**GraphPad PRISM ®**

Version 4.0

# Step-by-Step Examples

*Jerry R. Miller*

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To contact GraphPad Software, email support@graphpad.com or sales@graphpad.com.

# Table of Contents

*Contents*                                                                    *5*

# Introduction

*Step-by-Step Examples* shows, in exhaustive detail, typical applications of GraphPad Prism 4 for graphing and analyzing basic research data. The examples progress from simple graphs, through common statistical analyses, and finally to curve fitting and special topics. It is not necessary to read the examples in order, and you are encouraged to proceed directly to the examples of most interest. You should consult the table of contents entries, as well as the index, carefully to find techniques that are not obvious from the chapter headings alone.

While the directions and figures match the Windows version of Prism 4, all examples can be applied to Apple Macintosh systems with little adaptation.

We encourage you to read this manual at your computer, trying each step as you go. Before you start, use Prism's **View** menu to make sure that the Navigator and all optional toolbars are displayed on your computer.

This manual is one of four that come with Prism 4. All manuals are available for download as PDF files at www.graphpad.com.

# Bar Graphs with One Grouping Variable

There are three ways to make a bar graph in Prism, each using a different graph type.

- This chapter shows how to create and format a *column bar* graph, so named because each bar shows the mean of all Y values in a column (data set). This approach is recommended when you have one grouping variable, because the table format is the same as that necessary to do several other analyses, such as *t* tests, one-way ANOVAs, and column statistics.

- If you have two grouping variables, consult "Bar Graphs with Two Grouping Variables" on page 19.

- If you want to plot bars and lines together on the same graph, or to place bars along a true X axis where horizontal position of the bars is determined by X value, see "Combining a Bar Graph with a Line Graph" on page 43.

Histograms—bar graphs that illustrate frequency distributions—are covered separately, beginning on page 33.

## Creating Column Bar Graphs

When you launch Prism, the Welcome dialog appears. Select **Create a new project** and indicate that you will create the initial data table and graph by choosing the **Type of graph**.

Suppose we want to make a graph similar to the following:
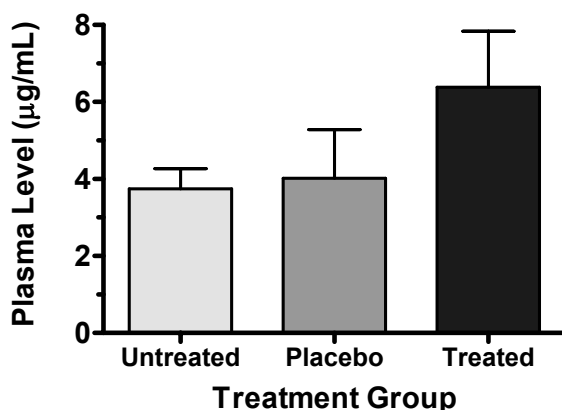
Our data are organized by one grouping variable, *Treatment Group*, which has three levels—Untreated, Placebo, and Treated. Therefore, in the Welcome dialog, select the tab for **One grouping variable**.
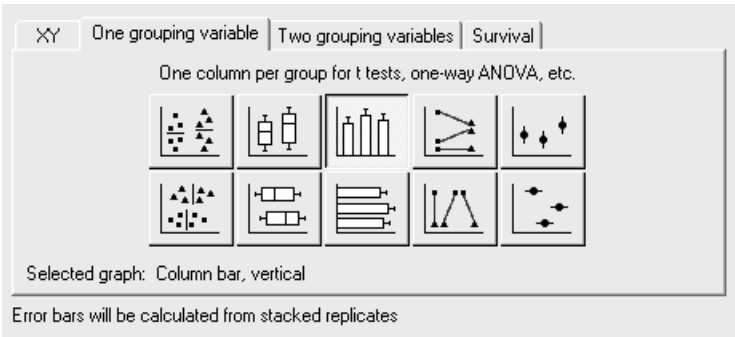


Prism displays 10 choices for the graph type. You can switch freely between the buttons, reading the "Selected graph" descriptions if you don't find the thumbnail illustrations clear. Note that you are not limited to using bars on a column (one-grouping-variable) graph; you may also choose dot plots (column scatterplots), box-and-whiskers configurations, etc. The top-middle button—for vertical bars—is the correct choice for our intended graph. Click **OK** to exit the Welcome dialog. Prism creates and displays the new table.

The table contains no X column, and for each data set (A, B, C,...; each corresponding to a level of the grouping variable), there is a single Y column. With no X values, there is no categorical distinction from row to row, so replicate values can now be stacked vertically within single columns.

Click the default table name in the drop-down list on the toolbar. Type a new name for the table. This name will be used as the title for your graph and in the names of other sheets linked to the table (note, however, that you are free to name any of those sheets independently).
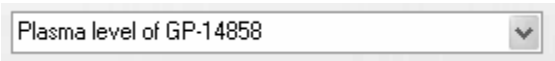


Enter the values shown below into your table. Be sure to include column headings. The name of the grouping variable is not included on the table, but the levels are identified in the Y-column headings "Untreated", "Placebo", and "Treated".

|   | A | B | C |
|---|---|---|---|
|   | Untreated | Placebo | Treated |
|   | Y | Y | Y |
| 1 | 3.4 | 2.3 | 4.2 |
| 2 | 4.3 | 5.2 | 7.8 |
| 3 | 3.0 | 4.5 | 5.9 |
| 4 | 3.9 | 3.1 | 6.4 |
| 5 | 4.1 | 5.0 | 7.6 |

You can change the number format if you wish. Select all of the columns you want to change, then choose **Number Format…** from the drop-down list under the **Change** button.

Prism creates the column bar graph automatically. In the Navigator, choose the sheet **Plasma level of GP-14858 graph** (or the name you gave to the data table earlier).
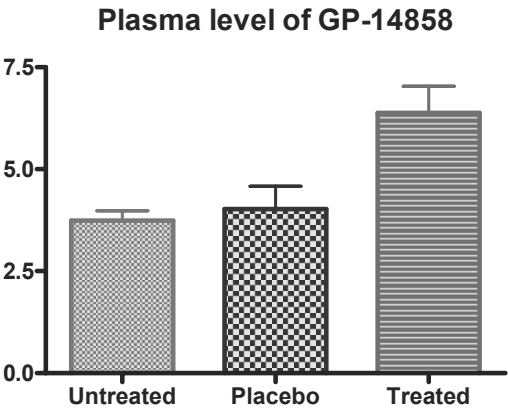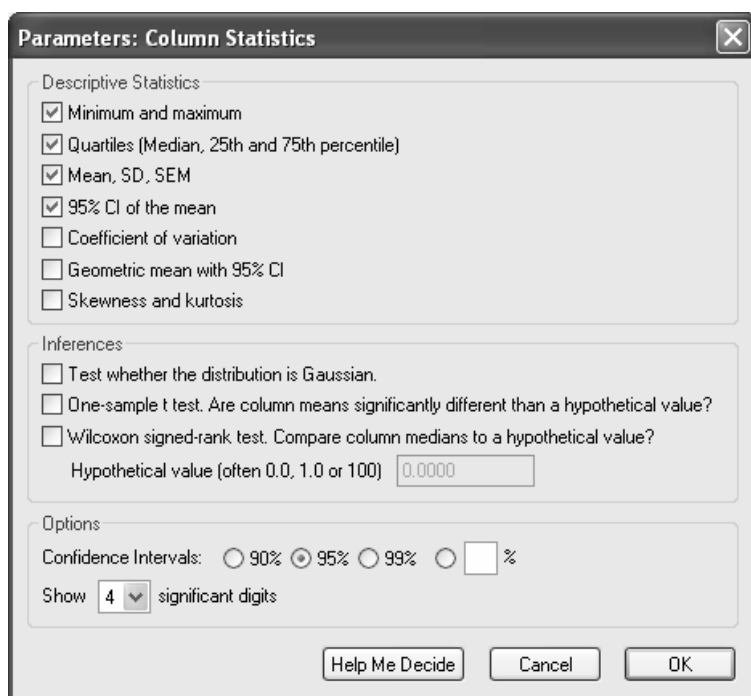


Note that Prism fills each bar with a different pattern—each bar in this graph represents a different data set (column), and Prism always colors/patterns bars or symbols according to data set. If you want to override that default to make all the bars look the same, follow the directions on page 15 for changing bar appearance, using the button for changing all data sets at once:

# Displaying Column (Descriptive) Statistics

At this point, you may want to see descriptive statistics for each column. Click the **Analyze** button. From the list of **Statistical analyses**, choose **Column statistics**.

In the **Parameters: Column Statistics** dialog, choose the **Descriptive Statistics** you want to see.



When you exit the dialog, Prism displays the Results sheet.

|  | Untreated | Placebo | Treated |
|---|---|---|---|
|  | Y | Y | Y |
| Number of values | 5 | 5 | 5 |
|  |  |  |  |
| Minimum | 3.000 | 2.300 | 4.200 |
| 25% Percentile |  |  |  |
| Median | 3.900 | 4.500 | 6.400 |
| 75% Percentile |  |  |  |
| Maximum | 4.300 | 5.200 | 7.800 |
|  |  |  |  |
| Mean | 3.740 | 4.020 | 6.380 |
| Std. Deviation | 0.5320 | 1.264 | 1.457 |
| Std. Error | 0.2379 | 0.5652 | 0.6515 |
|  |  |  |  |
| Lower 95% CI of mean | 3.079 | 2.451 | 4.571 |
| Upper 95% CI of mean | 4.401 | 5.589 | 8.189 |
|  |  |  |  |
| Sum | 18.70 | 20.10 | 31.90 |

Remember that this sheet is linked to the original data table, so that if you change that data, these results will be updated automatically.

# Formatting Column Bar Graphs

Click on the yellow **Graphs** tab to return to the graph. Refer to the finished graph at the beginning of this chapter as you make the following format changes:

| To: | Do this: |
|---|---|
| Change bar appearance | Double-click on a bar. In the **Format Columns** dialog, choose the **Appearance** tab. Verify the data set, then change **Fill**, **Pattern**, or **Border**. If you wish, change to a different method of representing your data, such as a box-and-whiskers plot (**Appearance** drop-down box). You can even mix different plotting methods on the same graph. To change all data sets together, click the **All** button *before you make any changes*:<br><br>All |
| Edit X axis title | Click on the default "XTitle". Type replacement title. Use the $\alpha$ button on the tool bar to enter Greek letters. When done, click elsewhere. |
| Adjust axis title position | Position the mouse pointer near the axis title (avoiding tick labels) to display a two-headed-arrow. Click and drag title away from, or closer to, the axis. |
| Adjust tick label positions | Double-click on an axis. Choose the appropriate **...axis** tab in the **Format Axes** dialog, then adjust the **Numbering/labeling...Distance from axis** setting. |
| Edit Y axis title | Click on the default "YTitle". The text will flip to horizontal. Type the replacement title. When done, click elsewhere to flip the title back. Use the $\alpha$ button to enter Greek letters. |
| Edit Y axis ticks and numbering | Double-click on the Y axis. Under **Range**, remove check from the **Auto** box. Adjust axis.  Adjust **Tick options** as desired. |
| Convert the error bars from *SEM* to *SD* | Double-click on a bar, then choose the **Appearance** tab. Click the **All** button to change all data sets at once. Set **Plot** to **Mean & SD**. |

# Reorganizing Column Bar Graphs

The changes discussed in this section are made by double-clicking any bar on the graph to open the **Format Columns** dialog, then making adjustments under the **Order and Direction** tab.
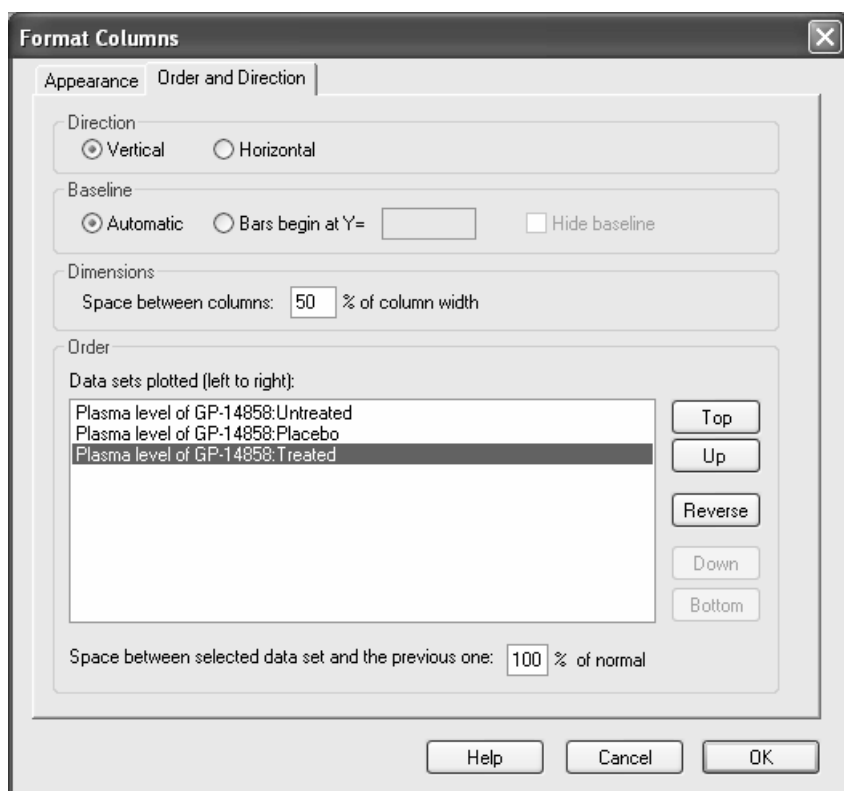


## Rearranging Bars

Under **Data sets plotted (left to right)**, Prism shows the order of the bars on the graph. Select the data set you wish to move, then click **Top**, **Up**, **Down**, or **Bottom** as appropriate. Or you can completely invert the order of the bars by clicking **Reverse**.

## Adjusting Spacing between Bars

Change the uniform spacing between bars by adjusting **Dimensions... Space between columns**. The overall width of the graph is not changed in the process, hence widening the gaps between bars will simultaneously narrow the bars.

Another quick way to expand the space between bars is to select the baseline, then drag the right "handle" to the right.



But this widens the bars at the same time. It is a good way to make more room for baseline labels that are so long that they overlap with adjacent labels.

You may wish to introduce additional spacing into a bar graph to isolate a particular bar or group of bars. In the **Data sets plotted** section, select the data set (bar) that is to immediately follow the additional gap, then change **Space between selected data set and the previous one**. For example, the settings below produce additional spacing between the second and third bars.
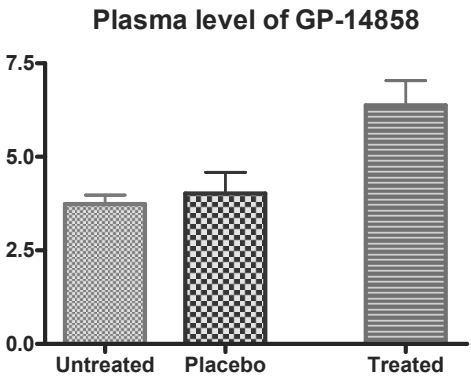
# Bar Graphs with Two Grouping Variables

There are three methods for making a bar graph in Prism, each using a different graph type:

- Most of this chapter is concerned with the method that you must use to graph data organized into two grouping variables (although you may apply the method to data organized by one grouping variable). Prism uses the term *bar graph* to refer specifically to a graph produced by this method.

- When your data has only one grouping variable, the preferred method is to create a *column graph* on which data are depicted using bars (as opposed to columns of point symbols or box-and-whiskers plots). See "Bar Graphs with One Grouping Variable" starting on page 11.

- A third method actually uses a variation of the *XY graph* on which point symbols are replaced with "spikes" widened so as to appear as bars. This method is discussed later in this chapter (see page 28) and in the chapter "Combining a Bar Graph with a Line Graph" on page 43.

Histograms—bar graphs that illustrate frequency distributions—are covered separately, beginning on page 33.

## Creating Bar Graphs

When you launch Prism, the Welcome dialog appears. Select **Create a new project** and indicate that you will create the initial data table and graph by choosing the **Type of graph**. Suppose we wish to create the following graph:
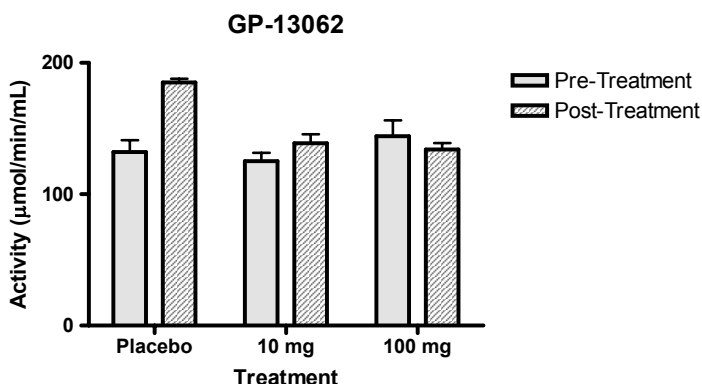
As the graph shows, our data are organized by two grouping variables:

1. *Treatment*, with three levels—placebo, 10 mg, and 100 mg
2. *Time*, with two levels—pre-treatment and post-treatment

Therefore, in the Welcome dialog, select the tab for **Two grouping variables**.
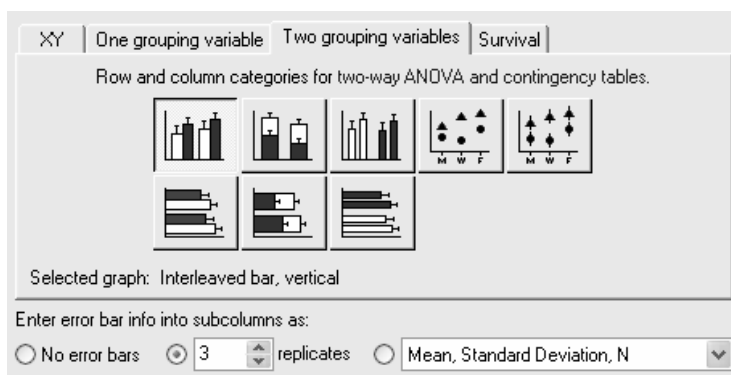


The dialog now displays eight choices for graph type. You can switch freely between the buttons, reading the "Selected graph" descriptions if you don't find the thumbnail illustrations clear. The upper-left button—for interleaved vertical bars—matches the organization and orientation of the bars in our intended graph.

> The difference between the interleaved bars and grouped bars is potentially confusing. In the thumbnail views, white bars and black bars represent different data sets. In an "interleaved" bar graph, Prism places bars for all data in a particular *row* together, mixing data from different columns in the process. In a "grouped" bar graph, Prism places bars for all data in a particular *column* together, mixing data from different rows in the process. To help you keep this straight, remember Prism's rule—it colors/patterns bars or symbols according to data set. All data in a particular data set (column) are colored/patterned in the same way.
>
> The interleaved arrangement is the most common. Grouping is used rarely, so avoid it unless you are sure that's what you want.

With the graph type selected, indicate that you will enter data into subcolumns as **3 replicates**. That means that you will enter your replicate measurements, and Prism will compute the means and standard deviations or standard errors automatically. Click **OK** to exit the dialog. Prism creates and displays the new table.

The table contains an X column, which will hold the levels of one grouping variable. Since we indicated in the previous dialog that we will enter data as 3 replicates, Prism subdivides each data set (A, B, C, …) to make room for side-by-side entry of replicates

(A:Y1, A:Y2, A:Y3; B:Y1, B:Y2, B:Y3; etc.). Each data set represents a level of the other grouping variable.

Click on the default table name (drop-down list on the toolbar) and type a new name for the table. That name will be used as the title for your graph and in the names of other linked sheets, although you are free to name those sheets independently.

GP-13062

Enter the values show below into your table, including column headings, maintaining the appropriate organization of grouping variable.

- Since there is an X column, each row corresponds to a different level of one of the grouping variables. The X-column heading "Treatment" identifies that grouping variable. The levels of that variable are identified by the text labels "Placebo", "10 mg", and "100 mg" in the X column.
- Similarly, each Y column (i.e., each data set A, B, C, ...) corresponds to a different level of the other grouping variable. The name of that grouping variable is not included on the table, but the levels are identified in the Y-column headings "Pre-treatment" and "Post-treatment".

| X Labels | A | | | B | | |
|---|---|---|---|---|---|---|
| Treatment | Pre-Treatment | | | Post-Treatment | | |
| X | A:Y1 | A:Y2 | A:Y3 | B:Y1 | B:Y2 | B:Y3 |
| 1 Placebo | 115 | 135 | 146 | 180 | 190 | 185 |
| 2 10 mg | 113 | 127 | 135 | 126 | 150 | 140 |
| 3 100 mg | 120 | 152 | 160 | 125 | 135 | 142 |

Which levels should you put in rows, and which in columns? Your choices will affect labeling, grouping, and appearance of the bars. Prism will use the levels in the X column as labels under the graph baseline (identifying the groups of bars) and the levels in the Y-column headings as elements in the legend. Bars from *different* columns of the data table will be graphed with *different* color/fill patterns, while bars from different rows of the *same* column are shown, repeated, with the *same* color/fill. If you don't get the arrangement you want, you can change that without redoing your data table, as we'll see in subsequent sections (see "Rearranging Bars" on page 24 and "Transposing Data" on page 25). If your data table contains three or more data sets, it will probably be easier to choose **Change... Graph Type** and select a different graph type.

You can change the number format if you wish. Select all of the columns you want to change, then choose **Number Format...** from the drop-down list under the **Change** button.

As soon as you have entered your data, Prism creates a graph automatically. In the Navigator, choose the sheet **GP-13062 graph**.
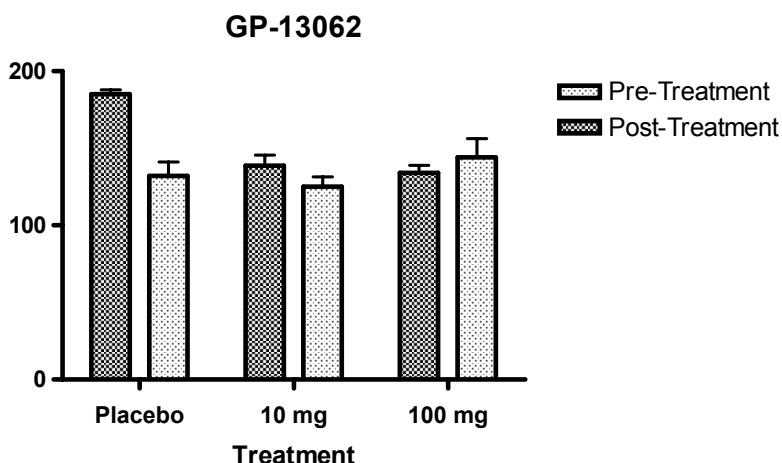


Prism automatically graphs all the data sets (columns A and B) on the data table. You may easily add or remove data sets from the graph (click the **Change** button, then choose the **Data on Graph** tab in the **Format Graph** dialog.

Note carefully how the information on your data table is used to create the bar graph:

- Text entries in the X column are placed beneath the appropriate group of bars.

- Labels for the data sets are placed in the legend, where they identify the data sets by color/pattern. As we noted earlier, Prism plots data from the same data set using the same color and pattern. To change colors, patterns, and borders of the bars, double-click on one of the bars to open the **Format Bars** dialog.

- The X column heading becomes the initial horizontal "axis" title, although you can edit the title later.

- The name of the data table becomes the title of the graph.

- Error bars are produced automatically. Prism averages replicate values and plots the mean and error bars.

# Formatting Bar Graphs

The following table shows tips for common format changes:

| To: | Do this: |
| --- | --- |
| Change bar appearance | Double-click on a bar. In the **Format Bars** dialog, choose the **Appearance** tab. Verify the data set (to change all data sets together, use the **All** button): <br><br> [ All ] <br><br> Change **Fill**, **Pattern**, and **Border**. |
| Move the legend | Click on one legend element, hold the **Shift** key, and click the other element. Drag both legend elements on the graph to move them. Do fine positioning using the arrow keys. |
| Adjust axis title position | Click on axis title (avoiding tick labels) to display two-headed-arrow cursor. Drag title away from, or closer to, the axis. |
| Adjust tick label positions | Double-click on an axis. Choose the appropriate **...axis** tab in the **Format Axes** dialog, then adjust the **Numbering/labeling...Distance from axis** setting. |
| Edit the Y axis title | Click on the default "YTitle". The text will flip to horizontal. Type the replacement title. When done, click elsewhere to flip the title back. Use the $\alpha$ button on the tool bar to enter Greek letters. |
| Convert the error bars from *SEM* to *SD* | Double-click on a bar, then choose the **Appearance** tab. Press the **All** button to change all data sets at once. Set **Error values** to **Mean & SD**. |

# Reorganizing Bar Graphs

## Rearranging Bars

To change how the bars are arranged, double-click on one of the bars to open the **Format Bars** dialog, then choose the **Order and Direction** tab.
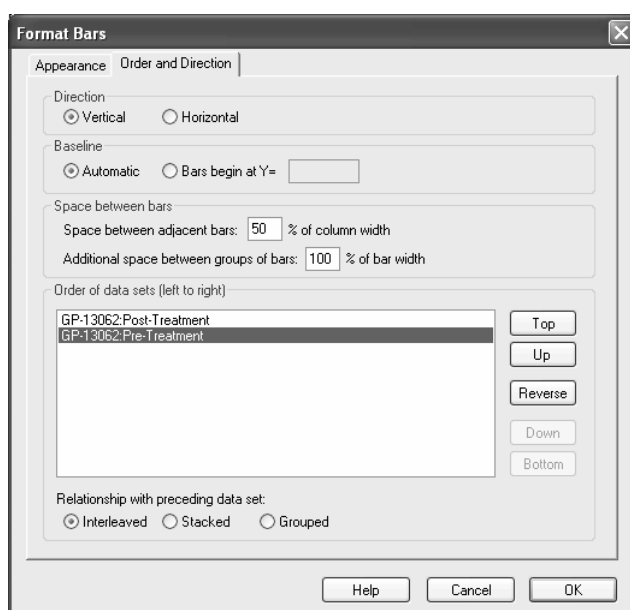


A dialog is displayed that allows you to change bar direction, spacing, and order. You can change the graphical relationship between data sets, choosing among **Interleaved**, **Stacked**, or **Separate** bar arrangements, to produce the following arrangements.
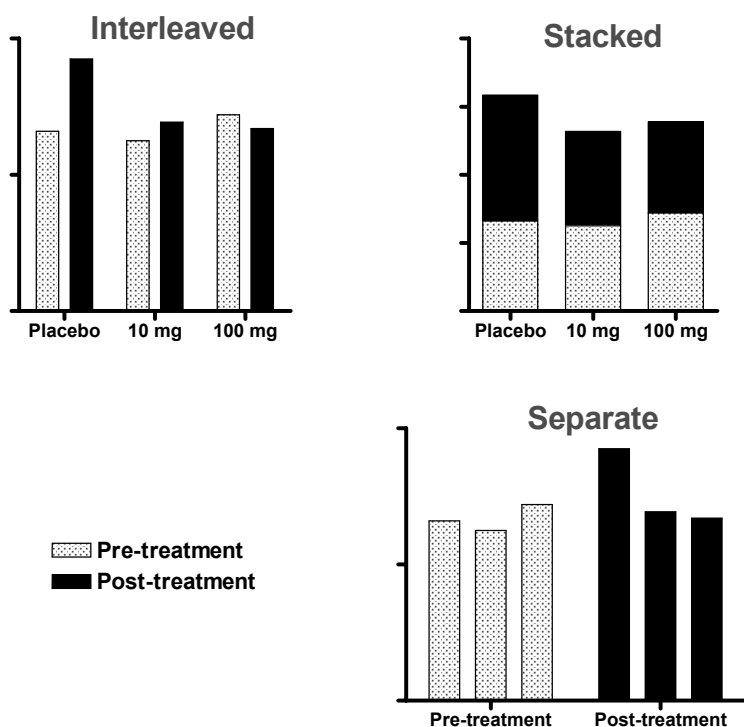
**Interleaved**

**Stacked**

**Separate**

Pre-treatment
Post-treatment

> If your data table contains three of more data sets, it will probably be easier to choose **Change... Graph Type** and select a different arrangement from the graph thumbnails.

## Transposing Data

Simply changing the relative arrangement of bars (interleaved, stacked, separate) may still not yield the graph you want. In that case, try transposing the data table. With the data table displayed, click **Analyze**, choose **Data manipulations... Transpose X and Y**, and check the option to **Create a new graph of the results**. Your original data table and graph are not modified, but the transposed table is produced on a Results sheet and a new graph of the transposed data is created. If, for example, you transform this data table...

| X Labels | A | B | C |
|---|---|---|---|
| X Labels | Control | 1 Week | 1 Month |
| X | Y | Y | Y |
| 1  No treatment | 144 | 138 | 143 |
| 2  Hypophysectomy | 150 | 99 | 51 |

to produce this Results sheet...

| X Labels | A | B |
|---|---|---|
| X Labels | No treatment | Hypophysectomy |
| X | Y | Y |
| 1  Control | 144 | 150 |
| 2  1 Week | 138 | 99 |
| 3  1 Month | 143 | 51 |

...Prism assigns colors and patterns to the bars in the same way before and after transposing, but since columns and rows are interchanged, so is the color/pattern scheme.
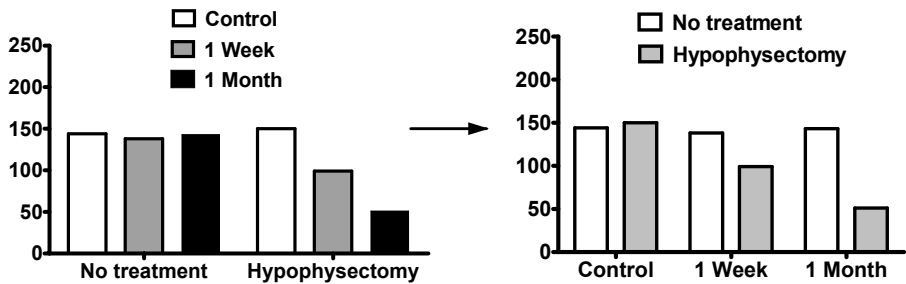


This may be useful even when your data has only one grouping variable, as in the following example. In this instance, you can use transposition to switch between having all bars shown in the same way and having bars shown differently.

*Prism Examples*

**Single Column**

| X Labels | Data Set-A |
|----------|-----------|
| One | 1 |
| Two | 2 |
| Three | 3 |
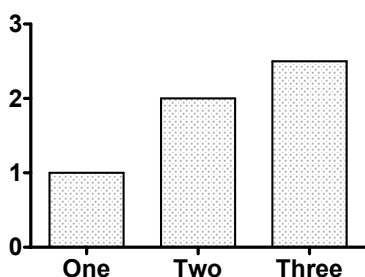
**Transposed to Single Row**

| X Labels | One | Two | Three |
|----------|-----|-----|-------|
| A | 1 | 2 | 3 |
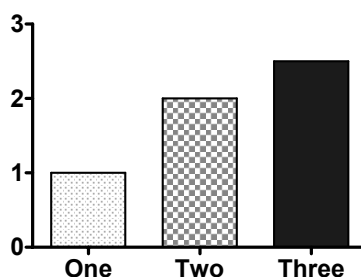
## Adjusting Spacing between Bars: Uniform Changes

You can change the horizontal spacing of bars from the **Format Bars** dialog (under the **Order and Direction** tab).

Space between bars
Space between adjacent bars: 15 % of column width
Additional space between groups of bars: 50 % of bar width

Here is the effect of a decrease in the gap between bars (note that the bars widen in the process):
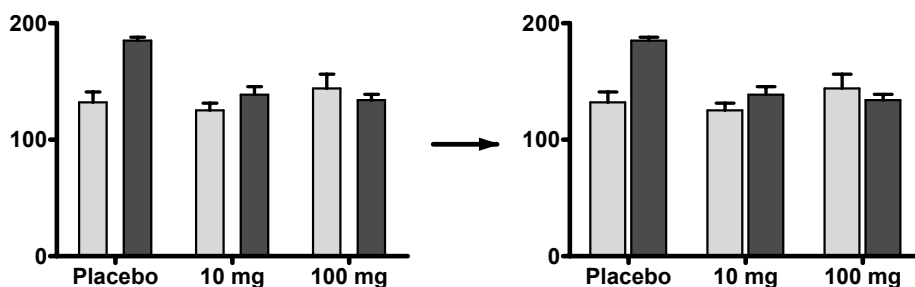
## Adjusting Spacing between Bars: Custom Changes

Many users wish to introduce additional spacing into a bar graph to isolate one group of bars in particular—not to be confused with the "additional space" offered in the dialog shown above, which is placed between all groups. You can create this extra space by inserting an empty row in the data table in the appropriate place. For example, inserting the empty row (2) in the following data table…

| | X Labels | A | | |
|---|---|---|---|---|
| | X Labels | Low Dose | | |
| | X | A:Y1 | A:Y2 | A:Y3 |
| 1 | Control | 115 | 135 | 146 |
| 2 | | | | |
| 3 | 5 mg | 113 | 127 | 135 |
| 4 | 25 mg | 120 | 152 | 160 |

…produces this change in the graph:
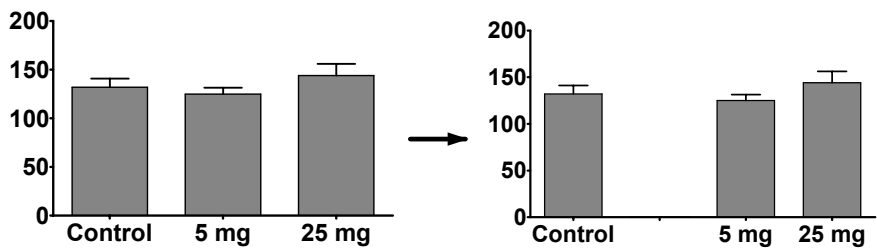


Note that this works only for a regular bar graph (text in the X column), not a *column* bar graph (no X column).

Remembering that *Prism shows values in any given data set in the same way* suggests another way to single out one, or a few, bars to be shown differently. You can always change a bar or symbol appearance by moving data to another column (data set). Thus this table…

| | X Labels | A | | B | |
|---|---|---|---|---|---|
| | X Labels | Data Set-A | | Data Set-B | |
| | X | Mean | SEM | Mean | SEM |
| 1 | Control | 119 | 8 | | |
| 2 | 2 hrs | | | 188 | 11 |
| 3 | 4 hrs | | | 141 | 12 |
| 4 | 8 hrs | | | 113 | 20 |
| 5 | Follow-up | 121 | 5 | | |

..leads to this graph (when the *interleaved* bar arrangement is selected):
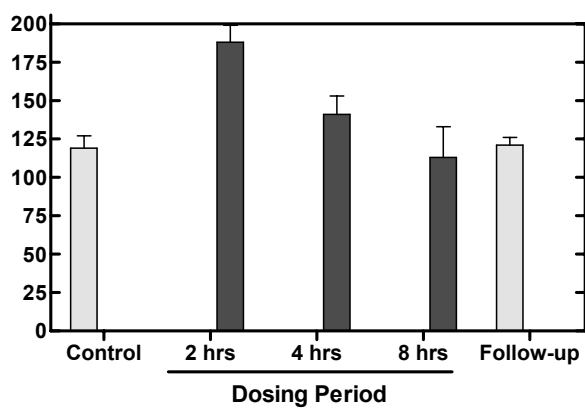


The problem with this approach is evident if you look closely at the positioning of the bars and the alignment of bars with text labels. Prism leaves space for the "missing" bars, that is, it interprets the empty cells as if they contained zeros. A better solution may be to set up your table to make an XY graph (format X column for **Numbers**), as shown below.

| | X Values | A | | B | |
| --- | --- | --- | --- | --- | --- |
| | X Title | Data Set-A | | Data Set-B | |
| | X | Mean | SEM | Mean | SEM |
| 1 | 1.0 | 119 | 8 | | |
| 2 | 3.0 | | | 188 | 11 |
| 3 | 4.0 | | | 141 | 12 |
| 4 | 5.0 | | | 113 | 20 |
| 5 | 7.0 | 121 | 5 | | |

Here, the X coordinate determines the horizontal position of the symbols. The resulting graph will show your values as point symbols, but you can change those symbols to "spikes" (in the **Format Symbols and Lines** dialog, open the **Shape** list box and make the fourth choice from the bottom).
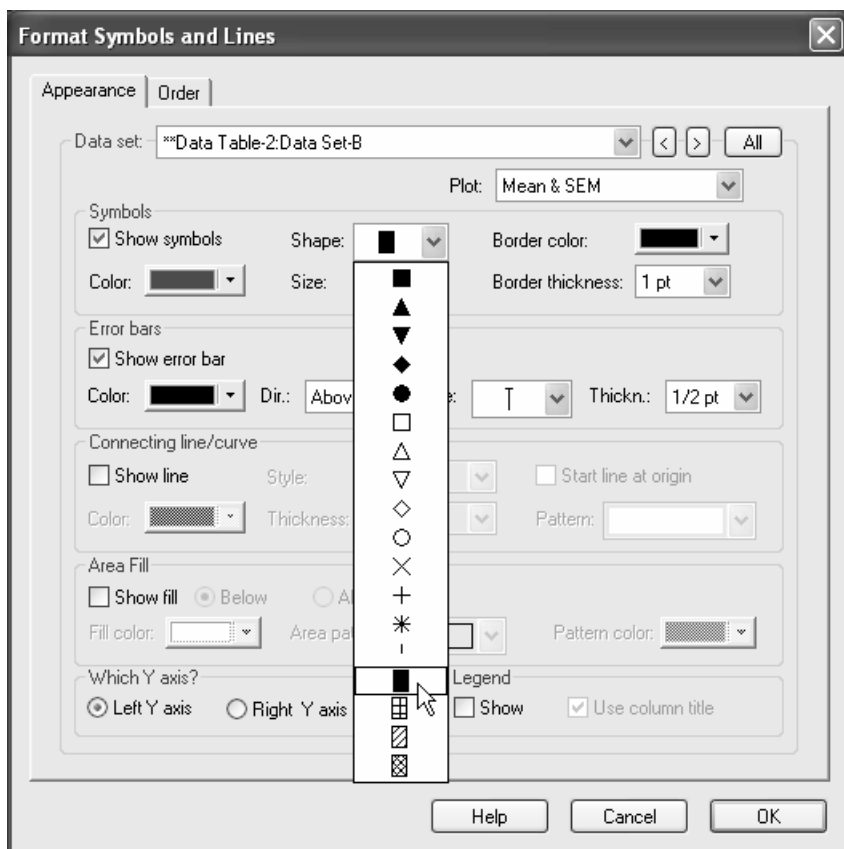
Finally, change the numbers along the X axis to text labels using "custom ticks" (double-click on the X axis, then choose **Custom Ticks**). In the **Customize Ticks and Gridlines** dialog, choose to show **Custom ticks only**. Enter the definitions for the five custom labels—for each, fill in the **Position (X=)**, **Label**, and **Tick Style** boxes and then click **Add**. As you proceed, the definitions are listed in the box below. When you're finished, the dialog should look like this:

**Customize Ticks and Gridlines**

Show
- ◉ Custom ticks only   ◯ Regular ticks only   ◯ Both

Define

| Position (X=) | Label | Tick Style |
|---|---|---|
| | | None |

| 1.0 | Control | None |
| 3.0 | 2 hrs | None |
| 4.0 | 4 hrs | None |
| 5.0 | 8 hrs | None |
| 7.0 | Follow-up | None |

[ Add ]   [ Change ]   [ Delete ]

[ Help ]   [ Sort ]   [ Cancel ]   [ OK ]

> A custom tick label can be blank space, that is, you can cover over a
> regular tick label with a "label" containing no text content. For
> details, see page 107.

