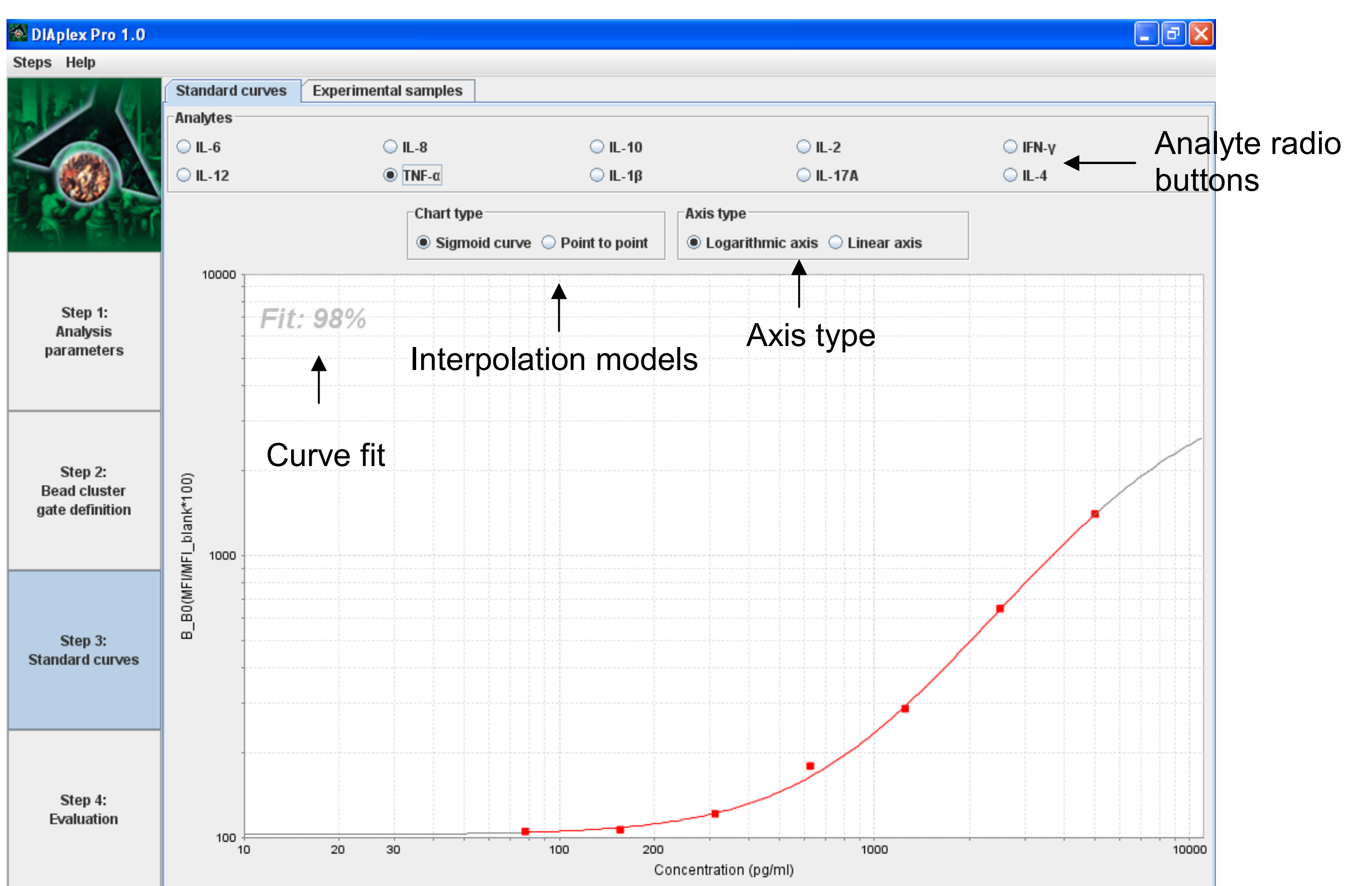


Figure 13: Standard curve

The program offers two interpolation models:

If you choose "Sigmoid curve" the program will find the best curve fit (log(y) = ((a - d)/(1 + (log(x)/c)^b)) + d). The grey part of the curve is the extrapolated part (outside the range of the standards used).

If you choose "Point-to-Point", the program will interpolate between two adjacent points using a line (y=ax+b).

On the charts, the curve fit is displayed to show if the curve is near the points.

The parameter of the x-axis is concentration, the parameter of the y-axis is given as MFI/MFI of blank * 100. You can choose between a linear and a logarithmic presentation of the standard curve by clicking on the buttons at the left of "Linear axis" or "Logarithmic axis".

Omitting / restoring data points:

In standard analysis, if you click on a red point you can omit it (it becomes grey) and the curve is recalculated without the deactivated points. If you click on a grey point, you restore the point (it becomes red) and the curve is recalculated (See Figure 14 and Figure 15 which represent the same data points but the second has a point removed).

