AlphaLISA® Data Analysis using the GraphPad Prism Software (Version 4.03)

A. Selection of graph type and data transfer

1. Open the GraphPad Prism 4.03 software.
2. Select **File** and **New Project…** from the menu bar to open the ***Welcome to GraphPad Prism*** window (this window may also open automatically).
   a. Select **To start: Create a new project**.
   b. Select **Choose: Type of graph** and the **XY** (e.g. **Points only**) graph format.
   c. Select **Choose: Format of data table** and **3 replicates to calculate error bars** (or as required).
3. In the **Data 1** table, enter the **X Title** (= title of X-axis) and **Titles** (= legend; tested conditions). (Note: The name of data tables may be changed by right-mouse clicking and select **Rename**. The name of the corresponding graphs is changed automatically. In this procedure, the default names are used.)
4. Enter the analyte concentrations (e.g. molar, M) used for the standard curve in the **X Values** column.
   a. Use the scientific notation to enter the analyte concentrations (e.g. concentration = 1×10^{-6} M; enter 1.0e-06 instead of 0.000001). (Note: The number format can be standardized by clicking on **X Values** (to select the column), followed by **Change** and **Number Format…** In the ***Number Format*** window, select **Always** under **Use scientific notation**.)
   b. Do not enter "0" as concentration for background (buffer) samples. Instead, enter a value two logs lower than the lowest analyte concentration of the standard curve (e.g. lowest analyte concentration: 3.0e-12; enter 3.0e-14 for all background samples).
5. Open the Excel file containing the Envision AlphaLISA data.
6. Copy and paste the data into the GraphPad table. (Note: To facilitate the data transfer, the GrahPad table and plate layout should be identical). The table should look like Example 1 below:

Example 1:

X Values	A		
[TNFα] (M)	AlphaLISA Signal (counts)		
X	A:Y1	A:Y2	A:Y3
1.00E-06	802729	801077	745013
3.00E-07	1380851	1440733	1435494
1.00E-07	2291553	2383819	2392411
3.00E-08	2526134	2658322	2645676
1.00E-08	1576856	1557610	1552331
3.00E-09	711402	657941	655692
1.00E-09	262733	232834	230468
3.00E-10	75125	72173	74629
1.00E-10	24078	26249	26576
3.00E-11	11085	10363	9902
1.00E-11	6153	5353	5677
3.00E-12	3945	3905	3893
3.00E-14	2898	2926	3167
3.00E-14	3037	2896	2941
3.00E-14	3149	2944	2774
3.00E-14	3145	3157	3094

7. Format the graph:
   a. In the ***Navigator*** window, select the **Data 1 graph** in the **Graphs** subfolder.
   b. To change any of the titles, click on **Y Title**, **X Title** or the graph title (**Data 1**) and enter the desired text.
   c. To format any of the axes, double-click on one of them to open the ***Format Axes*** window. Select the desired subfolder and adjust the settings as required.

B. Data conversion (log10)

1. In the ***Navigator*** window (**Data Tables** subfolder), select the **Data 1** table.
2. To convert the data table and graph to log10 scale, click on **Analyze** and select **Type: Data manipulations** and **Transforms** in the ***Analyze Data*** window.
3. In the ***Parameters: Transforms*** window, select **Transform X-values using** and choose **X=Log(X)** from the scroll-down menu. The converted data are shown in the **Transform of Data 1** table (***Navigator*** window, **Results** subfolder).
4. Select the **Transform of Data 1 graph** (**Graphs** subfolder) to format the graph layout:
   a. Double-click on the X-axis.
   b. In the ***Format Axes / X axis*** window, select **Gaps and Direction: Two segments (---//---)**.
   c. Select **Segment: Left** and adjust **Range Minimum / Maximum** so that only the background (buffer) reading of the standard curve is shown in the center of this segment of the X-axis (e.g.: Minimum: -14.0 ; Maximum: -13.0). Adjust the **Major ticks: Interval** to half of the segment length (e.g.: 0.5) to show one tick in the center of the left X-axis segment.
   d. Adjust **Length: 10 % of axis**.
   e. Select **Numbering or labeling**, **Location: None**.
   f. Select **Segment: Right** and appropriately adjust **Range**, **Minimum / Maximum** (e.g.: -12.0, -5.0), **Major ticks: Interval** (e.g.: 1.0) and **Starting at** (e.g.: -12.0).
   g. Insert the "-∞" symbol below the left segment of the X-axis:
      - Select the **T**-symbol in the menu bar (**Place text on the graph or page**) and click below the left segment of the X-axis (align with the numbering of the right segment).
      - Click on the **α▼**-symbol (menu bar), select **Insert Math…** and ∞ in the ***Insert Math Character*** window. Add "-" before the symbol.
   h. Refer to step A.7 for other adjustments.

