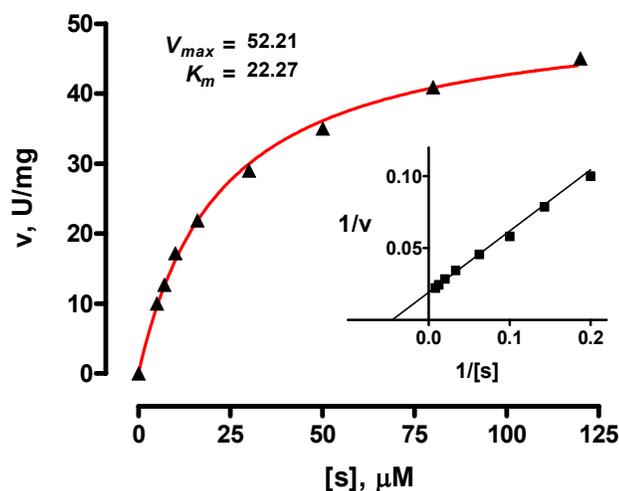


**Enzyme Activity Analysis**

**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. This article includes the following techniques:

- Automatic baseline subtraction
- Displaying the fitted kinetic constants on the graph
- Lineweaver-Burk transformation, one of Prism's built-in *Pharmacology and biochemistry transforms*
- Offset X and Y axes



<sup>1</sup> 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](http://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.

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:  $1/\text{velocity}$  vs.  $1/[\text{substrate}]$ . When catalytic activity follows Michaelis-Menten kinetics over the range of substrate concentrations tested, the Lineweaver-Burk plot is a straight line with

$$Y \text{ intercept} = 1/V_{max}$$

$$X \text{ intercept} = -1/K_m$$

$$\text{Slope} = K_m/V_{max}$$

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 Step-by-Step Example "Saturation Binding Curves and Scatchard Plots", making appropriate changes to axis titles and labels.

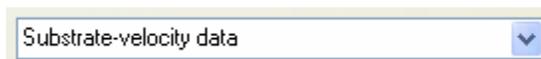
## Creating a Substrate-Velocity Curve

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:

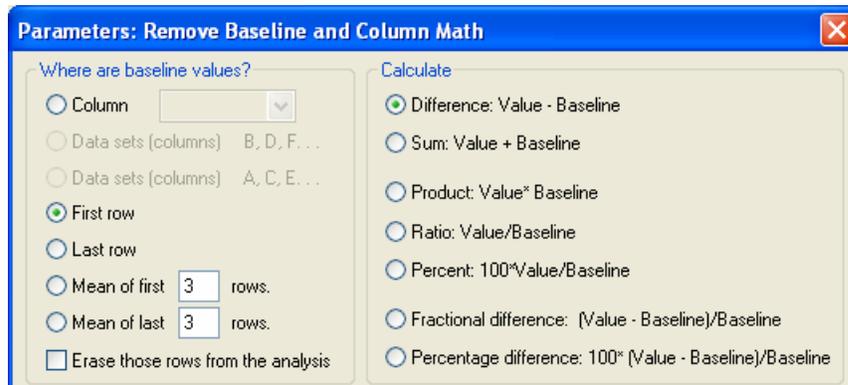
	X Values	A
	[s]. mM	v. U/mg
	X	Y
1	0.0	3.4
2	5.0	13.4
3	7.0	16.1
4	10.0	20.6
5	16.0	25.3
6	30.0	32.4
7	50.0	38.4
8	80.0	44.3
9	120.0	48.4