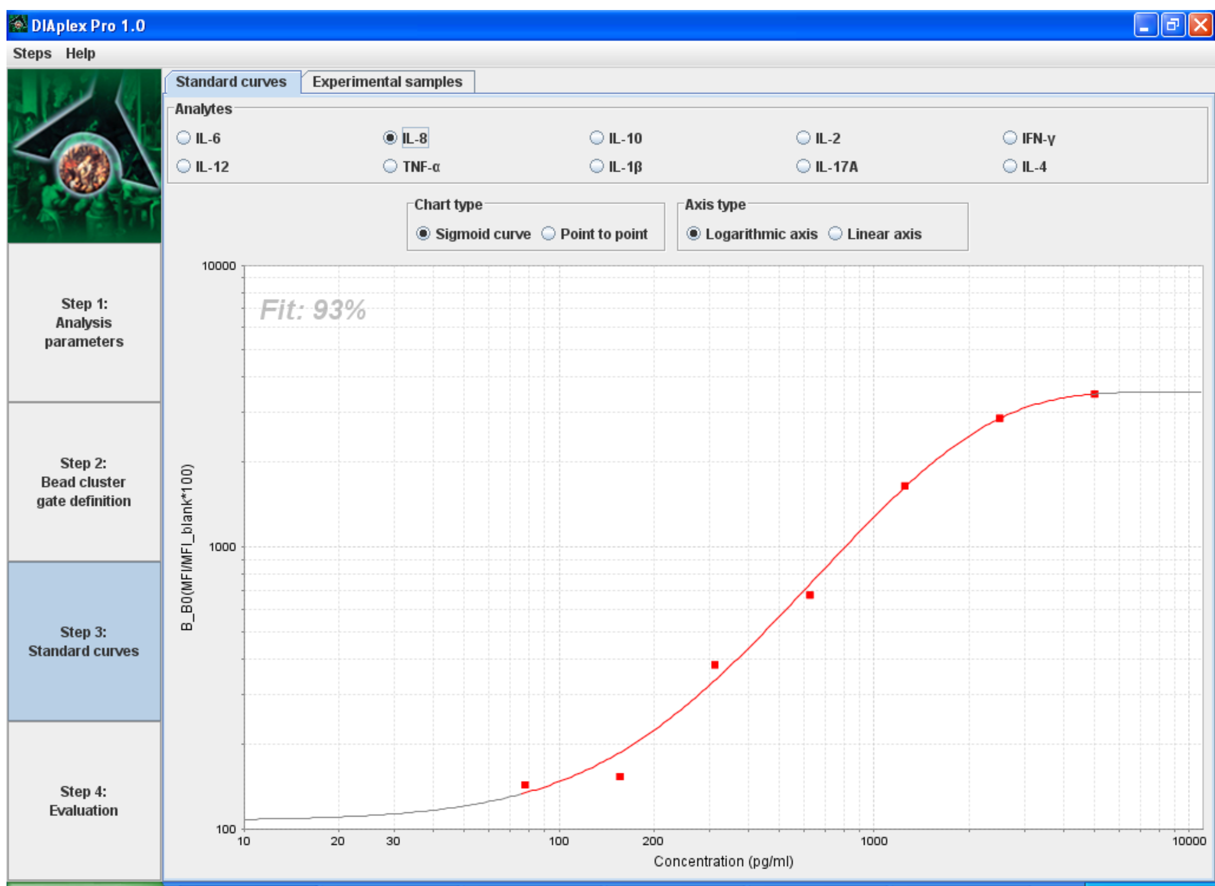


Figure 14: Standard curve with an incorrect value

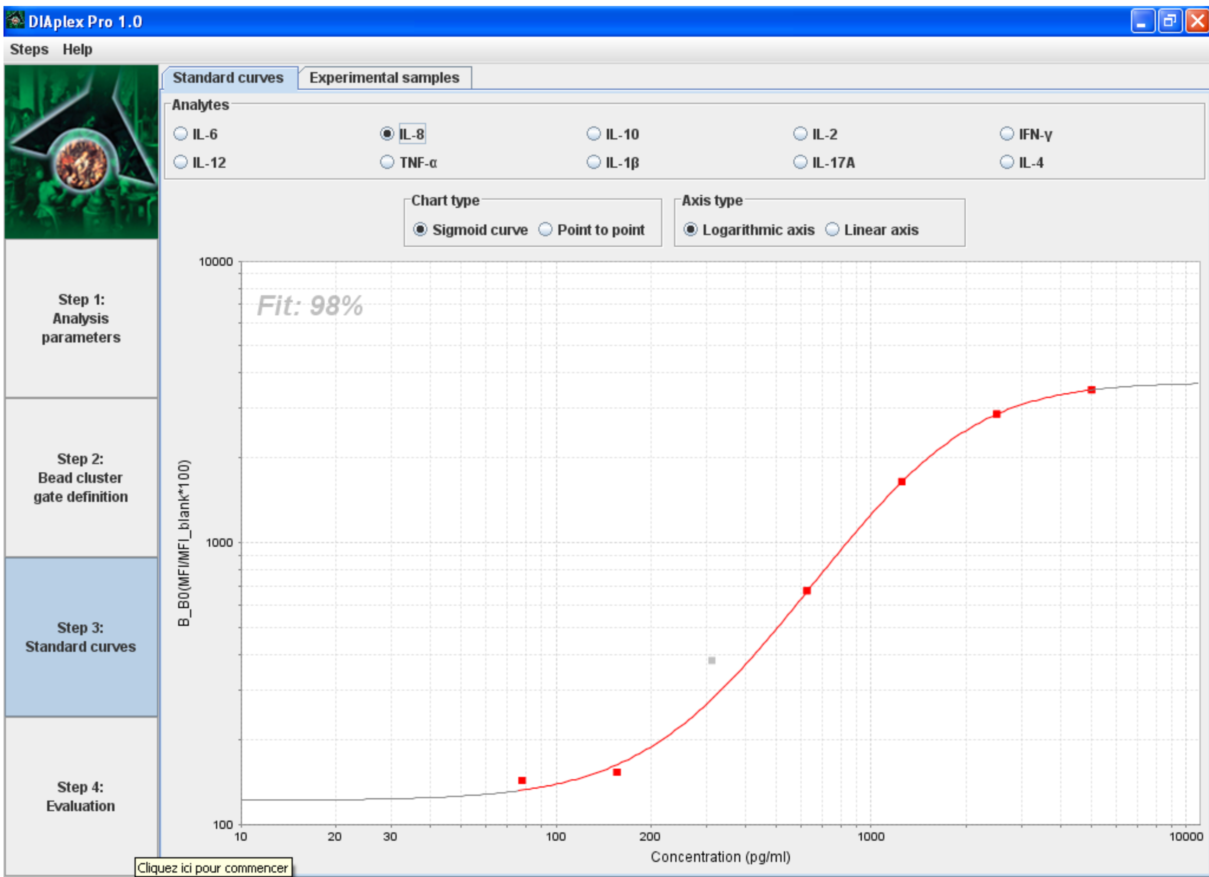


Figure 15: Standard curve with a point removed

Note: data points should only be omitted if they are known to be anomalous

In duplicate/triplicate analysis, the orange points correspond to a file, red points correspond to the B_B0 average of the orange and grey points correspond to omitted points. You can remove orange points.

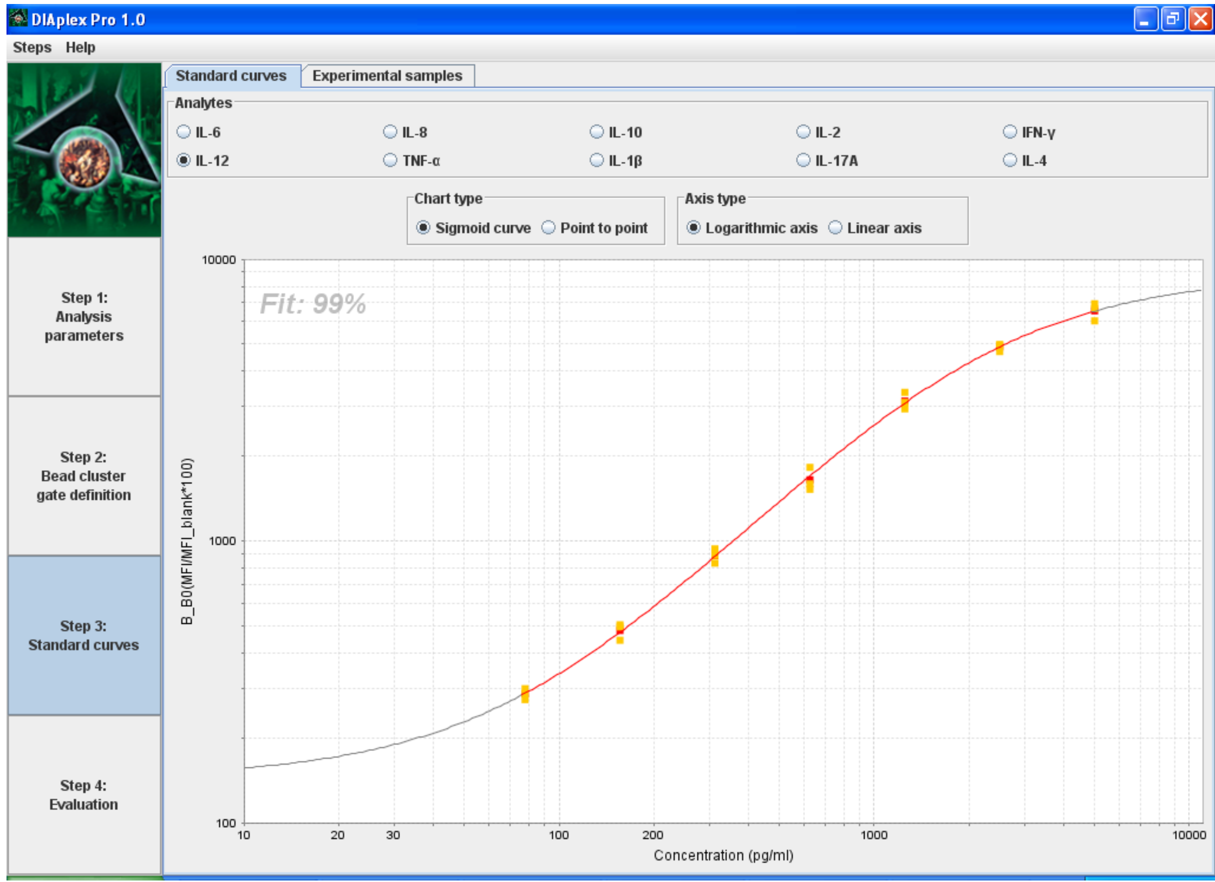


Figure 16: Standard curve in triplicate analysis

Once the standard curves are completed, the experimental sample files can be selected (FCS files).

6.2. Experimental samples

Experimental sample data files are imported as described for the importing of standard files (section 4.1).

Activate the button corresponding to the required analysis format: a single analysis (with one list of samples files), a duplicate analysis (two lists: Samples and Control 1) or a triplicate analysis (three lists: Samples, Control 1 and Control 2).

Click on the ✚ button to add the data files. Go to your data files directory and select the corresponding file.

There are three options to fill the list for a single analysis:

i) You can add files one by one by unselecting **Add consecutive files.**

ii) You can add all the consecutive files you have in your folder by selecting **Add consecutive files** and **All consecutive files**. E.g. your folder contains 10 consecutives samples files from Sample_01.FCS to Sample_10.FCS. If you select Sample_01.FCS, the

10 consecutive files will be automatically added in the lists.

iii) You can also limit the number of consecutive files by selecting **Add consecutive files**, unselecting **All consecutive files** and choosing the number of files by list in **Number of files**. E.g. your folder contains 10 consecutives samples files from Sample_01.FCS to Sample_10.FCS and your **Number of files** is 3. If you select Sample_01.FCS, the 3 consecutive samples, from Sample_01.FCS to Sample_03.FCS will be automatically added in the lists.

For a duplicate or triplicate analysis, select the appropriate standard files naming scheme in the multi-list options section:

- Select **Add consecutive files by lists:** The software will start from file Sample_01.FCS and will automatically fill with the following files. (Sample_02.FCS, Sample_03.FCS, ….)

- Select **Add consecutive files by concentration**: The software will sort the files by row.

c) Removing sample data files:

To remove files, select them and click on the button.

d) File information:

The button provides the information on the file's name, the file's version, the parameters number and the events number.

Once your samples selected enter the dilution (by default 1.0) and enter the sample name in the <Enter the sample name> sample label. When you enter a new dilution factor, the concentration values of the samples will be adjusted accordingly.

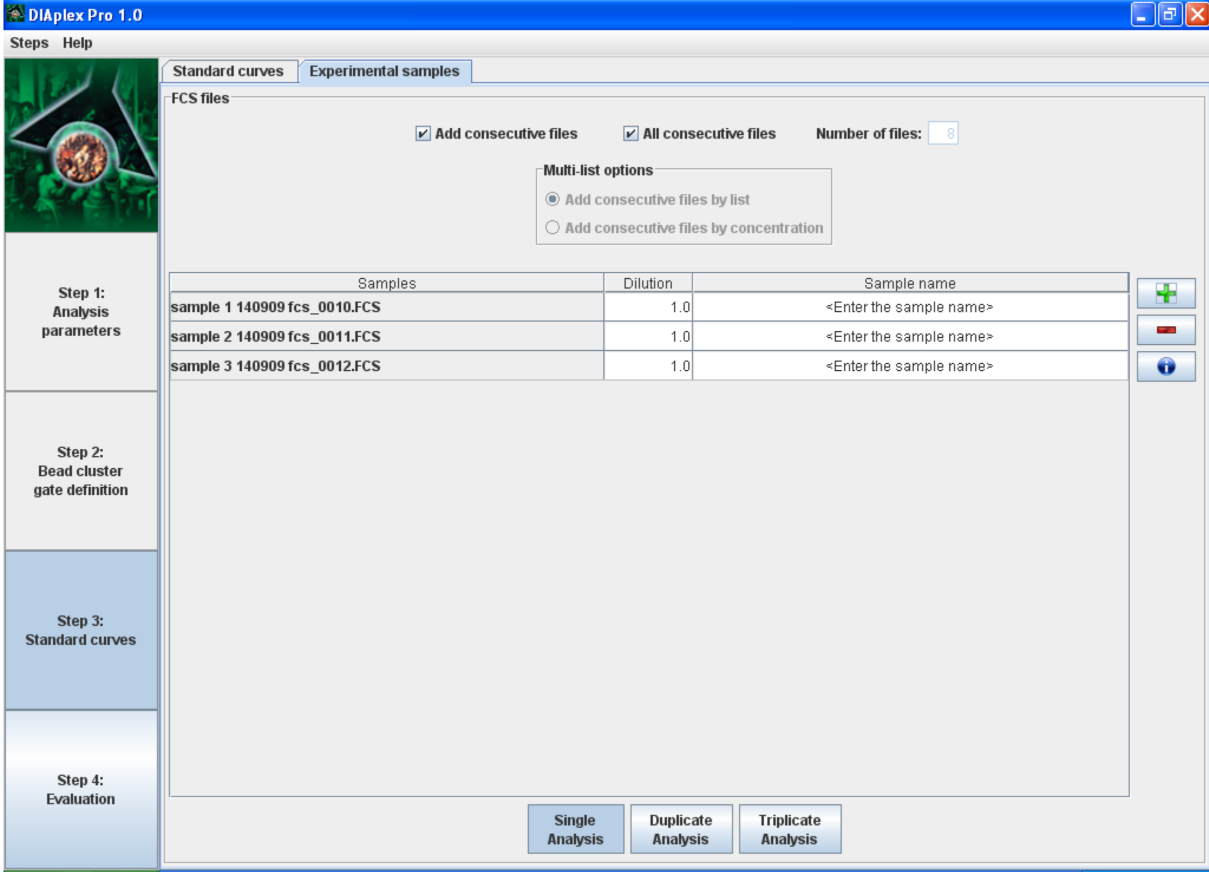


Figure 17: Experimental samples

Once samples have been selected and sample information entered proceed to **Step 4: Evaluation.**

7. Step 4: Evaluation

Report by experimental samples | Report by analytes | 2D-Clustering | Fluorescence Cluster 1 | Fluorescence Cluster 2 | Standard curves

The report presents the MFI and the concentration values in two different formats:

7.1. Report by experimental samples and report by analytes

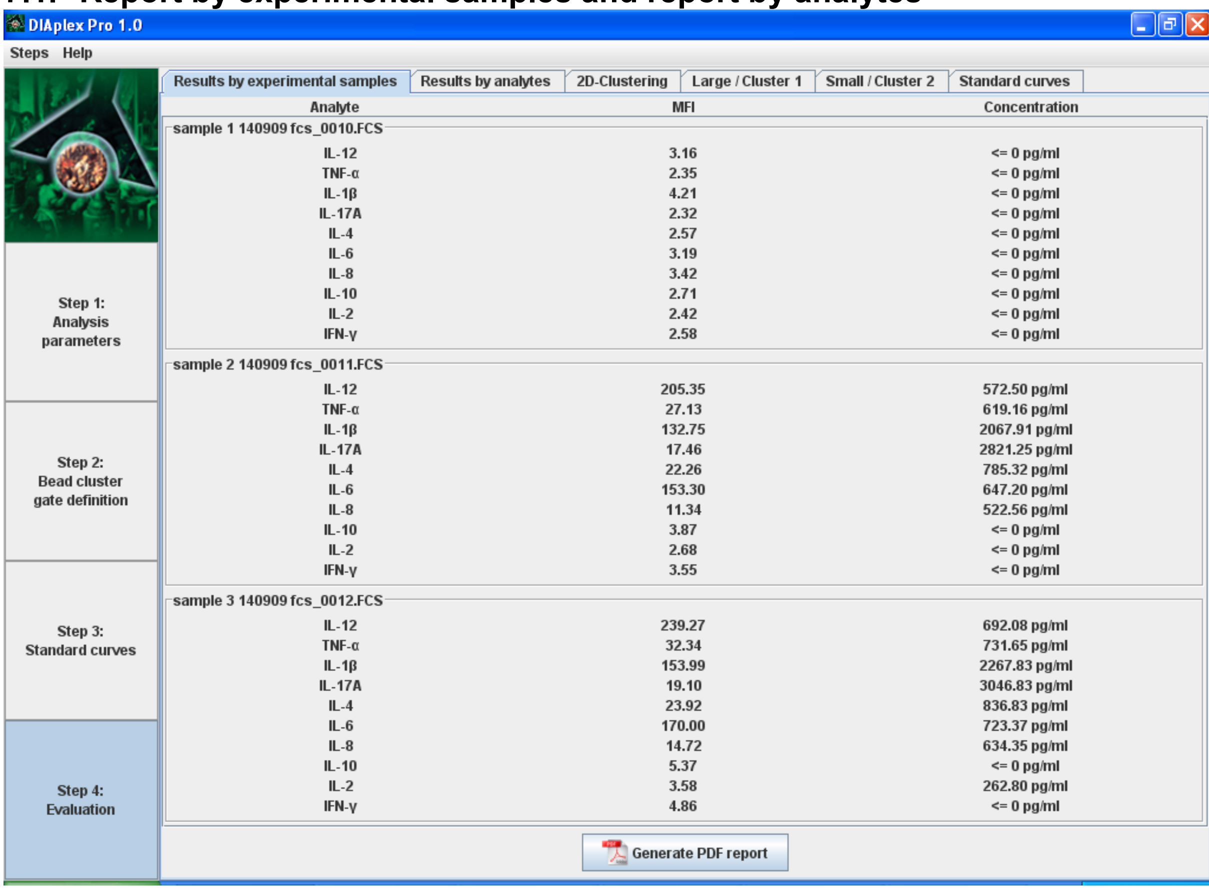


Figure 18: Report by experimental samples

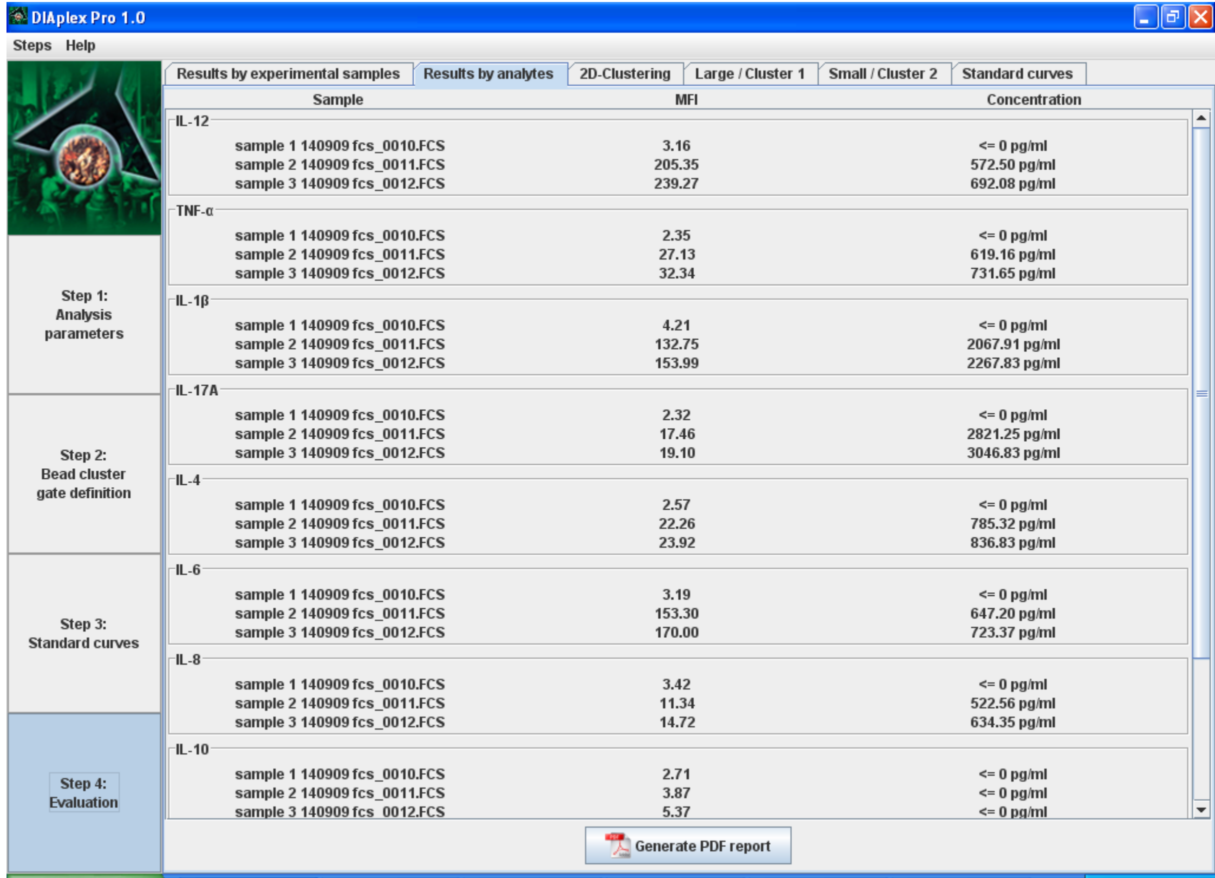


Figure 19: Report by analytes

Note: The (*) for some sample analyte data denotes that the result has been taken from an extrapolated point on the standard curve and therefore data should be interpreted with caution.

PDF report generation

At the bottom of these two report tabs, you have a “Generate PDF report” button to enable you to export the analysis data into a PDF file.

You can select specifically what you want to export into your PDF report.

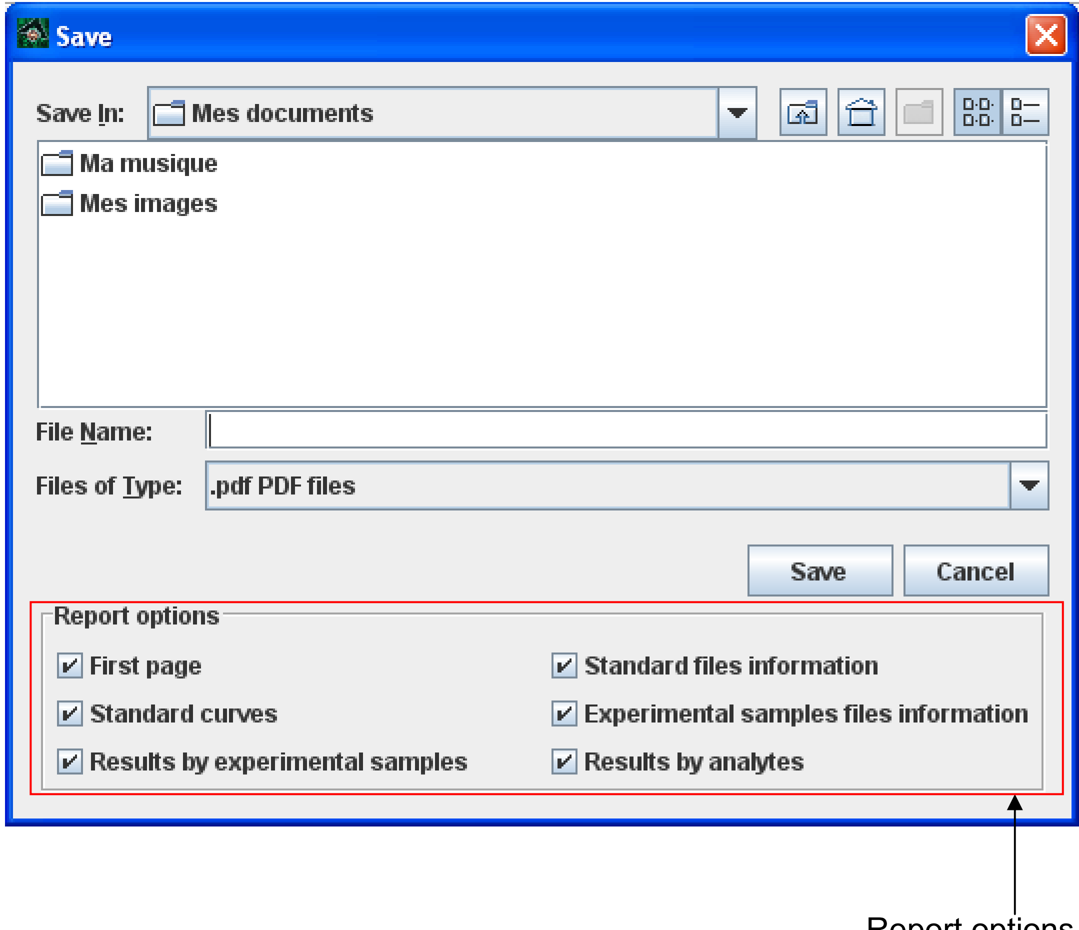


Figure 20: PDF report options

The PDF report options are as follows:

1. **First page:** Diaclone presentation page.
2. **Standard files information:** Information about the FCS files chosen in step 1.

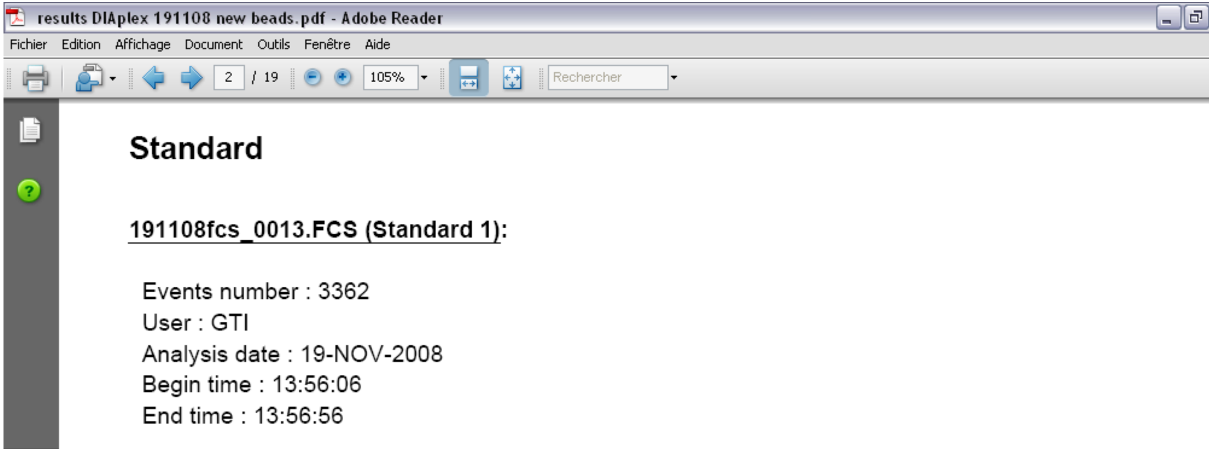
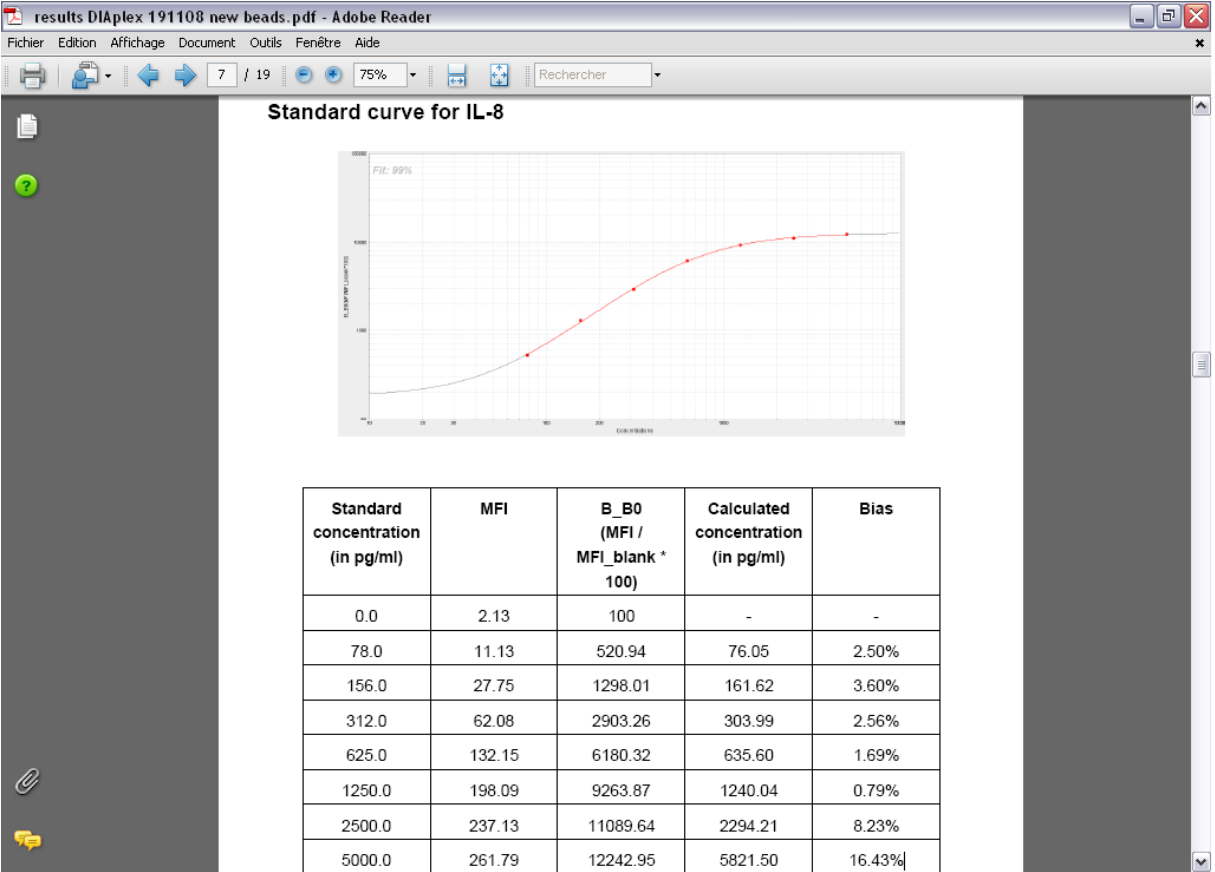