Here's the graph:
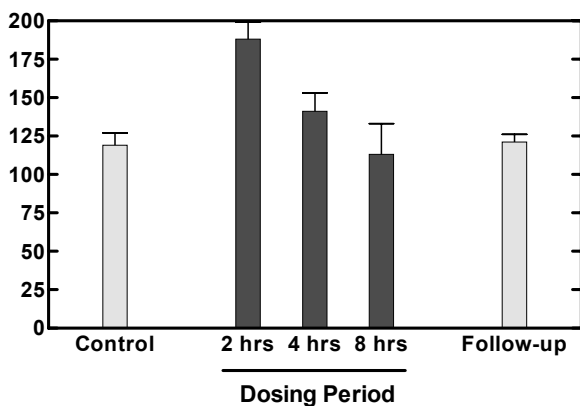
# Histograms and Frequency Distributions

This chapter shows how to create histograms—bar graph depictions of frequency distributions. We'll cover two alternative approaches:

- Tabulate and graph frequency data that you have already computed from your raw data, or
- Give Prism the raw data itself and let it compute and graph the frequencies automatically.

## Histograms from Pre-Computed Frequency Data

In this section, we'll discuss two methods for constructing histograms when you have already computed the frequency values and you intend to enter them manually. Histograms may be either bar graphs or XY graphs.

### Bar-Graph Histogram

Suppose we've measured the heights, in inches, of 55 male adults and we record the number of values in each of nine categories, or "bins", each representing a different 2-inch range.

In the Welcome dialog, choose **Type of graph**. Select the **Two grouping variables** tab and then the first thumbnail view.
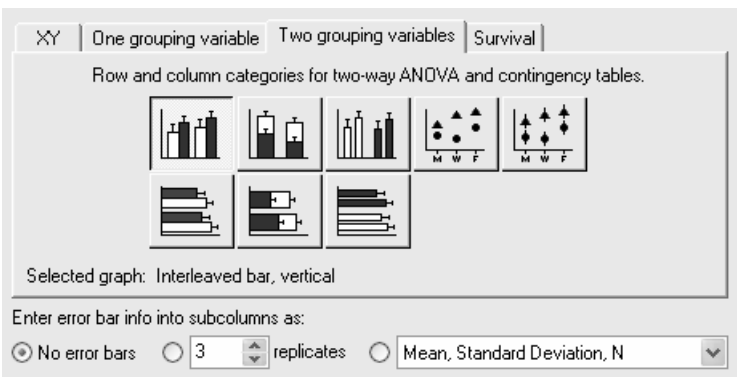


Enter the data and column headings as follows:

| | X Labels | A |
| --- | --- | --- |
| | Height | Frequency |
| | X | Y |
| 1 | 62-64 | 1 |
| 2 | 64-66 | 3 |
| 3 | 66-68 | 5 |
| 4 | 68-70 | 9 |
| 5 | 70-72 | 12 |
| 6 | 72-74 | 11 |
| 7 | 74-76 | 8 |
| 8 | 76-78 | 5 |
| 9 | 78-80 | 1 |

The histogram appears as soon as you click the yellow **Graphs** tab on the toolbar.
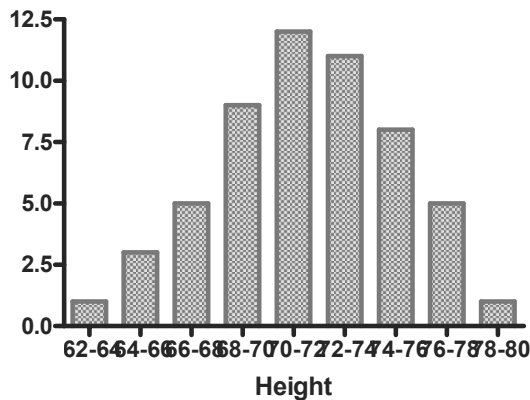
You can rotate the bar labels to avoid overlapping—double-click the baseline to open the **Format Axes** dialog, select the **X axis** tab, and adjust the **Numbering or labeling** settings as follows:
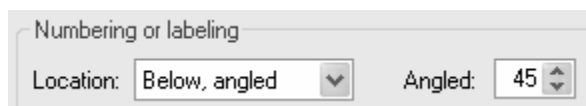
Below is the graph, with a few additional formatting changes. The baseline title "Height" has been lowered to increase readability. To do this, place your cursor just above or below the title so that it becomes a double-headed arrow, then click and drag downward.

**Height**
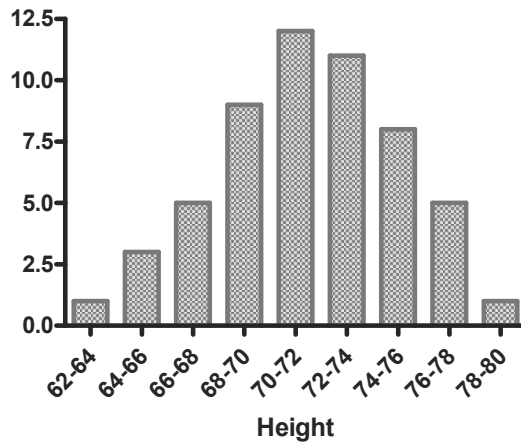
This is a quick and easy way to make a histogram for which you have already computed the frequency values. Because the bar labels are text (even if that "text" takes the form of numbers), and since you can change the angle of the labels, you have versatility in labeling. If you don't want angled labels but need to create a little extra clearance space between labels, select the baseline and then either drag one of the ends to make it longer or choose **Change… Selected Text…** to change the font size. You can also increase readability by omitting some of the labels at regular intervals (which wouldn't work well here, but might work where the labels are single numbers designating bin "centers"). To omit a label, simply omit the X-column entry for that row, entering the Y value only.

## XY-Graph Histogram

Here is another way to make a histogram using pre-computed frequencies. It's a bit more work, but it gives more versatility in labeling, allows you to use irregularly spaced bin centers if desired, and allows you to superimpose a line graph, if desired.

Click the yellow **Data** tab, then the **New** button. Choose **New Data Table (+Graph)**. In the **Create New Table** dialog, select **Create new table (choose X and Y format).** Format the X column for **Numbers (XY Graph)** and the Y columns for **A single column of values**. Enter this data:

| | X Values | A |
| | Bin Center | Frequency |
| | X | Y |
|---|---|---|
| 1 | 0 | 1 |
| 2 | 15 | 2 |
| 3 | 25 | 2 |
| 4 | 30 | 1 |
| 5 | 35 | 3 |
| 6 | 45 | 4 |
| 7 | 50 | 3 |
| 8 | 55 | 5 |
| 9 | 60 | 4 |
| 10 | 65 | 1 |
| 11 | 70 | 2 |
| 12 | 75 | 1 |
| 13 | 85 | 1 |

Click the yellow **Graphs** tab on the toolbar.
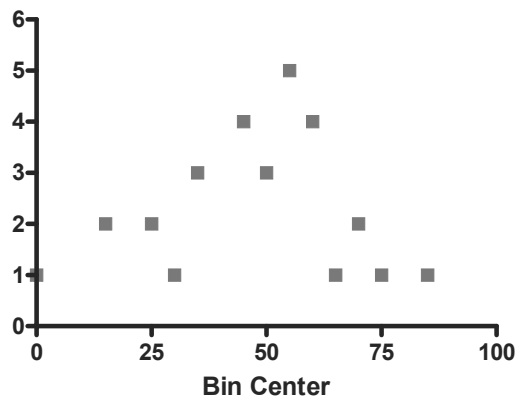


You could immediately change this XY plot to a bar graph, but instead we'll just change the point symbols to "spikes", which produces a pseudo bar graph. Since the graph is still an XY plot, we'll have the X-axis formatting latitude that comes with that graph type. Click **Change.. Symbols & Lines**. Under the **Appearance** tab, change the symbol shape to one of the last four choices to produce "spikes" (bars).
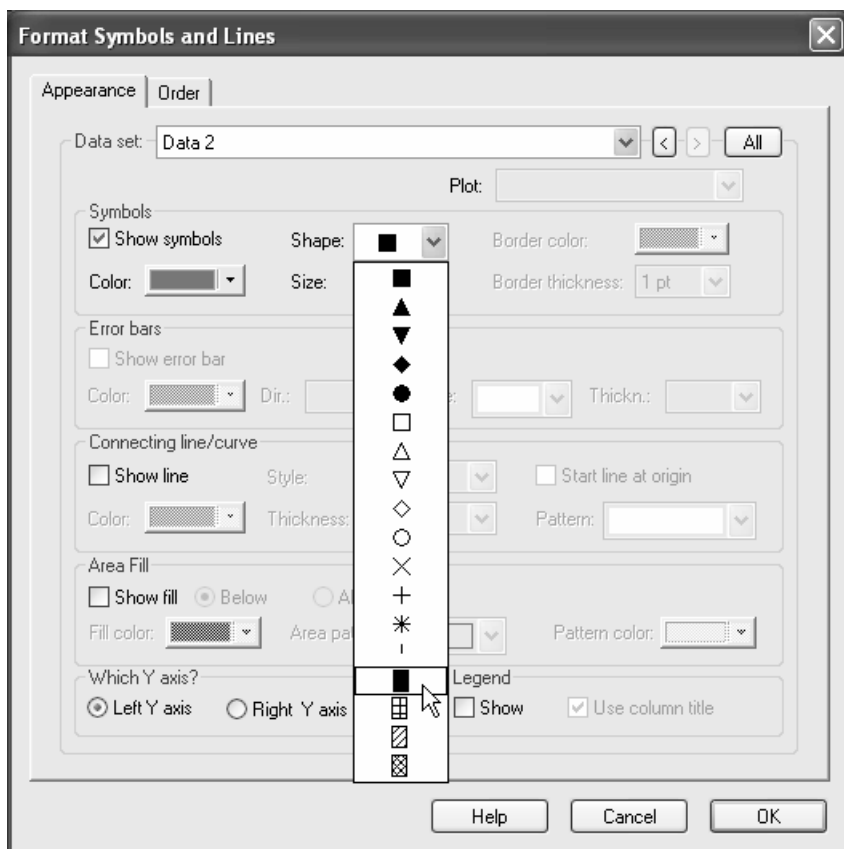
Increase the symbol size, which will widen the "bars", if desired.
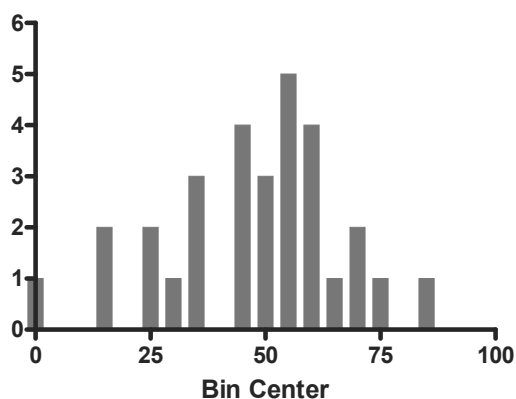


**Bin Center**

This results in easier-to-read (not crowded) bin center labeling, which you can adjust further by double-clicking the baseline, verifying that the **X axis** tab is selected, and making changes to the **Range** and **Tick options** settings (don't forget to deselect the **Auto** checkbox). The following settings will add an "offset" between the Y axis and the first bar, while keeping the bin labels centered. Note that we are also adding minor ticks by selecting **5** intervals from the **Minor ticks** drop-down list:

| Range | | Tick options | | | | |
|---|---|---|---|---|---|---|
| ☐ Auto | | Major ticks: | Interval: | 25.0 | Starting at | 0.0 |
| Minimum: | -4.0 | Minor ticks: | # intervals: | 5 | Spacing: | Equal |
| Maximum: | 104.0 | All ticks: | Direction: | Down | Length: | Short |

Let's also make the Y axis labeling more useful. Double-click on the Y axis. Make sure that in the resulting **Format Axes** dialog, the **Y axis** tab is selected. Change the **Range** and **Tick options** settings as follows:

| Range | | Tick options | | | | |
|---|---|---|---|---|---|---|
| ☐ Auto | | Major ticks: | Interval: | 1.0 | Starting at | 0.0 |
| Minimum: | 0.0 | Minor ticks: | # intervals: | 0 | Spacing: | Equal |
| Maximum: | 5.0 | All ticks: | Direction: | Left | Length: | Short |

In the graph below, we've also edited the Y-axis title.
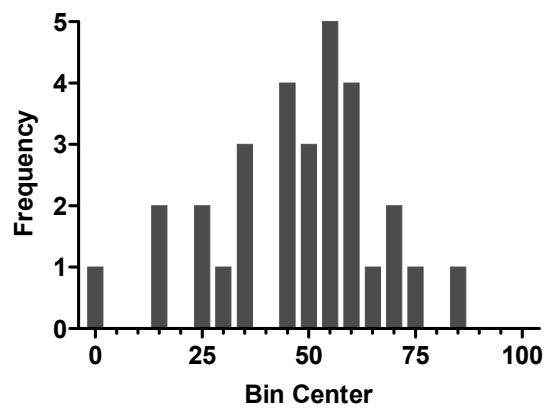
## Adding an Ideal Gaussian Distribution Line

To superimpose a normal-distribution line fitted to your data, click **Analyze... Curves & regression... Nonlinear regression (curve fit)** . In the **Parameters: Nonlinear Regression (Curve Fit)** dialog, choose **Gaussian distribution** from the list of **Classic equations**. The curve, by default, will only be plotted over the X range of the input data, so let's extend the rightward extent of the line. Select the **Range** tab and make the following settings:



Click **OK** to fit the curve.
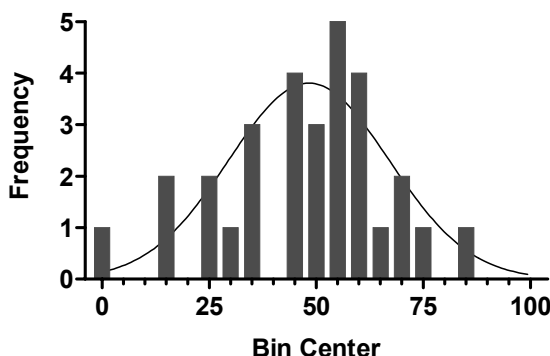


# Histograms from Raw Data, using Automatic Frequency Computation

Prism can provide a frequency distribution from raw data and draw the histogram automatically.

In the Welcome dialog, indicate that you will specify the **Format of data table** directly.

In the resulting data table, enter the raw data values into a *single* Y column.



Here are all the values for this example—but be sure to *put all 50 numbers into one column*, as shown above.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 30 | 17 | 22 | 19 | 23 | 17 | 22 | 8 | 19 | 49 |
| 37 | 28 | 61 | 18 | 60 | 46 | 74 | 69 | 4 | 61 |
| 23 | 71 | 66 | 24 | 42 | 78 | 64 | 60 | 70 | 63 |
| 83 | 74 | 2 | 31 | 57 | 20 | 80 | 23 | 15 | 57 |
| 16 | 24 | 9 | 58 | 67 | 10 | 54 | 35 | 52 | 76 |

Click **Analyze**, and choose **Frequency distribution** from the **Statistical analyses** list. The **Parameters: Frequency Distribution** dialog box appears.

In the **Define bins** section, you can choose to define the way Prism will sort the data into bins or you can allow Prism to do it automatically. Under **Exclude values**, note that Prism excludes from the analysis all values below those fitting the first bin. You can additionally choose to exclude all values larger than a value that you designate. Under **Options**, you have the option to choose **Relative frequencies** to have Prism plot the fraction of the total number of values contained in each bin, rather than the absolute number of values themselves. You also have the option to display a cumulative distribution, whereby each bar shows the number or fraction of value falling *in or below* that bin. If you make the settings shown in the figure above (don't forget **Create a new graph of the results**), the following line graph is produced (click the yellow **Graphs** tab):
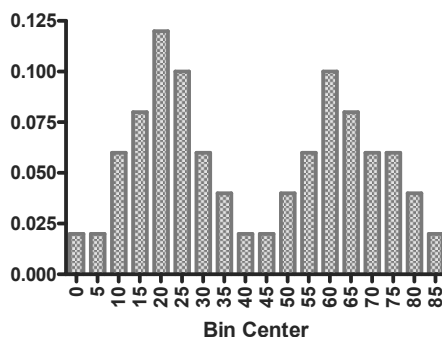
As we pointed out earlier, you can drag the end of the baseline to create more room for the labels,



and you can reduce the size of the labels (select baseline and choose **Change... Selected Text...**).
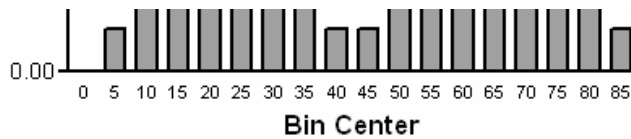


Finally, if we had chosen to display **Cumulative** relative frequencies (you can back up and try these options by clicking **Change... Analysis Parameters...**),
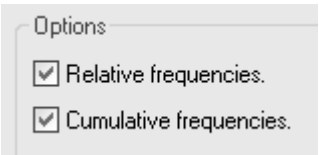


the graph would resemble the one below. In the illustration below, we made some additional formatting changes.
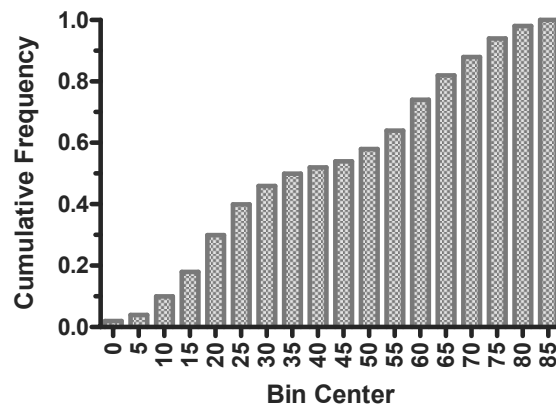
# Combining a Bar Graph with a Line Graph

Occasionally, you may wish to use both bars and lines on the same plot.
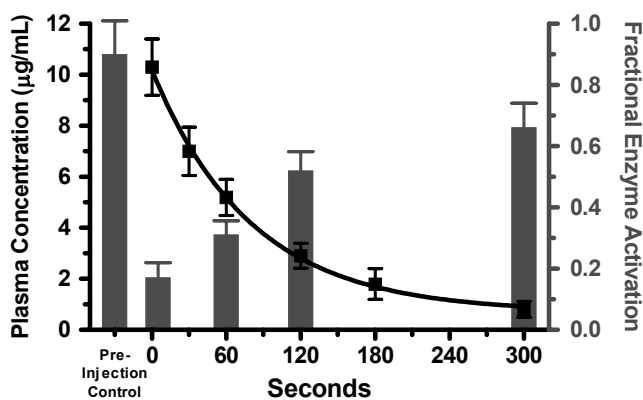


In this example, we'll use point symbols and a fitted exponential decay curve to show the time course for plasma concentration of an injected drug. On the same graph, we'll graph periodic chemical measurements (activity of a tissue enzyme), shown using vertical bars.

## Creating an XY Graph with Fitted Curve

In the Prism Welcome dialog, select **Create a new project** and indicate that you will create the data table and its linked graph by choosing the **Type of graph**.

To get "bars" on this graph, we will replace the point symbols with spikes—lines projected from each X,Y coordinate to the X axis—widened to look like bars. So our graph is entirely an XY plot. Choose the **XY** tab. Prism displays the four possible graph types.

Choose the second graph-type thumbnail, because we want to graph points combined with Y, but not X, error bars. Then indicate that you will enter data into subcolumns as **Mean, Standard Deviation**. Click **OK** to exit the Welcome dialog. Prism displays an appropriately formatted data table.

Enter the data below, including column headings. The X values are the times after drug injection, the first Y column contains plasma drug levels, and the second Y column contains the enzyme activity measurement. Measurements in this example don't always coincide—the first enzyme activity measurement is made 30 seconds before drug treatment, for example—creating some blank cells. Prism has no problem with that.

|  | X Values | A | | B | |
|---|---|---|---|---|---|
|  | Seconds | Plasma drug level | | Enzyme activity | |
|  | X | Mean | SD | Mean | SD |
| 1 | -30 | | | 0.90 | 0.110 |
| 2 | 0 | 10.3 | 1.10 | | |
| 3 | 5 | | | 0.17 | 0.050 |
| 4 | 30 | 7.0 | 0.94 | | |
| 5 | 60 | 5.2 | 0.71 | 0.31 | 0.046 |
| 6 | 120 | 2.9 | 0.49 | 0.52 | 0.062 |
| 7 | 180 | 1.8 | 0.60 | | |
| 8 | 300 | 0.8 | 0.31 | 0.66 | 0.080 |

To view the default graph, click the **Graphs** tab on the toolbar.
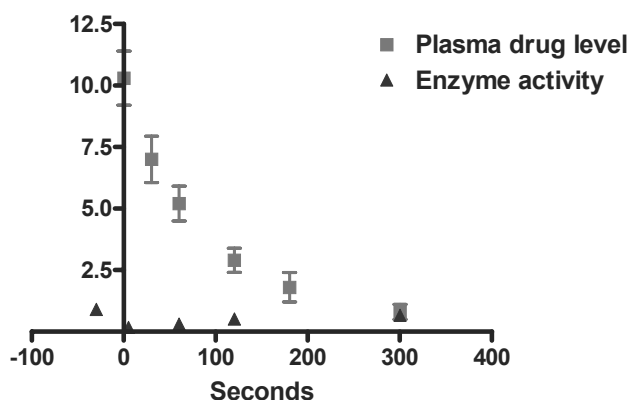
Move the Y axis so that it intersects the X axis at its leftmost point: Double click on the X axis, select the **General** tab, and set **Origin** to **Lower Left**.

Click the **Analyze** button to open the **Analyze Data** dialog box.  Under **Data to analyze**, choose **Selected data sets**, then click the **Select** button to open the **Select Data Sets** dialog box. Select **Data Table-1:Plasma drug level** and deselect **Data Table-2:Enzyme activity**. Click **OK** to return to the **Analyze Data** dialog. Finally, choose **Nonlinear regression (curve fit)** from the list of **Curves & regression** analyses.

Click **OK** to move to the **Parameters: Nonlinear Regression (Curve Fit)** dialog. With the **Equation** tab selected, choose **One phase exponential decay** from the **Classic equations** list. When you click **OK** to exit this box, Prism adds the fitted curve to your graph.

We won't use the legend in this example, so we can get rid of it. Select the two elements of the legend (click on one element, hold the **Shift** key, and click on the other). Press **Delete**.

## Adding a Second Y Axis

The activity data are shown as triangular point symbols. They are barely visible because the Y axis is scaled to accommodate the plasma concentration values, which are larger numbers. This is a good time to use dual Y axes, giving the enzyme activity data a separate, right-hand scale. Double-click on symbol for "Enzyme Activity" (triangle). In the **Format Symbols and Lines** dialog, select the **Right Y axis** radio button to assign this data to a right-hand axis, which Prism will add to the graph automatically.
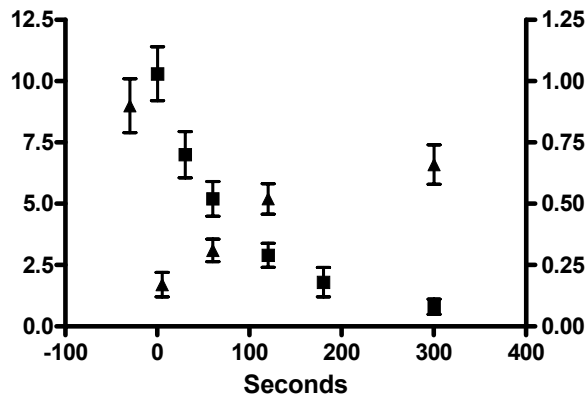
## Changing Point Symbols to "Bars"

Replacing the triangular point symbols with bars is merely a matter of choosing a different "symbol". Return, if necessary, to the **Format Symbols and Lines** dialog (**Change... Symbols & Lines**). Under the **Appearance** tab, make sure the data set chosen is **...Enzyme activity**, then choose the fourth shape from the bottom from the **Shape** drop-down list. Adjust the **Size** to **8** and assign this "bar" a color. Assign a **Color** and **Border**, if desired. Here are the selections:
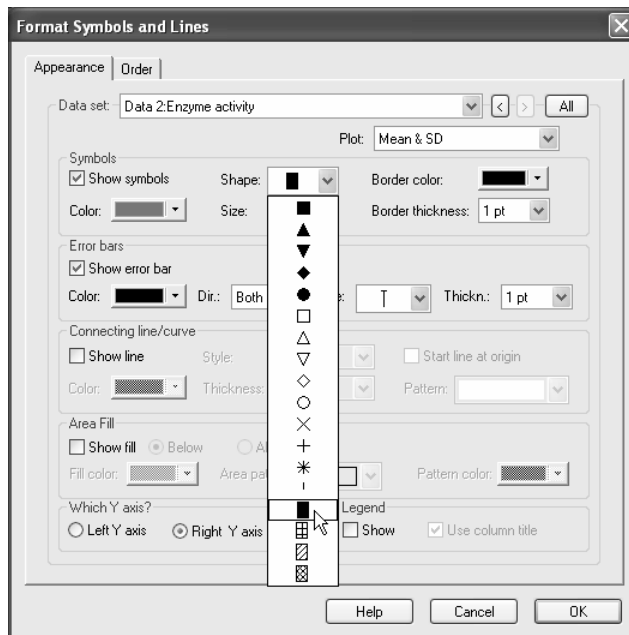
When you click **OK** to return to the graph, you may notice that the square symbol at X=120 is obscured by the newly created bar. Bring the symbol toward the "front" by choosing **Change... Symbols & Lines**. Select the **Order** tab, choose the data set **...Plasma drug level**, and click **Down**.

# Final Graph Formatting

Refer to the finished graph on page 43 as you go through the changes in this section:

Edit each axis title by selecting the default title and then typing a meaningful name. To insert Greek symbols, such as the *mu* in the left-hand Y axis title, click where you want the character, then use the α button on the toolbar.

The label "Pre-Injection Control" is simply a text object. Click the text tool, then click where you want to place the label and type. Click elsewhere to leave the text-editing mode. Select the label and then use the text editing buttons or **Format...Text** to change font size, center-justify, etc., and use your arrow keys to make fine adjustments in the position of the label.

If you are using colored bars, you may wish to color the right-hand Y axis to match. To do that, click once on that axis to select it, then choose **Change... Selected Object(s)...**. Assign a color under **Lines, Arrows & Arcs**. With the axis still selected, choose **Change... Selected Text...** to color the axis title.

To adjust tick spacing and labeling, double-click on the appropriate axis to open the **Format Axes** dialog. Verify the selection of the proper tab, then change the **Range** and **Tick options** settings as show in the following chart:

|  | X axis | Left Y axis | Right Y axis |
|---|---|---|---|
| Range settings: |  |  |  |
| Minimum | -50 | 0 | 0 |
| Maximum | 320 | 12 | 1 |
| Tick option settings: |  |  |  |
| Interval | 60 | 2 | 0.2 |
| Starting at | 0 | 0 | 0 |
| Minor intervals | 2 | 2 | 2 |

While you're in the **Format Axes** dialog, if you would like to move the tick labels further away from the axis, change the setting for **Numbering or labeling... In. from axis**.

To reposition an axis title, click on the title (avoiding tick labels) to display a two-headed-arrow cursor. Drag title away from, or closer to, the axis.

# Performing a *t* Test

A *t* test, typically done to compare observations made in two groups subjected to different experimental treatments, is probably the most common statistical analysis. As with other statistical tests, your experimental design and data should fulfill certain criteria, and the results should be interpreted carefully. Prism provides help in both cases.

## Entering and Graphing the Data

In the Welcome dialog box, select **Create a new project** and indicate that you will create the data table and its linked graph by choosing the **Type of graph**.

Data subject to analysis by the *t* test are divided into two groups using a single grouping criterion. Therefore, in the Welcome dialog, select the tab for **One grouping variable**.
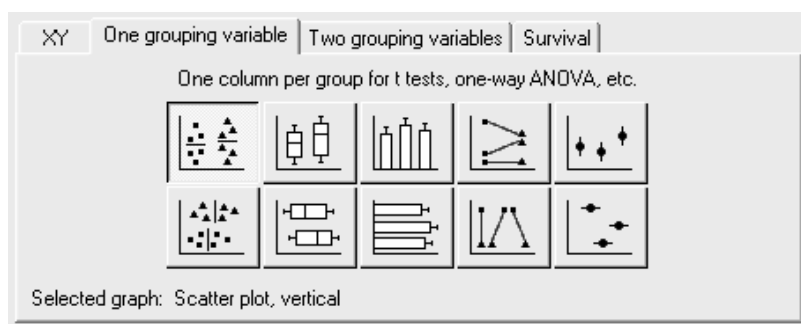


Your choice of graph type will depend on how you wish to graph it and, possibly, the relationship between the two data sets being analyzed. Suppose we want to show individual points grouped by experimental treatment, but that measurements are unpaired (so we don't want to connect points as in the "before-and-after" graph). Choose the upper-left thumbnail for a vertical column scatter plot. When you click **OK**, Prism displays an empty data table.

Enter the data into the table. We want to compare experimental observations made on two sample groups, "Control" and "Treated".

| | A | B |
|---|---|---|
| | Control | Treated |
| | Y | Y |
| 1 | 4.5 | 5.6 |
| 2 | 3.7 | 6.4 |
| 3 | 5.3 | 6.4 |
| 4 | 5.4 | 6.0 |
| 5 | 3.9 | 5.7 |

Many statistics programs expect you to enter data in an indexed format:

| Group | Value |
|---|---|
| 1 | 4.5 |
| 1 | 3.7 |
| 1 | 5.3 |
| 1 | 5.4 |
| 1 | 3.9 |
| 2 | 5.6 |
| 2 | 6.4 |
| 2 | 6.4 |
| 2 | 6.0 |
| 2 | 5.7 |

The first column designates the group, and the second contains all the data. Prism cannot analyze data entered in the index format, but it can import indexed or stacked data. Read the chapter "Importing and pasting data" in the companion *User's Guide*.

Click the **Graphs** tab on the toolbar to view the default graph.
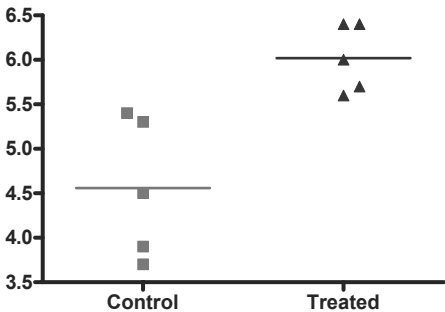
The graph is a column scatter plot, wherein all points are shown, grouped into columns according to level of the grouping variable. By default, the horizontal line across each group of points locates the mean for that group. You can change, for example, to an ordinary vertical error bar indicating *SEM* or *SD* by choosing **Change... Column Appearance...**, choosing the **Appearance** tab, and adjusting the setting in the **Line at** drop-down box.

If you change your mind about the column format on the graph—perhaps you would rather see box-and-whiskers configurations or bar--click **Change... Graph Type...**.

Note that for paired experimental data, a before-and-after graph is available. You can choose that graph type in the Welcome dialog (see above) or change to it later (**Change... Graph Type...**).
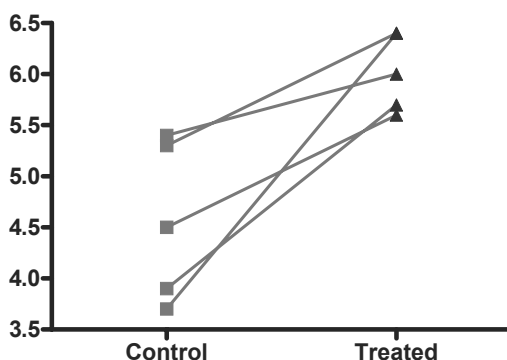


## Performing the Analysis

Click on the **Analyze** button. From the **Type** category, select **Statistical analyses** and then **t tests (and nonparametric tests)**. From the **Data to analyze** category, the choice of **All data sets** is all right for our example, since the data table has only two data sets. When your table has more than two data sets, you must choose **Selected data sets** and then click **Select** to tell Prism which two data sets to compare.

In the **Parameters: t Tests (and Nonparametric Tests)** dialog box, choose a test. You can do this in either of two ways:

- If you know what test you want, select it from the drop-down list. Prism automatically checks the option boxes corresponding to that test. You can get help deciding which test to do by clicking **Help Me Decide**.
- Check the appropriate option boxes. Prism then picks the test for you and displays its name.

If you want Prism to pick the test for you, you'll need to make some decisions about your data and to check the option boxes accordingly:

- Decide whether the data is "paired". Select a **Paired test** when you expect each value in one group to be closer to a particular value, rather than a randomly selected value, in the other group. When this is the case, it's usually obvious. For example, you've made one observation under "control" conditions (before treatment) and the "paired" observation during or after treatment in the same individual. There are more examples available on the help screen accessed by clicking **Help me decide**. In our example experiment, the data are not paired.

- Decide whether you are willing to assume that your data follows a Gaussian (normal) distribution. If not, check **Nonparametric test**. Prism does not make that choice for you; it's usually impossible to tell whether the population is Gaussian just by analyzing the distribution of a small sample. You must make the decision based on knowledge of any previous data and the sources of variability in your experiment. A *t* test is parametric. Click **Help Me Decide** for more information.

- Click **Welch's correction** only if you're sure you want it. This option is rarely used.

- Select a one-tailed or a two-tailed test. Usually, a two-tailed test is the correct choice. For more information, click **Help me decide**.

- You'll probably want to see descriptive statistics for each of the data groups, so check that box (near the bottom of the dialog, **Create a table of descriptive statistics**...).

For our example, make the following selections:

When you click exit the dialog, Prism switches to the Results section of your project and displays the analysis results.

## Viewing and Understanding the Results

If you requested them, the descriptive statistics appear when you select **Column statistics** from the drop-down box on the tool bar or in the Navigator.

```
☐ ☐ Results
   ☐ t  t test of Data 1
      t  Tabular results
      t  Commentary
      t  Column statistics
```
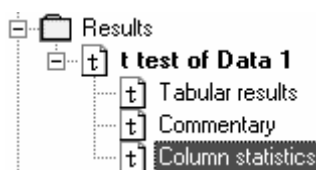
To see the results of the statistical analysis, change the view to **Tabular results**. The P value is 0.0055. That is, there is a 0.55% chance of measuring a difference as large as, or larger than, you saw simply as a consequence of random sampling. Since P is far lower than the traditional cutoff of 0.05, the difference is termed "statistically significant".

The difference between sample means is 1.46. The 95% confidence interval for the difference between population means is -2.35 to −0.57. This means we can be 95% sure that the difference in population means (essentially the difference we would see if we repeated the experiment many times) lies within that range.

All statistical analyses are based on assumptions. Press the "Analysis Check List" button on Prism's toolbar

☑

to read the Prism help screens that review these assumptions. Other Help screens review the meaning of the P value, statistical significance, and confidence intervals.

## Reconfiguring the Analysis

It's quite easy to change the parameters for, and then re-run, the *t* test or nonparametric analysis. In the Navigator, choose any of the sheets containing analysis results.

Click the **Change** button and then choose **Analysis Parameters…**. The **Parameters: t Tests (and Nonparametric Tests)** dialog reappears. Make your changes, then click **OK** to recompute the analysis and display the amended results.

# One-Way ANOVA and Nonparametric Analyses

Prism offers four ways to compare three or more sets of data grouped by a single factor or category: regular ANOVA, repeated-measures ANOVA, Kruskal-Wallis test, and Friedman test. Following ANOVA, Prism can perform the Bonferroni, Tukey, [Student] Newman-Keuls, or Dunnett's post test. Following nonparametric analysis, Prism can perform Dunn's post test.

In this step-by-step example, we'll demonstrate a simple repeated-measures ANOVA followed by the Tukey post test, but we'll point out other options as we go. To learn more about analysis of variance, consult the ANOVA sections of the companion *Statistics Guide*. If you have only two data groups to compare, see the chapter on *t* tests starting on page 49. If you have more than two groups, but the data are grouped by *two* factors, refer to "Two-Way ANOVA with Post Tests" on page 63.

## Entering and Graphing the Data

When you launch Prism, the Welcome dialog appears. Choose **Create a new project** and indicate that you will create the initial data table and linked graph by choosing **Type of graph**.

Suppose that you measure the mean blood pressures of six animals during a pre-treatment control period, then while administering a neural stimulus alone, in the presence of a placebo drug, and in the presence of a putative antagonist designed to inhibit the effect of neural stimulation. The data are matched, since each treatment is repeated in succession with each subject. Since the data are organized by one grouping variable, *Treatment*, which has four levels—Control, Stimulus alone, Stimulus plus placebo, and Stimulus plus antagonist, select the tab for **One grouping variable**.