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



## 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**.



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).

```

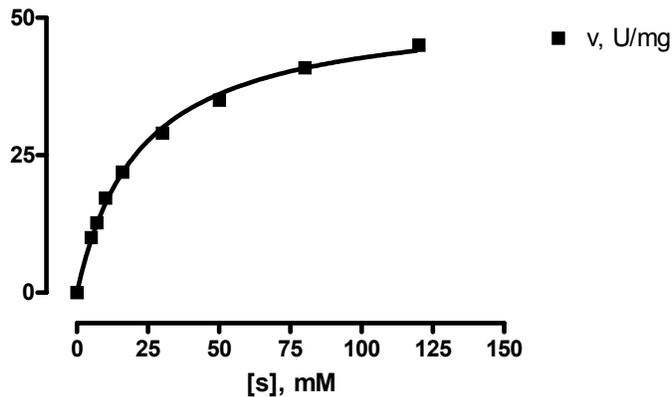
Y=Vmax*X/(Km+X)
;This describes an enzyme-catalyzed reaction obeying classical, or
;Michaelis-Menten, kinetics.
;Vmax is the maximal velocity at saturation.
;Km, or the Michaelis constant, is the substrate concentration required
;to reach half-maximal velocity (Vmax/2).
    
```

If you do not have the “Enzyme kinetics template” file, you can enter the equation shown above as a user-defined equation. See the Step-by-Step Example “Fitting Data to User-Defined Equations”. 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

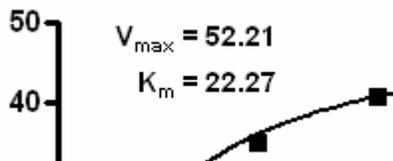
If you wish, you can paste cells from this results table to your graph, so that the kinetic constants will be displayed there and will also be linked to the Results sheet. That way, if you change the saturation binding data later,  $V_{max}$  and  $K_m$  will be automatically updated both on the Results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

T

...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.

**B I U X<sup>2</sup> X<sub>2</sub> A<sup>^</sup> A<sub>^</sub> 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:



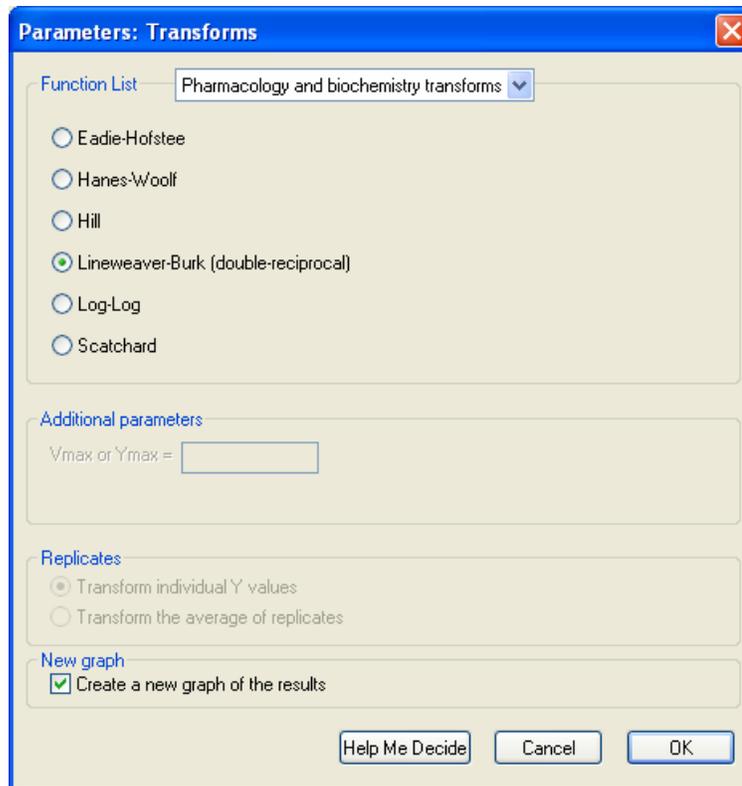
At this point, you may wish to change some of your original activity data (data table) and observe how the values of  $V_{max}$  and  $K_m$  change automatically on the Results sheet and on the graph.

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## 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**.