Figure 21: Standard file information

3. **Standard curves:** The curves chart of step 3 with a table containing the information on each red point of the chart.



results DIAplex 191108 new beads.pdf - Adobe Reader

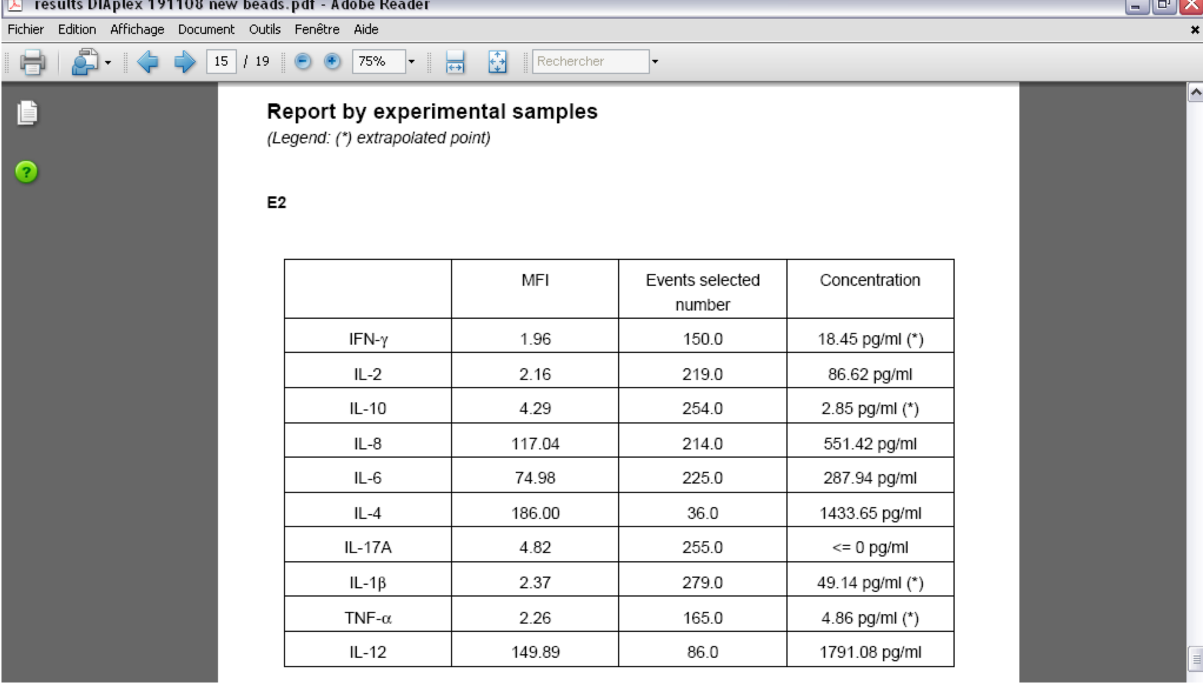
Standard curve for IL-8

Standard concentration (in pg/ml)	MFI	B_B0 (MFI / MFI_blank * 100)	Calculated concentration (in pg/ml)	Bias
0.0	2.13	100	-	-
78.0	11.13	520.94	76.05	2.50%
156.0	27.75	1298.01	161.62	3.60%
312.0	62.08	2903.26	303.99	2.56%
625.0	132.15	6180.32	635.60	1.69%
1250.0	198.09	9263.87	1240.04	0.79%
2500.0	237.13	11089.64	2294.21	8.23%
5000.0	261.79	12242.95	5821.50	16.43%

Figure 22: Standard curves report

4. **Experimental samples information:** Information about the FCS files chosen in step 3 - same information as in standard files information (See Figure 21).

5. **Report by experimental samples:** The data of the "Report by experimental samples" plus the number of events selected in the fluorescence cluster 1 and 2 rectangles.



results DIAplex 191108 new beads.pdf - Adobe Reader

Report by experimental samples

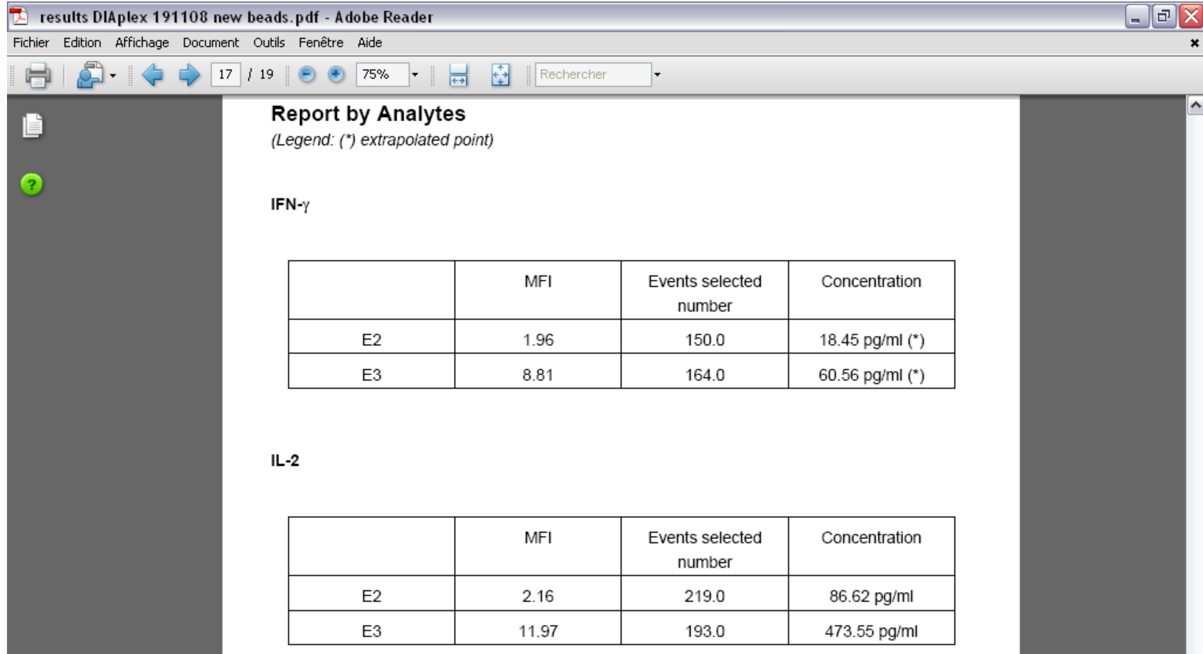
(Legend: (*) extrapolated point)