The Welcome dialog now displays 10 choices for the graph type. You can switch freely between the buttons, reading the "Selected graph" descriptions if you don't find the thumbnail illustrations clear. Note that you are not limited to using bars on a column (one-grouping-variable) graph; you may also choose dot plots, box-and-whiskers configurations, etc. The top-middle button—for vertical bars—is the correct choice for our intended graph. Click **OK** to exit the Welcome dialog. Prism creates and displays the new table.

The table contains no X column, and for each data set (A, B, C, ...; corresponding to a level of the grouping variable), there is a single Y column. With no X values, there is

no categorical distinction from row to row, so replicate values can now be stacked vertically within single columns.

Click the default table name in the drop-down list on the toolbar. Type a new name for the table. This name will be used as the title for your graph and in the names of other linked sheets (you may, however, name linked sheets independently).

| Effect of Neural Stimulation on Blood Pressure | ∨ |
|---|---|

Enter the values show below into your table. Be sure to include column headings. The name of the grouping variable is not included on the table, but the levels are identified in the Y-column headings "Control", "Stimulus", "+Placebo", and "+Antagonist".

| | A | B | C | D |
|---|---|---|---|---|
| | Control | Stimulus | +Placebo | +Antagonist |
| | Y | Y | Y | Y |
| 1 | 90 | 117 | 120 | 98 |
| 2 | 88 | 116 | 118 | 90 |
| 3 | 96 | 116 | 109 | 105 |
| 4 | 94 | 131 | 127 | 97 |
| 5 | 91 | 123 | 127 | 99 |
| 6 | 97 | 132 | 129 | 108 |

If you want to change the number format, select the columns you want to change, then choose **Change... Number Format...**.

> When you choose a graph type under the **One grouping variable** tab, Prism omits the X column from the table, but you may find it useful to add an X column simply to keep track of subject identities, as shown here:
>
> | | X Labels | A | B | C | D |
> |---|---|---|---|---|---|
> | | Animal No. | Control | Stimulus | +Placebo | +Antagonist |
> | | X | Y | Y | Y | Y |
> | 1 | W-1078 | 90 | 117 | 120 | 98 |
> | 2 | W-1079 | 88 | 116 | 118 | 90 |
> | 3 | W-1080 | 96 | 116 | 109 | 105 |
> | 4 | N-562 | 94 | 131 | 127 | 97 |
> | 5 | W-1129 | 91 | 123 | 127 | 99 |
> | 6 | N-588 | 97 | 132 | 129 | 108 |

> To include an X column without causing Prism to graph the data as if it had two grouping variables, start with a graph type under the **One grouping variable** tab, then from the data table, select **Change... Format Data Table ...** and change the X-column format to **Text (bar graph)**.

As soon as you have entered your data, Prism creates a graph automatically. In the Navigator, choose the sheet **Effect of Neural Stimulation on Blood Pressure graph** (or the name you gave to the data table earlier).

> You could also format your table for entry of mean, standard deviation or standard error, and N. Choose a graph type from the **One grouping variable** tab, then from the data table, select **Change.. Format Data Table ...** and change the Y Columns setting to **Mean, Standard Deviation, N** (or other desired format).
>
> | | A | | | B | | | |
> |---|---|---|---|---|---|---|---|
> | | Control | | | Stimulus | | | |
> | | Mean | SD | N | Mean | SD | N | M |
> | 1 | 92.7 | 3.6 | 6 | 122.5 | 7.5 | 6 | |
>
> With this format, you can't perform repeated-measures or nonparametric analyses, which require raw data.

Click on the yellow **Graphs** on the toolbar to view the default graph.



**Effect of Neural Stimulation on Blood Pressure**
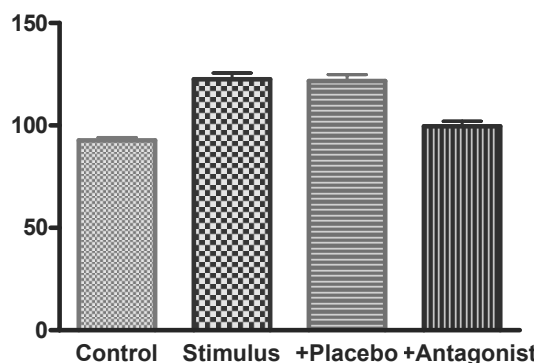
If the baseline labels overlap, you can either select the baseline and reduce the font size



or select and drag to elongate the baseline.



We won't discuss the graph further; for more information about working with one-grouping-variable graphs, see "Bar Graphs with One Grouping Variable" beginning on page 11.

## Performing the Analysis

Click on the yellow **Data** tab to return to the data table. Click **Analyze**. In the **Analyze Data** dialog, select **Statistical analyses** and **One-way ANOVA (and nonparametric)**.

Prism compares three or more sets of data grouped by one factor in four ways. The correct approach depends on whether observations are matched by subject at each factor level and whether you assume that measurements are drawn from a population that follows a normal (Gaussian) distribution. Your options are outlined below.

|  |  | **Assume Gaussian distribution?** | |
|---|---|---|---|
|  |  | Yes – Parametric test | No – Nonparametric test |
| **Repeated measures?** | No – data NOT matched by subject | Regular ANOVA | Kruskal-Wallis test |
|  | Yes – data matched by subject | Repeated-measures ANOVA | Friedman test |

You don't have to memorize this table; Prism can help you with your choice.

In the **Parameters: One-way ANOVA (and Nonparametric)** dialog box, you have two options for selecting the appropriate test.

One option is to set the checkboxes according to (a) whether or not you have repeated measures and (b) whether or not you're assuming that measurements are drawn from a population that follows a Gaussian distribution. Prism provides the test name automatically:

The other option is to select the desired test from the **Test Name** drop-down box. In this case, Prism sets the checkboxes for you:



Next, choose the post test, if any. Set the checkbox to tell Prism whether or not to do a post test when, on the basis of the *F* statistic computed in the course of the ANOVA or nonparametric analysis, it does not find an overall difference in group means. Then choose the post test from the **Test Name** drop-down list.



You can get help choosing both the basic analysis and the post test when you click **Help me decide** at the bottom of the Parameters dialog box.

Before leaving the Parameters dialog, check the box for the optional table of descriptive statistics.

# Viewing and Understanding the Results

When you leave the Parameters dialog, Prism runs the analysis. The Results page for this example consists of three views (you can switch among the views (**Tabular results**, **Commentary**, **Column statistics**) either in the drop-down box on the toolbar or in the Navigator.

Prism displays the **Tabular results** view first, indicating whether there is an overall difference in means

| Effect of Neural Stimulation on Blood Pressure | |
|---|---|
| Repeated Measures ANOVA | |
| P value | P<0.0001 |
| P value summary | *** |
| Are means signif. different? (P < 0.05) | Yes |
| Number of groups | 4 |
| F | 58.42 |
| R squared | 0.9212 |

and whether subject matching was effective (and hence increased the power to detect differences among means).

| Was the pairing significantly effective? | |
|---|---|
| R squared | 0.09355 |
| F | 3.927 |
| P value | 0.0178 |
| P value summary | * |
| Is there significant matching? (P < 0.05) | Yes |

The ANOVA table is presented

| ANOVA Table | SS | df | MS |
|---|---|---|---|
| Treatment (between columns) | 4198 | 3 | 1399 |
| Individual (between rows) | 470.3 | 5 | 94.07 |
| Residual (random) | 359.3 | 15 | 23.96 |
| Total | 5028 | 23 | |

as well as the post test results.

| Tukey's Multiple Comparison Test | Mean Diff. | q | P value |
|---|---|---|---|
| Control vs Stimulus | -29.83 | 14.93 | P < 0.001 |
| Control vs +Placebo | -29.00 | 14.51 | P < 0.001 |
| Control vs +Antagonist | -6.833 | 3.420 | P > 0.05 |
| Stimulus vs +Placebo | 0.8333 | 0.4171 | P > 0.05 |
| Stimulus vs +Antagonist | 23.00 | 11.51 | P < 0.001 |
| +Placebo vs +Antagonist | 22.17 | 11.09 | P < 0.001 |

You can get help understanding the results by clicking the "Analysis Check List" button on the toolbar:

If Prism cannot do the analysis (e.g., you request a repeated-measures analysis, but data are missing from one or more cells), this is indicated on the **Tabular results** view also, and you are referred to the **Commentary** view.

| Table Analyzed |
|---|
| Effect of Neural Stimulation on Blood Pressure |
| Error:See Commentary |

Results
  F₁ **1way ANOVA of Effect**
    F₁ Tabular results
    F₁ Commentary
    F₁ Column statistics
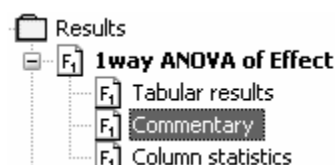
**ANOVA: Overview**
Table analyzed:Effect of Neural Stimulation on Blood Pressure
This test cannot be performed with missing values.

Finally, choose the **Column statistics** view.

| | Control | Stimulus | +Placebo | +Antagonist |
|---|---|---|---|---|
| | Y | Y | Y | Y |
| Number of values | 6 | 6 | 6 | 6 |
| Minimum | 88.00 | 116.0 | 109.0 | 90.00 |
| 25% Percentile | 89.00 | 116.0 | 113.5 | 93.50 |
| Median | 92.50 | 120.0 | 123.5 | 98.50 |
| 75% Percentile | 96.50 | 131.5 | 128.0 | 106.5 |
| Maximum | 97.00 | 132.0 | 129.0 | 108.0 |
| | | | | |
| Mean | 92.67 | 122.5 | 121.7 | 99.50 |
| Std. Deviation | 3.559 | 7.450 | 7.581 | 6.348 |
| Std. Error | 1.453 | 3.041 | 3.095 | 2.592 |
| | | | | |
| Lower 95% CI | 88.93 | 114.7 | 113.7 | 92.84 |
| Upper 95% CI | 96.40 | 130.3 | 129.6 | 106.2 |

# Reconfiguring the Analysis

You can easily change the parameters for, and then re-run, your ANOVA. In the Navigator, choose any of the sheets containing the analysis results. Click the **Change** button and then choose **Analysis Parameters…**. The **Parameters: One-way ANOVA (and Nonparametric)** dialog reappears. Make your changes, then click **OK** to recompute the analysis and display the new results.

# Two-Way ANOVA with Post Tests

Two-way analysis of variance may be used to examine the effects of two variables (factors), both individually and together, on an experimental response. Suppose you've studied the effects on heart rate of three experimental treatments (factor 1) before and during exercise (factor 2). Two-way ANOVA, in combination with post testing, can answer the following questions:

- Is there an effect of treatment on heart rate?
- Is there an effect of exercise on heart rate?
- Is there interaction between the factors? That is, does the affect of treatment differ between exercise states, or equivalently, does the effect of exercise differ among treatments?
- At which treatments is there a significant difference between exercise levels?

To learn more about two-way analysis of variance, consult the companion *Statistics Guide*. If your data are grouped by only one factor, see "One-Way ANOVA and Nonparametric Analyses" beginning on page 55.

## Entering and Graphing the Data

When you launch Prism, the Welcome dialog appears. Choose to **Create a new project** and indicate that you will create the initial data table and linked graph by choosing the **Type of graph**.

Our data are organized by two grouping variables, or factors:

- *Exercise*, which has two levels—Before exercise and During exercise
- *Treatment*, which has three levels—None, Lesion, and Lesion+Drug

Therefore, select the tab for **Two grouping variables**.

The Welcome dialog displays eight choices for the graph type. You can switch freely between the buttons, reading the "Selected graph" descriptions if you don't find the thumbnail illustrations clear. Choose the top-center button—for grouped vertical bars.

With the graph type selected, indicate that you will enter data into subcolumns as **3 replicates**. That means that you will enter your replicate measurements, and Prism will compute the means and standard deviations or standard errors automatically. Click **OK** to exit the Welcome dialog. Prism creates and displays the new table.

Data entry for two-way ANOVA in Prism differs from that in most other statistics programs. Other programs require you to enter data in a three-column indexed format—all measurements in one column and the levels of the two factors in two parallel columns. With Prism, you enter your data onto a table as you'd normally illustrate the results, with the row-and-column position of each datum indicating the factor level. The data table contains an X column, which holds the levels of one grouping variable. Since we indicated in the previous dialog that we will enter data as 3 replicates, Prism subdivides each data set (A, B, C, ...) to make room for our replicates (A:Y1, A:Y2, A:Y3; B:Y1, B:Y2, B:Y3; etc.). Each data set represents a level of the other grouping variable.

Enter the values show below into your table, including column headings.

| X Labels | A | | | B | | |
| Treatment | Before exercise | | | During exercise | | |
| X | A:Y1 | A:Y2 | A:Y3 | B:Y1 | B:Y2 | B:Y3 |
|---|---|---|---|---|---|---|
| 1 None | 75 | 70 | 73 | 131 | 125 | 140 |
| 2 Lesion | 69 | 80 | 81 | 175 | 174 | 169 |
| 3 Lesion+Drug | 78 | 75 | 76 | 134 | 126 | 137 |

Although we instructed Prism to format the table for "3 replicates", in this example, the values in subcolumns Y1, Y2, and Y3 are matched measurements, not simple replicates (we'll indicate that when we enter the two-way ANOVA analysis parameters later). This affects Prism's tolerance for missing values:

- If you request ordinary two-way ANOVA (no matching), Prism will work even if some values are missing. You must have at least one value for each condition in each row. For example, if your data table is formatted for triplicate values (as in the example), you can't leave all three values empty.

- If you specify repeated-measures two-way ANOVA (row or column matching), Prism will allow you to have a different number of subjects in one data set than you do in another, but within each data set you must have the same number of subjects on each row.

> In the Welcome dialog, we could have specified a data entry format of mean; standard deviation or standard error; and N (unless you want to do repeated-measures analysis, in which case you must provide individual replicate measurements).
>
> | | X Labels | A | | | B | | |
> |---|---|---|---|---|---|---|---|
> | | Treatment | Before exercise | | | During exercise | | |
> | | X | Mean | SD | N | Mean | SD | N |
> | 1 | None | 72.7 | 2.5 | 3 | 132.0 | 7.5 | 3 |
> | 2 | Lesion | 76.7 | 6.7 | 3 | 172.7 | 3.2 | 3 |
> | 3 | Lesion+Drug | 76.3 | 1.5 | 3 | 132.3 | 5.7 | 3 |

Click on the default table name (drop-down list on the toolbar). Type a new name for the table. That name will be used as the title for your graph, although you are free to name the graph independently.

Exercise and Treatment Effects

Click on the yellow **Graphs** tab on the toolbar to view the bar chart that Prism has produced automatically.



Exercise and Treatment Effects
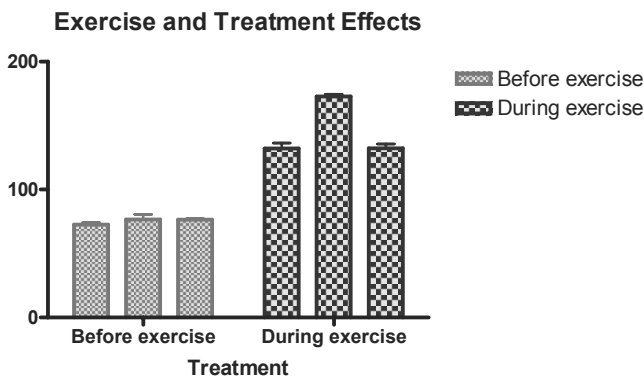
Note that the error bars on this graph will show standard error (*SEM*) rather than standard deviation (*SD*) if that is the default setting in Prism (to check this, choose **Edit... Preferences...**, select the **Graph** tab, and check the **Error Bar** settings). If you don't want to change the default setting to *SD* error bars but you do want the error bars to show *SD* on *this* graph, double-click on one of the bars to open the **Format Bars** dialog. Choose the **Appearance** tab, use the button to change all data sets at once,



and set **Error values** to **Mean & SD**.

# Performing the Analysis

Click **Analyze**. In the **Analyze Data** dialog, select **Statistical analyses** and **Two-way ANOVA**. Accept the default setting to analyze **All data sets**.

> If you have three or more data sets, you can choose to limit the analysis to only some of the data sets by choosing **Selected data sets** and clicking **Select** to open the **Select Data Sets** dialog and tell Prism which sets to analyze. If you later change your mind about which data sets to analyze, click the **Change** button and choose **Data Analyzed...**.

In the **Parameters: Two-Way ANOVA** dialog box, enter names for the factors (variable names) defining the columns and the rows. This step is optional. It doesn't affect the computations; it just makes the output easier to follow.



Now tell Prism whether or not you have repeated measures, and if so, whether the measurements are repeated row-wise or column-wise. We'll assume that subjects were assigned to only one of the three treatment groups (None, Lesioned, Lesioned + drug treated) but that measurements were made on each subject both before and during exercise. This is matching by row—the value in *row 1*, subcolumn A:Y1 was obtained from the same subject as the value in *row 1*, subcolumn B:Y1.

| X Labels | A | | | B | | |
|---|---|---|---|---|---|---|
| Treatment | Before exercise | | | During exercise | | |
| X | A:Y1 | A:Y2 | A:Y3 | B:Y1 | B:Y2 | B:Y3 |
| 1 None | 75.0 | 70.0 | 73.0 | 131.0 | 125.0 | 140.0 |
| 2 Lesion | 69.0 | 80.0 | 81.0 | 175.0 | 174.0 | 169.0 |
| 3 Lesion+Drug | 78.0 | 75.0 | 76.0 | 134.0 | 126.0 | 137.0 |

The dialog setting is...

Repeated measures (mixed model) ANOVA

○ No matching. Use regular two-way ANOVA (not repeated measures).

◉ Each column represents a different time point, so related values are spread across a row.

○ Each row represents a different time point, so related values are stacked into a subcolumn.

Placement of related values:

| | X Title | Time1 | | Time2 | | Time3 | |
|---|---|---|---|---|---|---|---|
| | X | A:Y1 | A:Y2 | B:Y1 | B:Y2 | C:Y1 | C:Y2 |
| 1 | | | | | | | |
| 2 | | | | | | | |
| 3 | | | | | | | |

Note that the **Repeated measures** section will be greyed-out if you do not provide individual replicate values.

Finally, select the Bonferroni post-test option.

Post tests

☑ Bonferroni post-tests to compare replicate means by row.

For each row:

○ Compare each column to all the other columns

◉ Compare each column to column  A ▾

> You can get more help with these settings by clicking **Help Me Decide** at the bottom of the Parameters dialog box.

## Viewing and Understanding the Results

When you leave the Parameters dialog, Prism runs the analysis. The Results page for this example consists of two views. You can switch between the views (**Tabular results** and **Narrative results**) in the drop-down box on the toolbar or in the Navigator. Prism displays the **Tabular results** view first, indicating significant effects of both exercise and treatment, and interaction between the two factors (the effect of one factor is influenced by the level of the other factor, e.g., the difference in

heart rate before exercise and heart rate during exercise is not the same with all treatments).

| Source of Variation | % of total variation | P value |
|---|---|---|
| Interaction | 5.70 | 0.0008 |
| Exercise | 7.06 | 0.0004 |
| Treatment | 86.07 | P<0.0001 |
| Subjects (matching) | 0.5858 | 0.5041 |
| | | |
| Source of Variation | P value summary | Significant? |
| Interaction | *** | Yes |
| Exercise | *** | Yes |
| Treatment | *** | Yes |
| Subjects (matching) | ns | No |

The results show that, in this case, matching of subjects was not effective in controlling for variability between subjects. If you click the "Analysis Check List" button

and request information on subject matching—choose **How to think about results from two-way ANOVA...Subject (matching)**—Prism advises you to reconsider using repeated-measures ANOVA.

Further down the **Tabular results** view, the ANOVA table is presented

| Source of Variation | Sum-of-squares | Mean square | F |
|---|---|---|---|
| Exercise | 1832 | 916.2 | 36.17 |
| Treatment | 22330 | 22330 | 873.8 |
| Subjects (matching) | 152.0 | 25.33 | 0.9913 |
| Residual | 153.3 | 25.56 | |

as well as the post test results.

| Bonferroni posttests | | | |
|---|---|---|---|
| Exercise | During exercise | Difference | 95% CI of diff. |
| None | 132.0 | 59.33 | 45.76 to 72.90 |
| Lesion | 172.7 | 96.00 | 82.43 to 109.6 |
| Lesion+Drug | 132.3 | 56.00 | 42.43 to 69.57 |
| | | | |
| Exercise | t | P value | Summary |
| None | 14.37 | P<0.001 | *** |
| Lesion | 23.26 | P<0.001 | *** |
| Lesion+Drug | 13.57 | P<0.001 | *** |

For an easy-to-understand synopsis of the results, switch to the **Narrative results** view.

Here's a partial illustration of the narrative:

Does Exercise have the same effect at all values of Treatment?
Interaction accounts for 5.70% of the total variance.
F = 28.91.  DFn=2 DFd=6
The P value = 0.0008
If there is no interaction overall, there is a 0.083% chance of randomly observing so much interaction in an experiment of this size.  The interaction is considered extremely significant.

Since the interaction is statistically significant, the P values that follow for the row and column effects are difficult to interpret.

The narrative points out the "extremely significant" interaction (very small interaction P value), which makes the P values for factor (row and column) effects hard to interpret. It is particularly worthwhile in that case to investigate the results of the post tests. If the interaction P value had been high, suggesting a consistent difference from before to during exercise among all treatments, the post tests would not have been very helpful.

If Prism cannot do the analysis (e.g., you request a repeated-measures analysis, but data are missing from one or more cells), the **Tabular results** view is empty, and you can read an explanation in the **Narrative results** view.

Prism cannot perform repeated measures two-way ANOVA when data points are missing for some subjects.

While Prism makes two-way ANOVA very simple, it does not offer all the options of a high-end statistics program. Specifically, Prism cannot do the following:

- Two-way nonparametric ANOVA
- Post testing between row means
- Post testing between column means

# Reconfiguring the Analysis

You can easily change the parameters for, and then re-run, your ANOVA. In the Navigator, choose any of the sheets containing the analysis results. Click the **Change** button and then choose **Analysis Parameters...**. The **Parameters: Two-Way ANOVA** dialog reappears. Make your changes, then click **OK** to recompute the analysis and display the new results.

# Kaplan-Meier Survival Analysis

With some experiments, the outcome is a survival time, and you want to compare the survival of two or more groups. Survival curves show, for each time plotted on the X axis, the portion of all individuals surviving as of that time.
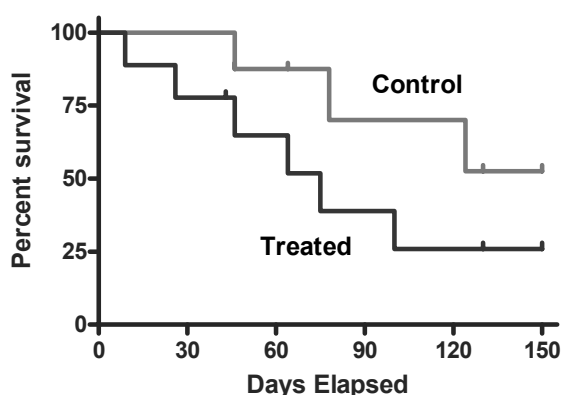


The term "survival" is a bit misleading; you can use survival curves to study times required to reach any well-defined endpoint (e.g., re-occlusion of a grafted blood vessel, first metastasis, discharge from the hospital).

Prism creates survival curves using the method of Kaplan and Meier and calculates the 95% confidence interval for fractional survival at any particular time. Prism can also compare two or more survival curves using the log-rank test.
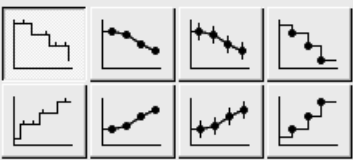
## Entering Survival Data

In the Welcome dialog, select **Create a new project** and **Type of graph**.

Select the **Survival** tab. Prism displays the possible graph types for survival analysis. As you click on the thumbnail views, you can read a **Selected graph** description for each below. For this example, we'll create a descending staircase graph with tick marks showing censored data and with no error bars. Therefore, select the top-left thumbnail view. Near the bottom of the dialog, choose to express the Y values as the **Percents** of subjects surviving at the designated times. Here are the selections:

Before leaving the Welcome dialog, note the instructions for data entry provided at the bottom. When you click **OK**, Prism displays the formatted data table.

For each subject in the study, enter the survival data as follows:

1. In the X column, enter the time elapsed (e.g., days, months) between entry of a subject into the study and the time of the "outcome" for that subject. You must enter a specific number of days (or other units of time), not a range of dates.

2. In the Y column, enter the binary code value specifying the outcome for the subject on that row. By default, the value "1" denotes occurrence of your designated experimental outcome (death or other specified endpoint), and the value "0" denotes that the subject was censored. Unless you change this default (see "Changing the Analysis", page 75), the only permissible values are 0 and 1. A subject is censored when you have no useful information about what happened to the subject after that time. Subjects alive at the end of the study are censored, because you do not know about their later survival. Subjects who leave the study are also censored, either because you do not know whether or not they survived, or because you can't use that information because they were no longer following the study protocol.

If you need to record two or more outcomes at the same time, use multiple rows with the same X value. Here is our example data:

| | X Values | A | B |
|---|---|---|---|
| | Days Elapsed | Control | Treated |
| | X | Y | Y |
| 1 | 9 | | 1 |
| 2 | 26 | | 1 |
| 3 | 43 | | 0 |
| 4 | 46 | 1 | |
| 5 | 46 | | 1 |
| 6 | 46 | 0 | |
| 7 | 64 | | 1 |
| 8 | 64 | 0 | |
| 9 | 75 | | 1 |
| 10 | 78 | 1 | |
| 11 | 100 | | 1 |
| 12 | 124 | 1 | |
| 13 | 130 | 0 | |
| 14 | 130 | | 0 |
| 15 | 150 | | 0 |
| 16 | 150 | 0 | |
| 17 | 150 | 0 | |

## Survival Data

*For this example transplant study, we'll describe just the Control data in column A. Subjects in the Control group died (code = 1) at 46, 78, and 124 days after transplant. One subject in the Control group was lost to follow-up (code = 0) 46 days after treatment, and another was dropped for noncompliance (code = 0) 64 days after treatment. At the end of the study, three subjects were still alive and enrolled in the study; one had survived for 130 days, and the other for 150 days, after treatment.*

You don't have to enter X values in order of occurrence. In fact, if it is more convenient, you can enter all of your data for a particular treatment group together, as shown in the following table. Just be sure to match each event code (Y-column entry) to its proper time (X-column entry).

| | X Values | A | B |
|---|---|---|---|
| | Days Elapsed | Control | Treated |
| | X | Y | Y |
| 1 | 46 | 1 | |
| 2 | 46 | 0 | |
| 3 | 64 | 0 | |
| 4 | 78 | 1 | |
| 5 | 124 | 1 | |
| 6 | 130 | 0 | |
| 7 | 150 | 0 | |
| 8 | 150 | 0 | |
| 9 | 9 | | 1 |
| 10 | 26 | | 1 |
| 11 | 43 | | 0 |
| 12 | 46 | | 1 |
| 13 | 64 | | 1 |
| 14 | 75 | | 1 |
| 15 | 100 | | 1 |
| 16 | 130 | | 0 |
| 17 | 150 | | 0 |

**Data Vertically Ordered by Group**

*All the data for the Control group are entered first (rows 1-8), followed by the data for the Treated group.*

Before proceeding, make sure that each subject in your study is represented exactly once (by either 1 or 0) in the Y data column. In other words, the total number of 1's and 0's entered in each column should equal the number of subjects in that group.

# Automatic Analysis and Graphing

Since it has been given all the information it needs in the Welcome dialog, Prism performs the survival analysis and graphs the survival curve automatically. Click the yellow **Results** tab on the toolbar. Prism displays the results on a Results page, which shows the coordinates for plotting percent survival vs time. You can see further results by clicking on any of four subsheets in the Navigator: **Survival proportions**, showing fractional survival at each plotted time; **# of subjects at risk**, number of subjects still alive up until each time point; **Curve comparison** using the logrank test; or **Data summary**.

Click on the yellow **Graphs** tab to view the survival graph. The following illustration includes some formatting changes not discussed here:

---
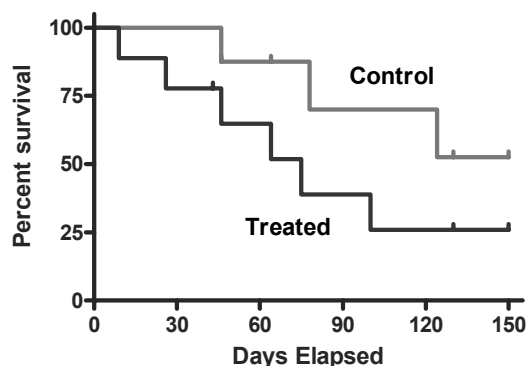
# Changing the Analysis

If the survival analysis is not configured as you wish, click **Change... Analysis Parameters...**.



The **Input** frame of the resulting parameters dialog shows the default binary coding for experimental outcomes—"1" indicating death or other specified endpoint, "0" indicating censored data. You may substitute other code numbers (but not letters) here.

In the **Output** frame, you can change how the survival data are graphed—whether Y coordinates indicate fractions or percentages, whether the graph shows survival (descending from 1.0 or 100%) or deaths (increasing from 0), etc. The output parameters were originally chosen when you selected the graph type, in fact, another way of changing these parameters is to choose **Change... Graph type...**.

# Analyzing Contingency Tables

A contingency table shows numerically the results of an experiment in which the outcome is a categorical variable. Commonly, two groups of subjects are studied, and there are two possible outcomes. Suppose you treat 126 subjects with either a placebo or a new drug designed to decrease the incidence of coronary artery restenosis within 6 months after angioplasty. The numbers of subjects experiencing each outcome may be arranged on a 2 x 2 contingency table as follows:

|  |  | Restenosis within 6 months after angioplasty? | |
| --- | --- | --- | --- |
|  |  | Yes | No |
| **Treatment** | Placebo | 23 | 41 |
|  | Drug-treated | 16 | 46 |

In this step-by-step example, we'll analyze the table using Fisher's exact test to answer the following question: If there were really no association between treatment and occurrence of restenosis within the observation period, what is the chance that random sampling would produce an apparent association as strong, or stronger, than that observed in this experiment?

Prism will analyze contingency tables not only for experiments, but also for cross-sectional, prospective, or retrospective case-control studies. To learn more about these distinctions or about contingency analysis in general, consult the companion *Statistics Guide*.

## Entering and Graphing Contingency Data

When you launch Prism, the Welcome dialog appears. Select **Create a new project** and indicate that you will create the initial data table and graph by choosing the **Type of graph**.

Suppose we wish to create the following graph:
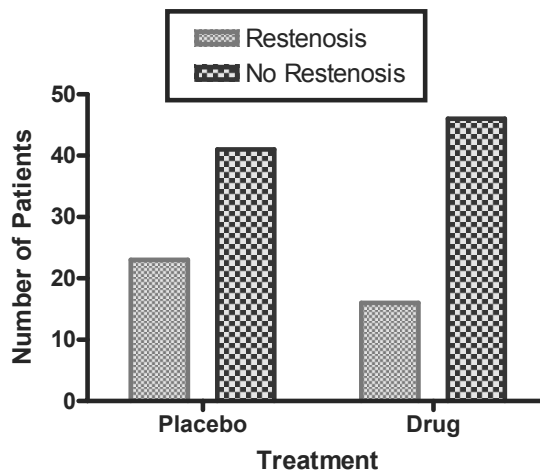
As the graph shows, our data are organized according to two grouping variables:

1. *Treatment*, with two levels—Placebo and Drug
2. *Outcome*, with two levels—Restenosis and No Restenosis

Therefore, in the Welcome dialog, select the tab for **Two grouping variables**.
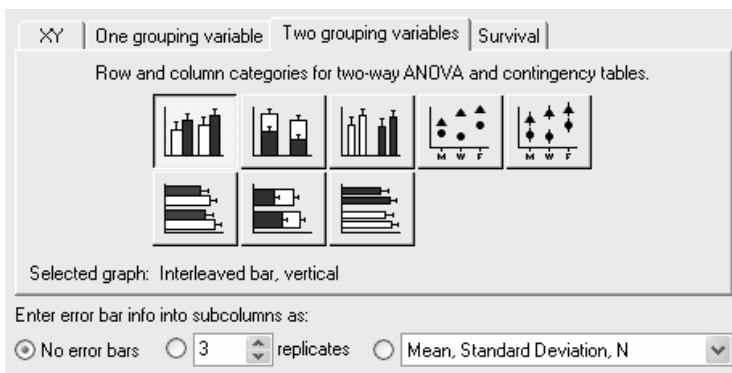


The dialog now displays eight choices for graph type. You can switch freely between the buttons, reading the "Selected graph" descriptions if you don't find the thumbnail illustrations clear. The upper-left button—for interleaved vertical bars—matches the organization and orientation of the bars in our intended graph.

Near the bottom of the dialog box, choose the radio button for **No error bars**—since the data table shows precise counts, error bars don't make sense on the graph. Click **OK** to exit the dialog. Prism creates and displays the formatted data table.

Enter the data—numbers of subjects falling into each mutually exclusive category, not fractions or percentages—as follows:

| | X Labels | A | B |
|---|---|---|---|
| | Treatment | Restenosis | No Restenosis |
| | X | Y | Y |
| 1 | Placebo | 23 | 41 |
| 2 | Drug | 16 | 46 |

> You must *type* in the four values for the contingency table. Prism cannot cross-tabulate raw data to create a contingency table.

Click on the yellow **Graphs** tab on the toolbar to view the automatic bar chart.

# Performing Fisher's Exact Test

Click on the yellow **Data** tab to return to the data table. Click the **Analyze** button. In the **Analyze Data** dialog, select **Contingency tables** from the list of **Statistical analyses**. Accept the default setting to analyze **All data sets**.

In the **Parameters: Contingency Tables** dialog, accept the choice of **Fisher's exact test**, which is the default when you're analyzing a 2 x 2 table.

> If your table has more than two rows or columns, Prism will only do Chi-square analysis**.**

In the same dialog, set the checkboxes to do the optional **Relative Risk** calculation. All the settings are shown below; you can get more help understanding these settings by clicking **Help me decide**.
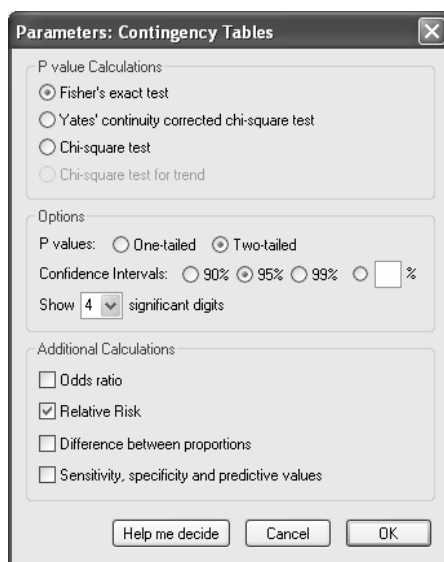
# Viewing and Understanding the Results

When you leave the Parameters dialog, Prism runs the analysis and displays the results page (you can get help understanding the results by clicking the "Analysis Check List" button).

For this example, the P value indicates a high (25%) likelihood that the apparent association between treatment and occurrence of restenosis within 6 months was due simply to random sampling.

| Fisher's exact test | |
|---|---|
| | |
| P value | 0.2508 |
| P value summary | ns |
| One- or two-sided | Two-sided |
| Statistically significant? (alpha<0.05) | No |

Further down the Results sheet, Prism reports the relative risk. That number simply indicates that *in this experiment* there was a 39 percent greater incidence of restenosis among the drug-treated subjects. The wide 95% confidence interval for relative risk, which includes the value 1.0 (no association), is consistent with the lack of statistical significance (P > 0.05) noted above.

| Strength of association | |
|---|---|
| Relative Risk | 1.393 |
| 95% confidence interval | 0.8163 to 2.376 |

# Reconfiguring the Analysis

You can easily reconfigure and re-run the analysis. In the Navigator, choose one of the sheets containing the analysis results. Click the **Change** button and then choose **Analysis Parameters…**. The **Parameters: Contingency Tables** dialog reappears. Make your changes and then click **OK** to display the new results.

# Saturation Binding Curves and Scatchard Plots

In this chapter, we'll make a commonly used combination graph—a saturation binding curve with an inset Scatchard plot.
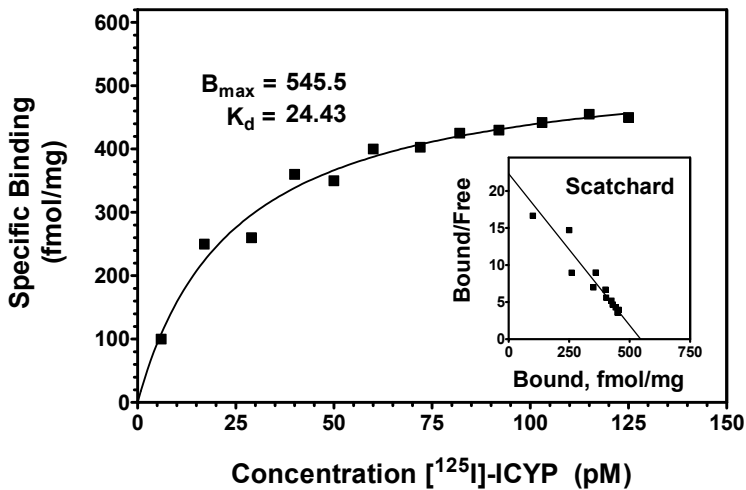


Scatchard analysis is a method of linearizing data from a saturation binding experiment in order to determine binding constants. One creates a "secondary" plot of specific binding/free radioligand concentration (Y axis) vs. specific binding (X axis). For each site where the ligand binds according to mass-action kinetics, the Scatchard plot is a straight line with

$$Y\ intercept = B_{max} / K_d$$
$$X\ intercept = B_{max}$$
$$Slope = -1 / K_d$$

Now that programs such as Prism easily do nonlinear regression, the best way to determine $K_d$ and $B_{max}$ is to fit a hyperbola directly to the saturation isotherm. Yet Scatchard analysis remains a good way to visualize saturation binding data. We'll see how to combine these techniques, creating a conventional Scatchard plot, but superimposing a line that reflects the best possible estimates of $K_d$ and $B_{max}$.