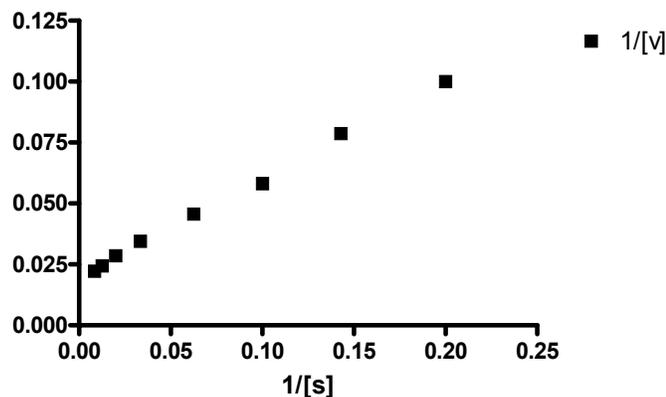
The transformed data are shown on a new Results sheet (the original data are unchanged in the Data Tables section of your project). Prism will carry over the original column titles, which will now be incorrect, so you can edit them if you wish:

	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

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

$$Y = 0$$

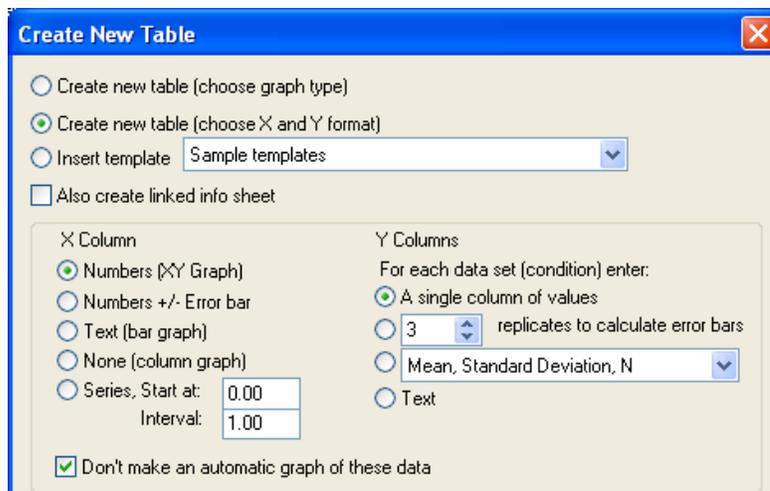
and the coordinates for the upper-right end of the line are

$$X = 1/[S_{min}] = 0.2$$

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

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

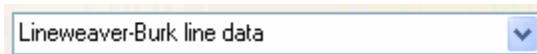
Switch to the Data Tables section of your project and click **New**. Create a **New Data Table (+Graph)...** by making the selections below (note that you will not be making a new graph).



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

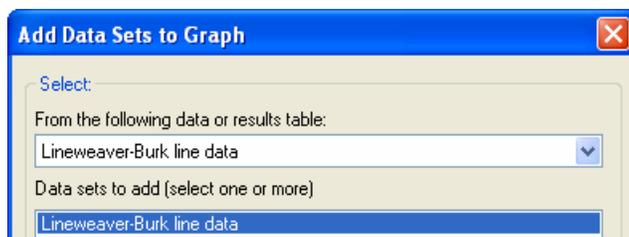
Rename this table.