E2

	MFI	Events selected number	Concentration
IFN-γ	1.96	150.0	18.45 pg/ml (*)
IL-2	2.16	219.0	86.62 pg/ml
IL-10	4.29	254.0	2.85 pg/ml (*)
IL-8	117.04	214.0	551.42 pg/ml
IL-6	74.98	225.0	287.94 pg/ml
IL-4	186.00	36.0	1433.65 pg/ml
IL-17A	4.82	255.0	<= 0 pg/ml
IL-1β	2.37	279.0	49.14 pg/ml (*)
TNF-α	2.26	165.0	4.86 pg/ml (*)
IL-12	149.89	86.0	1791.08 pg/ml

Figure 23: PDF report by experimental samples

6. **Report by analytes:** The data of the "Report by analytes" plus the number of events selected in the fluorescence cluster 1 and 2 rectangles.



Report by Analytes

(Legend: (*) extrapolated point)

IFN-γ

	MFI	Events selected number	Concentration
E2	1.96	150.0	18.45 pg/ml (*)
E3	8.81	164.0	60.56 pg/ml (*)

IL-2

	MFI	Events selected number	Concentration
E2	2.16	219.0	86.62 pg/ml
E3	11.97	193.0	473.55 pg/ml

Figure 24: Report by analytes

8. Charts

In step 4, you also have the opportunity to review the charts generated in preceding steps. This allows verification that your previous selections (ellipses and rectangles) are correct and to evaluate the points on the standard curves.

You can't change the selections, the scale or deactivate points here. These charts are only for consultation. If re-analysis is required than Steps can be returned to by selecting the preceding step under the 'Steps' menu option.

On the standard curves, the samples points appear in green (Figure 25)

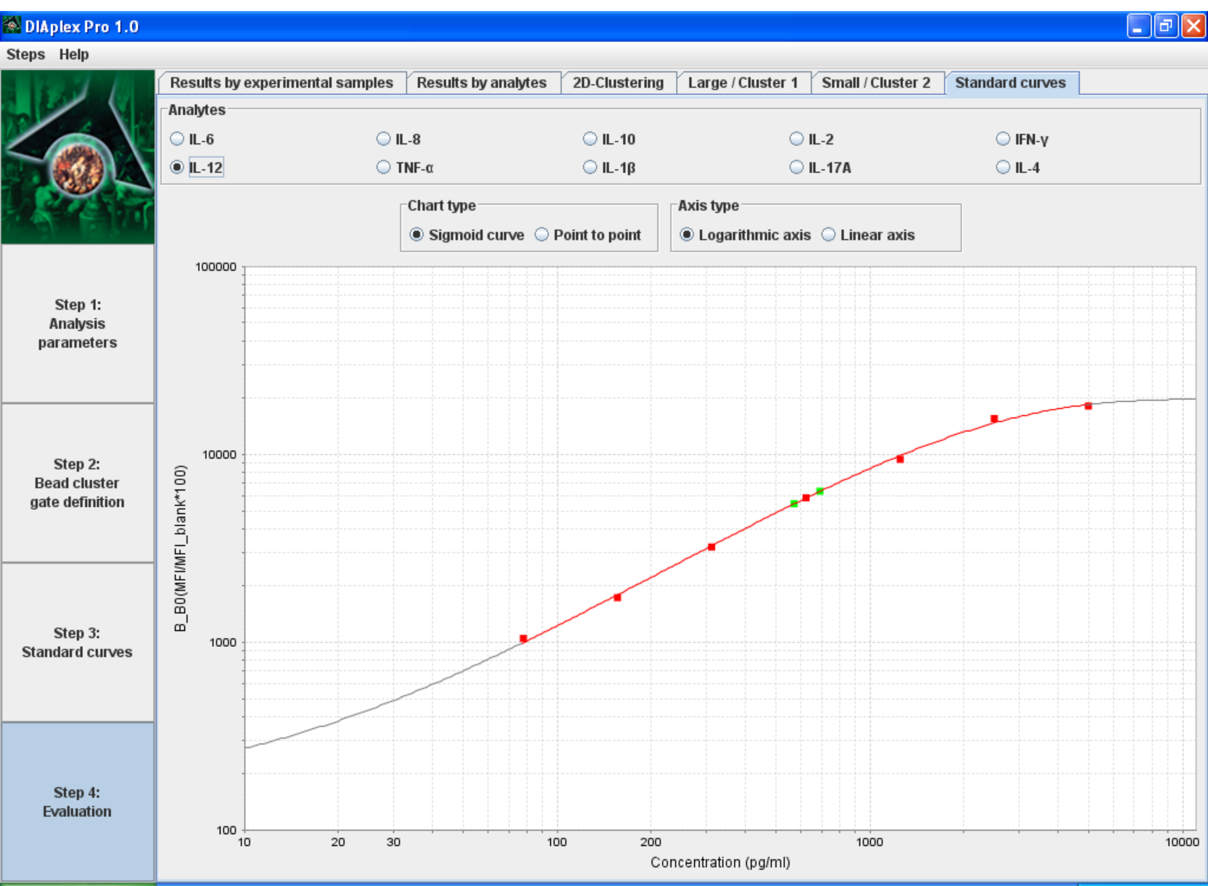


Figure 25: Standard curve with the samples points (in green)

9. Contact Details

Gen-Probe Diaclone SAS

France: +33 38 141 3838
UK: +44 161 946 2222
USA: +1 203 328 9599
Email: diaclone@gen-probe.com
Web: WWW.GEN-PROBE.COM

Gen-Probe Diaclone SAS. 1 Boulevard A. Fleming - BP 1985 25020 Besançon Cedex France T: +33 381413838 F: +33 381413636
UK office: +44 (0) 161 946 2222 F: +44 (0) 161 946 2224 USA office: +1 203-328-9500 F: +1 203-328-9599
E: diaclone@gen-probe.com WWW.GEN-PROBE.COM