## Creating a Saturation Isotherm

When you launch Prism, the Welcome dialog appears. It lets you create a new project (file) or open an existing one. Choose **Create a new project** and **Type of graph**. Choose the **XY** graph-type tab, then select the first thumbnail view, for "Points only".

For each point on the saturation binding curve, enter the concentration of ligand into the X column and the value for *specific* binding into the Y column. The units you use are up to you—use whatever is meaningful. Just remember that *Prism will graph the data and compute the binding parameters in whatever units you've used on your table.* Place labels over the X and Y columns as a reminder. Here are the data for our example:

| | X Values Conc (pM) X | A Binding (fmol/mg) Y |
|---|---|---|
| 1 | 6 | 100 |
| 2 | 17 | 250 |
| 3 | 29 | 260 |
| 4 | 40 | 360 |
| 5 | 50 | 350 |
| 6 | 60 | 400 |
| 7 | 72 | 403 |
| 8 | 82 | 425 |
| 9 | 92 | 430 |
| 10 | 103 | 442 |
| 11 | 115 | 455 |
| 12 | 125 | 450 |

> Prism can determine specific binding values for you, starting from total binding and nonspecific binding. For details, consult the appendix to this chapter.

In the sheet selection box on the toolbar, rename the table as follows:

Saturation Binding Data

Click on the yellow **Graphs** tab to view the graph of your data.

## Fitting a Saturation Binding Curve to Determine $K_d$ and $B_{max}$

Click on the **Analyze** button. From the **Curves & regression** category, select **Nonlinear regression (curve fit)**.

In the **Parameters: Nonlinear Regression (Curve Fit)** dialog box, choose **Classic equations** and, from the list below, select **One site binding (hyperbola).** Click **OK** to exit the parameters set-up and complete the curve fit.

**Saturation Binding Data**
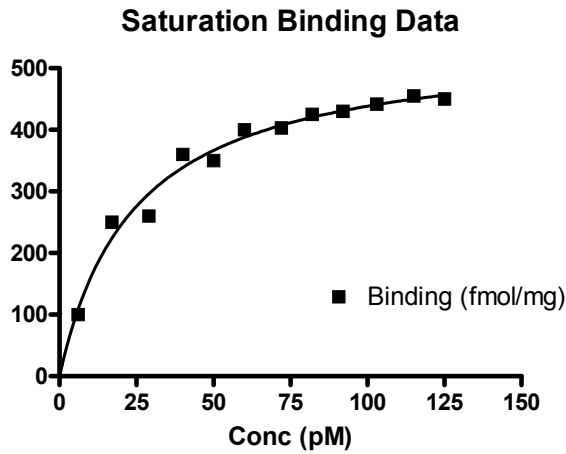
Choose the **Table of results** subsheet in the Results section of the Navigator. Prism displays the best-fit values for the binding parameters.

| Equation 1 | |
|---|---|
| Best-fit values | |
| BMAX | 545.5 |
| KD | 24.43 |

The units for $B_{max}$ are the units that you used when entering Y values on the original data table. The units for $K_d$ are the units you used when entering the X values.

## Displaying Binding Constants on the Graph

If you wish, you can paste cells from this results table to your graph, so that your binding constants will be displayed there and will also be linked to the Results sheet. That way, if you change the saturation binding data later, $B_{max}$ and $K_d$ will be automatically updated both on the Results sheet and on the graph. If you don't like the way the pasted data are displayed, you can change that. Select a cell on the

Results sheet containing the value you want to paste. Choose **Edit... Copy**. Switch to the graph, and choose **Edit... Paste Table**. The value is placed on the graph, and you can drag it wherever you want. Now use the text tool (bottom row of the toolbar)...

$$T$$

...to add customized labels. Click first on the text tool button, then on the graph where you want the label. Type your label. You can make the label boldface, or resize it, or add subscripting as desired, using the text editing tools.

**B** *I* U X² X₂ Á A⁺ α ⁻

Click elsewhere to exit the text insertion mode, then select the label so that you can drag it into position (use the "arrow" keys on your keyboard to make the final adjustments). Here is a finished example:
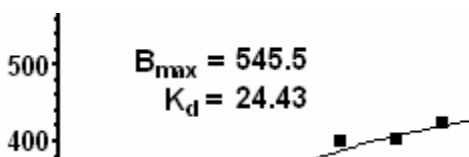


At this point, you may wish to change some of your original saturation binding data (data table) and observe how the values of $B_{max}$ and $K_d$ change automatically both on the Results sheet and on the graph.

# Creating a Scatchard Plot

The original data table has the "free" ligand concentration in the X column and the "bound" ligand concentration in the Y column. To get "bound" in the X column and "bound/free" in the Y column, we need to do two transformations.

## Transforming the Data

From the original table (**Saturation Binding Data**), click **Analyze** and select **Data manipulations**, then **Transforms**. In the next dialog, **Parameters: Transforms**, choose **Pharmacology and biochemistry transforms** from the **Function List** drop-down box, then set the **Scatchard** radio button. Near the bottom of the dialog, choose to **Create a new graph of the results**.

When you click **OK** to exit this dialog, Prism creates and displays the Results sheet (named **Scatchard of Saturation Binding Data** in this example) containing the transformed data.

| X Values | A |
|---|---|
| Bound | Bound/Free |
| X | Y |
| 100.000 | 16.667 |
| 250.000 | 14.706 |
| 260.000 | 8.966 |
| 360.000 | 9.000 |
| 350.000 | 7.000 |
| 400.000 | 6.667 |
| 403.000 | 5.597 |
| 425.000 | 5.183 |
| 430.000 | 4.674 |
| 442.000 | 4.291 |
| 455.000 | 3.957 |
| 450.000 | 3.600 |

## Plotting the Points

The default Scatchard plot appears as the most recently generated graph. Click the yellow **Graphs** tab on the toolbar.

**Scatchard of Saturation Binding Data: Transformed data**
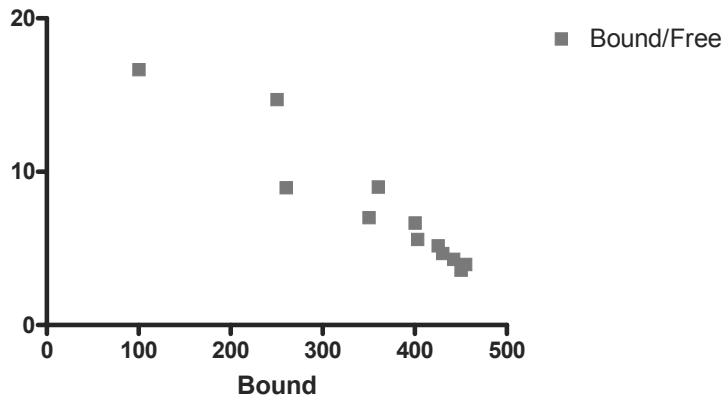


## Adding the Line

So far, there is no line superimposed over the points. We advise against using linear regression to produce the line, because linear regression of the transformed data would violate several of the assumptions of linear regression. Instead, we'll add a line connecting X and Y axis intercepts that accurately reflect the $K_d$ and $B_{max}$ values that we have just found by *nonlinear* regression.

Referring to the Results sheet for your nonlinear regression analysis, compute the Y coordinate for the Y axis intercept manually as

$$B_{max} / K_d = 545.5 / 24.43 = 22.33$$

Switch to the **Data Tables** section of your project and click **New**. Create a **New Data Table (+Graph)** by making the selections below (note that you will not be making a new graph).

Fill in the table using the values for $B_{max}$ and $B_{max}/K_d$ as follows:

| Into row 1 enter: | X = 0 | Y = $B_{max}/K_d$ |
| Into row 2 enter: | X = $B_{max}$ | Y = 0 |

Here's the table:



Rename this table:



We now have the elements for the final Scatchard plot on two sheets. The graph labeled **Scatchard of Saturation Binding Data graph** contains the points from the saturation binding experiment, and the table labeled **Scatchard Y and X intercepts** contains two points marking the Y and X intercepts ($B_{max}/K_d$ and $B_{max}$, respectively). We need to combine the two data sets on the same graph.

Select the **Scatchard of Saturation Binding Data graph** sheet to display it. In the Navigator, click and drag the data table sheet labeled **Scatchard Y and X intercepts** onto the displayed graph.

In the dialog that appears, verify that you are adding the correct data set and then click **OK**.

**Add Data Sets to Graph**

Select:

From the following data or results table:

Scatchard Y and X intercepts

Data sets to add (select one or more)

Scatchard Y and X intercepts
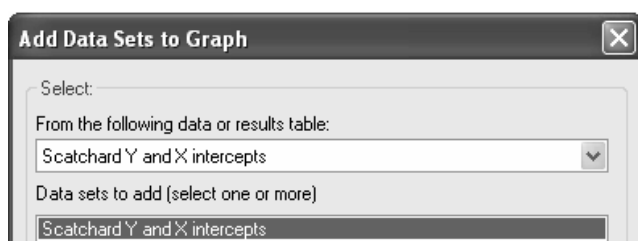
It should now be evident that the Y and X intercepts (the ends of the Scatchard line) are plotted using symbols distinct from those for the saturation binding points (labels and arrows pointing to the Y and X intercepts will not be on your graph, and your axis formatting may be different).
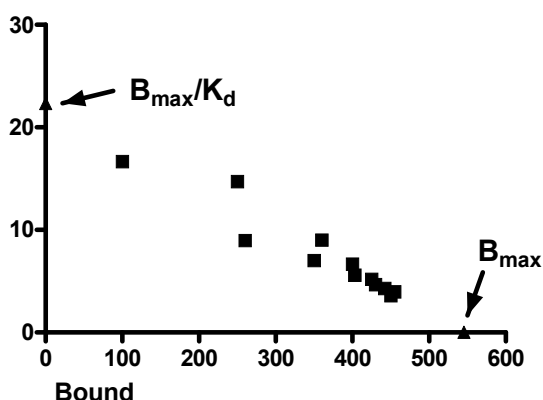


Format the Scatchard Line data set to make the points invisible but to connect them with a line: Click **Change...Symbols & Lines**. Under the **Appearance** tab, set **Data set** to **Scatchard Y and X intercepts**. Choose to plot no symbols, but to connect them with a line.
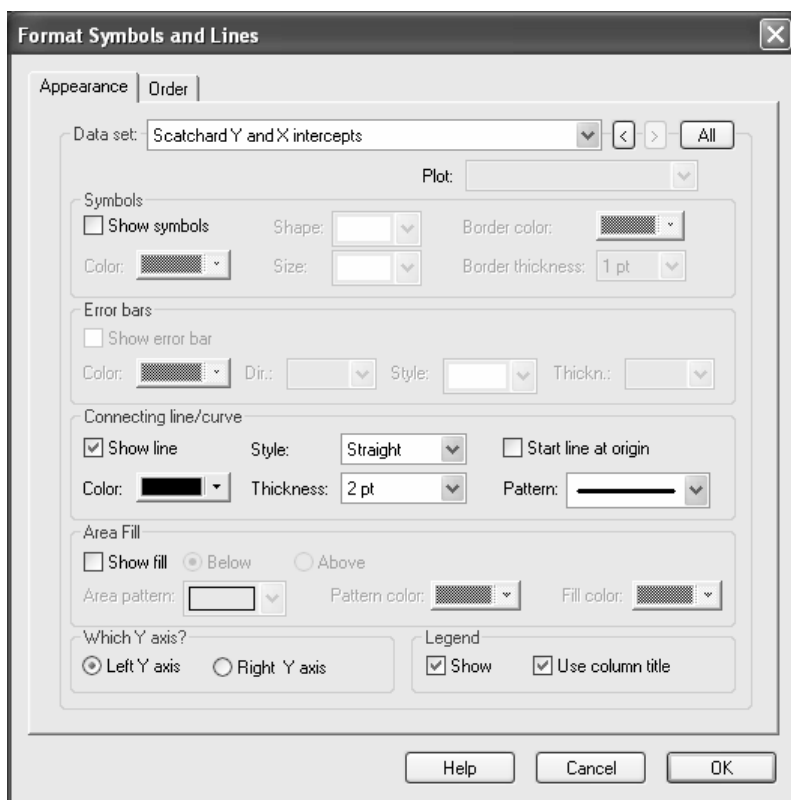
Here is the Scatchard plot:
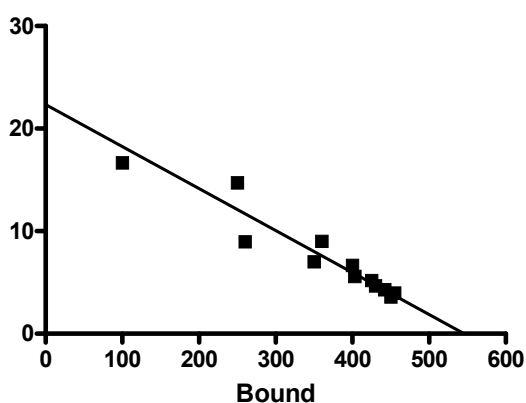


Following is the final Scatchard graph for the inset plot, after a few a formatting changes not covered here:

---
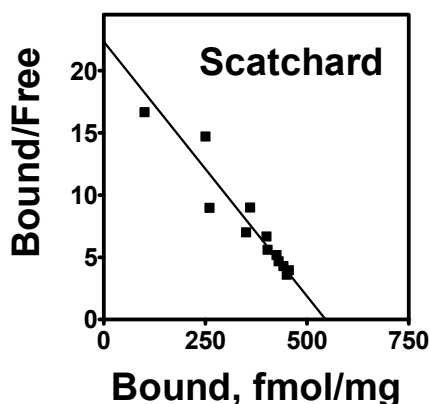
## Combining Saturation Isotherm and Scatchard Plot

Switch to the **Layout** section of your project, and if necessary, click the **New** button and choose **New Layout...**. In the **Create New Layout** dialog, choose the **Landscape** page orientation and the **Inset graph** arrangement.
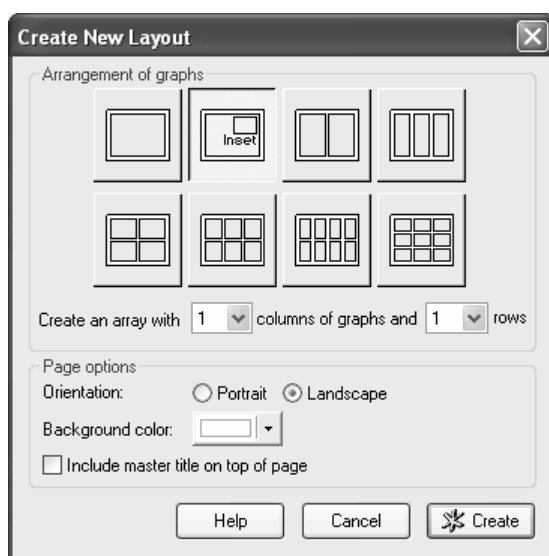


When you click **Create**, you'll see a layout with two placeholders. Click and drag the appropriate Graph sheet in the Navigator onto its placeholder, assigning your saturation binding plot to the larger, and your Scatchard plot to the smaller, placeholder.

Now move and resize the Scatchard plot:
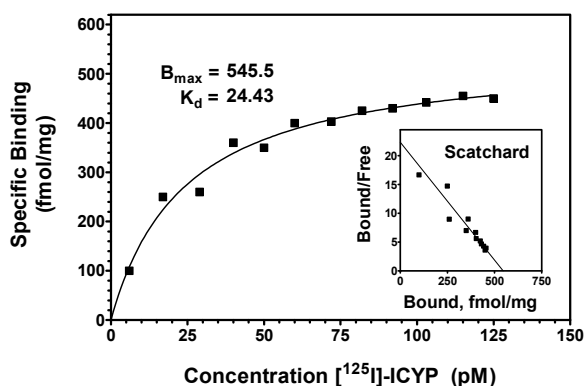


If, after viewing your layout, you want to resize individual objects/labels to enhance readability, switch back to the appropriate Graph sheet and make the changes there. Test the changes by switching back to the layout, which updates automatically. One exception to this is when you want to change the size of the overall layout or change the sizes of the two graphs on the layout relative to one another. Do that directly on the layout, by selecting the inset graph, or the entire layout, and then dragging as appropriate.

# Appendix: Working with Total and Nonspecific Binding

Earlier in this chapter, we fitted a saturation isotherm after computing specific binding manually:

$$Specific\ binding = Total\ binding - Nonspecific\ binding$$

Thus, we provided the *specific* binding (Y) and added ligand (X) values and then let Prism fit the curve relating the two. If you don't want to preprocess the data in this way, you may enter *total* and *nonspecific* binding values at each added ligand concentration and then proceed in at least two ways:

- Have Prism perform the subtractions to obtain nonspecific binding values, then proceed as discussed before.
- Perform a *global* curve fit to both total and nonspecific binding data, estimating $K_d$ and $B_{max}$ from the total binding curve, while finding the best value for nonspecific binding for the two curves taken together.

## Direct Subtraction at Each Added Ligand Concentration

By this method, you simply let Prism do the subtraction that you'd otherwise do yourself, then fit the nonspecific binding data as already discussed. Tabulate total and nonspecific binding values:

| | X Values | A | B |
|---|---|---|---|
| | Conc (pM) | Total (fmol/mg) | NS (fmol/mg) |
| | X | Y | Y |
| 1 | 6 | 100 | 3.3 |
| 2 | 17 | 239 | 8.1 |
| 3 | 29 | 286 | 12.5 |
| 4 | 40 | 360 | 21.0 |
| 5 | 50 | 382 | 22.4 |
| 6 | 60 | 410 | 31.8 |
| 7 | 72 | 432 | 34.0 |
| 8 | 82 | 476 | 37.7 |
| 9 | 92 | 479 | 46.5 |
| 10 | 103 | 500 | 52.1 |
| 11 | 115 | 529 | 59.9 |
| 12 | 125 | 519 | 64.0 |

Prism graphs the data as follows:
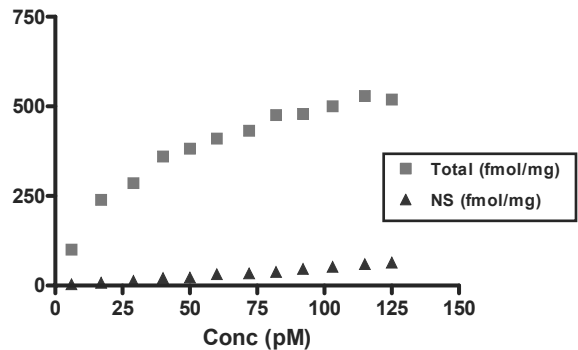


Click **Analyze** and then choose **Remove baseline and column math** from the **Data manipulations** list.

In the Parameters dialog, choose to subtract the values in column B from column A.

**Parameters: Remove Baseline and Column Math**

Where are baseline values?

- ○ Column [ ▾ ]
- ⦿ Data sets (columns)   B, D, F. . .
- ○ Data sets (columns)   A, C, E. . .
- ○ First row
- ○ Last row
- ○ Mean of first [ 3 ] rows.
- ○ Mean of last [ 3 ] rows.
- ☐ Erase those rows from the analysis

Calculate

- ⦿ Difference: Value - Baseline
- ○ Sum: Value + Baseline
- ○ Product: Value* Baseline
- ○ Ratio: Value/Baseline
- ○ Percent: 100*Value/Baseline
- ○ Fractional difference:  (Value - Baseline)/Baseline
- ○ Percentage difference: 100* (Value - Baseline)/Baseline

Linear baseline

- ○ Assume the baseline is linear with X. Use the values predicted from the regression line.
- ⦿ Don't assume linear baseline. Use the baseline values entered.

At the bottom of the dialog (not shown above), choose to **Create a new graph of the results**.

Prism tabulates the differences (B-A) on the Results sheet.

| | X Values | A |
|---|---|---|
| | Conc (pM) | A-B |
| | X | Y |
| 1 | 6.000 | 96.700 |
| 2 | 17.000 | 230.900 |
| 3 | 29.000 | 273.500 |
| 4 | 40.000 | 339.000 |
| 5 | 50.000 | 359.600 |
| 6 | 60.000 | 378.200 |

Here is the new graph of specific binding:



*Binding Analysis*
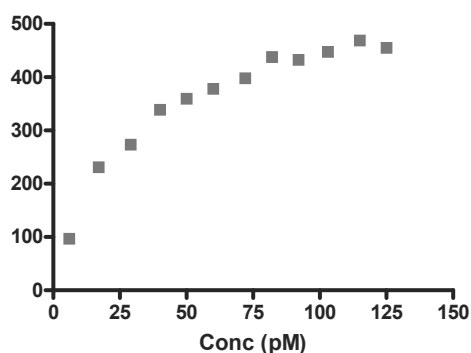
## Global Fitting of Total and Nonspecific Binding Curves

*Start from the initial table of total and nonspecific binding values shown in the previous section.* We will fit a different equation to the data in each Y column on the data table as follows:

- To the total binding data (column A), we'll fit the sum of a hyperbolic curve (one-site binding model, corresponding to specific binding) and a straight line (nonspecific binding),

$$Y = \frac{B_{max}X}{K_d + X} + NS \cdot X$$

- To the nonspecific binding data (column B), we'll fit the linear equation

$$Y = NS \cdot X$$

The parameter in common to be equations (NS) is to be fitted "globally", i.e., to the best-fit value for the two data sets taken together.

> While this approach gives better estimates for the equation parameters, note that you will not be able to draw a Scatchard plot, since nonspecific binding is not expressly computed.

Click **Analyze**. Select **Nonlinear regression (curve fit)** from the **Curves & regression** list.

In the **Parameters: Nonlinear Regression (Curve fit)** dialog, select the **Equation** tab if necessary. Choose the radio button for **More equations**, then select **[Enter your own equation]**.

> This technique requires curve fitting to user-defined equations, and we'll cover the steps perfunctorily. For more information about user-defined functions, see the chapter "Fitting Data to User-Defined Equations" beginning on page 151.

In the **Use-defined Equation** dialog, give your equation system a name (e.g., "Total and nonspecific binding curves"). In the **Equation** window, enter the sequence shown in the next figure.

Note that the equations denoted by "<A>" and "<B>" are fitted to data in columns A and B, respectively, so the order of the Y columns on the data table is important.

The two-equation system shown above is discussed in more detail in the chapter entitled "Analyzing saturation radioligand binding data" of the companion manual *Fitting Models to Biological Data using Linear and Nonlinear Regression.*

Click the **Rules for Initial Values** tab. Prism has identified the parameters of the curve-fit equations. Fill in the rules for computing their initial values as shown:



Click the **Default Constraints** tab. Specify that the parameter NS is to be shared—fitted to the same best value for both data sets A and B taken together.

**User-defined Equation**

Enter Equation | Rules for Initial Values | Default Constraints

Define a set of constraints to use as the default every time you curve fit with this equation.

Fix, constrain or share a parameter

| Parameter | Constraint | Value |
|-----------|------------|-------|
| BMAX | No constraint | |
| KD | No constraint | |
| NS | Shared value for all data sets | |

Click **OK** twice to perform the curve fit. Here is a partial view of the Results sheet:

| | Total (fmol/mg) | NS (fmol/mg) |
|---|---|---|
| | Y | Y |
| Total and nonspecific binding curves | | |
| Best-fit values | | |
| BMAX | 570.1 | (not used) |
| KD | 28.63 | (not used) |
| NS | 0.5050 | 0.5050 |
| Std. Error | | |
| BMAX | 11.12 | (not used) |
| KD | 1.629 | (not used) |
| NS | 0.03002 | 0.03002 |

The graph is shown below. To extend the curves leftward to the origin, click **Change... Analysis Parameters...**. Select the Range tab, then choose to **Start the curve at X=0**.
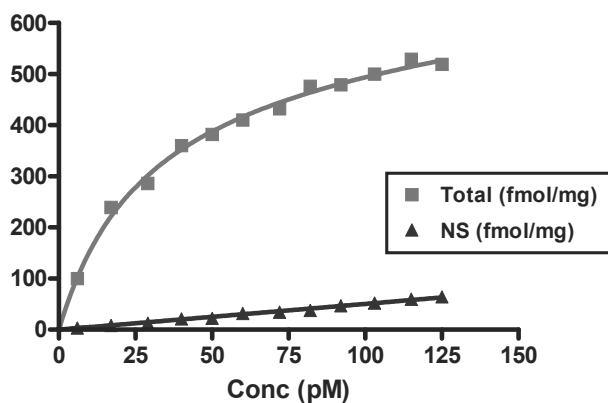
# Substrate-Velocity Curves and Lineweaver-Burk Plots

In this example, we'll make a combination graph commonly used to characterize enzyme activity—a curve of initial velocity vs. substrate concentration, sometimes referred to as a Michaelis-Menten plot, with an inset Lineweaver-Burk plot.
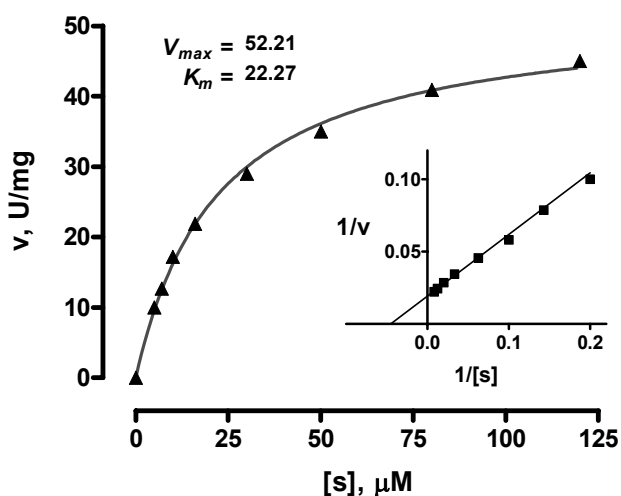


Lineweaver-Burk analysis is one method of linearizing substrate-velocity data so as to determine the kinetic constants $K_m$ and $V_{max}$. One creates a secondary, reciprocal plot: 1/velocity vs. 1/[substrate]. When catalytic activity follows Michaelis-Menten kinetics over the range of substrate concentrations tested, the Lineweaver-Burk plot is a straight line with

$$Y\ intercept = 1/V_{max}$$
$$X\ intercept = -1/K_m$$
$$Slope = K_m/V_{max}$$

Now that programs such as Prism easily do nonlinear regression, the best way to determine $K_m$ and $V_{max}$ is to fit a hyperbola directly to the substrate-velocity data. Yet the Lineweaver-Burk plot continues to be a useful visual tool, particularly because of its characteristic shifts in the presence of various types of inhibitors. So we'll create a Lineweaver-Burk plot with data points derived from double-reciprocal transformation, but we'll superimpose a line based upon nonlinear regression analysis, so that it reflects the best possible estimates of $K_m$ and $V_{max}$.

> A different secondary plot, such as Hanes-Woolf or Eadie-Scatchard, is just as easy to create with Prism. Simply modify the data transformation discussed below. In fact, for Eadie-Scatchard, you may prefer to follow the example in the chapter on radioligand binding analysis, page 81, making appropriate changes to axis titles and labels.

## Creating a Substrate-Velocity Curve

When you launch Prism, the Welcome dialog appears. It lets you create a new project (file) or open an existing one. Choose **Create a new project** and **Type of graph**. Choose the **XY** graph-type tab, then select the first thumbnail view, for "Points only".

For each point on the substrate-velocity curve, enter the concentration of substrate into the X column and the value for initial velocity into the Y column. The units are up to you—use whatever is meaningful. Just remember that Prism will graph the data and compute the binding parameters in whatever units you've used on your table. Place labels over the X and Y columns as a reminder. Here are the data for our example:

|   | X Values [s]. mM X | A v. U/mg Y |
|---|---|---|
| 1 | 0.0 | 3.4 |
| 2 | 5.0 | 13.4 |
| 3 | 7.0 | 16.1 |
| 4 | 10.0 | 20.6 |
| 5 | 16.0 | 25.3 |
| 6 | 30.0 | 32.4 |
| 7 | 50.0 | 38.4 |
| 8 | 80.0 | 44.3 |
| 9 | 120.0 | 48.4 |

In the sheet selection box on the toolbar, rename the data table:

| Substrate-velocity data | ⌄ |
|---|---|

## Automatic Baseline Correction

Suppose that this data is uncorrected for a constant background value of 3.4, which you determined from your assay measurement in the absence of substrate (row 1). You can use Prism to make the background correction (subtract 3.4 from each Y value). Click **Analyze**, then choose **Remove baseline & column math** from the **Data manipulations** list. In the **Parameters: Remove Baseline and Column Math** dialog, tell Prism where the baseline is (in this example, **First row**; note that you also have the option to put "baseline" values in the bottom row(s) and that, when appropriate, you may exclude those rows from the analysis). Choose to calculate **Difference: Value - Baseline** and, at the bottom of the dialog, to **Create a new graph of the results**.

**Parameters: Remove Baseline and Column Math**

Where are baseline values?
- ○ Column [　　　⌄]
- ○ Data sets (columns)　B, D, F. . .
- ○ Data sets (columns)　A, C, E. . .
- ⦿ First row
- ○ Last row
- ○ Mean of first [3] rows.
- ○ Mean of last [3] rows.
- ☐ Erase those rows from the analysis

Calculate
- ⦿ Difference: Value - Baseline
- ○ Sum: Value + Baseline
- ○ Product: Value* Baseline
- ○ Ratio: Value/Baseline
- ○ Percent: 100*Value/Baseline
- ○ Fractional difference:  (Value - Baseline)/Baseline
- ○ Percentage difference: 100* (Value - Baseline)/Baseline

 The corrected data are produced on a Results sheet labeled **Baseline-corrected of Substrate-velocity data**.

|   | X Values | A |
|---|---|---|
|   | [s], mM | v, U/mg |
|   | X | Y |
| 1 | 0.000 | 0.000 |
| 2 | 5.000 | 10.000 |
| 3 | 7.000 | 12.700 |
| 4 | 10.000 | 17.200 |
| 5 | 16.000 | 21.900 |
| 6 | 30.000 | 29.000 |
| 7 | 50.000 | 35.000 |
| 8 | 80.000 | 40.900 |
| 9 | 120.000 | 45.000 |

Since we requested it as part of the baseline subtraction analysis, Prism makes a new graph of the corrected data. To view it, choose the last (most recently created) graph listed in the Graphs section of the Navigator.

## Fitting a Curve to Find $K_m$ and $V_{max}$

You can quickly fit a curve to the data using the One-site binding (hyperbola) model from the **Classic equation** list, but then the kinetic constants will be reported as "VMAX" and "KM". Instead, we'll import an equation from a template included with Prism.

With the *baseline-corrected* Michaelis-Menten plot (the last graph listed in the "Graphs" section of the Navigator) in view, click the **Analyze** button. Choose the **Curves & regression** category, and select **Nonlinear regression (curve fit)**. Click **OK**.

In the **Parameters: Nonlinear Regression (Curve Fit)** dialog box, choose **More equations**. From the window below, select **[Import an equation from a Prism file or template]**. The **Equation Import** dialog appears. Select **Michaelis-Menten** from the file **Enzyme kinetics template** (if you don't see these choices, click the **Browse…** button, then select **Enzyme kinetics template.PZT** from the **..\Templates\Sample templates** folder within your Prism program folder).

> Y=Vmax*X/(Km+X)
> ;This describes an enzyme-catalyzed reaction obeying classical, or
> ;Michaelis-Menten, kinetics.
> ;Vmax is the maximal velocity at saturation.
> ;Km, or the Michaelis constant, is the substrate concentration required
> ;to reach half-maximal velocity (Vmax/2).

> If you do not have the "Enzyme kinetics template" file, you can enter the equation shown above as a user-defined equation. See the chapter entitled "Fitting Data to User-Defined Equations" starting on page 151 for details. The rules for initial values are
>
> | | |
> |---|---|
> | VMAX: | 1*YMAX |
> | KM: | 0.3*XMID |

Click **OK** twice to exit the parameters set-up and complete the curve fit. The curve appears on the graph. You can produce the offset between X and Y axes shown below by choosing **Change… Size & Frame…**, then selecting **Offset X & Y axes** under **Frame & axes**.

**Baseline-corrected of Substrate-velocity data:Subtract baseline data**
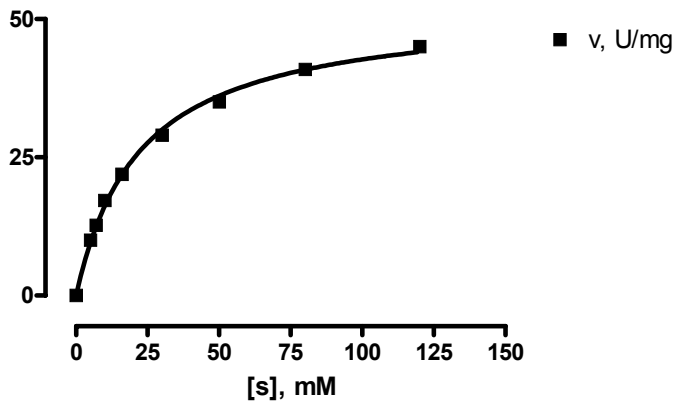


A fitted curve is treated by Prism as a separate data set (different from the underlying points). In this instance, we started the curve-fit analysis from the graph, so Prism knows where we want the curve to go. At other times, that may not be the case. When you do a curve fit and get a corresponding Results sheet, but can find no curve on your graph, click **Change.. Add Data Sets...** Select the data set (curve) and add it to the graph.

Click the yellow **Results** tab, or choose the **Nonlin fit...** Results sheet in the Navigator. Prism displays the best-fit values for the kinetic constants.

| Michaelis-Menten | |
|---|---|
| Best-fit values | |
| VMAX | 52.21 |
| KM | 22.27 |

If your results don't agree with those shown here, check to be sure that you did the curve fit starting from the baseline-corrected Michaelis-Menten graph.

The units for $V_{max}$ are the units that you used when entering Y values on the original data table. The units for $K_m$ are the units you used when entering the X values.

## Displaying Kinetic Constants on the Graph

If you wish, you can paste cells from this results table to your graph, so that the kinetic constants will be displayed there and will also be linked to the Results sheet. That way, if you change the saturation binding data later, $V_{max}$ and $K_m$ will be

automatically updated both on the Results sheet and on the graph. If you don't like the way the pasted data are displayed, you can change that. Suppose that you wish to paste the data, but make your own labels and customize the display. Select a cell on the Results sheet containing the value you want to paste. Choose **Edit...Copy**. Switch to the graph, and choose **Edit...Paste Table**. The value is placed on the graph, and you can drag it wherever you want. Now use the text tool (bottom row of the toolbar)...



...to add customized labels. Click on the text tool button, then click on the graph approximately where you want the label, and type your label. You can make the label boldface, resize it, or add subscripting as desired, using the text editing tools.
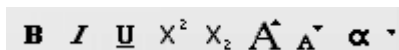


Click elsewhere to exit the text insertion mode, then select the label so that you can drag it into position (use the arrow keys on your keyboard to make the final adjustments). Here is a finished example:
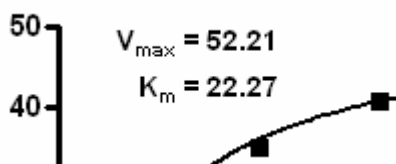


At this point, you may wish to change some of your original activity data (data table) and observe how the values of $V_{max}$ and $K_m$ change automatically on the Results sheet and on the graph.

# Creating a Lineweaver-Burk Plot

## Transforming the Data

From the Results sheet **Baseline-corrected of Substrate-velocity data**, click **Analyze**. Select **Transforms** from the **Data manipulations** analysis list. In the next dialog, **Parameters: Transforms**, choose **Pharmacology and biochemistry transforms** from the **Function List** drop-down box, then set the **Lineweaver-Burk (double-reciprocal)** radio button. Near the bottom of the dialog, choose to **Create a new graph of the results**.

The transformed data are shown on a new Results sheet (the original data are unchanged in the Data Tables section of your project). Prism will carry over the original column titles, which will now be incorrect, so you can edit them if you wish:

| | X Values | A |
|---|---|---|
| | 1/[s] | 1/v |
| | X | Y |
| 1 | | |
| 2 | 0.200 | 0.100 |
| 3 | 0.143 | 0.079 |
| 4 | 0.100 | 0.058 |
| 5 | 0.063 | 0.046 |
| 6 | 0.033 | 0.034 |
| 7 | 0.020 | 0.029 |
| 8 | 0.013 | 0.024 |
| 9 | 0.008 | 0.022 |

Notice that the top row of the results table is blank. Prism leaves individual cells blank when it has been asked to perform an illegal math operation, in this case to evaluate the quantity 1/0, in that cell.

## Plotting the Points

The default Lineweaver-Burk plot appears as the most recently generated graph (now the last graph listed in the Navigator).
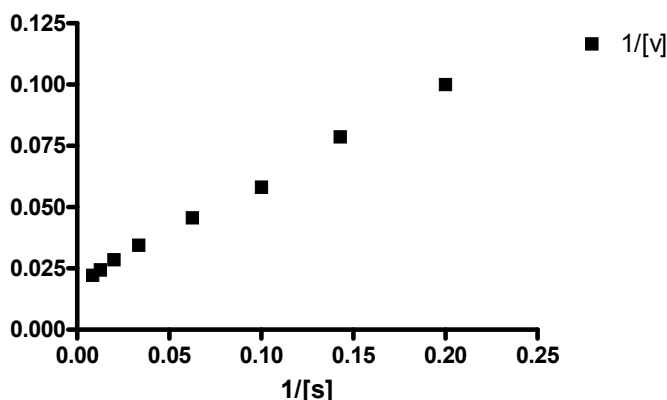


## Adding the Line

Thus far, there is no line superimposed over the points. We recommend that you add a line to your graph that accurately reflects the $K_m$ and $V_{max}$ values that we found using nonlinear regression. This will be a straight line running from the X intercept to the point predicted by the nonlinear regression analysis for the lowest non-zero substrate concentration (i.e., the highest value of 1/[s]). Referring to the Results sheet for your nonlinear regression analysis and the original substrate-velocity data table, note that the coordinates for the X-axis intercept are

$$X = -1/K_m = -1/22.27 = -0.0449$$
$$Y = 0$$

and the coordinates for the upper-right end of the line are

$$X = 1/[S_{min}] = 0.2$$
$$Y = (1/V_{max})(1 + K_m/[S_{min}]) = (1/52.21)(1 + 22.27/5) = 0.1045$$

where $S_{min}$ is the minimum substrate concentration tested.