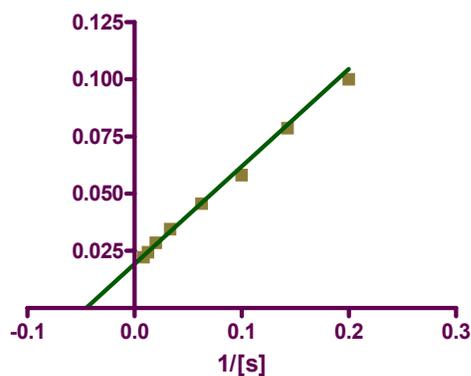
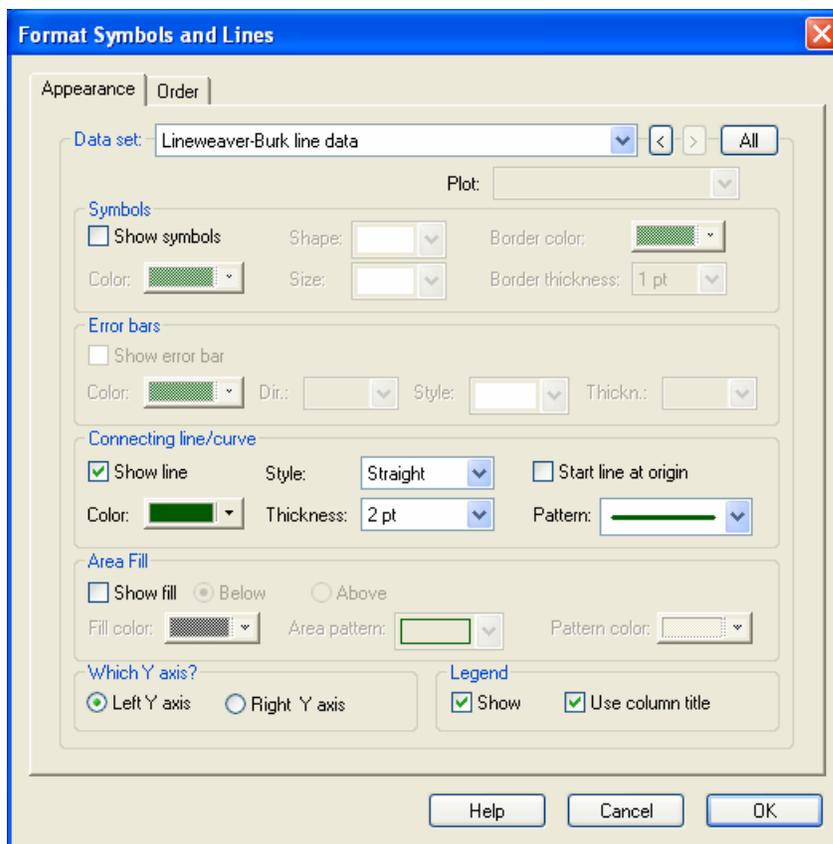
We now have the elements for the final Lineweaver-Burk plot on two sheets. The graph labeled **Lineweave-Burk...** contains the transform activity data, while the table labeled **Lineweaver-Burk line data** contains two points marking the Y and X intercepts ( $V_{max}/K_m$  and  $V_{max}$ , respectively).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

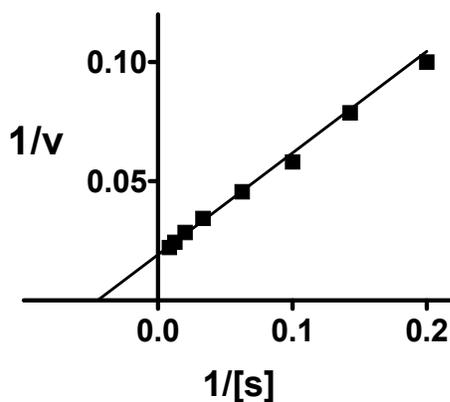
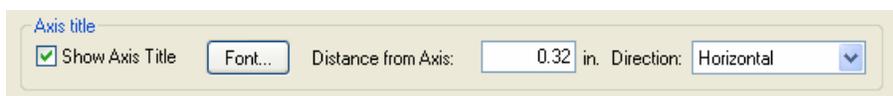
In the **Customize Ticks and Gridlines** dialog, make the entries shown below. Important: Don't leave the **Label** box blank. With your cursor in the **Label** box, press the spacebar to produce a "blank" label.



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.

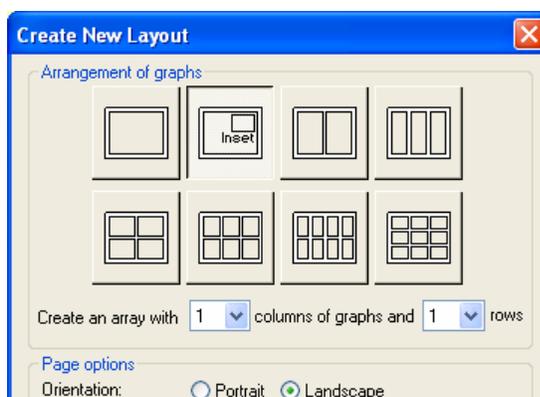


The Y-axis title can be rotated to the horizontal position by double-clicking on the Y axis and changing the **Direction** setting for the **Axis title**.



## 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 reportioning 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.