C. Curve fit

1. If the graph includes hook points, all points after the maximum counts have to be removed before performing the curve fit analysis.
2. In the **Data 1** table (***Navigator*** window, **Data Tables** subfolder), highlight all hook points, then right-mouse click and choose **Exclude Values**. Refer to Example 2 below:

Example 2:

X Values	A		
[TNFα] (M)	AlphaLISA Signal (counts)		
X	**A:Y1**	**A:Y2**	**A:Y3**
1.00E-06	*802729**	*801077**	*745013**
3.00E-07	*1380851**	*1440733**	*1435494**
1.00E-07	*2291553**	*2383819**	*2392411**
3.00E-08	2526134	2658322	2645676
1.00E-08	1576856	1557610	1552331
3.00E-09	711402	657941	655692
1.00E-09	262733	232834	230468
3.00E-10	75125	72173	74629
1.00E-10	24078	26249	26576
3.00E-11	11085	10363	9902
1.00E-11	6153	5353	5677
3.00E-12	3945	3905	3893
3.00E-14	2898	2926	3167
3.00E-14	3037	2896	2941
3.00E-14	3149	2944	2774
3.00E-14	3145	3157	3094

3. Select the **Transform of Data 1** table (***Navigator*** window, **Results** subfolder).

4. Click on **Analyze** and choose **Type: Curves & regression** and **Nonlinear regression (curve fit)** in the ***Analyze Data*** window.
5. In the ***Parameters: Nonlinear Regression (Curve Fit)*** window choose:
   a. **Equation** subfolder: **Sigmodial dose-response (variable slope)**, **Unknowns from standard curve**.
   b. **Weighting** subfolder: **Weight by 1/Y2 (minimize relative distance squared)**.
6. The curve fit results are shown in the **Nonlin fit of Transform of Data 1** table (***Navigator*** window, **Results** subfolder).

D. Calculation of LDL (Lower Detection Limit) and interpolation of unknowns from the standard curve

1. Determine the LDL value:
   a. Calculate the average and standard deviation (SD) counts of all background (buffer) wells using e.g. an Excel spreadsheet.
   b. Calculate the average + 3×SD counts and enter this value in the **Data 1** table in the first Y-column below the standard curve data. Refer to Example 3 below:

Example 3:

X Values	A		
[TNFα] (M)	AlphaLISA Signal (counts)		
X	**A:Y1**	**A:Y2**	**A:Y3**
1.00E-06	*802729**	*801077**	*745013**
3.00E-07	*1380851**	*1440733**	*1435494**
1.00E-07	*2291553**	*2383819**	*2392411**
3.00E-08	2526134	2658322	2645676
1.00E-08	1576856	1557610	1552331
3.00E-09	711402	657941	655692
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3.00E-11	11085	10363	9902
1.00E-11	6153	5353	5677
3.00E-12	3945	3905	3893
3.00E-14	2898	2926	3167
3.00E-14	3037	2896	2941
3.00E-14	3149	2944	2774
3.00E-14	3145	3157	3094
	3404		

2. Enter the counts to calculate the concentration of the unknown:
   a. Copy the triplicate readings obtained for the unknown sample from the Envision AlphaLISA Excel file in the **Data 1** table below the standard curve data (or alternatively, enter the average count). See Examples 4a and 4b below:

Example 4a:

	3404		
	83544	87569	85682

Example 4b:

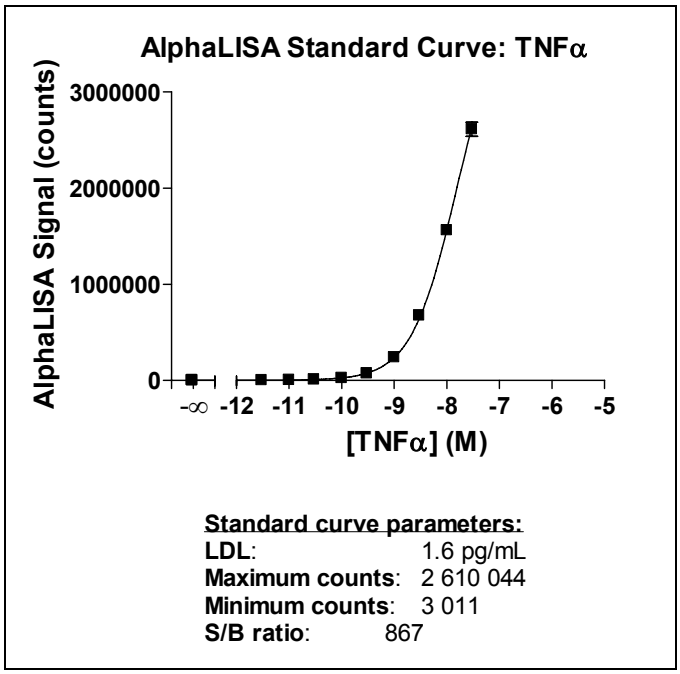
	3404		
	85598		

3. To re-convert the LDL and unknown data to linear scale, proceed as follows:
   a. Select the table **Non-lin fit of Transform of Data 1**, then **Interpolated X mean values** (***Navigator*** window, **Results** subfolder).
   b. Click on **Analyze** and selecting **Type: Data manipulations** and **Transforms** in the ***Analyze Data*** window.
   c. In the ***Parameters: Transforms*** window, select **Transform X values using X=10^X**.
   d. The **Transform of Nonlin fit of Transform of Data 1** table opens showing the calculated results. If no data are displayed in the X-axis column of this table, the counts entered in step 1 and 2 do not fall in the range of the standard curve and can thus not be interpolated.
4. To convert the data into a more user-friendly format, perform the following steps:
   a. Click again **Analyze** and select **Type: Data manipulations** and **Transforms**.
   b. In the ***Parameters: Transforms*** window, select **Transform X values using X=K×X** and **K= 1e12** (if converting from M to pM; or adjust accordingly).
   c. The converted results for the LDL and unknown concentrations are shown in the **Transform of Transform of Nonlin fit of Transform of Data 1** table (***Navigator*** window, **Results** subfolder).

E. Calculation of maximum counts and signal-to-background (S/B) ratio

1. Select the **Data 1** table in the ***Navigator*** window (**Data Tables** subfolder).
2. Click on **Analyze** and select **Type: Statistical analyses** and **Row means/totals** in the ***Analyze Data*** window.
3. In the ***Parameters: Row Means/Totals*** window, select **Calculate: Row means with SD**.
4. The average counts (mean) and standard deviations (SD) for each standard curve concentration are shown in the **Row Stats of Data 1** table (***Navigator*** window, **Results** subfolder).
   a. Take note of the average maximum counts in this table.
5. Calculate the S/B ratio by dividing the average maximum counts (refer to step 4.a) by the average background counts (refer to step D.1.a).
6. In the **Transform of Data 1 graph** (***Navigator*** window, **Graphs** subfolder) enter the following information using the text **T**-symbol in the menu bar:
   a. LDL: refer to step D.4.c
   b. Maximum counts: refer to step 4.a of this section
   c. Minimum counts: refer to step D.1.a
   d. S/B ratio: refer to step 5 of this section
7. The final graph with a linear Y-axis should look like the Example 5 below:

Example 5:



8. To create a copy of this graph with a log10 Y-axis, perform the following steps:
   a. Select the **Transform of Data 1 graph** (***Navigator*** window, **Graphs** subfolder).
   b. From the main menu, select **Insert** and **Duplicate Current Sheet** to create the **Copy of Transform of Data 1 graph**.
   c. Double-click on the Y-axis and select **Appearance: Scale: Log 10** in the Format Axes (***Left Y axis***) window. Refer to Example 6 below:

Example 6: