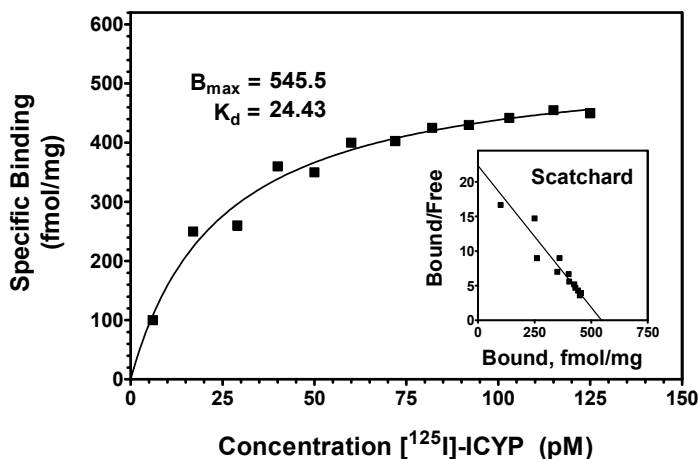


Radioligand Binding Analysis

Saturation Binding Curves and Scatchard Plots

In this article, we'll make a commonly used combination graph—a saturation binding curve with an inset Scatchard plot. The article includes the following techniques:

- Graphing a saturation isotherm starting with either precomputed specific binding values or from total and nonspecific binding values
- Computing specific binding using automatic baseline subtraction
- Improving estimates of K_d and B_{max} by fitting total and nonspecific binding *globally*
- Displaying binding constants on the saturation isotherm graph
- Scatchard transformation, one of Prism's built-in *Pharmacology and biochemistry transforms*



¹ Adapted from: Miller, J.R., *GraphPad Prism Version 4.0 Step-by-Step Examples*, GraphPad Software Inc., San Diego CA, 2003. *Step-by-Step Examples* is one of four manuals included with Prism 4. All are available for download as PDF files at www.graphpad.com. 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 print this article and read it 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.

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

$$Y \text{ intercept} = B_{max} / K_d$$

$$X \text{ intercept} = B_{max}$$

$$\text{Slope} = -1 / K_d$$

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

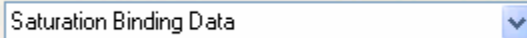
When you launch Prism, the Welcome dialog appears. It lets you create a new project (file) or open an existing one. Choose **Create a new project** and **Type of graph**. Choose the **XY** graph-type tab, then select the first thumbnail view, for “Points only”.

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	A
	Conc (pM)	Binding (fmol/mg)
	X	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:

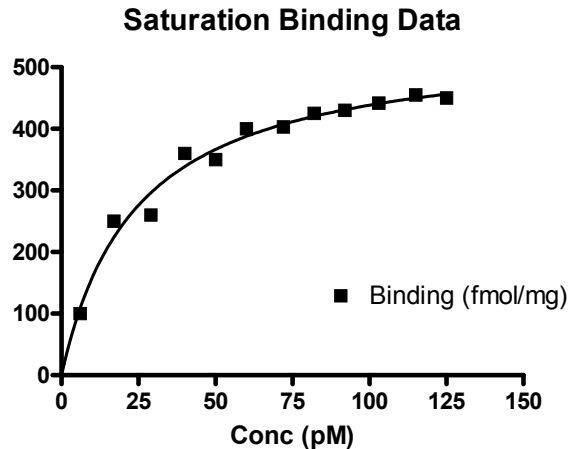


Click on the yellow **Graphs** tab to view the graph of your data.

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.



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

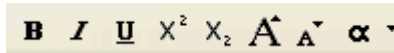
The units for B_{max} are the units that you used when entering Y values on the original data table. The units for K_d are the units you used when entering the X values.

Displaying Binding Constants on the Graph

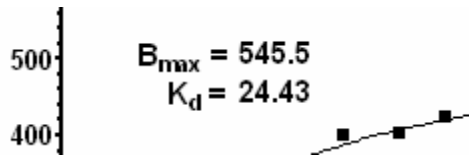
If you wish, you can paste cells from this results table to your graph, so that your binding constants will be displayed there and will also be linked to the Results sheet. That way, if you change the saturation binding data later, B_{max} and K_d will be automatically updated both on the Results sheet 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)...

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...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.



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:



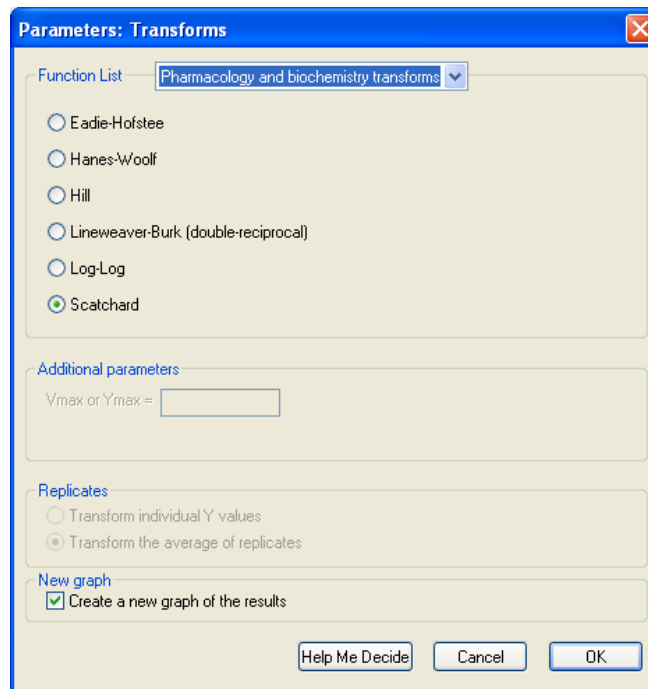
At this point, you may wish to change some of your original saturation binding data (data table) and observe how the values of B_{max} and K_d change automatically both on the Results sheet and on the graph.

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**.



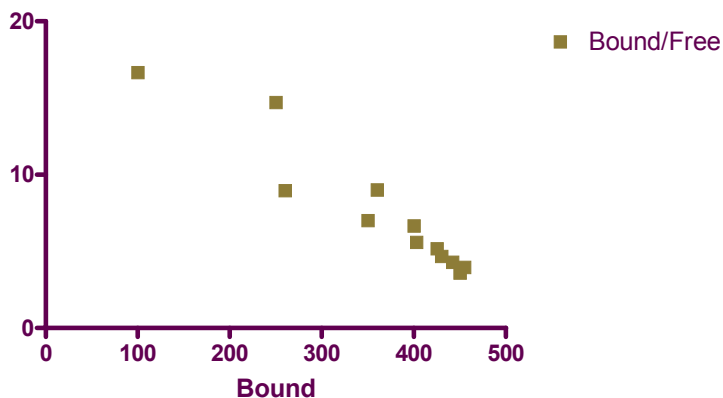
When you click **OK** to exit this dialog, Prism creates and displays the Results sheet (named **Scatchard of Saturation Binding Data** in this example) containing the transformed data.

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



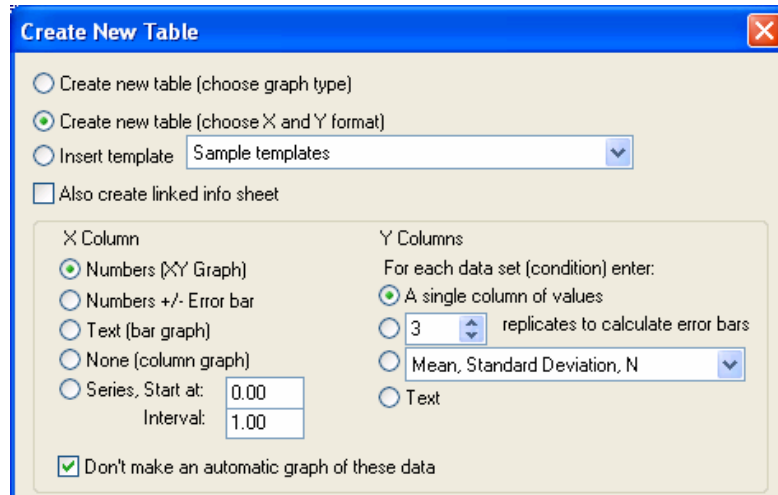
Adding the Line

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).



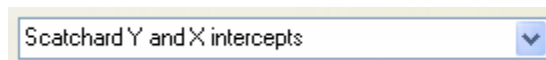
Fill in the table using the values for B_{max} and B_{max}/K_d as follows:

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

Here's the table:

	X Values	A
	X Title	Data Set-A
	X	Y
1	0.0	22.33
2	545.5	0.00

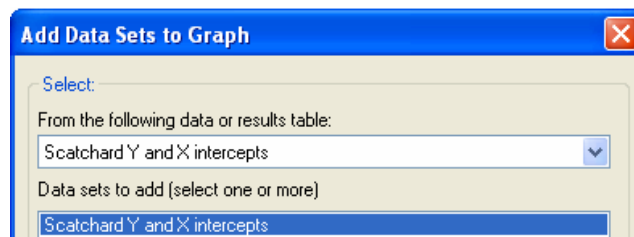
Rename this table:



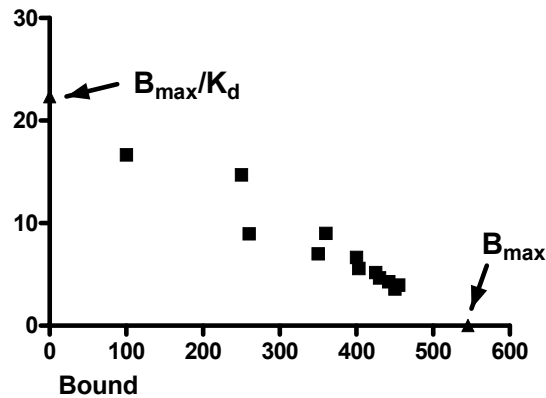
We now have the elements for the final Scatchard plot on two sheets. The graph labeled **Scatchard of Saturation Binding Data graph** contains the points from the saturation binding experiment, and the table labeled **Scatchard Y and X intercepts** contains two points marking the Y and X intercepts (B_{max}/K_d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

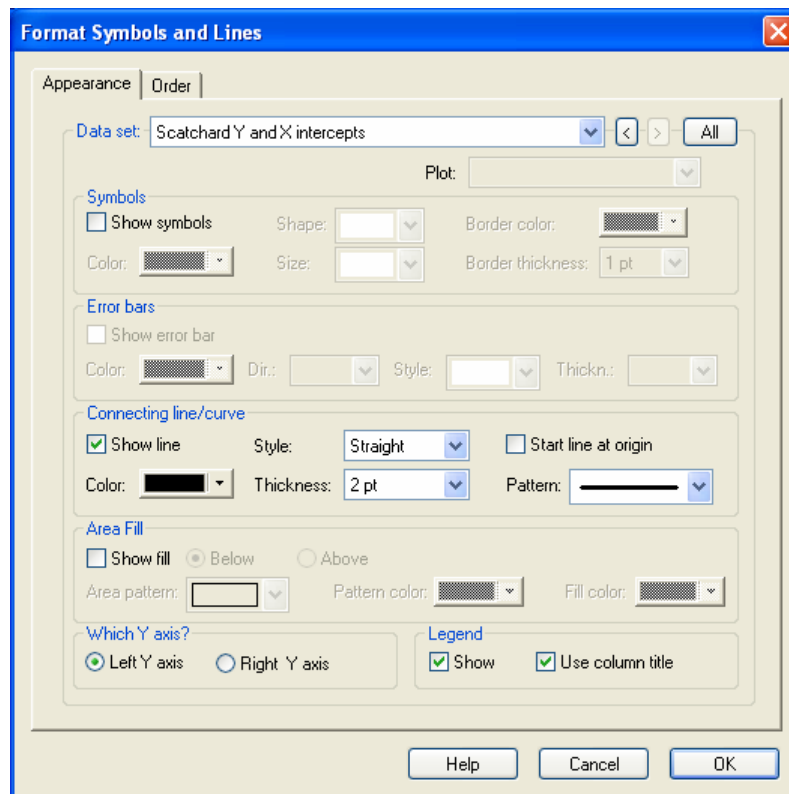
In the dialog that appears, verify that you are adding the correct data set and then click **OK**.