Switch to the Data Tables section of your project and click **New**. Create a **New Data Table (+Graph)...** by making the selections below (note that you will not be making a new graph).



Fill in the table using the coordinates above:

| | X Values | A |
|---|---|---|
| | X Title | Data Set-A |
| | X | Y |
| 1 | -0.0449 | 0.0000 |
| 2 | 0.2000 | 0.1045 |

Rename this table.



Lineweaver-Burk line data

We now have the elements for the final Lineweaver-Burk plot on two sheets. The graph labeled **Lineweave-Burk...** contains the transform activity data, while the table labeled **Lineweaver-Burk line data** contains two points marking the Y and X intercepts ($V_{max}/K_m$ and $V_{max}$, respectively). We need to combine the two data sets on the same graph.

Select the **Lineweave-Burk...** graph to display it. In the Navigator, click and drag the data table sheet labeled **Lineweaver-Burk line data** onto the displayed graph.

In the dialog that appears, verify that you are adding the correct data set and then click **OK**.
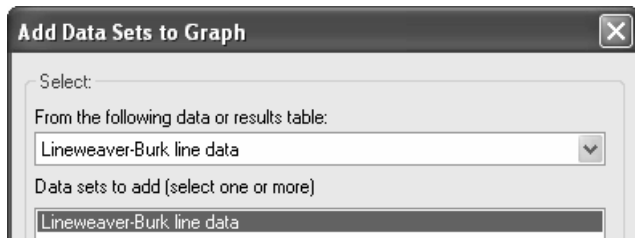
This results in the X and Y intercepts being plotted as distinctive points. Format this data set to make the points invisible but to connect them with a line: Click **Change...Symbols & Lines**. Under the **Appearance** tab, set **Data set** to **Lineweaver-Burk line data**. Choose to plot no symbols, but to connect them with a line.
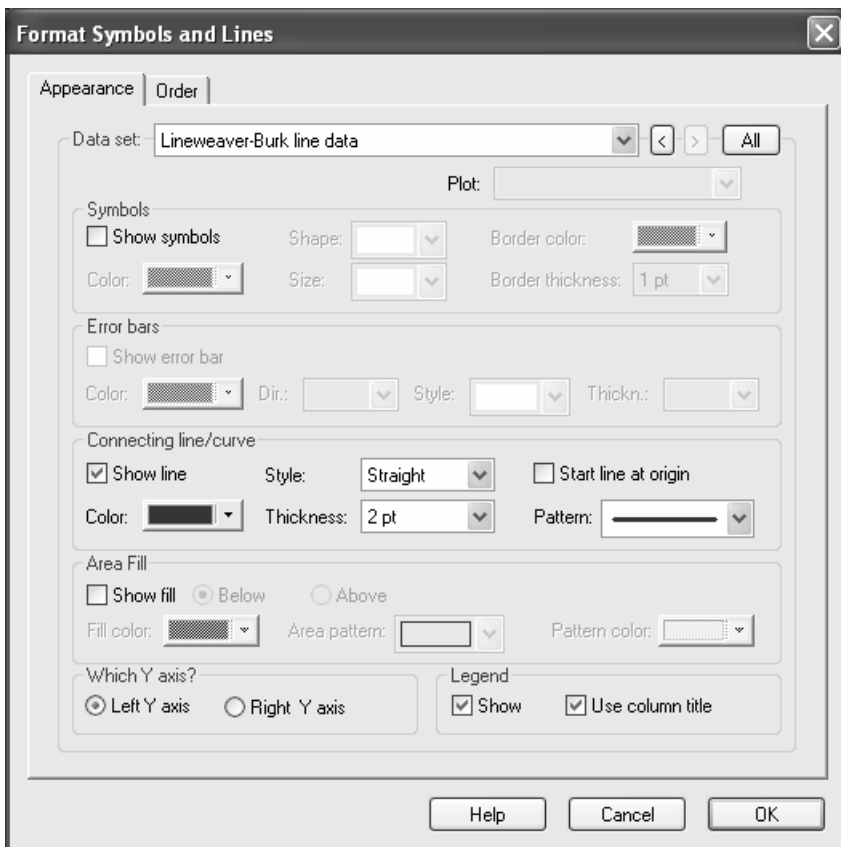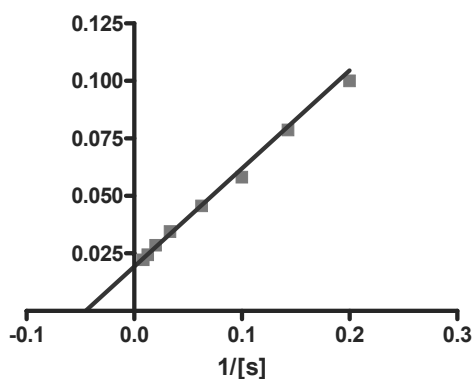
Since the value of −0.1 shown on the X axis doesn't make sense, we can effectively erase that value by replacing it with a custom tick label. Double-click on the X axis to open the **Format Axes** dialog. Make sure the **X axis** tab is selected, then click the **Custom ticks...** button.

In the **Customize Ticks and Gridlines** dialog, make the entries shown below. Important: Don't leave the **Label** box blank. With your cursor in the **Label** box, press the spacebar to produce a "blank" label.



When the label definitions have been entered, click **Add**, then **OK** twice to return to the graph. In the graph below, additional formatting changes were made—the axis ranges were changed, the legend was removed, the default Y-axis title was changed, and titles and axis labels were enlarged using one of the text editing buttons.

The Y-axis title can be rotated to the horizontal position by double-clicking on the Y axis and changing the **Direction** setting for the **Axis title**.
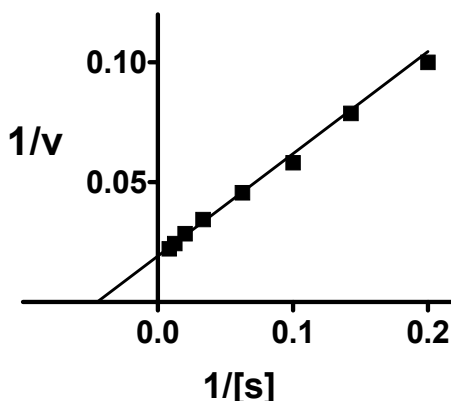




## Combining Substrate-Velocity and Lineweaver-Burk Plots

Switch to the **Layout** section of your project, and if necessary, click the **New** button and choose **New Layout....** In the **Create New Layout** dialog, choose the **Landscape** orientation and the **Inset** arrangement:

When you click **Create**, you'll see a layout with two placeholders. Double-click on each in turn, assigning your substrate-velocity plot to the larger placeholder, and your Lineweaver-Burk plot to the smaller placeholder. Alternatively, you may click and drag the appropriate Graph sheets from the Navigator onto the correct placeholders.

Now you can individually select, then move and resize, each graph so that the two fit together nicely. Hint: choose the larger graph (v vs. [s]) first and make it as large as you can.

As you resize the Lineweaver-Burk plot, Prism does a certain amount of reproportioning of the graph—adjusting font sizes and line thicknesses—as appropriate. But you will probably want to make some modifications of your own to further improve its readability. In most cases—changing line thicknesses, symbol sizes, label sizes and positions—you must make the changes on the original graphs. Those changes will then appear on the layout. Example: return to the Michaelis-Menten plot graph (the larger graph) and lengthen the Y axis to make more room for the inset Lineweaver-Burk plot. Click once to select the Y axis, then drag the top "handle" upward. When you return to the composite layout, you should see the alteration there.
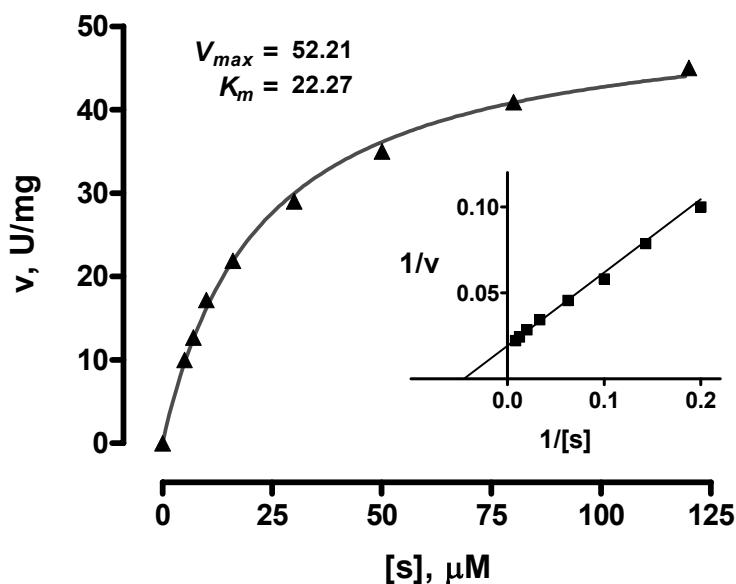
# Computing $K_i$ for a Competitive Enzyme Inhibitor

A competitive enzyme inhibitor interferes with binding of substrate to enzyme so as to raise the $K_m$ value without affecting $V_{max}$. Competitive inhibition is overcome by increasing substrate concentration. A competitive inhibitor I increases the "apparent" value of $K_m$ according to the relationship

$$K_{m_{app}} = K_m \left( 1 + \frac{[I]}{K_i} \right)$$

where $K_i$ is the dissociation constant for the enzyme·inhibitor complex. $K_i$ is particularly useful for expressing the potency of an inhibitor because, unlike $IC_{50}$, it is independent of substrate concentration.

There are several graphical methods for estimating $K_i$, but the underlying experiment is the same—the investigator generates substrate-velocity curves in the presence of various concentrations of inhibitor.
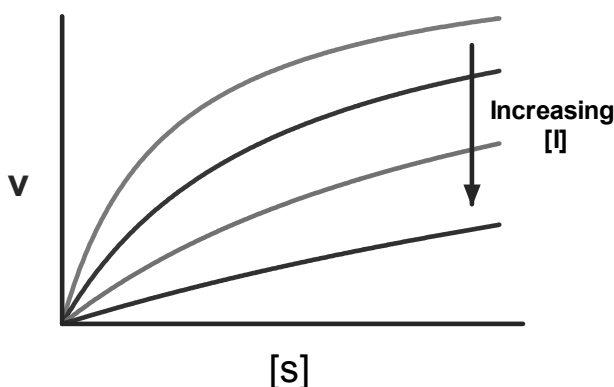


Formerly, $K_i$ estimation required linearizing transforms and replots, but Prism offers two improvements. First, the kinetic constants are more quickly and reliably estimated directly from nonlinear regression. Second, part of the function of replotting is replaced by global (shared-parameter) curve fitting. The rate equation is

$$v = \frac{V_{max}[S]}{K_m\left(1 + \dfrac{[I]}{K_i}\right) + [S]}$$

In the family of substrate velocity curves, [S] and [I] are independent variables, [I] changing with each curve in the family. But $V_{max}$, $K_m$, and $K_i$ are intrinsic to the system and therefore common to all the curves. Prism can perform a global curve fit, finding the single best-fit estimates of $V_{max}$, $K_m$, and $K_i$ for all curves taken together.

# Entering and Graphing the Data

In the Welcome dialog, make the settings shown below.
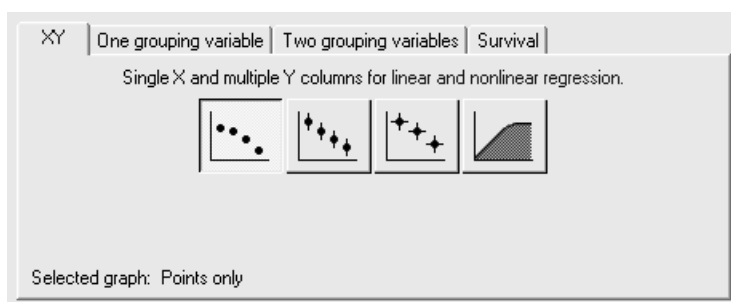


Complete the data table as shown below. The X column holds substrate concentrations, while each Y column holds the velocity value measured at the inhibitor concentration shown in the heading for that column.

| | X Values | A | B | C | D |
|---|---|---|---|---|---|
| | [S], nM | 0 nM | 3 nM | 10 nM | 30 nM |
| | X | Y | Y | Y | Y |
| 1 | 0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 2 | 50 | 17.0 | 7.2 | 3.6 | 0.0 |
| 3 | 100 | 22.2 | 13.3 | 8.1 | 0.1 |
| 4 | 150 | 30.6 | 17.8 | 13.6 | 4.8 |
| 5 | 200 | 33.6 | 25.1 | 10.8 | 5.2 |
| 6 | 400 | 50.4 | 38.0 | 21.9 | 14.6 |
| 7 | 600 | 58.4 | 45.0 | 25.5 | 13.5 |
| 8 | 800 | 62.7 | 50.5 | 31.8 | 15.9 |
| 9 | 1000 | 63.5 | 52.6 | 38.1 | 20.9 |
| 10 | 10000 | 81.0 | 74.3 | 70.9 | 61.7 |

> For this example, we're assuming that the units of [S] and [I] are nM, and the units of velocity are µmol/min/mg protein. The units notations in the column headings above are useful but optional. Prism will show those units in the graph legend, but simply extract the numeric portion of the column heading for its curve-fitting analysis. Prism is indifferent to these units—it is for you to remember that data on Results sheets are in units that match your input data (thus, nM for $K_m$ and $K_i$, µmol/min/mg protein for $V_{max}$).

Click the yellow **Graphs** tab to display the graph. Most of the points are crowded at the left-hand side of the graph. We'll fix that later.

Click **Analyze**. In the **Analyze Data** dialog, choose **Nonlinear regression (curve fit)** from the **Curves & regression** list.

In the **Parameters: Nonlinear Regression (Curve Fit)** dialog, verify that the **Equation** tab is selected and then choose **More equations**. In the list below, select **[Enter your own equation]**. The **User-defined Equation** dialog opens.

Give your user-defined equation a name, then enter the two-line system shown:



The first line defines an intermediate variable equal to the apparent $K_m$ as discussed earlier.

$$K_{m_{app}} = K_m \left( 1 + \frac{[I]}{K_i} \right)$$

The right-hand side of that equation will be substituted for "Kmapp" in the second line, and Prism will fit the resulting equation to each data set. The second line is the same as the velocity equation given earlier,

$$v = \frac{V_{max}[S]}{K_m \left( 1 + \dfrac{[I]}{K_i} \right) + [S]}$$

after substituting "X" for the independent variable [S].

> The multiplication operator * in the first line isn't strictly necessary, but it's good practice to always include it. Prism allows implied multiplication, but it must not lead to ambiguity. For example, Prism will read "AB" as a parameter with that name, not as the product of A and B. Refer to the Prism *User's Guide* for more information about the rules for writing user-defined equations.

We must now tell Prism (a) what parameters to treat as "shared" among data sets and (b) what values to use for [I] in the case of each data set.

## Designating Global (Shared) Parameters

Select the **Default Constraints** tab. Prism identifies and displays the equation parameters to be fitted by nonlinear regression.

$K_m$, $K_i$, and $V_{max}$ are common to all data sets, so select **Shared value for all data sets** for those parameters.

> Remember that, in the presence of inhibitor, the *apparent* $K_m$ changes, not $K_m$ itself. $K_m$ is a global parameter.

The settings are shown below. *Leave this dialog open* as you proceed to the next section.

> Note that we have just specified shared parameters and constants in the **User-defined Equation** dialog. That means that these settings will apply whenever we use this user-defined model. You can also make these settings under the **Constraints** tab in the **Parameters: Nonlinear Regression (Curve Fit)** dialog. In that case, the settings will apply only to the analysis you're running when you make the settings and will not be part of the equation definition.

## Assigning Parameter Values from Y-Column Headings

Next to "I" (see dialog above), select **Data set constant (=column title)**. This causes Prism to fit a different equation to each data set, in that it will set [I] equal to the numerical portion of the Y column heading for that data set.

| A | B | C | D |
|---|---|---|---|
| 0 nM | 3 nM | 10 nM | 30 nM |
| Y | Y | Y | Y |

Column heading constants are a powerful feature that effectively allows you to have a second independent variable.

# Completing the Curve Fit

Select the **Rules for Initial Values** tab. Enter initial-values rules as shown below (for more on setting for initial values, see "Entering Initial Values for Curve-Fit Parameters", page 154).



Click **OK** twice to exit the Parameters dialogs and perform the curve fit. Here is a partial view of the Results sheet:

| | 0 nM | 3 nM | 10 nM | 30 nM | Global (shared) |
|---|---|---|---|---|---|
| | Y | Y | Y | Y | Y |
| Competitive inhibition | | | | | |
| Best-fit values | | | | | |
| KM | 236.7 | 236.7 | 236.7 | 236.7 | 236.7 |
| I | 0.0 | 3.000 | 10.00 | 30.00 | |
| KI | 2.744 | 2.744 | 2.744 | 2.744 | 2.744 |
| VMAX | 79.92 | 79.92 | 79.92 | 79.92 | 79.92 |

The values for fitted (remember that I is constrained) parameters in all columns are the same because they are all shared parameters. The object here is to get the best estimate for $K_i$, and that has been done by fitting the parameters globally.

If you want to change any of the parameter settings and re-run the curve fit, choose **Change... Analysis Parameters**.

## Graph of the Fitted Data

Click on the yellow **Graphs** tab to display the graph.

The curves measured at higher inhibitor concentrations are not covered as uniformly by the data points. We wanted to keep the data table in this example simple; in an actual experiment, consider using a different distribution of substrate concentrations at different concentrations of inhibitor. You don't have to use the same X values for each data set on your table. For an example of using different X values, see "Entering Dose-Response Data" beginning on page 121.
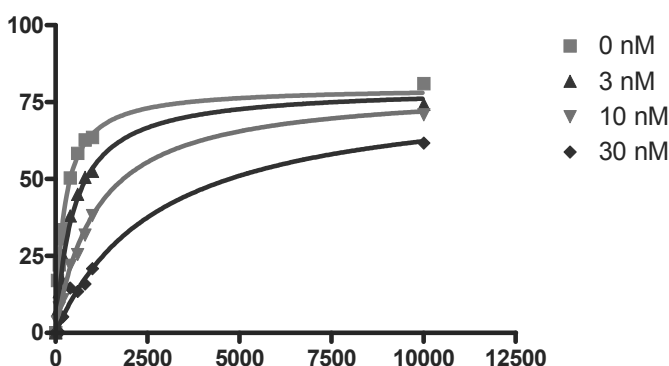


We can address the crowding of points into the left-hand side of the graph by dividing the X axis into two segments.

## Placing a Gap in the X Axis

In this section, we'll segment the X axis to eliminate the space between X = 1050 and X = 9000 and to use different scales for each segment:
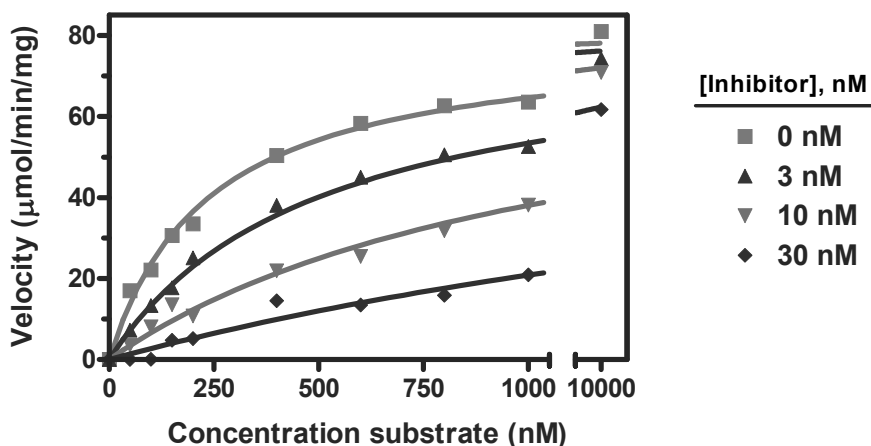
***Family of Substrate-Velocity Curves with Gapped (Segmented) X Axis***

*See text for instructions on producing the gap. The graph includes other formatting changes covered elsewhere in this book.*

Double-click on the X axis to open the **Format Axes** dialog. Make sure the **X axis** tab is selected. Under **Gaps and Direction**, choose to divide the axis into **Two segments**. Now you must set range and tick options for each segment. Select **Left** from the list box labeled **Segment**. Set the range and tick options for the left segment as follows:



Now select **Right** from the **Segment** list box, then set the range and tick options for the right segment as follows:

For other graphs, these settings may require some experimentation. A particularly tricky part may be to decide on the relative lengths of the segments (**Length % of axis**). An easy way to adjust this is to switch to the graph, click to select either of the segments, then drag the end of that segment to adjust its length. Prism then adjusts the **Length % of axis** setting automatically.

# Analyzing Dose-Response Data

A dose-response curve describes the relationship between response to drug treatment and drug dose or concentration. Sensitivity to a drug acting at a specific, saturable receptor typically spans a large concentration range, so dose-response curves are usually semi-logarithmic, i.e., the amount of drug is plotted as the log of drug concentration, giving them their familiar sigmoidal shape.
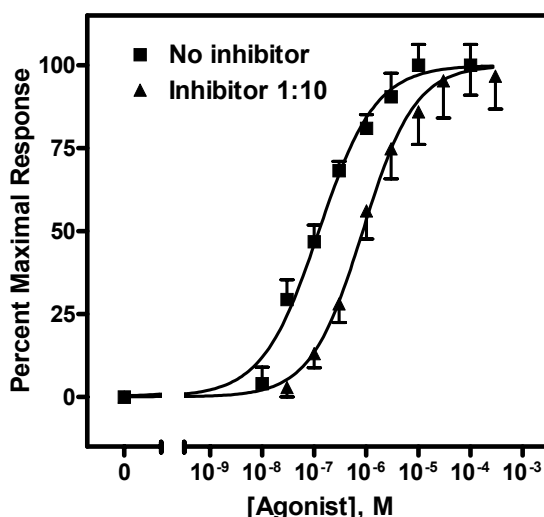


This example will show you (a) how to use Prism to fit sigmoidal (also known as "logistic") curves to your dose-response data and (b) one way to compare two dose-response curves statistically.

Suppose we want to plot two dose-response curves, showing the average in vitro response to an agonist, in the presence and in the absence of an antagonist, measured in triplicate. Further, we wish to include on the graph a zero-concentration control value.

## Entering Dose-Response Data

In the Welcome dialog, make the following settings:

To start:    ⊙ Create a new project    ○ Open an existing file

Choose:    ⊙ Type of graph    ○ Format of data table    ○ Template

XY | One grouping variable | Two grouping variables | Survival |

Single X and multiple Y columns for linear and nonlinear regression.

Selected graph:  Points with Y error bars

Enter error bar info into subcolumns as:

○ No error bars    ⊙ 3    ⇕ replicates    ○ Mean, Standard Deviation, N

Enter the data as shown below. The X values (agonist dose) are given as molar concentration, expressed in exponential notation. The Y values (response, in arbitrary units) go in subcolumns Y1 to Y3.

| | X Values | A | | | B | | |
|---|---|---|---|---|---|---|---|
| | Agonist | No inhibitor | | | Inhibitor 1:10 | | |
| | X | A:Y1 | A:Y2 | A:Y3 | B:Y1 | B:Y2 | B:Y3 |
| 1 | 1.00e-010 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 2 | 1.00e-008 | 1.0 | 0.0 | 4.0 | | | |
| 3 | 3.00e-008 | 12.0 | 10.0 | 15.0 | 1.0 | 0.0 | 2.0 |
| 4 | 1.00e-007 | 19.0 | 18.0 | 22.0 | 5.0 | 3.0 | 6.0 |
| 5 | 3.00e-007 | 28.0 | 30.0 | 28.0 | 8.0 | 10.0 | 12.0 |
| 6 | 1.00e-006 | 32.0 | 35.0 | 35.0 | 17.0 | 20.0 | 23.0 |
| 7 | 3.00e-006 | 35.0 | 41.0 | 38.0 | 23.0 | 28.0 | 29.0 |
| 8 | 1.00e-005 | 39.0 | 43.0 | 44.0 | 27.0 | 31.0 | 34.0 |
| 9 | 3.00e-005 | | | | 30.0 | 34.0 | 38.0 |
| 10 | 1.00e-004 | 39.0 | 43.0 | 44.0 | 32.0 | 37.0 | 38.0 |
| 11 | 3.00e-004 | | | | 32.0 | 37.0 | |

Several points about this data are worth noting:

▪ The dosage increment (10 nM, 30 nM, 100 nM, etc) shown in the X column is typical. These values will be log-transformed, giving roughly equal horizontal spacing of data points on the dose-response curve.

▪ These data are a good illustration of Prism's use of only one X column. Although the X values for data sets A and B are not the same, we can combine all the X values into a single column and leave Y-value cells empty where appropriate.

- The values in row 1 represent the zero-concentration (no agonist) data. Since our X coordinates will be plotted as logarithms, and since the log of 0 is undefined, we approximate this point with an X coordinate of 1.0e-010, about 2 log units below the lowest "real" X value. Later on, we'll show how to place a zero on the X axis for this data point.

- For this example, we're assuming that the Y values do not require correction by subtraction of a baseline—the minimum Y values are already zeroes. If you wish to subtract a baseline value from each Y value in a data set, read about automatic baseline corrections on page 99. Later in this example, we'll demonstrate normalization of the Y values, which can also serve as a means of "baseline correction".

- You can enter the Y values in any convenient units, then transform to other units if you wish. We'll use Prism to normalize (i.e., transform to percent of maximal response) our Y values later.

- Particularly when dosing with an impure preparation, you may wish to enter X values in terms of "dilution factor" or in mass/volume (e.g., mg/mL) units. Using mass/volume units is not a problem.

# Data Manipulations

This section covers the data modification necessary to produce a conventional dose-response graph, with response values normalized to span the range from 0% to 100% plotted against the logarithm of agonist concentration

## Log Transforming the X Values

To convert the values in the X column to their respective logarithms, click **Analyze**. Choose **Built-in analysis**. From the **Data manipulations** category, select **Transforms**.

In the **Parameters: Transforms** dialog, choose to **Transform X values using X=Log(X)**.

> At the bottom of this dialog, there is an option to **Create a new graph of the results**. But we're not ready to do that; we have one more manipulation, namely normalization, to do before making the final graph.

Prism displays a new Results sheet (partly illustrated as follows) with the log-transformed X values.

| X Values | A | | | B | | |
| Agonist | No inhibitor | | | Inhibitor 1:10 | | |
| X | A:Y1 | A:Y2 | A:Y3 | B:Y1 | B:Y2 | B:Y3 |
|---|---|---|---|---|---|---|
| 1 | -10.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 2 | -8.000 | 1.000 | 0.000 | 4.000 | | | |
| 3 | -7.523 | 12.000 | 10.000 | 15.000 | 1.000 | 0.000 | 2.000 |
| 4 | -7.000 | 19.000 | 18.000 | 22.000 | 5.000 | 3.000 | 6.000 |
| 5 | -6.523 | 28.000 | 30.000 | 28.000 | 8.000 | 10.000 | 12.000 |

> In a system following mass-action kinetics, the dose-response curve
> becomes sigmoidal only when response is plotted against the
> *logarithm* of dose, and Prism's built-in sigmoidal dose-response
> curve-fit models presuppose that your X values are in that form. But
> users occasionally find that their dose-response relationships are
> sigmoidal before log transformation. In that case, consider using the
> user-defined model
>
> $$Y=BOTTOM+(TOP-BOTTOM)*X^H/(EC_{50}{}^H+X^H)$$
>
> where EC50 is the midpoint of the curve, and H is the Hill slope. For
> an example of implementing user-defined equations, see page 151.

## Normalizing the Y Values

In this section, we'll convert the Y values in data sets A and B to a common scale. This
is useful when you want to compare the shape or position ($EC_{50}$) of two or more
curves and don't want to be distracted by different maximum and minimum values.

With the log-transformed Results sheet in view, click **Analyze**.

In the **Analyze Data** dialog, select the **Data manipulations** category, then choose
**Normalize**.

In the **Parameters: Normalize** dialog, specify how Prism will normalize the Y
values. For this exercise, we'll accept the default settings, which will define 0% and
100% as the smallest and the highest values, respectively, for each of the data sets.

Normalizing the data in this way converts it to "Percent of maximum response". Note two points: (a) Normalization will cause the dose-response curve to extend vertically from 0 to 100 by definition, and we'll constrain the curve fit later to ensure this. Therefore, you should be fairly confident of the accuracy of your minimum and maximum values. (b) In this example, the minimum Y values were already zeroes, so no alteration of the minimum values will be made. Note, however, that had these values been greater than 0, the normalization would effectively have produced a "baseline correction".

Choose to present the results as **Percentages**. And since the results of this "analysis" are the values that we will finally graph, check the box to **Create a new graph of the results**. *Overlooking this checkbox is a very common mistake.* The settings are shown here:



The Results sheet is displayed. Because we have replicate Y values, 0% and 100% are defined by the mean of the replicates. It is neither possible nor desirable to normalize each subcolumn separately. This is why none of the upper Y values in the table is exactly 100.

| X Values | A | | | B | | |
| Agonist | No inhibitor | | | Inhibitor 1:10 | | |
| X | A:Y1 | A:Y2 | A:Y3 | B:Y1 | B:Y2 | B:Y3 |
|---|---|---|---|---|---|---|
| -10.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| -8.000 | 2.381 | 0.000 | 9.524 | | | |
| -7.523 | 28.571 | 23.810 | 35.714 | 2.804 | 0.000 | 5.607 |
| -7.000 | 45.238 | 42.857 | 52.381 | 14.019 | 8.411 | 16.822 |
| -6.523 | 66.667 | 71.429 | 66.667 | 22.430 | 28.037 | 33.645 |
| -6.000 | 76.190 | 83.333 | 83.333 | 47.664 | 56.075 | 64.486 |
| -5.523 | 83.333 | 97.619 | 90.476 | 64.486 | 78.505 | 81.308 |
| -5.000 | 92.857 | 102.381 | 104.762 | 75.701 | 86.916 | 95.327 |
| -4.523 | | | | 84.112 | 95.327 | 106.542 |
| -4.000 | 92.857 | 102.381 | 104.762 | 89.720 | 103.738 | 106.542 |
| -3.523 | | | | 89.720 | 103.738 | |

Now click the yellow **Graphs** tab on the toolbar to switch to the graph of the log-transformed and normalized data.
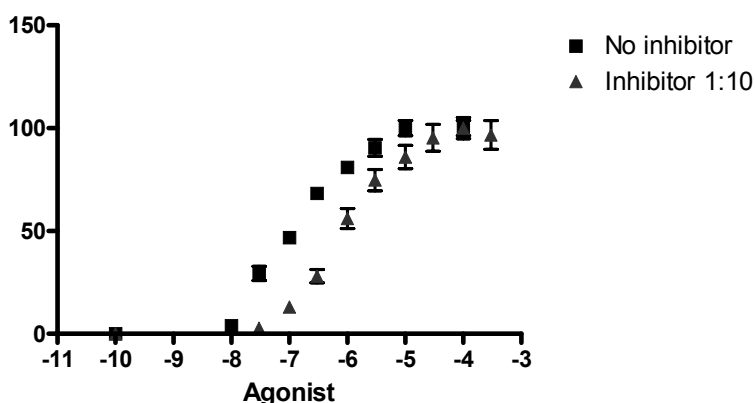


Note that, unless you specify otherwise, Prism plots the averages of replicate data and adds error bars automatically.

# Fitting a Sigmoidal (Four-Parameter Logistic) Curve

With the graph displayed, click on the **Analyze** button. From the **Curves & regression** category, select **Nonlinear regression (curve fit)**.

In the **Parameters: Nonlinear Regression (Curve Fit)** dialog box, choose **Classic equations**. Select **Sigmoidal dose-response (variable slope)**.

> Which sigmoidal dose-response curve-fit model should you use? When you choose **Sigmoidal dose-response**, Prism assumes that the curve has "standard" steepness (a Hill slope of +/- 1) and fits only the Top, Bottom, and log $EC_{50}$. Other programs may refer to this model as "three-parameter logistic". If you suspect that your curve has more or less than normal steepness, choose **Sigmoidal dose-response (variable slope)**, and Prism will also fit the Hill slope. Other programs refer to the variable-slope model as "four-parameter logistic".

This is normally all you need do to prepare Prism to fit the curves, and if you were to click **OK** at this point, Prism would perform a four-parameter (bottom, top, log $EC_{50}$, and Hill slope) sigmoidal curve fit and add the curves to the graph. But we'll choose two options.

## Constraining Curve-Fit Parameters

Since we normalized the original data such that the vertical range extends by definition from 0 to 100, it doesn't make sense to fit the "bottom" and the "top" of the curves. So we'll fix those parameters, leaving only the midpoint (log $EC_{50}$) and slope (Hill slope) of each curve to be fitted by Prism. Select the **Constaints** tab. Constrain the parameters BOTTOM and TOP to 0 and 100, respectively:



## Comparing Curves Statistically

Instruct Prism to compare the fitted midpoints (log $EC_{50}$) of the two curves statistically. Click the **Comparison** tab and make the settings shown in the following figure:

Only two of the four parameters from the variable-slope sigmoidal model show up in the list box, since the other two parameters were constrained—therefore no longer to be fitted—in the previous section.

While you're looking at the **Comparison** page of the **Parameters: Nonlinear Regression (Curve Fit)** dialog, note the variety of comparisons available.

- **For each data set, which of two equations (models) fits best?**

  Select different curve-fit equations and allow Prism to decide which better fits your data.

- **Does the best-fit value of a selected parameter differ between data sets?**

  Compare data sets. Select (a) one or more curve-fit parameters and allow Prism to decide whether their values differ statistically among data sets, i.e., whether the curves statistically different with respect to the specified parameter(s). That's what we're doing in this example, using log $EC_{50}$ as our criterion for comparison. Or select (b) *all* parameters and have Prism decide on the basis of the overall curve whether the data sets differ.

- **For each data set, does the best-fit value of a selected parameter differ from a hypothetical value?**

Experiment with these settings, observing how Prism changes the dialog and reading the **Explanation** frame for each option.

When you click **OK** to exit the **Parameters: Nonlinear Regression (Curve Fit)** dialog, Prism fits the curves and places them on the graph.
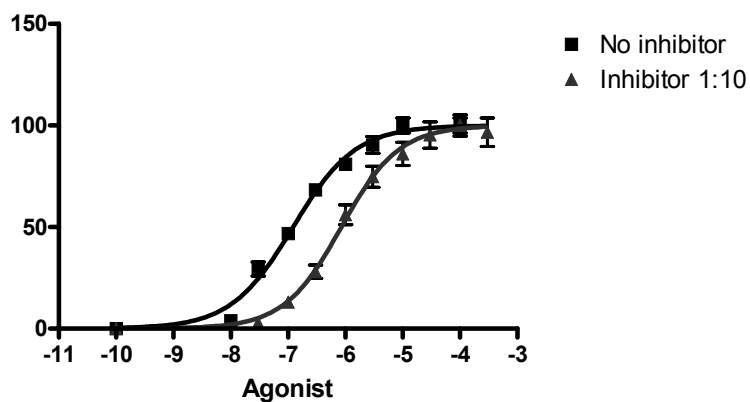


The Results sheet is extensive; we'll show the highlights. Select the **Table of results** subsheet in the Navigator.

Prism summarizes the result of the *F* test giving the hypotheses tested, in the first rows of the table (in the illustration, we juxtaposed the first and fourth columns by horizontal scrolling).

|  | Global (shared) |
| --- | --- |
|  | Y |
| Comparison of Fits |  |
| Null hypothesis | LOGEC50 same for all data sets |
| Alternative hypothesis | LOGEC50 different for each data set |
| P value | P<0.0001 |
| Conclusion (alpha = 0.05) | Reject null hypothesis |
| Preferred model | LOGEC50 different for each data set |
| F (DFn, DFd) | 163.1 (1,52) |

Further down, Prism reports the values fitted under the assumptions of each hypothesis. When Prism is free to fit log $EC_{50}$ for each data set separately (the alternate hypothesis)...

|  | No inhibitor | Inhibitor 1:10 |
|---|---|---|
|  | Y | Y |
| LOGEC50 different for each data set |  |  |
| Best-fit values |  |  |
| BOTTOM | 0.0 | 0.0 |
| TOP | 100.0 | 100.0 |
| LOGEC50 | -6.912 | -6.069 |
| HILLSLOPE | 0.7978 | 0.8652 |
| EC50 | 1.2244e-007 | 8.5320e-007 |
| Std. Error |  |  |
| LOGEC50 | 0.04262 | 0.04777 |
| HILLSLOPE | 0.05848 | 0.07374 |

When Prism fits a common value for log $EC_{50}$ to both data sets…

|  | No inhibitor | Inhibitor 1:10 | Global (shared) |
|---|---|---|---|
|  | Y | Y | Y |
| LOGEC50 same for all data sets |  |  |  |
| Best-fit values |  |  |  |
| BOTTOM | 0.0 | 0.0 |  |
| TOP | 100.0 | 100.0 |  |
| LOGEC50 | -6.466 | -6.466 | -6.466 |
| HILLSLOPE | 0.6947 | 0.7618 |  |
| EC50 | 3.4198e-007 | 3.4198e-007 | 3.4198e-007 |
| Std. Error |  |  |  |
| LOGEC50 | 0.06972 | 0.06972 | 0.06972 |
| HILLSLOPE | 0.07409 | 0.08668 |  |

In both cases, the fixed values of 0 and 100 for BOTTOM and TOP are included in the table; since they weren't fitted, no standard errors are shown for them. The $EC_{50}$ shown in the table is simply the antilog of the parameter that is fitted—LOGEC50.

As we noted earlier, the null hypothesis was rejected on the basis of the $F$ test. It appears that the experimental intervention, addition of a putative inhibitor, shifts the dose-response curve to the right.

Users frequently object to the fact that the standard error for $EC_{50}$ is not reported. These standard error values express the uncertainty inherent in estimating the parameters by curve fitting. For the equation fitted here, the midpoint of the curve is *log $EC_{50}$*, not $EC_{50}$. Don't try to express the standard error for $EC_{50}$ obtained in this way using a single number—in particular, it is not the antilog of the standard error of log $EC_{50}$. We suggest that you use the 95-percent confidence interval for $EC_{50}$, which Prism does report.

| 95% Confidence Intervals | | |
|---|---|---|
| LOGEC50 | -7.000 to -6.824 | -6.167 to -5.971 |
| HILLSLOPE | 0.6773 to 0.9182 | 0.7139 to 1.017 |
| EC50 | 1.0002e-007 to 1.4987e-007 | 6.8080e-007 to 1.0692e-006 |

Additional help for understanding your results is available when you click the Analysis Check List button.



# Adjustments to the Y Axis

Click on the yellow **Graphs** tab to return to the graph. If you'd like a frame around your graph, double-click one of the axes to open the **Format Axes** dialog. Click the **General** tab, then choose **Plain Frame** in the **Frame & axes** drop-down box. We can also increase readability by adjusting the range and tick intervals for the Y axis. Select the **Left Y axis** tab. Deselect the Auto option under **Range**. Set the **Range** and **Tick options** as follows:



Select the **General** tab again, then set **Origin** to **Lower Left**. This lowers the X axis to coincide with the frame around the graph.

# Adjustments to the X Axis

Now we'll format the X axis, introducing a break, or gap, in the axis; labeling our zero-agonist control point; and expressing the X-axis values in different units.

## Segmenting the X Axis

Double-click on the X axis to reopen the **Format Axes** dialog box. Choose the **X Axis** tab. Carefully set the **Appearance** and **Segment** options as follows:

Now reset **Segment** to **Right** and enter or verify the following settings:



Your graph should now look like this:
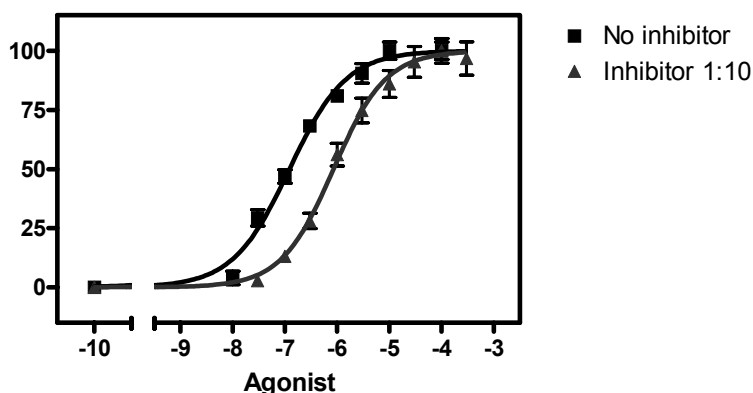


You may have to resort to some trial and error to get the look you want. One hint: Try selecting, then "dragging", the ends of the X axis segments directly on the graph, which will adjust the value for **Length... % of total axis** automatically.

## Labeling the Zero-Concentration Control Point

Now we'll substitute the value "0" for "-10" on the left axis segment. Double-click on the left-hand segment of the X axis to reopen the **Format Axes** dialog. Verify that the **X axis** tab is selected and that **Segment** is set to **Left**. Set the checkbox to **Plot only one tick on this segment** and direct Prism to insert the label "0" at X = -10.
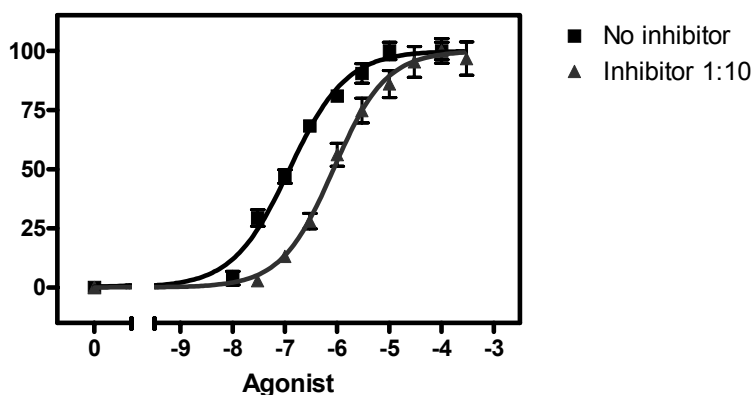




Note that you don't have to use a numeric tick label where we substituted for "-10". You could just as well label the tick "Control" or "Vehicle".
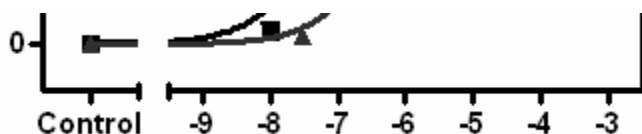


## Displaying Doses or Concentrations in Different Ways

To change how Prism expresses the values on the right-hand segment of the X axis, return to the **Format Axes** dialog (**X axis** tab). Set the axis **Segment** to **Right**.

Now change the setting in the **Format** drop-down list under **Numbering or labeling**. Only two of the four choices produce usable results in this example:

**Decimal**:



log [Agonist], log M

or **Power of 10**:



[Agonist], M
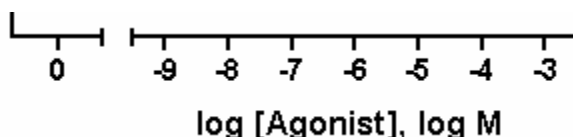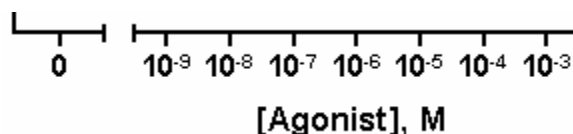
If you choose the **Power of 10** tick labeling option, you may also wish to add logarithmic minor ticks. In the **Format Axes** dialog (**X axis** tab), adjust **Minor ticks** and **Spacing** as shown here:





> Prism doesn't alter the axis *titles* to reflect the tick-labeling format—we did that manually for this illustration.

When you want to use one of the other (longer) formats, such as **Antilog**, you may find that the labels are long and overlapping each other. Try one of the following:

- If the crowding isn't too severe, create more room for the labels by clicking on the X axis to select it and then "dragging" the right-hand "handle" to the right.

- Enter your original data onto the data table in different units. You may be able to eliminate several place-holding zeros for tick labels in the Antilog format by entering data in micromolar or nanomolar units. Remember to modify the X-axis title if necessary, and remember that log $EC_{50}$ and $EC_{50}$ values reported on the curve-fit results will be in the same units that you used on the data table.

■ Show the tick labels at an angle. In the **Format Axes** dialog (**X axis** tab), try these settings:
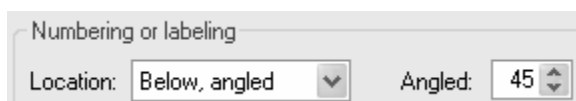


# Displaying Error Bars in Only One Direction

If you'd like to prevent overlapping of closely spaced error bars, double-click on one of the symbols for the "No inhibitor" data to open the **Format Symbols and Lines** dialog. Adjust the **Error bars...Dir.** to show only the top half (**Above**) of the bar. And for this example, switch to standard deviation error bars: **Plot... Mean & SD**.

Repeat for the "Inhibitor 1:10" data, choosing this time the lower half of the bar. Your graph should now look something like this (we edited the axis titles and relocated the legends):
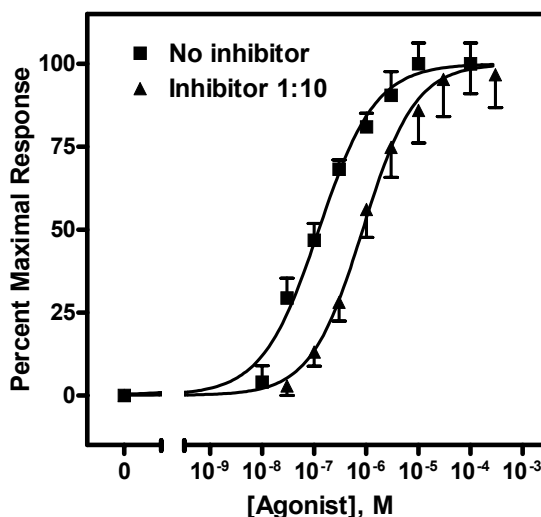


You wish to display some error bars associated with a data set in one direction only, and the rest in both directions. On the data table, choose the **Mean, +Error, -Error** format for the Y columns. Prism then leaves room for you to add differing positive and negative error limits. Enter zeroes on the table wherever you don't want that half of the error bar to be displayed.

# Displaying Curve-Fit Parameters on the Graph

We'll now transfer some information about the best-fit parameters from the Results page directly to the graph. The simplest technique is to return to the nonlinear regression Results sheet—remember to switch back to the **Table of results** subpage—and drag your cursor to select the results of interest:

| EC50 | 1.2240e-007 | 8.5320e-007 |
|------|-------------|-------------|

Choose **Edit...Copy**, switch to the graph, and choose **Edit...Paste Table**. You can now select the embedded table and drag it to a convenient open spot (in the illustration below, we removed the frame around the plot area).

With some ingenuity, you can improve the cosmetics and narrow the information down to what's important. In the illustration below, the $EC_{50}$ values were pasted individually and situated next to the legend elements. The heading "$EC_{50}$" was generated using the text tool,

T

and the line below "$EC_{50}$" was made with the line-drawing tool.

\
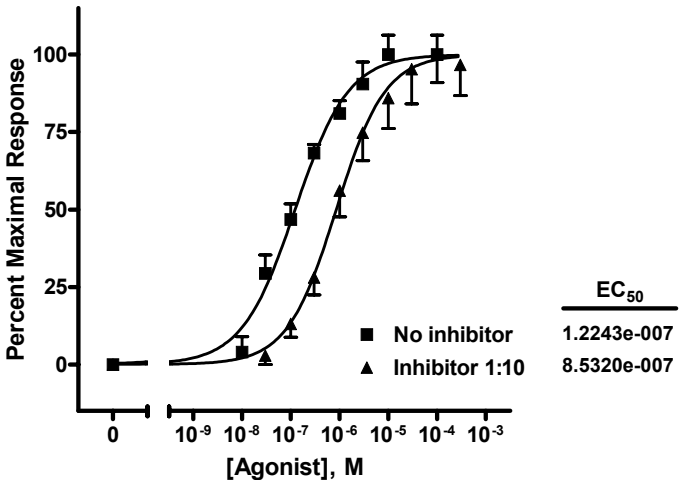
The beauty of pasting results from the Results sheet to your graph is that, since all related steps in your project are linked, changing your data will result in all corrections being made on the graph automatically—including the pasted values for $EC_{50}$.Try it! This saves effort not only when you need to correct data errors, but also when you need to construct a new dose-response curve. In fact, once you've settled on the design of your dose-response curve, consider saving your entire project, for use with new data, as a template.

# Linear Standard Curves

A standard curve is a graph relating a measured quantity (radioactivity, fluorescence, or optical density, for example) to concentration of the substance of interest in "known" samples. You prepare and assay "known" samples containing the substance in amounts chosen to span the range of concentrations that you expect to find in the "unknown" samples. You then draw the standard curve by plotting assayed quantity (on the Y axis) vs. concentration (on the X axis). Such a curve can be used to determine concentrations of the substance in "unknown" samples. Prism automates this process.

Prism can fit standard curves using nonlinear regression (curve fitting), linear regression, or a cubic spline (or LOWESS) curve. This chapter shows how to find "unknown" concentrations using a standard curve obtained using linear regression. For an example of a nonlinear standard curve, see page 145.

## Entering Linear Standard Curve and "Unknown" Data

In the Welcome dialog, make the settings shown below.
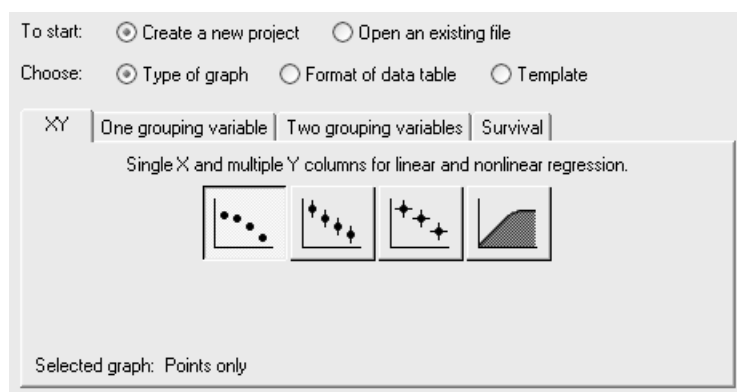


Enter data for the standard curve. In our example, shown below, we entered concentrations for the "known" samples into the X column, rows 1-5, and the corresponding assay results into the Y column. Just below the standard curve values, starting in row 6, enter the assay results for the "unknown" samples into the Y column, leaving the corresponding X cells blank.

| Protein (mg/mL) | Optical Density |
| --- | --- |
| X | Y |
| 1 | 0.05 | 0.097 |
| 2 | 0.10 | 0.183 |
| 3 | 0.20 | 0.290 |
| 4 | 0.30 | 0.371 |
| 5 | 0.40 | 0.462 |
| 6 | | 0.141 |
| 7 | | 0.190 |
| 8 | | 0.258 |
| 9 | | 0.423 |

Click on the default table name (drop-down list on the toolbar). Type a new name for the table. That name will be used as the title for your graph.

Protein standard curve

## Linear Regression with "Unknown" Interpolation

Click **Analyze**. From the **Curves & regression** category, select **Linear regression**.

In the **Parameters: Linear Regression** dialog, check the box labeled **Unknowns from standard curve**, because we want our unknown concentrations to be provided. Prism's default is to draw a linear regression line from the first to the last X coordinate on the data table. In our case, the line would therefore start at X = 0.05. To extend the line leftward to the Y axis, choose to **Start regression line at X= 0.0** in the **Output options** frame. Set the number of **Significant Digits** (to appear on the Results sheet) to **3**.

When you click **OK** to leave the linear regression parameters dialog, Prism performs the fit and displays the best-fit values.

| Best-fit values | |
| --- | --- |
| Slope | 1.01 ± 0.0686 |
| Y-intercept | 0.0690 ± 0.0169 |
| X-intercept | -0.0685 |
| 1/slope | 0.992 |
| 95% Confidence Intervals | |
| Slope | 0.789 to 1.23 |
| Goodness of Fit | |
| $r^2$ | 0.986 |
| Sy.x | 0.0196 |
| Is slope significantly non-zero? | |
| F | 216 |
| DFn, DFd | 1.00, 3.00 |
| P value | 0.0007 |
| Deviation from zero? | Significant |

Switch to the **Interpolated X values** subpage of the Results sheet to view the "unknown" values computed on the basis of the linear regression.
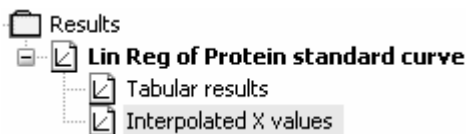


Prism reports the corresponding X value for each unpaired Y value on your Data sheet.

| Protein (mg/mL) | Optical Density |
|---|---|
| X | Y |
| 0.071 | 0.141 |
| 0.120 | 0.190 |
| 0.188 | 0.258 |
| 0.351 | 0.423 |

## Adding "Unknown" Data Points to the Graph

Prism's automatic graph includes the data from the Data sheet and the curve. To add the "unknowns" to the graph, click the yellow **Graphs** tab on the toolbar. Click the **Change** button and then select **Add Data Sets...**.
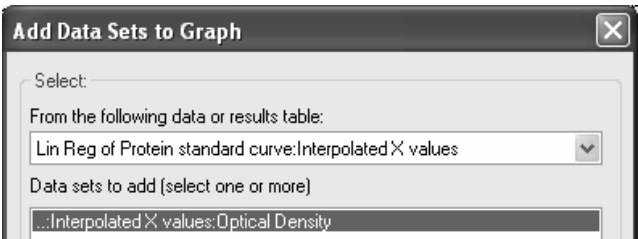


Using this dialog, you can add any appropriate data to any graph. Verify the settings shown above and click **OK** to return to the graph. The graph now includes the "unknown" values plotted in distinctive symbols (here, triangles).
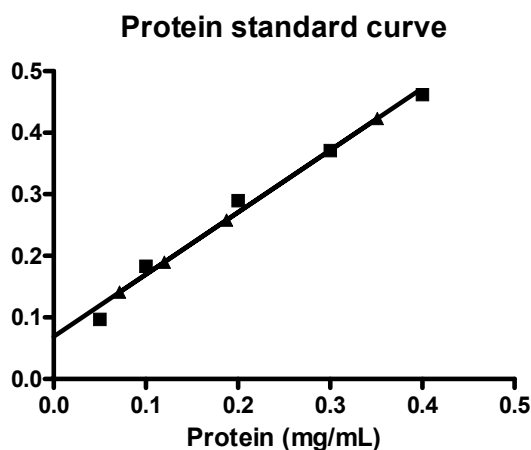
**Protein standard curve**



If you want the "unknowns" represented as spikes projected to the X axis (rather than data points), click on the **Change** button and select **Symbols & Lines...**. Verify that the **Appearance** tab is selected, and from the **Data set** drop-down list, select **Lin Reg of Protein standard curve:Interpolated X values**. Change the symbol **Shape** to one of the last four options (spikes) and set the **Size** to **0**.



*Linear Standard Curves*                                                                 *143*
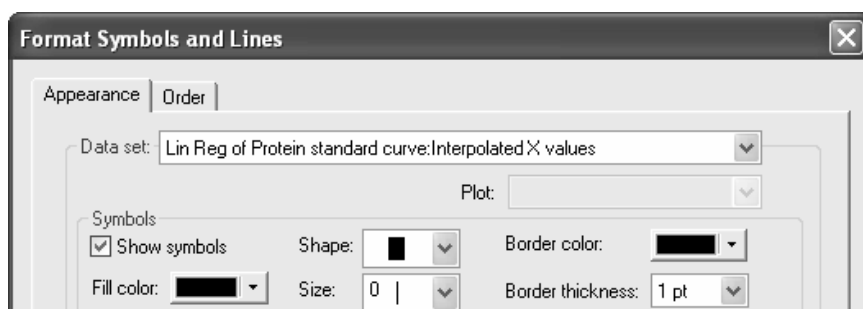
## Protein standard curve

| Protein (mg/mL) | Optical Density |
|---|---|
| 0.071 | 0.141 |
| 0.120 | 0.190 |
| 0.188 | 0.258 |
| 0.351 | 0.423 |
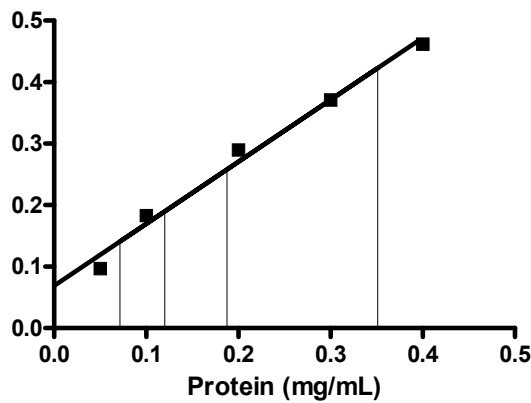


*Standard Curve*

*"Unknowns" are represented as spikes on the graph; their numerical values are shown on an embedded table pasted from the* **Interpolated X values** *subpage of the Results sheet.*

# Nonlinear Standard Curves: RIA and ELISA

Analyzing radioimmunoassay (RIA) or an enzyme-linked immunosorbent assay (ELISA) data is a two-step process:

- Prepare and assay a set of "known" samples, i.e., samples that contain the substance of interest in amounts that you choose and which span the range of concentrations that you expect to be present in your "unknown" samples. The quantity that you measure (radioactivity, optical density, or luminescence, for example) is related graphically to sample concentration to produce a standard curve.

- Assay the "unknown" samples. Then, for each measurement obtained, use the standard curve to find the corresponding substance concentration. Graphically, this amounts to finding the X-axis value (concentration) for a given Y-axis value (measured quantity).

Prism automates creation of a standard curve and interpolating unknown concentrations from it. For an example of a linear standard curve, see page 139.

## Entering Standard-Curve and "Unknown" Data

In the Welcome dialog, choose **Create a new project** and **Type of graph**. Under the **XY** tab, choose the leftmost thumbnail, for "Points only".

RIA/ELISA data often span such large concentration ranges that concentrations are plotted as their logarithms. We'll follow that convention here and let Prism compute the logarithms for us.

Enter data for the standard curve. For our example, enter "known" sample concentrations into the X column, rows 1-6, and the corresponding assay results into the Y column. Sometimes you'll want to include data from a zero-concentration sample. Since our data will be graphed on a semi-log plot and it's impossible to plot the log of 0, we approximate the zero-concentration point in row 1 with an X coordinate of 1.0e-014, two log units below the lowest non-zero X value. Including this data point will help to define the top of the fitted curve more accurately.

Just below the standard curve values, enter the assay results (Y values, rows 7-10) for the "unknown" samples, leaving the corresponding X cells blank. Later, Prism will fit the standard curve and then report the unknown concentrations using that curve.

| | X Title | Data Set-A |
|---|---|---|
| | X | Y |
| 1 | 1e-014 | 100.0 |
| 2 | 1e-012 | 97.2 |
| 3 | 1e-011 | 93.1 |
| 4 | 5e-011 | 67.9 |
| 5 | 1e-010 | 30.7 |
| 6 | 5e-010 | 4.8 |
| 7 | | 35.0 |
| 8 | | 53.0 |
| 9 | | 78.0 |
| 10 | | 90.0 |

## Log Transforming the X Values

Click **Analyze**. From the **Data manipulations** category, select **Transforms**.

In the **Parameters: Transforms** dialog, choose to **Transform X values using X=Log(X)**. At the bottom of this same dialog box, choose to **Create a new graph of the results**. When you click **OK** to exit this dialog, Prism displays a new Results sheet with the log-transformed X values.

| | X | Y |
|---|---|---|
| 1 | -14.000 | 100.000 |
| 2 | -12.000 | 97.200 |
| 3 | -11.000 | 93.100 |
| 4 | -10.301 | 67.900 |
| 5 | -10.000 | 30.700 |
| 6 | -9.301 | 4.800 |
| 7 | | 35.000 |
| 8 | | 53.000 |
| 9 | | 78.000 |
| 10 | | 90.000 |

Click on the yellow **Graphs** tab to view the plot of the log-transformed data.

## Analysis and Curve Fitting

Click the **Analyze** button. From the **Curves & regression** category, select **Nonlinear regression (curve fit)**.

In the **Parameters: Nonlinear Regression (Curve Fit)** dialog box (**Equation** tab), choose **Classic equations**. Select **Sigmoidal dose-response (variable slope)**.

> The sigmoidal model should work well for a one-site competitive binding system following mass-action kinetics. You have other options: choose another equation, or enter a user-defined equation (see page 151). Or consider a cubic spline or LOWESS, which provides a smoothed curve without fitting the data to a specific equation.

Since we want our unknown concentrations to be provided, check the box to **Also calculate…Unknowns from standard curve**. Select the **Range** tab, and under **Minimum X value**, choose to **Start the curve at X = -12** (we are truncating the curve at the lowest non-zero X coordinate).

> We will format the graph so as not to show the 100-percent-binding control point at X = -14. Many users would prefer to include that point and to substitute a value of "0" at X = -14, with or without breaking (segmenting) the X axis. For an example of this technique, see "Labeling the Zero-Concentration Control Point" on page 133.

When you click **OK** to leave the parameters dialog, Prism performs the fit and creates a Results sheet.
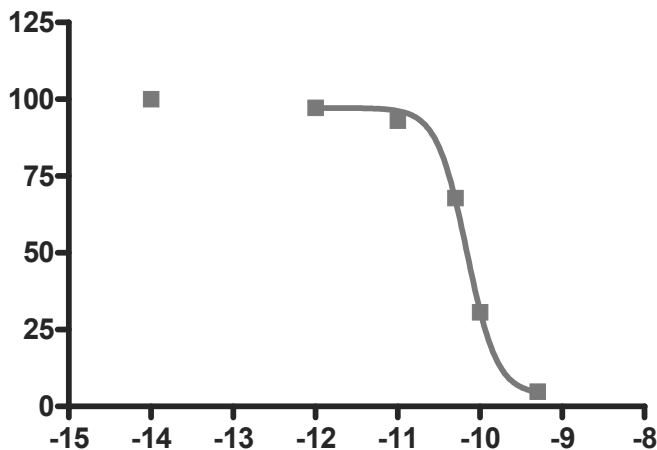


# Finding "Unknown" Values

Prism displays the results of the curve fit on the **Table of results** subpage of the **Results-x:Nonlinear regression (curve fit)** sheet. But for a routine RIA, you

may not care too much about the information contained there. Switch to the **Interpolated X values** subpage.
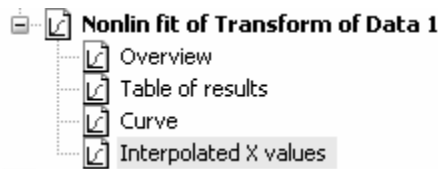


Prism reports the corresponding X value for each unpaired Y value on your Data sheet. These values are expressed as logarithms of concentration.

| X | Y |
|---|---|
| -10.036 | 35.000 |
| -10.182 | 53.000 |
| -10.410 | 78.000 |
| -10.617 | 90.000 |

> If your data table contains replicate Y values, Prism will report interpolated X values both on the basis of the mean of replicates and of each individual replicate.
>
> 
> Interpolated X mean values
> Interpolated X replicates

Although it's generally easiest to plot and analyze semi-logarithmic RIA/ELISA data, we'd like to read our unknown concentrations directly, not as logarithms. Click **Analyze.** From the **Data Manipulations** category, select **Transforms**.

In the **Parameters: Transforms** dialog, check the box for **Transform X values using...**. Select the equation **X=10^X** from the drop-down list.

Click on **OK**. Prism creates another Results sheet, on which the X values are now expressed as concentration. In this figure, column headings were added and the number format was changed (**Change... Number Format...**).

| Concentration | Binding |
|---|---|
| X | Y |
| 9.209e-011 | 35 |
| 6.580e-011 | 53 |
| 3.892e-011 | 78 |
| 2.414e-011 | 90 |

# Showing "Unknowns" on the Graph

Prism's automatic graph includes the data from the Data sheet and the curve. To add the "unknowns" to the graph:

Switch to the graph **Transform of Data 1 graph**. Choose **Change... Add Data Sets...**. From the top drop-down box in the **Add Data Sets to Graph** dialog, select the data set containing the **...Interpolated X values**. Click **OK** to return to the graph, which now includes the unknown values plotted using a different symbol.

If you want the "unknowns" represented as spikes projected to the X axis (rather than data points), click **Change** and select **Symbols & Lines...**. From the **Data set** drop-down list, select **...Interpolated X values**. Change the symbol **Shape** to one of the last four options (spikes), and set the **Size** to **0** or **1**.

# Final Graph Formatting

Double-click on the X axis, make sure the X axis tab is selected, and make the following settings. The axis scale remains linear, so that the horizontal positions of the data points are still determined by their logarithms. But as an aid to the viewer, the ticks labels are changed to the corresponding **Power of 10** values, and the tick marks themselves are spaced logarithmically.



The final graph, with some formatting changes not discussed here, is shown in the next figure. The table has been paste-embedded from the last Results sheet. To that, switch to the Results sheet and choose **Edit... Copy**. Then switch to the graph, choose **Edit... Paste Table**, and position the copy of the table where you want it.

| Concentration | Binding |
|---|---|
| 9.209e-011 | 35 |
| 6.580e-011 | 53 |
| 3.892e-011 | 78 |
| 2.414e-011 | 90 |
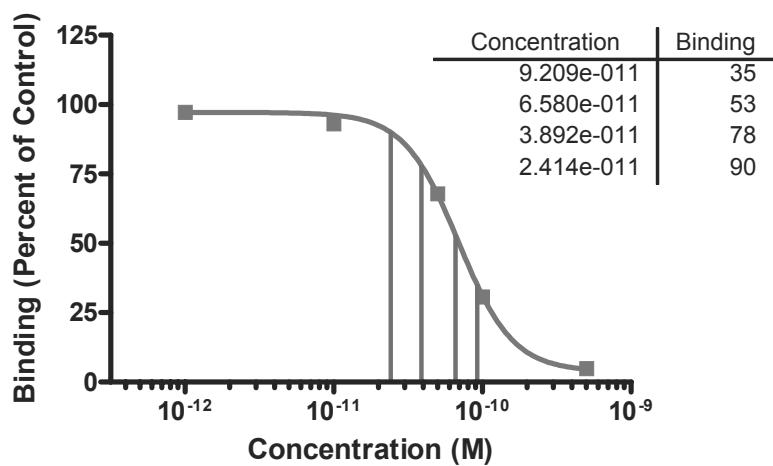
# Fitting Data to User-Defined Equations

Within the *Classic equations* and the *Prism equation library* are most of the curve-fit models that a biologist needs. Yet occasionally you may want to customize. For example, you may (a) need an equation of a form not built into Prism, (b) need to modify an existing equation, e.g., changing the two-site binding model to accommodate three sites, or (c) simply wish to rename the parameters in a built-in equation so that the curve-fit results will be labeled more appropriately.

In this example, we'll enter and fit data to a "user-defined" equation of a form not preprogrammed into Prism. The time course for total systemic absorption of a drug, expressed as the fraction (F) of the amount administered at a subcutaneous depot at time 0, is given by[1]

$$F = \frac{1}{(k_e/k_a)-1}\left[ e^{-k_e t/(k_e/k_a)} - e^{-k_e t} \right]$$

where t = time (min), and $k_e$ and $k_a$ are the first-order elimination and absorption rate constants (min⁻¹), respectively. We'll plot an example time course and fit the curve to the data to obtain an estimate of the rate constants.

In the Welcome dialog, make the settings shown below.



---
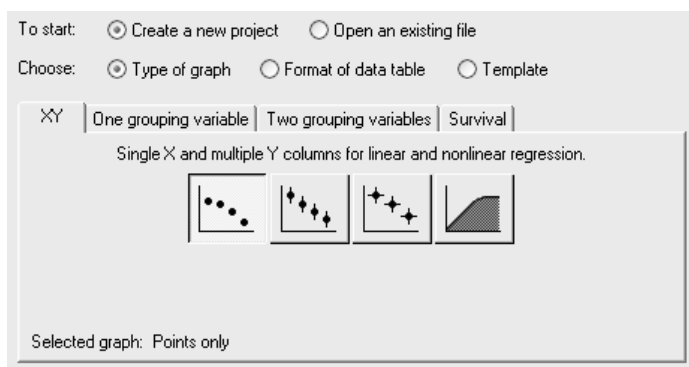
[1] Neubig, R. R., The Time Course of Drug Action, in *Principles of Drug Action* (W.B. Pratt and P. Taylor, eds.), 3rd Edition, Churchill Livingtone Inc., 1990.

Enter the data as shown below.

| | X Values | A |
|---|---|---|
| | Time (min) | Frac Abs |
| | X | Y |
| 1 | 0.0 | 0.00 |
| 2 | 2.5 | 0.05 |
| 3 | 5.0 | 0.11 |
| 4 | 10.0 | 0.20 |
| 5 | 20.0 | 0.25 |
| 6 | 30.0 | 0.27 |
| 7 | 45.0 | 0.27 |
| 8 | 60.0 | 0.20 |
| 9 | 90.0 | 0.10 |
| 10 | 120.0 | 0.06 |

For this example, we're assuming that the Y values do not require correction by subtraction of a baseline—the minimum Y value is already 0. If you wish to subtract a baseline value from each Y value in a data set, read about automatic baseline correction beginning on page 99.

Click the yellow **Graphs** tab on the toolbar to view the graph:
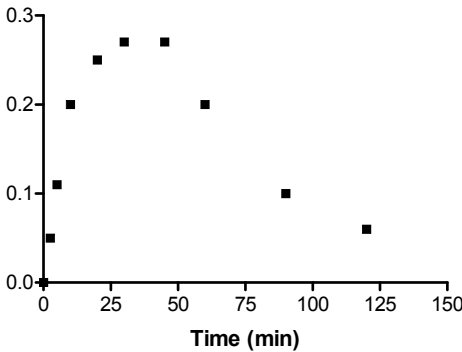


## Entering the User-Defined Equation

With the graph displayed, click on the **Analyze** button. From the **Curves & regression** category, select **Nonlinear regression (curve fit)**.

In the **Parameters: Nonlinear Regression (Curve Fit)** dialog box (**Equation** tab), choose **More equations** and, from the list below, click **[Enter your own equation]**.

The **User-defined Equation** dialog is displayed. With the **Equation** tab selected, enter a **Name** for this curve-fit analysis (which will be added to the **More equations** list the next time you view it). Now enter your equation, in single-line form, into the **Equation** box. You are limited to certain types of equations and must adhere to a few simple syntax rules. Your equation must be of the form

$$Y = f(x)$$

Thus, you must designate the dependent and independent variables as Y and X, respectively, and you must write the equation so that Y is isolated on the left-hand side. The Prism *User's Guide* lists all the rules for entering user-defined equations in Prism, including the functions, or operators, that you may use. Our equation can be written on a single line as follows (the equation is not reduced to simplest terms, but that will help illustrate the use of an "intermediate" variable below):

Y=(exp(-Ke*X/(Ke/Ka))-exp(-Ke*X))/(Ke/Ka-1)
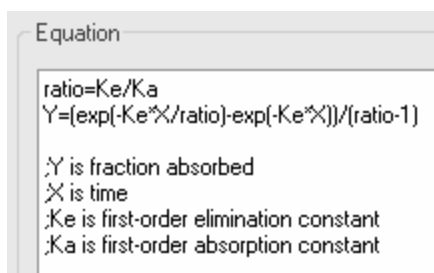
You can enter comments to prompt your memory or to help other users understand your equation by writing lines beginning with a semicolon.
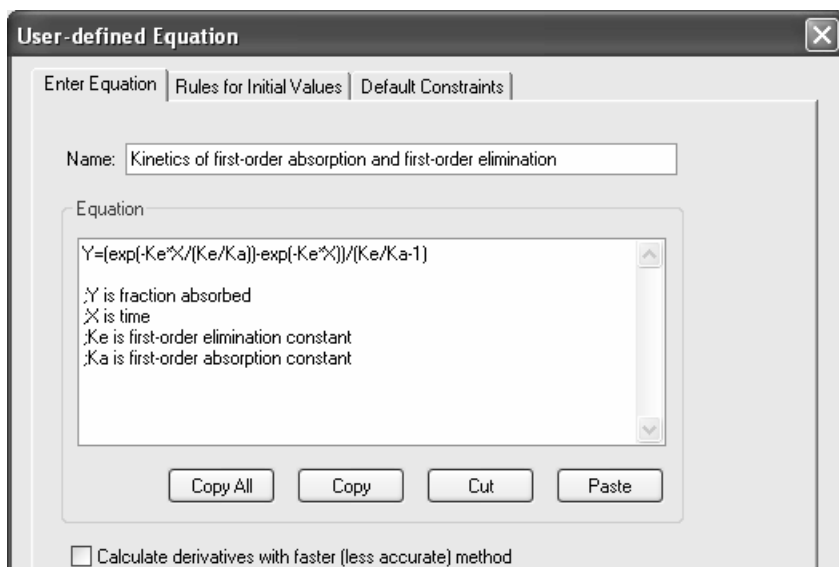


If you prefer, you can break an equation into two or more expressions to make the resulting system simpler or more readable. Below, we define an intermediate variable

$$ratio = k_e / k_a$$

Prism will read that definition in the first line and then substitute $k_e/k_a$ for "ratio" in the final equation:

```
Equation

ratio=Ke/Ka
Y=(exp(-Ke*X/ratio)-exp(-Ke*X))/(ratio-1)

;Y is fraction absorbed
;X is time
;Ke is first-order elimination constant
;Ka is first-order absorption constant
```

Your user-defined equation may be a variation of one of Prism's pre-programmed, or "classic", equations. In that case, you can keep manual entry of your equation to a minimum by copying the classic equation into the equation box. In the **Parameters: Nonlinear Regression (Curve Fit)** dialog box, choose an equation from the **Classic equations** list, then click the **View Equation...** button. In the **Classic Equation** dialog box, click **Copy All** to place the contents of the **Equation** window on the clipboard. Now you can move to the **User-defined Equation** dialog (from **Parameters: Nonlinear Regression (Curve Fit)**, choose **More equations...[Enter your own equation]**). Make sure the **Equation** tab is selected, then click **Paste** to enter the classic equation into the **Equation** window. Now type your modifications.

# Entering Initial Values for Curve-Fit Parameters

When you're done entering your equation, click the **Rules for Initial Values** tab. Prism reads your equation and, after eliminating X, Y, and operators, arrives at the "parameters" to be fit—in this case, $k_e$ and $k_a$. Curve fitting is an iterative, trial-and-error process of substituting values for the parameters in your equation in order to maximize the goodness of fit between your experimental data and the resulting substituted equation. Prism therefore must have an initial "guess" for the value of each parameter, that is, it must have a starting point for the process. When you use one of the *Classic equations*, Prism derives its own initial values, choosing numbers that are related to your experimental data by programmed "rules". For *User-defined equations*, Prism has no such rules, so you must provide either those rules or explicit initial values.

For each parameter, enter a constant value into the first column and a relational expression chosen from the drop-down list into the second column. Your choices fall into two categories:

### Initial value, to be fit

This choice indicates that the value in the first column is to be used explicitly. Select this option when there is no rule in the second column that reasonably

allows derivation of the initial value from your experimental data. The drawback of this choice is that you *may* have to provide a new initial value each time you run this analysis. For this step-by-step example, we will select this option, since no reasonable rule is available.

The initial value estimate generally does not have to be very close to the actual fitted value, particularly when the equation has few parameters to be fit and your data doesn't have too much scatter.

The initial values that you enter might come from (a) your past experience or the experience of other investigators, including published values, (b) alternative methods of computation, or (c) a trial process, whereby you allow Prism to generate a curve using the values you supply and check to see if the result is reasonable. We'll illustrate the latter process below.

Don't forget to avoid entering "illegal" initial values. In our example, the equation is undefined for $k_e = 0$, $k_a = 0$, and $k_e = k_a$.

**A rule**

If you can find a rule on this list that allows Prism *consistently* to derive an initial value from your data (i.e., if the rule holds as your data varies within reason from experiment to experiment), this is the best choice. You won't have to enter a new initial value each time you run the curve-fit analysis.

---

**Example**

When collecting data for a sigmoidal dose-response curve, the input data are the logarithms of drug concentrations tested, and investigators typically choose those concentrations such that their logarithms will be distributed evenly over the curve. And the lower and upper plateaus of the idealized curve can be approximated by the lowest and highest responses measured, respectively. So good initial value rules for the for a three-parameter sigmoidal fit would be

| | |
|---|---|
| LOGEC50: | 1*Value of X at YMID |
| BOTTOM: | 1*YMIN |
| TOP: | 1*YMAX |

---

Here are the entries for our example:
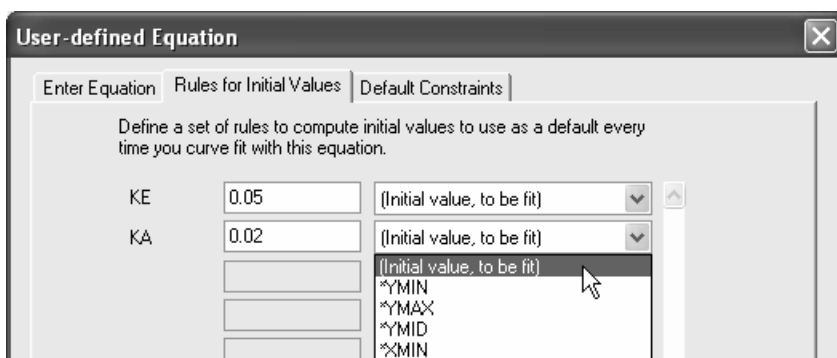
Click **OK** to return to the Parameters dialog.

## Trying out Initial Values

Before doing the curve fit at all, we might like to know how close a curve generated using specific parameter values will come to our data.

> Generating a trial curve is *not* mandatory. If the initial values produce a trial curve *very* far away from the data, Prism may not be able to figure out how to adjust the parameters for the next iteration, and it may abort the process. But ordinarily, the initial values need only be rough approximations of the fitted values, and it will not help to "fine-tune" them. If you are confident of your initial value settings, just go on to the next section.

In the **Parameters: Nonlinear Regression (Curve Fit)** dialog box, choose to **...Plot the curve defined by the initial values**.
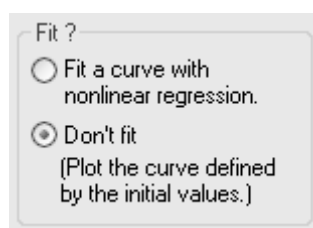


Click **OK** to generate the curve with $k_e$ and $k_a$ fixed to the specified initial values. Here is a partial view of the Results sheet.

| Best-fit values | |
| --- | --- |
| KE | 0.0500 |
| KA | 0.0200 |

Below is the graph (notations shown below were added using the text and drawing tools).
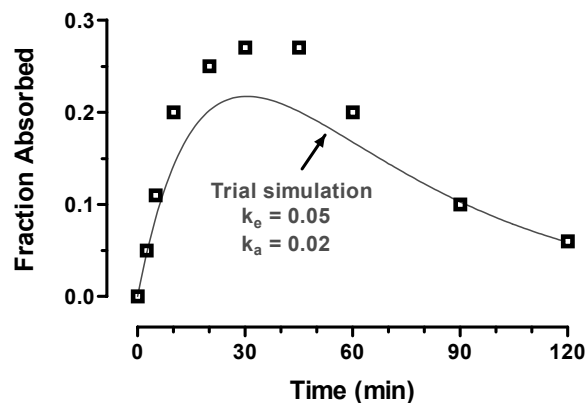


Since the curve is reasonably close to the points, you're ready to do the actual curve fit. Click the "Analysis parameters" button.



Reset the radio button to fit the curve.



## Completing the Curve Fit

From the **Parameters: Nonlinear Regression (Curve Fit)** dialog, click **OK** to fit the curve.

Click the yellow **Results** tab to display the fitted values for $k_e$ and $k_a$.

| Kinetics of first-order absorption | |
|---|---|
| Best-fit values | |
| KE | 0.04381 |
| KA | 0.02628 |
| Std. Error | |
| KE | 0.001352 |
| KA | 0.0009958 |
| 95% Confidence Intervals | |
| KE | 0.04069 to 0.04693 |
| KA | 0.02399 to 0.02858 |

Switch to the graph (notations in the figure below were added manually using the text and drawing tools).
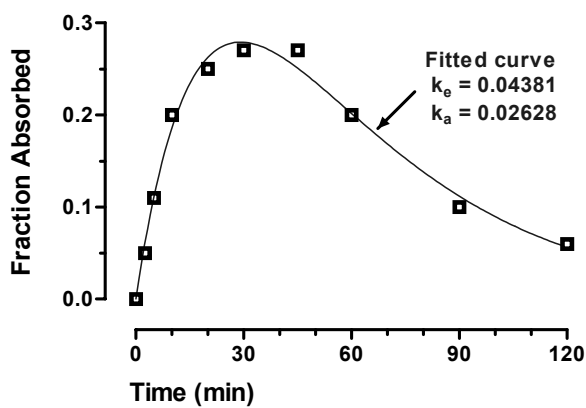


## Constraining Parameters

You may place limits on the value that a parameter can take on in the course of curve fitting. From the **User-defined Equation** dialog, select the **Default Constraints** tab. Prism displays the parameters for your equation. In the **Constraint** and **Value** boxed next to the parameter to be constrained, set a "rule" for the constraint. You may:

▪ Fix (hold constant) a curve-fit parameter. Prism will substitute the specified value as if you had written it directly into your equation, and it will fit only the remaining parameters. You may fix the value in the **Value** box...

...or direct Prism to use, for each data set it fits, a value taken from the Y column heading on the table holding that data set (for an illustration, see "Assigning Parameter Values from Y-Column Headings", page 115).

| Parameter | Constraint | Value |
|---|---|---|
| KE | Data set constant (=column title) | |

- Constrain a parameter to fall within a range.

Must be less than
Must be greater than
Absolute value must be less than
Must be between zero and

For example, make these settings to preclude the possibility of fitting either $k_e$ or $k_a$ to a negative value (physically unfeasible):

| Parameter | Constraint | Value |
|---|---|---|
| KE | Must be greater than | 0.0 |
| KA | Must be greater than | 0.0 |

- Stipulate that a parameter or parameters be shared, i.e., fitted to all data sets taken together.

| Parameter | Constraint |
|---|---|
| KE | Shared value for all data sets |

For an example of shared parameters, see "Designating Global (Shared) Parameters" beginning on page 114.

- Require that a parameter value both be shared over multiple data sets *and* fall within a range.

Shared, and must be less than
Shared, and must be greater than
Shared, and abs. value less than
Shared, and must be between zero and

- Limit the value of one parameter relative to another fitted parameter. Specify, for example, that the value of $k_e$ must be at least twice that of $k_a$:

Setting constraints in the **User-definded Equation** dialog is only one of two ways to constrain parameters. It is appropriate when you want the constraint to apply each time you use that particular model. You can also set and change constraints for a particular nanalysis using the **Constraints** tab in the **Parameters: Nonlinear Regression (Curve Fit)**.

# Creating a Family of Theoretical Curves

It is common to show relationships between three or more variables as a set, or "family" of curves on a single graph. For each curve, one independent variable is plotted continuously, while the other is held constant. The curve set is produced by incrementing the second variable arbitrarily. An example is the acid-base diagram[2], a family of curves relating $[HCO_3^-]$, $PCO_2$, and pH in blood.
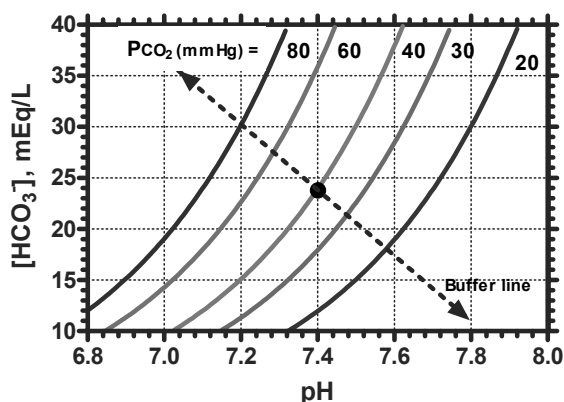


**Acid-Base Diagram**

*Physicians plot blood gas and chemistry measurements on the diagram and analyze acid-base disturbance/compensation based on displacement from the normal point (black circle). Respiratory and metabolic mechanisms are deduced by resolving displacements into components parallel to the "buffer line" and parallel to the $PCO_2$ isobars, respectively.*

Prism's curve generation analysis allows you to produce a family of theoretical curves quickly and easily. In this chapter, we'll create the graph shown above.

## Generating and Graphing the Curves

It isn't necessary to have a data table containing numbers in order to generate theoretical curves in Prism; you need only an open project file. If you're beginning a

---

[2] Davenport, H.W., *The ABC of Acid-Base Chemistry*, Chicago, University of Chicago Press, 1975.

new file, accept the default settings in the Welcome dialog, click **OK**, then click the **Analyze** button.

In the **Analyze Data** dialog, choose the **Simulate and generate** radio button, then select **Create a family of theoretical curves** from the list to the right.

Prism can generate curves based upon either its built-in equations (curve-fit models) or user-defined equations. We'll do the latter. In the **Parameters: Create a Family of Theoretical Curves** dialog, select the **More equations** radio button and then choose **Enter your own equation** from the list below.

Prism opens the **User-defined Equation** dialog. Give your user-defined equation a name.

Name: Acid-base diagram

Each curve we'll generate is based upon a modified Henderson-Hasselbalch equation.

$$pH = pK + \log \frac{[HCO_3^-]}{0.03 \cdot P_{CO_2}}$$

Before entering an equation into Prism, we must isolate the intended ordinate (Y value) on the left,

$$[HCO_3^-] = 0.03 \cdot P_{CO_2} \cdot 10^{(pH-pK)}$$

then indicate the dependent and independent variables by substitution with Y and X.

$$y = 0.03 \cdot P_{CO_2} \cdot 10^{(x-pK)}$$

$PCO_2$ will assume a different value for each curve in the family. We could substitute the constant value 6.1 for pK, but for the sake of illustration later, we'll treat it as a variable parameter.

In the **User-defined Equation** dialog (**Enter Equation** tab), type the equation and then click **OK** to return to the Parameters dialog.

Equation

Y=0.03*(PCO2)*10^(X-pK)

Prism identifies $PCO_2$ and pK as the parameters whose values you must fix for each curve. Choose to **Simulate a family of 5 curves**.

Enter the parameter values for the first curve (**Curve A**).



Switch to **Curve B**, then click **Copy Previous Parameters**, which restores the last-entered parameter values in order to save typing. Change the value of PCO₂.



Continue in this manner until $PCO_2$ values have been entered for all five curves, A-E. The values for this example are 20, 30, 40, 60, and 80.

> We left pK as a variable parameter in the user-defined equation just to illustrate the **Copy Previous Parameters** feature. We could have fixed that value in the **User-defined Equation** dialog.
>
> Equation
>
> Y=0.03*(PCO2)*10^(X-6.1)

At the top of the dialog, choose to plot each curve from **X = 6.8** to **X = 8.0**. Here are all the dialog settings after entry of all curve parameters:

Click **OK** to generate the curves and display the graph.



Theoretical curve:Curve
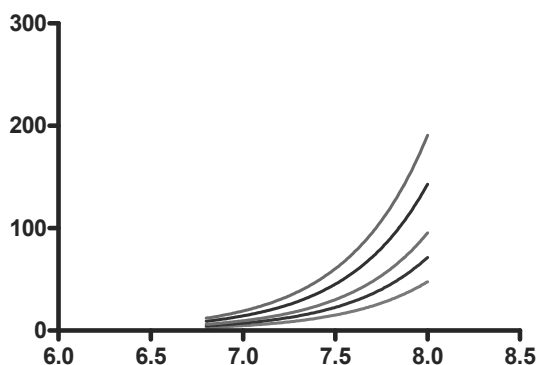
If the curves don't look right, and you think you made a mistake, click the "Analysis parameters" button to return to the Parameters dialog.

# Completing the Graph

Here is another look at our intended graph:
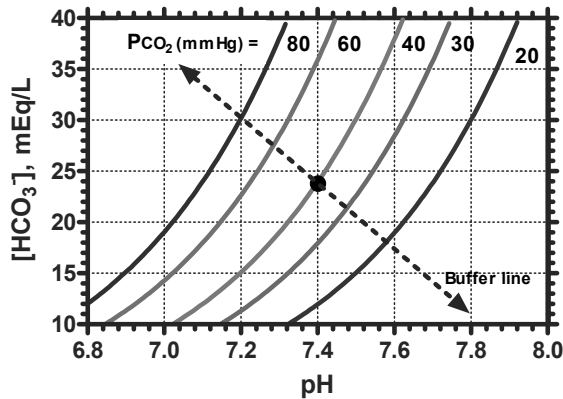


Click **Change... Size & Frame** to open the **Format Axes** dialog. Verify that the **General** tab is selected, then choose **Frame with grid** from the drop-down list under **Frame & axes**.

In the same dialog, choose the **X axis** tab. Remove the check from the **Auto** box under **Range** and change the values for **Minimum** and **Maximum**. Under **Tick options**, set the values for major and minor ticks as shown below:



Choose the **Left Y axis** tab, then adjust the range and tick settings as follows:



Delete or modify the graph title as desired, and click once on the X and Y titles to edit them, using the superscript and subscript text buttons when necessary.

$x^2 \quad x_2$

The text labels in the plot area are made using the text tool.

T

Click on the text tool button, then click once on the graph where you wish to insert the label. Then type the text. When done typing, click somewhere else to leave the text-insertion mode. Remember that you can then click the text label once to select it and make fine position changes using the arrow keys on your keyboard.

You may wish to place a white background behind the text labels in the plot area to avoid clashing with the gridlines. Click to select the text item and then choose **Change... Selected text...**. In the Format Text dialog, click the **Borders and Fill...** button. In the **Format Object** dialog, Set **Interior...Fill** to match the plot area color.

The "buffer line" may be added using the arrow drawing tool.

After you draw, position, and adjust the length of the arrow, double-click it to open the **Format Object** dialog and adjust **Thickness**, **Style**, and **Direction**.

You can add a "normal" point to the graph by switching to the data table and entering coordinates for the point (e.g., X = 7.4, Y = 24). Return to the graph and choose **Change... Add Data Sets...**. In the **Add Data Sets to Graph** dialog, verify that the correct data table (Data 1) is listed under **Data sets to add**, and click **OK**. Finally, double-click on the point symbol to open the **Format Symbols and Lines** dialog and change the size and shape of the symbol, if desired.

# Creating a PowerPoint® Slide from a Prism Graph

Prism and Microsoft PowerPoint® are a popular combination for producing 35-mm slides, overheads, or on-screen slide presentations. Although Prism can send images to slide makers directly, PowerPoint offers additional graphical formatting options, including more elaborate backgrounds. PowerPoint also allows users to project a series of slides, including those assembled from different program sources. This chapter should help you use Prism and PowerPoint together effectively. In the discussion to follow, when we refer to moving and editing graphs, the information applies equally well to layouts. The steps in PowerPoint discussed here match the commands found in PowerPoint 97 (and newer versions) for Windows; they may differ slightly from commands for other versions of PowerPoint.

## Moving your Graph or Layout to PowerPoint

### Paste-Embedding in Windows

In Prism, display the graph or layout you wish to move to PowerPoint. Choose **File... Send to PowerPoint** or click the "Create a new PowerPoint slide" button.

If PowerPoint is not currently running, Prism will launch it and start a new project with a new slide. If PowerPoint is running, Prism will simply direct it to create a new slide. Prism will then automatically paste the graph to the PowerPoint slide, and it will direct PowerPoint to make the background color of the slide match the background color of the original Prism graph or layout. The copy is embedded, i.e., a copy of all data, analyses, and graphical settings needed to create the graph are inserted into PowerPoint. You can double-click on the pasted graph in PowerPoint to edit it in a Prism environment. Changes that you make in this way affect only the PowerPoint file, not the source Prism file.

### Paste-Linking or Pasting a Picture in Windows

Two other copying techniques are available for Prism Windows, but are comparatively seldom used.

- ▪ <u>Linking</u>  The image is copied to PowerPoint, but a link to the source Prism file is also maintained. As with embedding, you can double-click on the pasted graph to edit it in a Prism environment, but now, changes also alter the source file.

- ▪ <u>Pasting a picture</u>  Only the graph is pasted — no links and no editing information. It's simple and space-efficient, but you can only edit the pasted image in PowerPoint. If you want to edit it in a Prism environment, you'll have to start again.

The transfer must be made manually. In Prism, display the graph you want to copy. Choose **Edit... Copy**. There is no need to "select" the graph before copying — everything on the page will be copied to the clipboard (in the case of layouts, the entire layout will be transferred).

Switch to PowerPoint. Open, or navigate to, the slide where you wish to place the image. If that's a blank slide, there is no need to select an insertion point. If the destination slide contains placeholders, select the appropriate one by clicking on it once—if you click twice, PowerPoint inserts a "chart" from Microsoft Graph.



Choose **Edit... Paste Special**, which produces the **Paste Special** dialog. Choose to **Paste Link** or **Paste... Picture**.

- ▪ When the **Paste** radio button is selected, you have a choice of pasting formats. Pasting a Prism Project Object results in the embedding already described. The other formats—pictures or bitmaps—can be useful for minimizing the size of your file or when you are pasting to a program that is not an OLE (Object Linking and Embedding) container. Since PowerPoint is OLE-capable, the bitmap choices will seldom, if ever, be best. Choosing **Picture** (Enhanced Metafile) gives best results when you wish to paste without embedding.

- ▪ When the **Paste Link** radio button is selected, the pasted image will be linked to the original Prism file.

For more information on embedding and linking, consult the Appendix to this chapter on page 176.

When you complete the **Paste Special** command, handles—small squares forming an outer boundary for the pasted graph—appear first on the PowerPoint slide, followed by the graph. As long as the handles are visible, indicating that the image is selected, you can click and drag within the area bounded by the handles to move the entire graph, or click and drag on a particular handle to resize the graph. Click elsewhere to deselect the graph.

A handy way to make fine adjustments to the position of your graph on the slide is to select it, then press and hold one of the "arrow" keys. This will "nudge" the image up, down, or to the left or right.

As discussed earlier, simple pasting embeds the graph. If you used that method, you can verify the embedding by double-clicking on the pasted graph. You are returned to Prism, and a copy of the graph appears, ready for editing. You are not limited to editing the graph directly; you may also edit the data in an embedded file, and any consequent changes to the graph, or to analyses linked to the graph, will be made automatically. Remember that editing changes you make here affect only the embedded file, not the source Prism file. As long as the graph is open for editing in Prism, it is unavailable in PowerPoint. This is evident from a hatched pattern over the PowerPoint image:
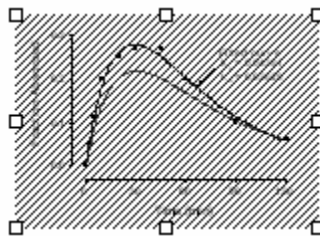


Close Prism (or close the current window in Prism, or choose **File… Close and Return to <PowerPoint file name>** in Prism). You are returned to the normal image in PowerPoint. The editing changes you made in Prism now appear in the PowerPoint copy.

If you pasted with linking rather than embedding, you can still edit the pasted graph in Prism by double-clicking on the PowerPoint copy. In this case, you'll be modifying the source Prism file.

## Pasting a Picture on the Mac

The Mac has no Object Linking and Embedding (OLE) capability. You can only paste a picture. And there is no **Send to PowerPoint** command in Prism for the Mac, so follow the same procedure presented earlier for manual pasting in Windows systems. The PowerPoint file will not contain data, Prism graphical settings, or results. If you want to edit the image, either do it in PowerPoint or return to Prism to edit, then paste a new copy.

The background color of the PowerPoint slide is not automatically matched to the original Prism image.

## Export/Import in Windows or Mac

This option moves a graph as a file, rather than via the clipboard. For transfers to PowerPoint, the option is probably useful only when moving the image to a different computer.

With the Prism Graph sheet in view, choose **File… Export**. In the **Export** dialog, assign a file name, choose a format type (preferably WMF for Windows and PICT for Mac), and choose the resolution if necessary.

In PowerPoint, with the destination slide in view, choose **Insert… Picture…**, then choose the appropriate file.

# Special Techniques and Considerations

## Background Effects

You can create colored backgrounds and/or colored plot areas within Prism, but if your graph is destined for PowerPoint, there may or may not be a point to doing so. When you paste a Prism graph to PowerPoint, backgrounds and plot areas are handled differently.

- The background is not pasted, so that users can easily exploit the more extensive background options available within PowerPoint without interference from a pasted background.

- Plotting areas are pasted, but when you choose to make the plotting area the same color as the background, Prism version 3 gives you the option of making the plotting areas transparent in Prism 3. This doesn't affect how the graph looks in Prism, but it prevents the plotting area color from showing up when the graph is pasted to PowerPoint.

Special effects such as "gradient" backgrounds must be done in PowerPoint.

## Reverse-Contrast Slides: an Illustration

If you're making 35-mm slides or an on-screen presentation, you'll likely be using light-colored (white or yellow) axes and text on a dark background. Here we show an easy method for making a white-on-blue slide with a simple "gradient" background, starting with the following Prism graph:
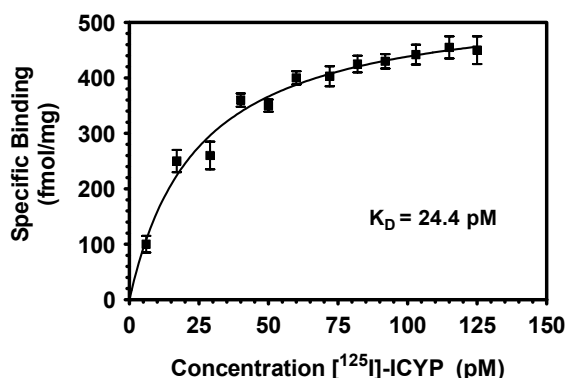
We recommend doing as much editing as you can in Prism before you move the image to PowerPoint. The final slide background will be created entirely in PowerPoint. However, both to facilitate editing and to help you see what you're doing when you actually paste the image, we'll begin by giving the graph a white-on-blue color scheme in Prism. Choose **Change... Define Color Scheme....** In the **Color scheme** frame, select **Diazo** from the drop-down list. When you click **OK** to exit the dialog, most or all of your graph is changed to the "reverse-contrast" scheme.

> For this example, we are changing the color scheme as the quickest way to make a white-on-blue slide. But you should be aware that you can change the color of any element of your graph independently by clicking the **Change** button and then selecting **Background Color**, **Plotting Area Color**, **Selected Object(s)...**, or **Selected Text....**

If any object remains black, change it individually as discussed in the previous note box.
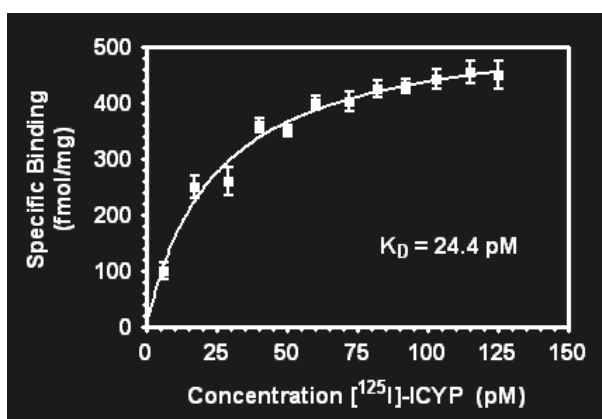
This slide actually contains two areas—a blue outer background and a blue plot area. Remember that when you paste this slide to PowerPoint, the background will not be transferred. But you must decide whether you want the plot area color to be opaque (to show up in the PowerPoint copy) or transparent (so that the background of the PowerPoint slide "shows through" the plot area). We'll leave the plotting area opaque. If you want a transparent plotting area, choose **Change... Plotting Area Color**, then select blue. Since the plotting area and the background area color selections are the same, Prism asks if you want to make the background transparent. Answer **Yes**.

> Prism won't offer to make the plotting area transparent unless you select a color for the plotting area that matches the background. Here we are not making the plotting area transparent, so that you can see the effect of pasting the plotting area.

At this point, it may be evident that the thickness or "weight" of some objects need to be adjusted to increase readability. For text, an easy way to do this is to select the text—for tick labels, select the axis, then choose the appropriate text-editing button...

 **B** or **A A**

For axes or frames, choose **Change... Size & Frame** and set **Thickness of axes** under the **General** tab. For symbols and lines, double-click on the desired object.

Now open a blank, white slide in PowerPoint. Choose **Format... Background...**. In the resulting dialog, click the "down" arrow of the drop-down box and select **Fill Effects...**. Make settings in the next dialog as follows:
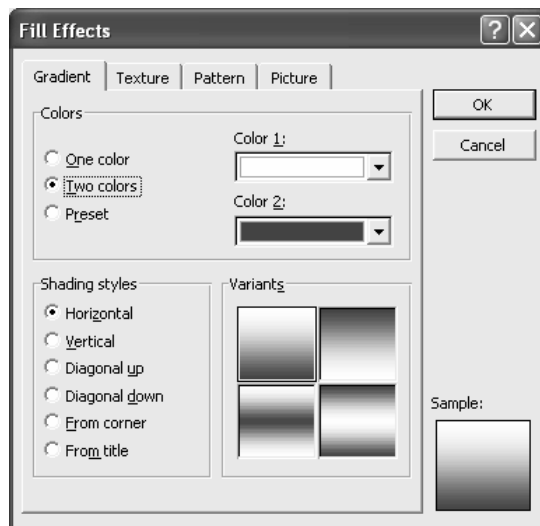
You can get an extensive color palette by selecting **More Colors...** from either the
**Color 1** or **Color 2** drop-down. When you have the colors you want, return to the
**Background** dialog and **Apply** them.
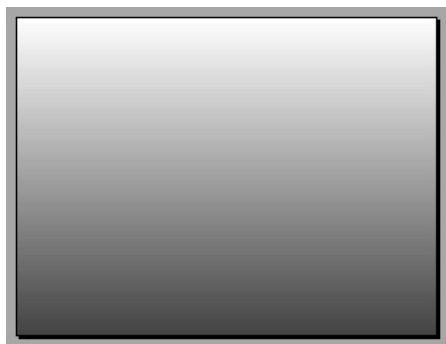


> If you prefer a simple, uniformly colored blue background, that's
> easier to set up. From a blank, white slide, simply choose **Format...
> Slide Color Scheme...**. and apply the blue color scheme.

Now you're ready to transfer the Prism graph to this slide. Return to your Prism
graph and choose **Edit... Copy**. Switch again to PowerPoint and choose **Edit...
Paste**. Move (remember to make fine adjustments with the arrow keys) and resize
the pasted graph as necessary.
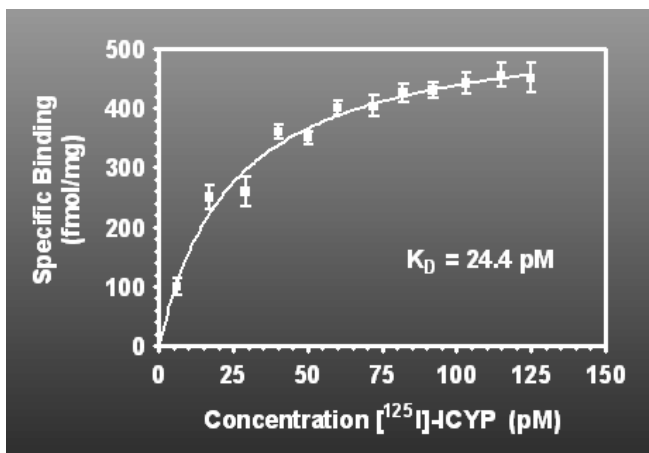


Note that unless you make it transparent in Prism, the plotting area from the Prism
graph shows up, blocking out the gradient effect in that area of the graph.

## Editing in the Pasted Image in PowerPoint

Again, we suggest editing your image to the extent that you can in Prism. If you transferred the image to PowerPoint with a simple **Paste** command, it is embedded in PowerPoint, so that you can return to Prism for editing the image merely by double-clicking on the pasted image on the PowerPoint slide.

Once you're done editing in Prism, you may need to modify individual objects on your graph using features available only in PowerPoint. For that, you must "ungroup" the image. This converts it to a Microsoft Office drawing before splitting it into individual, editable objects. The image will no longer be linked either to embedded data or to an external file, so make sure you're done editing in Prism before you "ungroup". To ungroup, *select the graph* and then choose **Ungroup** from the **Draw** menu or click on the **Disassemble Picture** button on the **Drawing+** toolbar.



Ungrouping splits the image an individually selectable, editable objects:
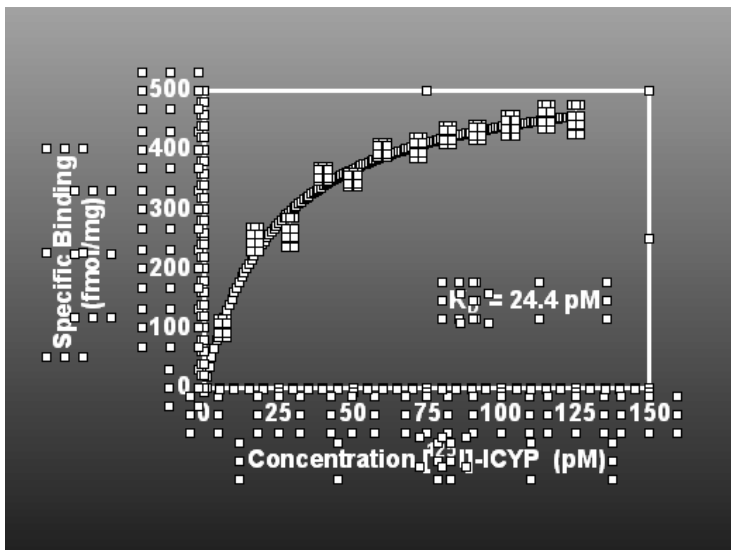


## Displaying Graph Objects Progressively

Here is a simple technique for making individual objects appear, one at a time, on a Prism graph being presented in a PowerPoint slide show. This technique utilizes OLE, so it only works on Windows systems.

Suppose you have pasted (i.e., embedded) the following graph, complete with all of the objects that you want to appear, into PowerPoint:

Effect of Competitive Antagonist
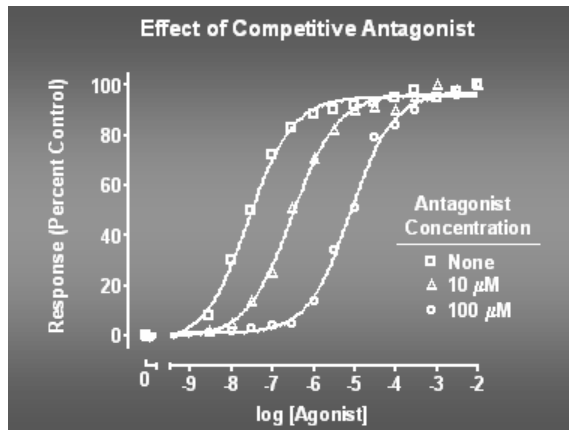
You'd like the three curves to appear one at a time, from left to right. Simply prepare a series of three slides — showing first only the left — hand curve, then the first two curves, and finally all three curves — then project them in sequence.

In PowerPoint, choose **View... Slide Sorter**, which allows you to see all of the slides in your "presentation". Now make two copies of the slide: select the slide, then choose **Edit... Duplicate**.

> Once you've duplicated the slides, to avoid a shifting effect when projecting the sequence, try not to reposition any of the images.

Remember that since you transferred this graph to PowerPoint using the **Paste** command, it is embedded in the PowerPoint file. And when you duplicate the resulting PowerPoint slide, multiple sets of embedded data are created and can be edited separately. Therefore, simply remove the appropriate curves from each copy by editing that copy in the embedded Prism file. Double-click a slide in the sorter to switch to the single-slide view, then double-click again to open the appropriate embedded Prism file. Remove the undesired symbols and curves using **Change... Remove/Replace Data Sets...**.

> In the example, the name of each data set was included in the Y column heading of the Prism data table. Unless you change the default setting for the legend (in the **Format Symbols and Lines** dialog), that name is placed on the legend automatically, and the legend is corrected automatically when you remove data sets from your graph.

When you're done, close the Prism window. You are returned to PowerPoint, and the slide reflects the editing changes that you made in Prism. Return to the Slide Sorter screen to begin editing to next slide. Here is the final series:
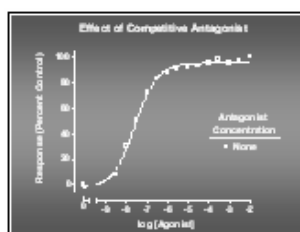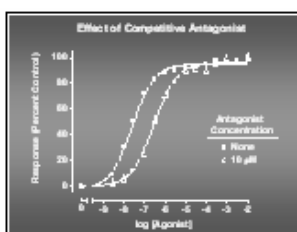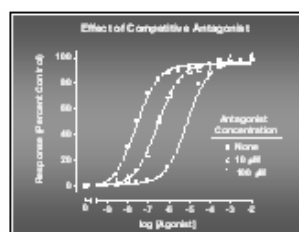
**1**     **2**     **3**

# Appendix: More about Embedding and Linking (Windows Only)

## Advantages and Disadvantages of Embedding

The advantage of embedding is that the PowerPoint file is self-contained, as it contains the Prism data and settings. You can later edit the graph with Prism, without having to find any other file. You can e-mail the file to a colleague with Prism, and (s)he will be able to open and edit the Prism graphs.

One problem with embedding is that you end up with two or more copies of your data. For example, assume you start with a completed Prism project. Now you embed a Prism graph into Word, creating an embedded copy of the data and results shown on that graph. Later, you also embed another graph that includes the same data, creating a third copy of the data. Then you embed that same graph into PowerPoint for a presentation, creating a fourth copy. If you then notice a mistake in data entry, you'll need to edit all four copies.

There are two other less important disadvantages to embedding. First, your files can get large because the embedded object includes Prism data and results. The second problem is that OLE does not always work perfectly. On rare occasions, the object will be saved incorrectly, and you'll lose the ability to edit the data. If the embedded object is the only place you saved data, you'll lose data if OLE fails.

## Advantages and Disadvantages of Linking

When you paste-link a Prism graph into PowerPoint, you paste the picture along with a link back to the Prism file. The original data exists only in the original file. You don't have to cope with the confusion of having multiple copies of the data (as you would with embedding).

The problem with linking is that you must keep track of the linked files. If you move or rename the Prism file, the link is broken. If you move the PowerPoint file to another computer, you need to remember to also move the Prism file(s).

# Analyzing Experiments Repeatedly

If you must perform the same analytical and graphing tasks many times, varying only the input data, Prism helps with a number of tools. Scripting, a means of automating data input and output via a simple programming language, is discussed in another chapter (see page 193). Three manual techniques are covered in this chapter:

- <u>Templates</u> allow you to duplicate an entire project in one step. You create the template the first time you set up the project, but store it without the data. To replicate your work, open the template, add the new data, and store the file under a new name. Or insert the template into another file.

- <u>Duplicating a family of sheets</u> replicates your work within the same project file. Simply duplicate a sheet and all sheets linked to it—formatted graphs, analyses, info, and layouts—using a single Prism command. Then replace the input data for the duplicate family.

- <u>Applying a method</u> effectively duplicates a family of sheets, but in this case, the replicated work can come from the same project or a different project. Add a new input data table, then either (a) apply a *saved* method or (b) point to another table in the project to apply *by example* all sheets linked to the latter.

We'll demonstrate these three techniques as applied to a family of linked sheets that produce a simple biochemical activity computation. To make it easier to see the linkage between sheets in the Navigator, open the **Edit** menu and choose **Preferences…**. In the Preferences dialog, select the **View** tab, and then activate the **Data+Results view**.
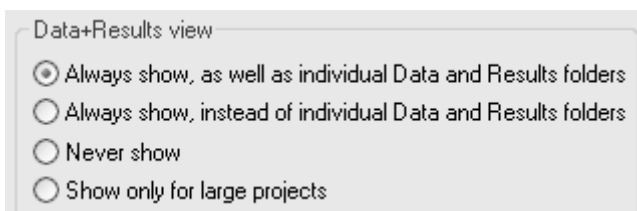


## Quantifying Uptake into Cells

Suppose you want to measure the effects of various inhibitors on uptake of a radiolabeled substrate into suspended cells. You have developed a method for removing aliquots at two times within the "linear" phase of uptake, quenching uptake

in the aliquots, and measuring radioactivity in the cell fraction. You are testing a number of inhibitors on the same day using the same cell preparation.

## Creating the Data Table

The variables recorded for each uptake measurement are

- Radioactivity at the beginning of the uptake interval ($CPM_{Time\ 1}$)
- Radioactivity at the end of the uptake interval ($CPM_{Time\ 2}$)

Their values will be recorded on the data table.

In the Prism Welcome dialog (if Prism is already running, choose **File... New project...**. Make the settings shown below.



Enter the data on the resulting table. The X column holds test article names. One test article is evaluated on each line, with column A and column B holding duplicate radioactivity measurements made 3 minutes apart, at 10 sec and 190 sec after initiation of uptake, respectively.

| | X Labels | A | | B | |
|---|---|---|---|---|---|
| | Test article | CPM Time 1 | | CPM Time 2 | |
| | X | A:Y1 | A:Y2 | B:Y1 | B:Y2 |
| 1 | Vehicle control | 4292 | 4334 | 109152 | 108930 |
| 2 | GP-10838 | 4228 | 4285 | 107590 | 109244 |
| 3 | GP-10839 | 4122 | 4027 | 65542 | 66425 |
| 4 | GP-10842 | 3588 | 3511 | 24518 | 26533 |
| 5 | GP-10843 | 4085 | 1463 | 68872 | 69244 |

Type a name for this table in the box on the toolbar.

## Creating an Info sheet

Variables whose values change from experiment to experiment, but are constant within each experiment, are

- Specific radioactivity of the uptake substrate (cpm/nmol)
- Time interval over which uptake is measured (min)
- Dry weight of cells in each aliquot (mg)

These variables will be recorded in Prism as *Info Constants*.

