Glucose-6-phosphate dehydrogenase kinetic assay

Analysis of your data:

- In this experiment, there are two blanks to consider: 1) the absorption of the reagents themselves, measured at 0 min; 2) the negative control (0 mM G6P). Growing absorption values in the negative control indicate other sources of NADPH or other compounds absorbing light at 340 nm, originating from the yeast homogenate.
  - Substract the 0 min blank from all other measurements for each well.
  - Calculate mean ± standard deviations (s.d.) for all conditions.
  - Substract the mean values of the negative control from the mean values of all other G6P concentrations. Recalculate s.d. (error propagation!).
- Plot absorbance (mean value ± standard deviations (s.d.)) against time for each condition: one graph for one ATP concentration at a time showing seven lines of each G6P concentration (**graph 1**).
- Convert the raw absorbance values into [mM NADPH] using Lambert-Beer law. The extinction coefficient of NADPH at 340 nm is 6.22 $mM^{-1} \cdot cm^{-1}$. The layer thickness d is 0.78 cm (calculated by using the pipetted volume in the well and the radius of the well bottom). Substract the blanks as described before.
- Plot [mM NADPH] against time (mean values + s.d.) (**graph 2**).
- Calculate the slope (see appendix), which gives the initial reaction rate [mM NADPH/s], for all conditions (use the corrected mean values!). For this, it is important to inspect linearity for concentration versus time. Concentration should increase linearly over time shortly after starting the reaction. Cut off some time in the beginning and at the end if it improves linearity.
  - Note: for a proper determination of kinetic constants, the experiment should be repeated at least three times. In this practical course, we do only one measurement, therefore, we only get one value for the reaction rate for each substrate concentration and no s.d. can be calculated here.
- Use this data for analysis in GraphPad Prism: Perform nonlinear regression analysis (see appendix) to analyze v_{max}, K_m, and K_i for each ATP concentration. Have a look into the appendix to see how to arrange the data and how to proceed in GraphPad Prism. Plot reaction rates against G6P concentrations (**graph 3**; Michaelis-Menten saturation curve) together with the regression curve.
- Linearize the graph by converting the reaction rates and the G6P concentrations so that you can plot a Hanes plot ([S] as x, $[S]/v$ as y) in Excel (**graph 4**). Determine v_{max} and K_m mathematically by using the linear equations (see appendix for linear regression analysis) for each line. Show in your protocol how you calculated v_{max} and K_m.
- Calculate K_i by using this equation:

$$v_{G6PDH} = v_{G6PDH}^{max} \frac{[G6P]}{(K_{m.G6P} + [G6P])\left(1 + \frac{[ATP]}{K_{i.ATP}}\right)}$$

  Show how you rearranged the equation to calculate K_i. For the calculation of K_i you always have to insert the values you obtained from the kinetics without inhibitor for v_{max} and K_m. All the other values depend on the particular condition you're looking at.

Report

- All the aforementioned graphs.
- Table with reaction rates for all conditions and the respective linear range of the curve.
- υ_{max}, K_m, and K_i you gained by different ways of analysis.
- All calculations you had to do.
- Discussion: Compare the results.
- Appendix: a table with raw absorbance values, mean values, and s.d.

Nonlinear regression analysis

For the analysis of your plots with reaction rates against G6P concentrations you need nonlinear regression analysis. Softwares like GraphPad Prism (free demo for 30 days: http://www.graphpad.com/demos) offer this function. By fitting a curve to your data the software can determine the kinetic parameters by considering the equation for noncompetitive inhibition.

- Open a new file. Choose for x values 'Numbers' and for y values 'Enter and plot a single Y value for each point'.
- Arrange your data in this way:

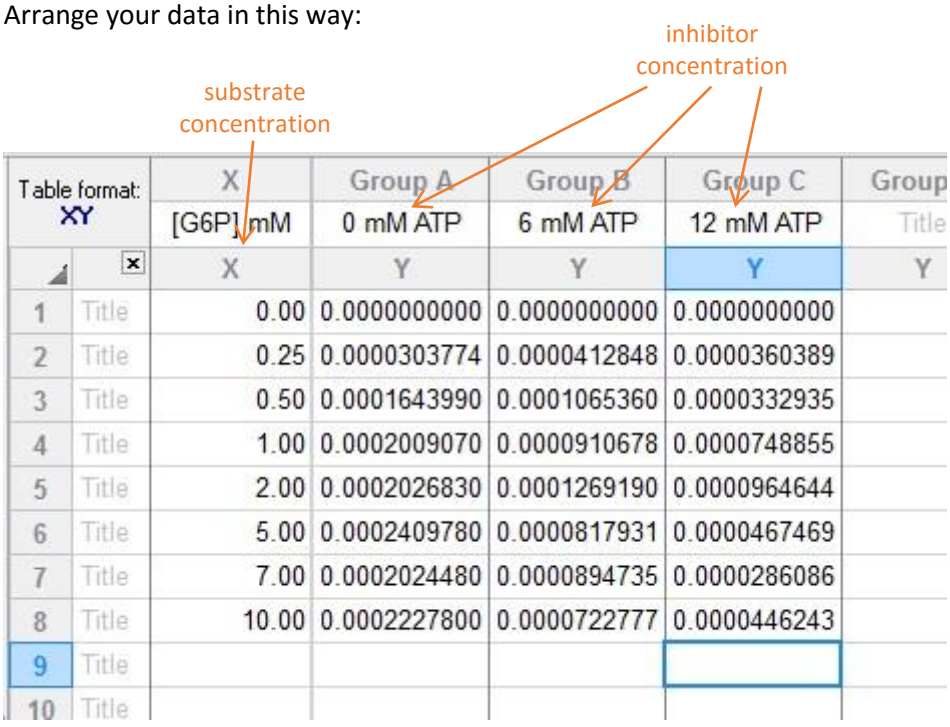

Table format: XY	X	Group A	Group B	Group C	Group
	[G6P] mM	0 mM ATP	6 mM ATP	12 mM ATP	Title
	X	Y	Y	Y	Y
1 Title	0.00	0.0000000000	0.0000000000	0.0000000000	
2 Title	0.25	0.0000303774	0.0000412848	0.0000360389	
3 Title	0.50	0.0001643990	0.0001065360	0.0000332935	
4 Title	1.00	0.0002009070	0.0000910678	0.0000748855	
5 Title	2.00	0.0002026830	0.0001269190	0.0000964644	
6 Title	5.00	0.0002409780	0.0000817931	0.0000467469	
7 Title	7.00	0.0002024480	0.0000894735	0.0000286086	
8 Title	10.00	0.0002227800	0.0000722777	0.0000446243	
9 Title					
10 Title					

- Choose 'Analyze' in the tool bar.
- A window opens where you can choose 'XY analyses' and then 'nonlinear regression (curve fit)'.
- For K_m and υ_{max} check only the data without inhibitor. Choose 'Enzyme kinetics – Velocity as a function of substrate' and then 'Michaelis-Menten'.
- For K_i check all data columns. Choose 'Enzyme kinetics – Inhibition' and then 'noncompetitive inhibition'.