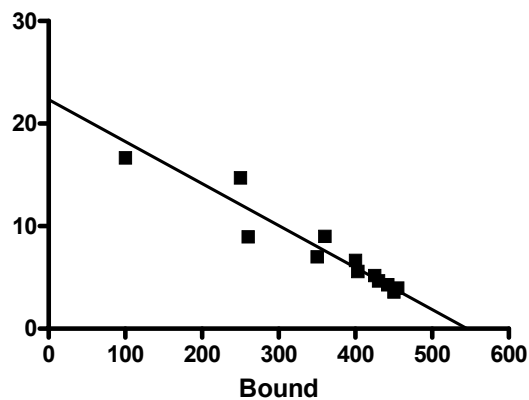
It should now be evident that the Y and X intercepts (the ends of the Scatchard line) are plotted using symbols distinct from those for the saturation binding points (labels and arrows pointing to the Y and X intercepts will not be on your graph, and your axis formatting may be different).



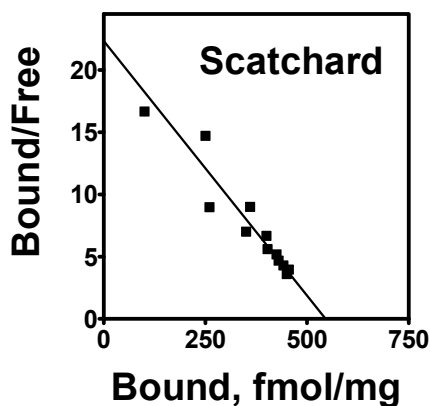
Format the Scatchard Line data set to make the points invisible but to connect them with a line: Click **Change...Symbols & Lines**. Under the **Appearance** tab, set **Data set** to **Scatchard Y and X intercepts**. Choose to plot no symbols, but to connect them with a line.



Here is the Scatchard plot:

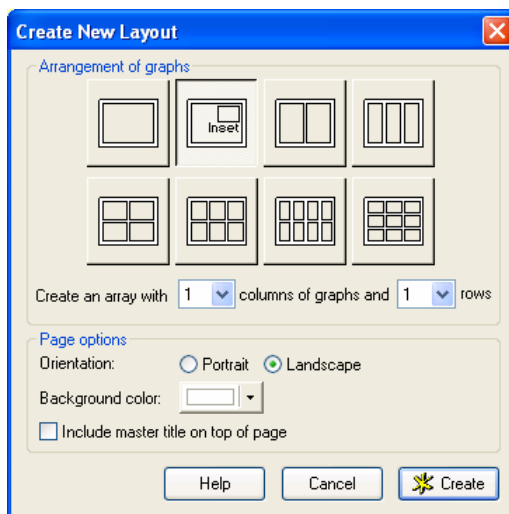


Following is the final Scatchard graph for the inset plot, after a few a formatting changes not covered here:



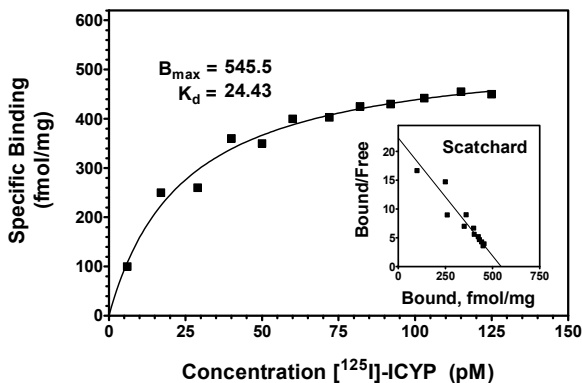
Combining Saturation Isotherm and Scatchard Plot

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:

$$\text{Specific binding} = \text{Total binding} - \text{Nonspecific binding}$$

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:

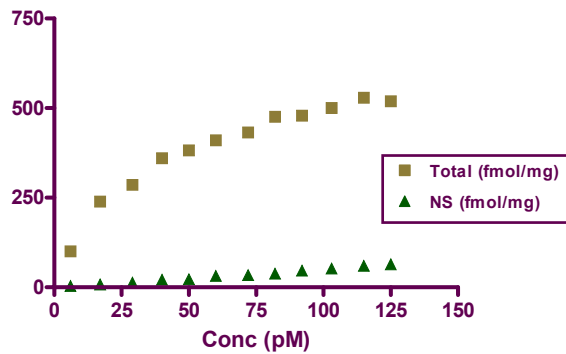
- Have Prism perform the subtractions to obtain nonspecific binding values, then proceed as discussed before.
- Perform a *global* curve fit to both total and nonspecific binding data, estimating K_d and B_{max} from the total binding curve, while finding the best value for nonspecific binding for the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

Direct Subtraction at Each Added Ligand Concentration

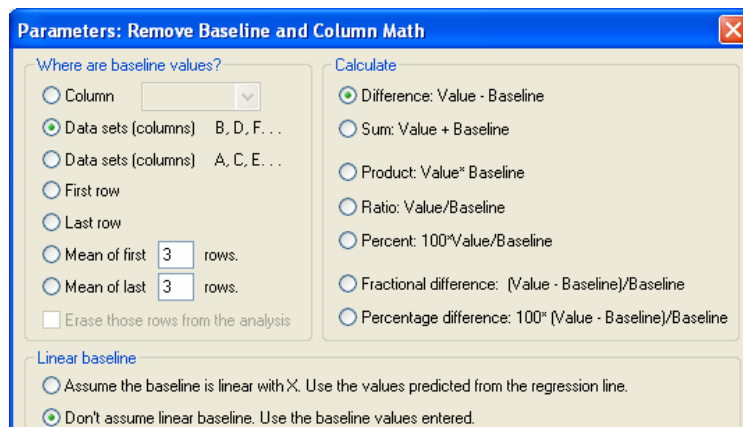
By this method, you simply let Prism do the subtraction that you'd otherwise do yourself, then fit the nonspecific binding data as already discussed. Tabulate total and nonspecific binding values:

	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:



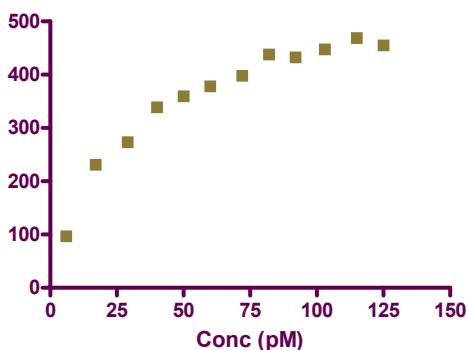
Click **Analyze** and then choose **Remove baseline and column math** from the **Data manipulations** list. In the Parameters dialog, choose to subtract the values in column B from column A.



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:



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.

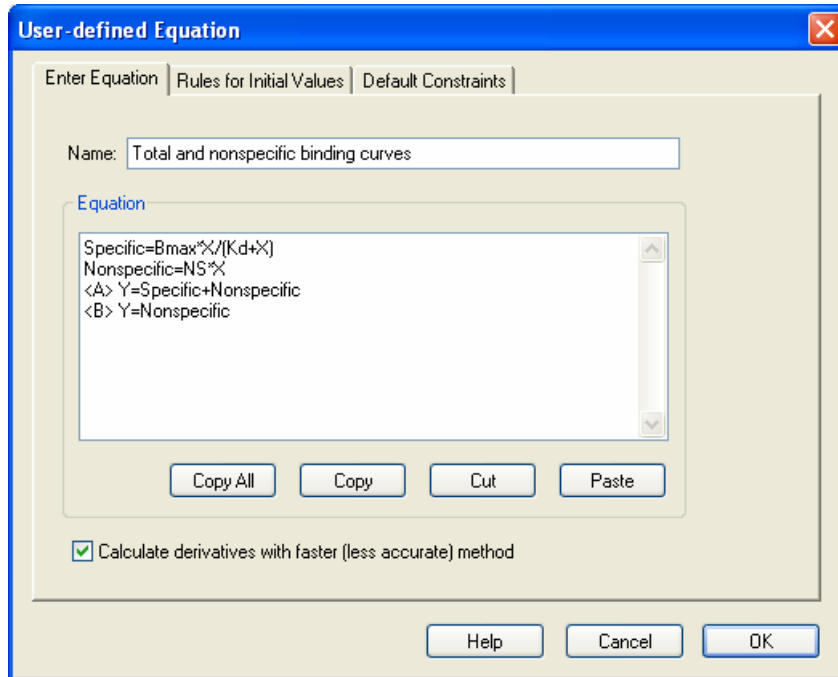
While this approach gives better estimates for the equation parameters, note that you will not be able to draw a Scatchard plot, since nonspecific binding is not expressly computed.

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 Step-by-Step Example "Fitting Data to User-Defined Equations".

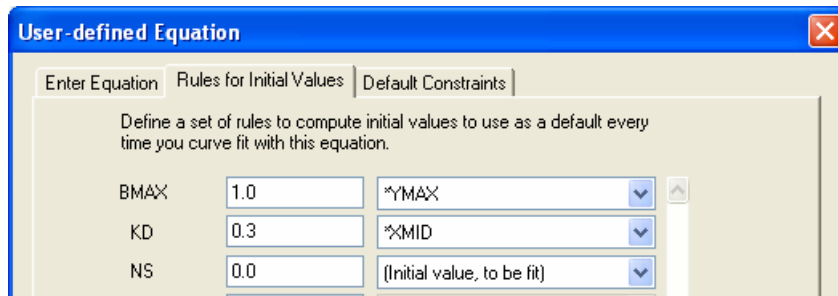
In the **Use-defined Equation** dialog, give your equation system a name (e.g., "Total and nonspecific binding curves"). In the **Equation** window, enter the sequence shown in the next figure.



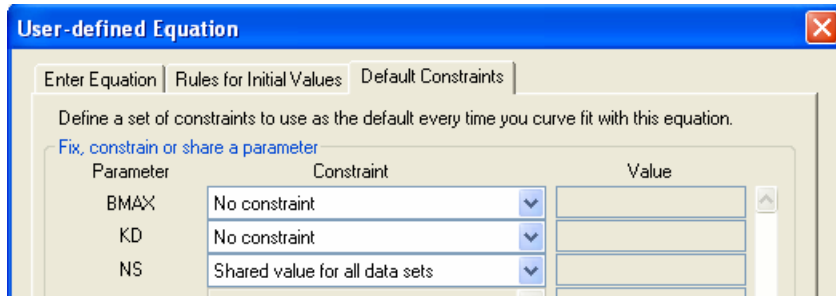
Note that the equations denoted by "<A>" and "" are fitted to data in columns A and B, respectively, so the order of the Y columns on the data table is important.

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.



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**.