From the data table, click the **New** button and choose **New Info…**. In the **Create New Info** dialog, choose to link the Info page to the data table (Input data) we just created.

In the **Constant** column of the Info page, make new entries for specific activity, uptake measurement interval, and dry weight of the cells in one aliquot. Wording isn't critical—just use terms you'll recognize later.

| Constant | Value |
|---|---|
| Experiment Date | Jan-27-2003 |
| Experiment ID | |
| Notebook ID | |
| Project | |
| Experimenter | |
| Protocol | |
| SA (cpm/nmol) | 511 |
| Time (min) | 3 |
| Dry weight (mg) | 6.3 |

In the **Value** column, enter the numbers shown. These are values are common to all measurements represented on the data table (Raw CPM). They will be used to perform the conversion and normalization of raw CPM values to uptake in nmol substrate/min/mg dry weight.

## Computing Uptake

We will compute uptake as

$$\frac{CPM_{Time\,2} - CPM_{Time\,1}}{Specific\ Activity \cdot Uptake\ Interval \cdot Dry\ Weight}$$

We'll compute this quantity for each pair of CPM measurements (CPM at 10 sec and CPM at 190 sec) on the original data table using two linked analyses. We'll compute $CPM_{Time\,2} - CPM_{Time\,1}$ using a Column Math analysis, then divide the results by the

values for specific activity (SA in cpm/nmol), uptake interval (Time in min), and dry weight (in mg) found on the Info page using a User-defined Y transform.

### Column Math Analysis

From the data table, click the **Analyze** button. In the **Analyze Data** dialog, choose **Remove baseline and column math** from the **Data manipulations** list.

Prism performs a **Column Math** analysis by computing the differences between values in pairs of adjacent columns (A and B, C and D, etc.) all the way across a data table or Results sheet, ignoring empty cells, and placing the differences on a new Results sheet. In the Parameters dialog, indicate that the subtrahends (numbers to *be* subtracted, also termed "baseline values") are in column A. This means that Prism is to subtract values in columns A, C, E... from values in B, C, F... respectively.

Complete the selections in the Parameters dialog as follows:



When you leave the dialog, Prism creates a Results table to hold the differences. Note that the distinction between duplicate determinations has been maintained, and

Prism has treated the replicates as "matched" per our setting in the Parameters dialog.

| Test article | B-A | |
|---|---|---|
| X | A:Y1 | A:Y2 |
| Vehicle control | 104860.000 | 104596.000 |
| GP-10838 | 103362.000 | 104959.000 |
| GP-10839 | 61420.000 | 62398.000 |
| GP-10842 | 20930.000 | 23022.000 |
| GP-10843 | 64787.000 | 67781.000 |

Name this Results sheet:

CPM2-CPM1

### User-Defined Transform

From this Results sheet, click **Analyze**. In the **Analyze Data** dialog, choose **Transforms** from the **Data manipulations** list.

In the **Parameters: Transforms** dialog, indicate that you will be using a user-defined Y transform.

Function List — User-defined Y functions

Click the **Add...** button to open the **Equation** dialog. Give your transform a name, and enter the transform equation in the **Equation** box, as shown below.

Name: Adjustment for spec act, time, weight

Equation

Y=Y/SpecAct/Time/Weight

The parameters "SpecAct", "Time", and "Weight", on the right-hand side of the equation are arbitrary—use terms that make sense to you. We'll specify their values in the next dialog. Click **OK** to return to the **Parameters: Transforms** dialog.

Prism analyzes the user-defined transform and displays the three parameters that it finds. Click in the value box next to SPECACT, and choose **SA (cpm/nmol)** from the **Link and Info Constant** dialog. Fill in the values for TIME and WEIGHT similarly.

Choose to **Transform the average of replicates** and check the box to **Create a new graph of the results**. A new Results sheet appears with the final transformed values. You can change the heading for column A to increase readability.

| Test article | nmol/min/mg |
|---|---|
| X | Y |
| Vehicle control | 10.844 |
| GP-10838 | 10.785 |
| GP-10839 | 6.410 |
| GP-10842 | 2.275 |
| GP-10843 | 6.863 |

Rename the Results sheet:



> Instead of applying one user-defined transform, you could do three successive divisions using the **Y=Y/K** transform from the **Standard functions** list, filling in the value for K from the **Link an Info Constant** dialog each time. That would avoid having to enter the user-defined transform and would allow you to see all intermediate values, but would also clutter the project with additional Results sheets.

Click the **Graphs** tab to view the graph of the transformed data. In the figure below, some additional editing has been done. You may be particularly interested in showing the baseline labels at an angle—double-click on the baseline to open the **Format Axes** dialog, make sure the **X axis** tab is selected, and choose angled labeling.
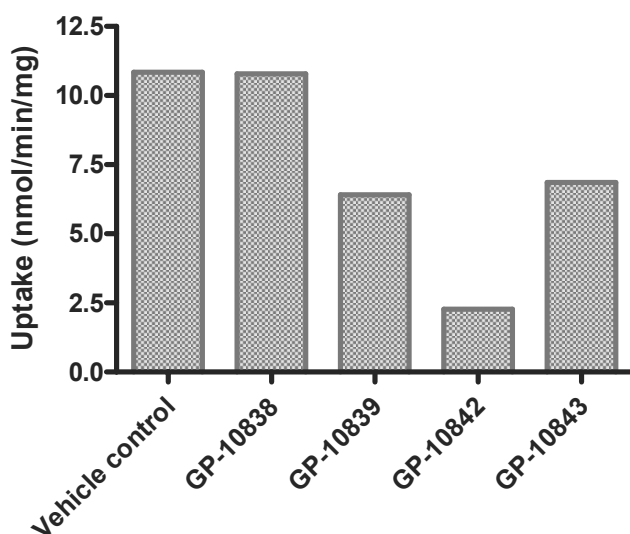
# Templates

## Using a Template Alone

The project file we just created will the basis for our template. Before saving a template, it's usually a good idea—although not strictly necessary—to erase any data on the table *that will change during subsequent use of the template*.

Delete the values for specific activity, time, and dry weight on the Info page if you wish. This is not mandatory.

Switch to the data table ("Input data"), select all data below the column headings.

| | X Labels | A | | B | |
|---|---|---|---|---|---|
| | Test article | CPM Time 1 | | CPM Time 2 | |
| | X | A:Y1 | A:Y2 | B:Y1 | B:Y2 |
| 1 | Vehicle control | 4292 | 4334 | 109152 | 108930 |
| 2 | GP-10838 | 4228 | 4285 | 107590 | 109244 |
| 3 | GP-10839 | 4122 | 4027 | 65542 | 66425 |
| 4 | GP-10842 | 3588 | 3511 | 24518 | 26533 |
| 5 | GP-10843 | 4085 | 1463 | 68872 | 69244 |
| 6 | | | | | |

Press the **Delete** *key*—don't use the **Delete** command on the **Edit** menu; you want to maintain the data cells, but without the data. If you see a message that tells you about the data being linked to the graph, disregard it and click **OK**.

> Don't switch to the graph at this point. That could break the link to the graph and cause the template not to work correctly.

Place the cursor in the top cell (row 1) of the X column, then select **File... Save Special... Save Template**.

Fill out the dialog box as shown below. Note that you may indicate here whether you wish the template to be stored on your computer or on another computer networked to it. You may also generate different categories under which templates are stored. The description at the bottom of the dialog is optional; this message will be displayed as an aid to any user opening the template.



Now close the file, and we'll see how to use the template.

Choose **File...New Project** to open the Welcome dialog. Set the radio button for a **Template** and select the template that you just created.
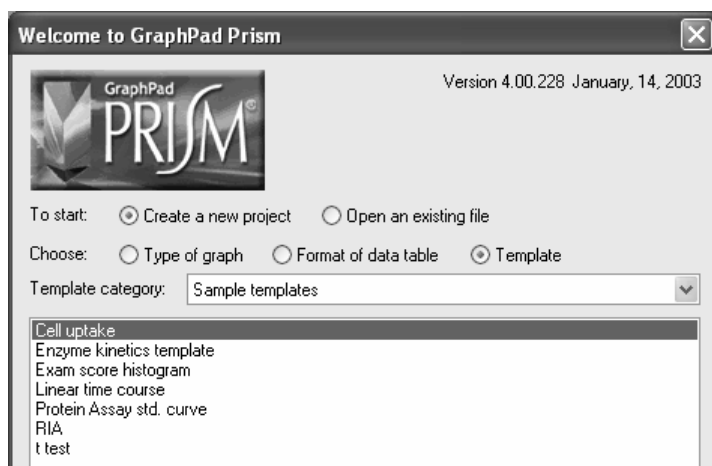


Click **OK**, and you'll get a new project formatted as your template. If you created a description, you will see it now. When you close the description box, the cursor should be at the top of the X column (note that the column headings are still there, if you didn't delete them before you saved the template).
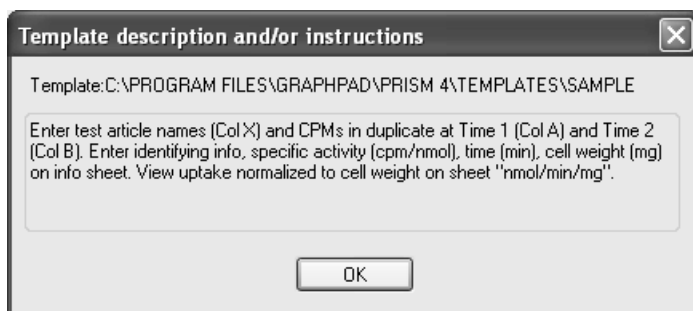


You can also insert a template into a project that you have open. With any data table in view, click the **New** button and then choose **New Data Table (+ Graph)...**. Choose **Insert template** and proceed as described above.

Now enter some new data:

| X Labels | A | | B | |
|---|---|---|---|---|
| Test article | CPM Time 1 | | CPM Time 2 | |
| X | A:Y1 | A:Y2 | B:Y1 | B:Y2 |
| 1 Vehicle control | 6378 | 6539 | 162140 | 163934 |
| 2 GP-10844 | 5936 | 5877 | 134951 | 133710 |
| 3 GP-10845 | 5216 | 5455 | 130946 | 131687 |
| 4 GP-10846 | 5819 | 5710 | 91859 | 93122 |
| 5 GP-10848 | 4938 | 4897 | 36834 | 39886 |

Switch to the Info page and fill in or replace the values in the last three rows.

| | |
|---|---|
| SA (cpm/nmol) | 642 |
| Time (min) | 3 |
| Dry weight (mg) | 7.4 |

Now switch to the Results page named nmol/min/mg to view the automatically updated uptake values,

| | X Labels | A |
|---|---|---|
| | Test article | nmol/min/mg |
| | X | Y |
| 1 | Vehicle control | 10.986 |
| 2 | GP-10844 | 9.011 |
| 3 | GP-10845 | 8.839 |
| 4 | GP-10846 | 6.085 |
| 5 | GP-10848 | 2.346 |

then click on the **Graphs** tab to view the graph:
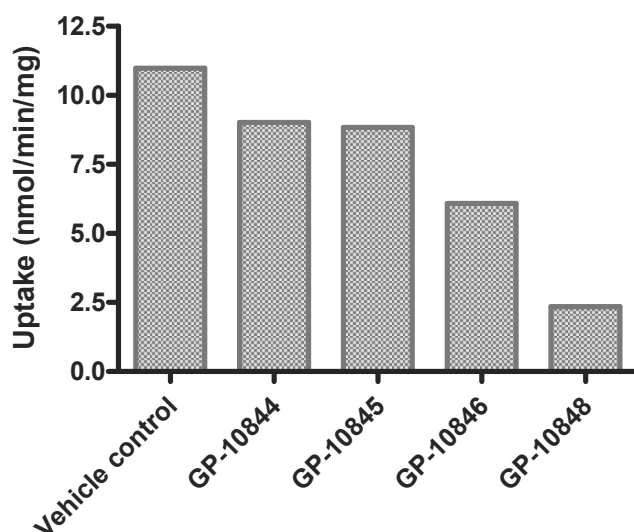
When you save your work, remember to use the **File... Save As...** command and give the file a new name.

## Inserting a Template into Another Project

We have just covered one way to use a template—alone, opening the template with its empty table, filling the table with data to produce updated results, and storing the file under a new name.

You may also insert a template into an existing file. Click the **New** button, then choose **New Data Table (+Graph)...**. In the **Create New Table** dialog, choose **Insert template**, change the template category if necessary, and select the template. The contents of the template are merged into the open file.

# Applying a Method

If you use the same treatment (analyses or graphs, or both) with various data tables, try applying a method.

## Saved Method

When you *save* a method, you can apply it later within the same project file or to another project file. From a data table that is linked to analyses and/or graphs that you wish to reproduce (you may use the template that we've created in this example), click **File... Save Special... Save Method**. Make entries in the **Save Method** dialog as shown below, and then click **OK** to save the method.
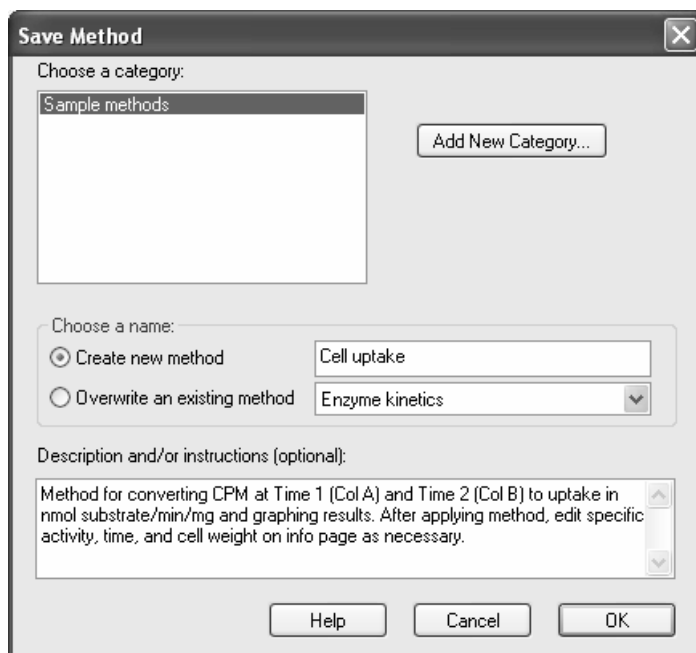
To use the method, start with the project file created earlier in this article (see "Quantifying Uptake into Cells" earlier in this chapter) or a similar file. Add a new table, with new input data, and rename each data table so that they can be distinguished easily. The organization of the project file might look like this:
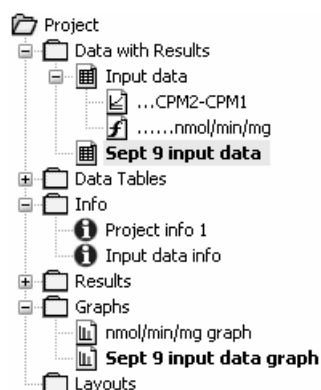


With the new table selected, click **Analyze**. In the **Analyze Data** dialog, choose to use a saved method, then select the category and specific method. If desired, enter a description of the method and a text string to be prefixed to the names of each newly generated sheet.
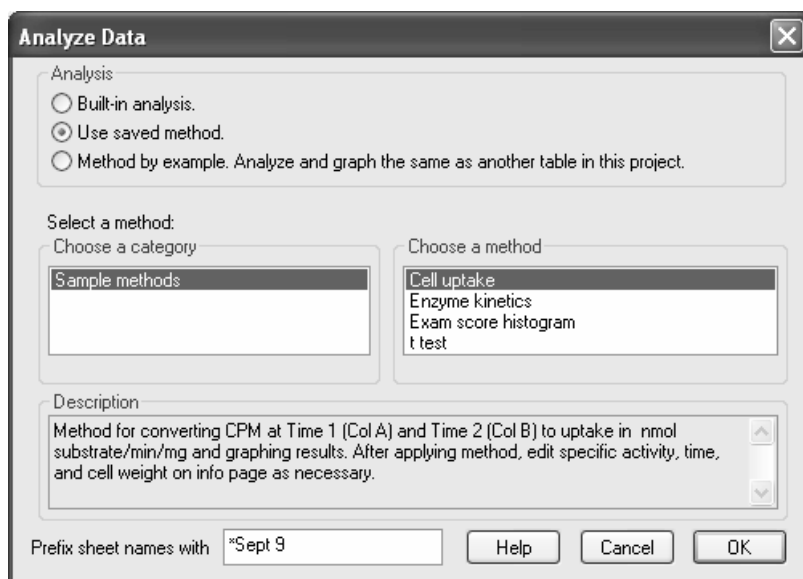
Click **OK** to apply the method to the data table you selected earlier. Prism applies the analyses and generates a new graph based on the new data.
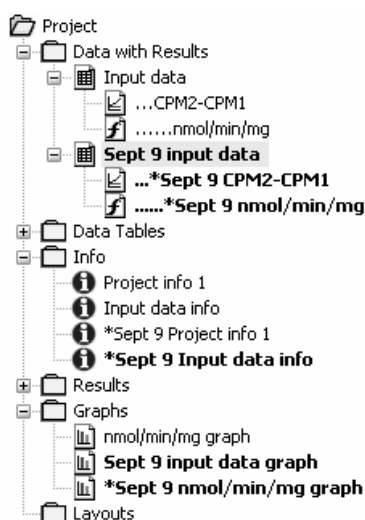


If any of the information is to be amended on the info page (including the date), do that now, then display the appropriate Results sheets and graph to verify that the results have been updated.

## Method by Example

If you wish to apply to a data table work that is associated with another data table in the *same project*, you can apply that work by example. Let's return to the project file in the previous section as it appeared before applying the saved method (at that point we had just created the new data table "Sept 9 input data" and entered new data on that table.
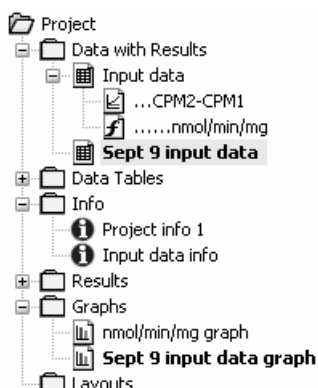


Click **Analyze**, then choose **Method by example**. Select the data table to which the method (analyses and graph) is linked, i.e., indicate the desired method by pointing to an example of its application. At the bottom of the dialog, provide a distinguishing prefix for the new sheet names if desired.
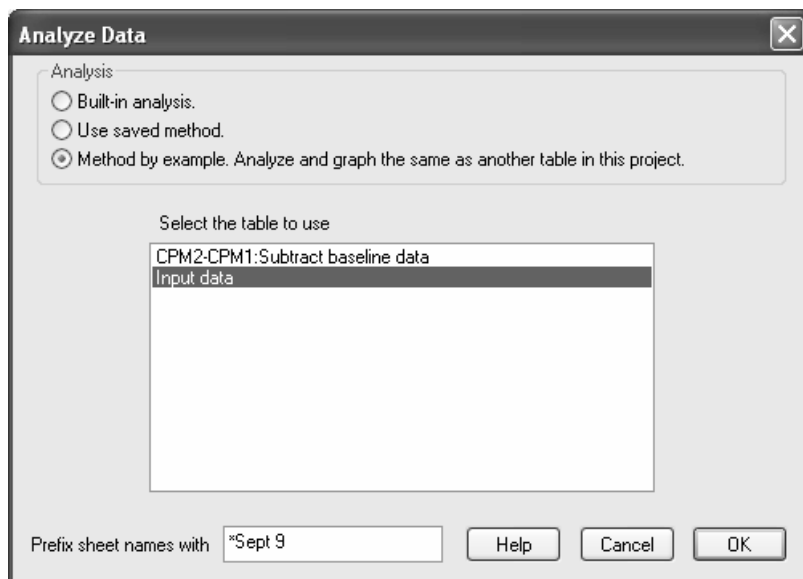
When you exit this dialog, Prism applies the analyses and creates a new graph, just as it did when the saved method was applied.
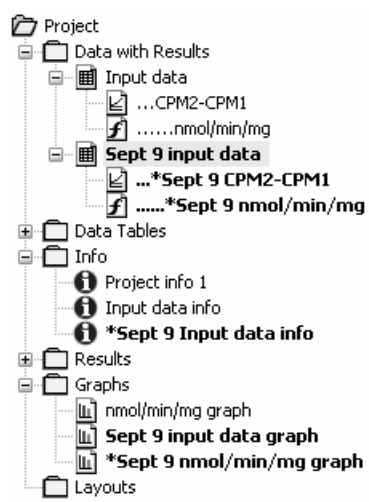
# Creating and Running a Prism Script

A Prism script is a set of instructions, written in a language unique to Prism and held in an ordinary text file, for moving raw data into, and output from, a Prism project file.

Prism scripts do not contain instructions for analyzing and graphing data—those procedures inhere in the project file itself. When new data are imported into the project file according to instructions in the script, the project file's internal links bring about reanalysis and regraphing automatically.

In this article, we'll create a Prism project file for graphing dose-response data and fitting a sigmoidal curve-of-best-fit to that data. We'll then create and use a Prism script to

1.  Transfer dose-response data from a series of input text files to the Prism project file,

2.  Save a list of $IC_{50}$ values to a single output text file, and

3.  Save the dose-response graph in a series of files, one for each test article.

So that you will be able to use the script in this chapter, first prepare a Prism project file for evaluating binding inhibition data. The procedure in the following section is condensed from the chapter "Analyzing Dose-Response Data" beginning on page 121.

## A Simple Project File for Computing $IC_{50}$

Suppose that you are designing a secondary screen for a new-drug-discovery program. You are evaluating entities found active in a primary screen for their effect on binding of a standard agonist to an isolated receptor preparation. The endpoint of your screen is the $IC_{50}$ of a curve fitted to the plot of agonist binding vs. log of agonist concentration. To keep the example simple, we'll assume that binding measurements are made in singlet and that data have already been normalized to percent of maximal binding (although you may use Prism to normalize the data automatically—see page 124 for details).

In the Welcome dialog, indicate that you will choose the **Format of data table** and format the table for **Numbers** in the X column and **A single column of values** for each data set.

On the data table, enter some example data describing a binding inhibition curve. Enter final test article concentrations in the X column and corresponding percent maximal binding values in the Y column.

| X Values | A |
|---|---|
| X Title | Data Set-A |
| X | Y |
| 1.0e-011 | 100.0 |
| 1.0e-010 | 100.0 |
| 3.0e-010 | 90.0 |
| 1.0e-009 | 75.0 |
| 3.0e-009 | 50.0 |
| 1.0e-008 | 35.0 |
| 3.0e-008 | 20.0 |
| 1.0e-007 | 5.0 |
| 1.0e-006 | 0.0 |

It doesn't matter what data you initially put on the table, as long as it is of sufficient quality for Prism to successfully log-transform the X values and then fit a sigmoidal curve to the points—the example data will be removed from the table later, when we run the script.

Now log-transform the values in the X column. Click **Analyze**. Choose **Built-in analysis**. From the **Data manipulations** category, select **Transforms**.

In the **Parameters: Transforms** dialog, choose to **Transform X values using X=Log(X)**. At the bottom of the dialog, choose to **Create a new graph of the results**. Prism displays a new Results sheet (partly illustrated below) with the log-transformed X values.

Click **Analyze**. From the **Curves & regression** category, select **Nonlinear regression (curve fit)**.

In the **Parameters: Nonlinear Regression (Curve Fit)** dialog box, choose **Classic equations**. Select **Sigmoidal dose-response (variable slope)**. Click **OK** to perform the curve fit. The Results sheet is displayed (illustrated in part below).

| Sigmoidal dose-response (variable slope) | |
|---|---|
| Best-fit values | |
| BOTTOM | -1.737 |
| TOP | 104.4 |
| LOGEC50 | -8.444 |
| HILLSLOPE | -0.7176 |
| EC50 | 3.5977e-009 |
| Std. Error | |
| BOTTOM | 3.781 |
| TOP | 3.581 |
| LOGEC50 | 0.08040 |
| HILLSLOPE | 0.09042 |

Select the yellow **Graphs** tab to display the graph of the log-transformed data and fitted curve. Customize the graph as desired; in the example below, we removed the graph title and edited the X- and Y-axis titles.
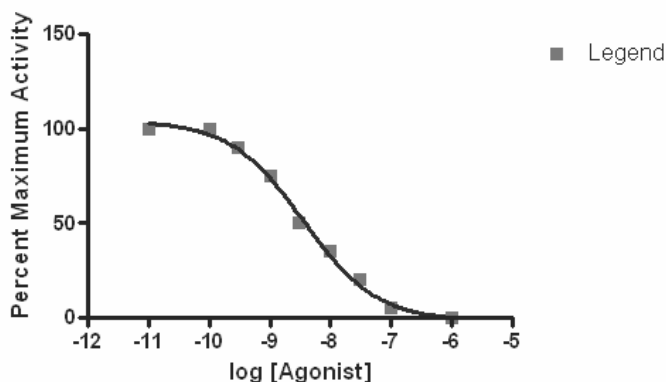


Click the yellow **Data** tab to return to the data table. Select all the data on the table, then press the **Delete** key to remove it.

Using Windows Explorer, create or designate a folder for holding this Prism project file, the Prism script file that we will write later, and the input and output text files associated with running the script. The folder can be anywhere, even on another drive, and you can store the different kinds of files in different folders, but it will be easier to follow this example literally. To do so, create a folder named **Assays** and place it in **c:\Documents and Settings**.

Now return to Prism and store the project file just created. Choose **File...Save As...**, name the project file **Dose-response**, verifying that Prism will save the file with the extension **PZF**, and store it in **c:\Documents and Settings\Assays** (or other path designated in the previous paragraph). Close the Prism project file, but leave Prism open.

# Writing the Script

From the Prism menu bar, choose **File...Run Script...** to display the **Open Script** dialog. Press the **New** button.

At the top of the **New Script** dialog, navigate to the folder **c:\Documents and Settings\Assays**. We'll store the script file there.

> We're placing all the files for this example in a single location— c:\Documents and Settings\Assays. You may wish to organize your files differently. For example, you could keep all scripts (for different purposes) in one folder designated for scripts only, perhaps in **...\Program Files\Prism 4\Scripts**.

Name the file that will contain your script, and make sure that it is of the type **PZC**.

| File name: | Process dose-response data.pzc | | Save |
|---|---|---|---|
| Save as type: | Prism script (*.PZC) | ⌄ | Cancel |
| | | | Help |

Click **Save** to save the empty script file and open the default text editor for this file type. Type the script shown below, then save it, using the **File...Save** command on the menu bar of the text editor.

```
Process dose-response data.pzc - Notepad

File  Edit  Format  View  Help

SetPath "c:\Documents and Settings\Assays"
OpenOutput Results.txt
WDate
WText
WText Compound ;
WText "log IC50";
WText "SE log IC50";
WText IC50
WText
Open Dose-response.pzf
ForEach GP*.txt
    GoTo D
    ClearTable
    Import
    GoTo R, 2, V2
    WText %F ;
    WText " ";
    WCell 5,1;
    WText " ";
    WCell 9,1;
    WText " ";
    WCell 7,1
    GoTo G, 2
    ExportWMF %F.wmf
Next
Close
CloseOutput
Beep
```

We won't discuss every line of this script—you can learn more about the syntax of Prism scripts in the GraphPad Prism *User's Guide*—but below are comments for selected lines of the script.

| Script command: | Comment: |
|---|---|
| SetPath "c:\Documents and Settings\Assays" | Default path—location of any file for which path is not specified. In this example, the location for all files. If you decide to store any files in other folders, be sure to specify the full path where appropriate in subsequent commands. |
| OpenOutput Results.txt | Create and open an output text file named "Results.txt". |
| WText Compound | The first of four commands to write column headings into the output file. |
| Open Dose-response.pzf | Open the Prism project file for computing $IC_{50}$ and creating the dose-response graph. |
| ForEach GP*.txt | Beginning of a loop to be executed once for each input text file with name starting with "GP". Each file contains raw data for one dose-response curve. |
| GoTo D | In the Prism project file, switch to data table. |
| ClearTable | Erase the data table. |
| Import | Transfer contents of the currently open GP*.txt file to the Prism data table. Since the test article name is in the second tab field of the first line, it becomes the heading for the second column—i.e., the first Y column—of the Prism data table and is therefore carried over to the graph legend. |
| GoTo R, 2, V2 | Switch to the second results page, second "view". Prism has automatically refitted the dose-response curve, and the new curve parameters are displayed. |
| WTtext %F ; | Write the current input file name (without the extension) into the first column of the output text file. This identifies the test article. Move to the next tab stop. |
| WText " "; | Move to the next tab stop—necessary to maintain alignment of the output columns. |

| | |
|---|---|
| WCell 5,1; | Write the contents of the fifth row, first Y column, of the displayed Results view. Move to the next tab stop. |
| WCell 7,1 | Write the contents of the seventh row, first Y column, of the displayed Results view. Insert carriage return/line feed (there is no ";" operator). |
| GoTo G, 2 | Switch to the second graph. Prism has automatically redrawn the graph with the new data points and curve, and the test article name appears in the legend. |
| ExportWMF %F.wmf | Save the graph as a Windows Meta File with the same name as the input text file (= test article name). |
| Next | As long as input text files remain to be processed (i.e., in the folder c:\Documents and Settings\Assays), return to the top of the loop. |
| Close | Close the Prism project file without saving changes. |
| CloseOutput | Close the output text file, saving changes. |

When fitting a sigmoidal dose-response curve, Prism reports the horizontal midpoint of the curve as LOGEC50—it doesn't consider whether you're studying an agonist or an antagonist. The script copies the value for this parameter from the Results sheet and writes it into the output file under the heading IC50.

## Preparing the Input Files

Using Microsoft Notepad (or a similar text editor), prepare two or more input text files, named GP0001.txt, GP0002.txt, etc. and stored in c:\Documents and Settings\Assays. Below are the contents for the three files in our example. Note that in each file, the test article name is in the second tab field of the first line, so that it will be written into the first Y column heading of the Prism data table. That will "push" the name to the legend of the dose-response graph.

```
GP0001.txt - Notepad
File  Edit  Format  View  Help
            GP0001
1.0e-012            100.0
1.0e-011            100.0
1.0e-010            100.0
3.0e-010            97.4
1.0e-009            84.7
3.0e-009            66.4
1.0e-008            34.4
3.0e-008            17.3
1.0e-007            5.0
3.0e-007            0.0
1.0e-006            0.0
1.0e-005            0.0
```

```
GP0002.txt - Notepad
File  Edit  Format  View  Help
            GP0002
1.0e-012            100.0
1.0e-011            100.0
1.0e-010            100.0
3.0e-010            100.0
1.0e-009            100.0
3.0e-009            96.7
1.0e-008            83.0
3.0e-008            66.4
1.0e-007            32.7
3.0e-007            16.8
1.0e-006            5.0
1.0e-005            1.7
```

```
GP0003.txt - Notepad
File  Edit  Format  View  Help
            GP0003
1.0e-012            100.0
1.0e-011            99.4
1.0e-010            84.7
3.0e-010            66.4
1.0e-009            34.5
3.0e-009            17.8
1.0e-008            5.0
3.0e-008            2.0
1.0e-007            0.0
3.0e-007            0.0
1.0e-006            0.0
1.0e-005            0.0
```

# Running the Script

At this point, the contents of **c:\Documents and Settings\Assays** should be as shown in the following display from Windows Explorer:



| Name | Size | Type ▲ |
|---|---|---|
| Dose-response.pzf | 32 KB | GraphPad Prism 4 Project |
| GP0001.txt | 1 KB | Text Document |
| GP0002.txt | 1 KB | Text Document |
| GP0003.txt | 1 KB | Text Document |
| Process dose-response data.pzc | 1 KB | Text Document |

There are no output files yet. They'll be created automatically as the script runs.

Launch Prism and then close the Welcome dialog. Choose **File...Run Script...**. In the Open Script dialog, navigate to the **c:\Documents and Settings\Assays** folder, select **Process dose-response data.pzc**, and click **Open**.

In the **GraphPad Prism Script** dialog, click the **Run** button to execute the script without interruption...
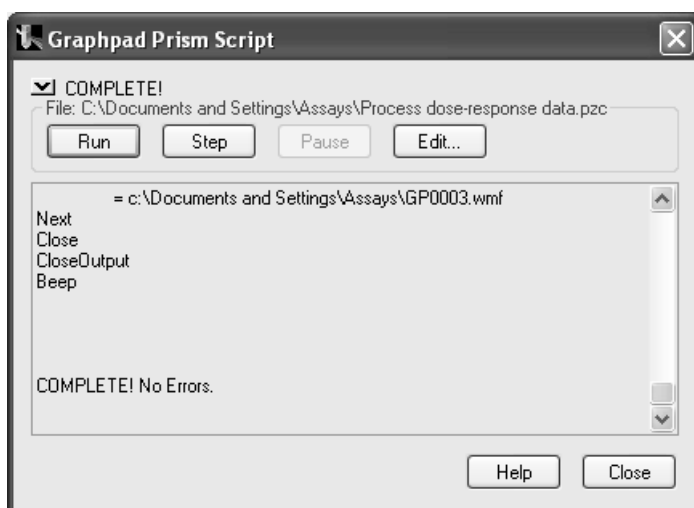


...or click **Step** repeatedly to run the script one step at a time and to see the results as you go. If you have made no errors writing the script or creating the Prism project file or input files, Prism reports "COMPLETE! No Errors." If you obtain an error message and the difficulty is in the script, click **Edit...** to open the script file in your text editor and make the necessary changes.

# The Results Files

The contents of **c:\Documents and Settings\Assays** should now be as follows:



| Name | Size | Type ▲ |
|---|---|---|
| Dose-response.pzf | 32 KB | GraphPad Prism 4 Project |
| GP0001.txt | 1 KB | Text Document |
| GP0002.txt | 1 KB | Text Document |
| GP0003.txt | 1 KB | Text Document |
| Process dose-response data.log | 2 KB | Text Document |
| Process dose-response data.pzc | 1 KB | Text Document |
| Results.txt | 1 KB | Text Document |
| GP0001.wmf | 3 KB | WMF Image |
| GP0002.wmf | 3 KB | WMF Image |
| GP0003.wmf | 3 KB | WMF Image |

The log file, **Process dose-response data.log**, shows each script command as Prism encounters it, plus interspersed notations on how it responded.

**Results.txt** is the output file containing tabulated curve-fit results for all test articles.



```
Results.txt - Notepad
File  Edit  Format  View  Help
Monday, December 09, 2002

Compound        log IC50        SE log IC50     IC50

GP0001          -8.250          0.7864          5.6207e-009
GP0002          -7.287          1.302           5.1630e-008
GP0003          -9.252          0.4795          5.6019e-010
```

Finally, **GP0001.wmf** through **GP0003.wmf** contain the dose-response graphs. Here is the graph for GP0001:

# Index

Scatchard analysis: Data transformation, 84; Plot, 81–96
Scripting, 193–202
Secondary plot, 81, 97, 98
Segmented axis. *See* Gap in axis
Semi-logarithmic plot, 121
Sensitivity. *See* Contingency analysis
Shared curve fit parameters, 114. *See* Nonlinear regression, Global curve fitting
Shared parameters. *See* Nonlinear regression, shared parameters. *See* Nonlinear regression, shared parameters
Sigmoidal curve, 121; Variable-slope model, 126, 146
Simulating a theoretical curve, 156
Simulating theoretical curves, 161–66
Slides. *See* PowerPoint® slides
Specific binding. *See* Saturation binding analysis
Specificity. *See* Contingency analysis
Spikes. *See* Bars on XY graph, Projection lines
Stacked data entry, 50
Staircase graph, 71
Standard curves: Linear, 139–44; Nonlinear, 145–50
Standard deviation, 64, 65; Manual entry, 44, 57; Of column of numbers, 14, 15, 62
Standard error, 64, 65; For column of numbers, 14; For curve-fit parameters, 130; For log $EC_{50}$, 130; Manual entry, 57; Of column of numbers, 15, 62
Subscript. *See* Text
Substrate-velocity curve, 97–109
Sum of column of numbers, 15
Survival analysis, 71–75; Ascending vs. descending curves, 71; Automatic analysis and graphing, 74; Censored data, 72, 75; Curve comparison (log-rank test), 74; Data entry, 71–74; Number of subjects at risk, 74; Survival proportions, 74
Survival curves. *See* Survival analysis
$S_{y.x}$, 141
Symbols: Appearance, 30; Border, 46; Fill color, 37, 46; Overlay order, 47; Shape, 29, 36, 46, 143; Size, 37, 46, 143

*t* Test, 11, 49–53; Automatic test or parameter selection, 51; One- or two-tailed, 52; Paired, 52
Table: Contingency, 77; Formatting manually, 35, 39, 57, 86, 105; Naming, 12, 21, 56, 65, 82, 98, 140
Template, 137, 183–87; Enzyme kinetics, 100; Inserting into a project, 187
Template description, 184, 185
Text: Bolding, 84, 102; Font size, 47; Justification, 47; Manual entry, 47; Positioning, 47; Subscripting, 84, 102, 165
Text tool, 47, 102, 136, 166
Theoretical curve. *See* Simulating a theoretical curve
Theoretical curves. *See* Simulating theoretical curves
Tick: *Antilog* labeling format, 133; Crowded, overlapping labels, 134; Custom labels. *See* Custom tick labels; *Decimal* labeling format, 133; Formatting, 38, 47, 165; Interval, 16, 47, 131, 165; Label color, 47; Label format, 133; Label position, 16, 23, 47; Logarithmic spacing, 134, 149; Minor, 38, 47, 165; *Power of 10* labeling format, 133, 149; *Scientific* labeling format, 133
Title: Graph, 12, 21, 22, 56, 65, 140; Rotating, 108
Transform, user-defined, 181–82
Transposing data, 21, 25
Tukey post test, 59, 60
Unknowns: Adding to standard curve graph, 142, 144; Computed from standard curve, 139–44, 145–50; Showing on standard curve graph, 149
Uptake into cells, analyzing, 177–82
User-defined equations, 94, 113–15, 151–60, 162
User-defined transform, 181–82
$V_{max}$, 97–109; Displaying on graph, 101
Welch's correction, 52
X-axis labels: Angled, 34; Crowded, overlapping, 58, 134
Y axis: Additional (second), 45; Color, 47