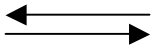
Reaction and physical basis of the assay:

The enzyme in the wet lab is ribose-5-phosphate isomerase (RpiA from spinach), which catalyzes this reaction:

ribose-5-phosphate  ribulose-5-phosphate

that is, an aldose is converted to a ketose, or the other way around. Ribulose-5-phosphate absorbs UV light (and ribose-5-phosphate doesn't), so one can measure the reaction in either direction by following the increase or decrease of absorbance at 290 nm. In the lab, we'll use this direct spectrophotometric assay with ribose-5-phosphate as substrate, so we'll be measuring an increase in absorbance. (You could also use fluorescence, which is more sensitive, but we don't have the right equipment during the course.)

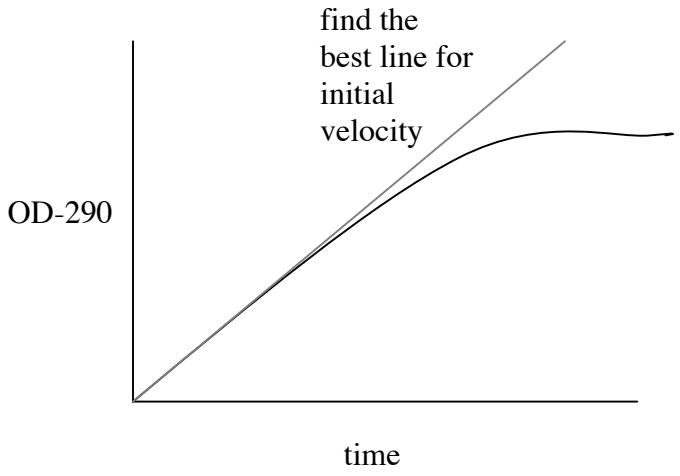
In the wet lab:

You will start with a control using wild-type enzyme, to be sure everything is working.

You will mix in a quartz cuvette:
50 ul of 1 M Tris-HCl, pH 7.5
50 ul of 0.1 M ribose-5-phosphate
(Here you would also add whatever else you want in the assay, e.g. inhibitor / salts /whatever)
then de-ionized water is added to give a final volume of 1000 ul

You will start the reaction by adding (e.g.) 5 ul of enzyme (RpiA), then mix quickly, and put into the spectrophotometer. You'll watch the change in absorbance with time (writing down the observed OD_{290} at 10 second intervals may be appropriate; it depends on how fast your reaction is going). Stop measuring when you are sure the initial (linear) phase is over.

Each experiment will look like this:



From your data, you need to find the line that best describes the linear phase of the reaction. That is, you will find the change in absorbance as a function of time ($\Delta A/\Delta t$).

Using Beer's law ($A = \varepsilon \times l \times c$, where l = path length (usually 1 cm), c = concentration of ribulose-5-phosphate, and ε = extinction coefficient, in this case 72 / cm-M for ribulose-5-phosphate), you can convert this to $\Delta c/\Delta t$. This $\Delta c/\Delta t$ is also known as *v*, the velocity of the reaction. Different experiments will give you different values for *v*, e.g. if you change substrate concentration.

Sample data to plot now:

Here, we will consider the reaction of RpiB from *Mycobacterium tuberculosis*, alone and in the presence of two different concentrations of the inhibitor 4PEH (4-phospho-D-erythronohydroxamic acid). This enzyme is completely different from RpiA, but happens to catalyze the same reaction.

The data here were obtained from a series of experiments like the one described above:

	0 mM 4PEH	0.01 mM 4PEH	0.05 mM 4PEH
[r5p] mM	ΔA/min	ΔA/min	ΔA/min
0.5	0.004	0.0037	0.0031
1	0.008	0.0061	0.0042
2	0.0099	0.0075	0.0071
5	0.016	0.013	0.011
10	0.0219	0.0182	0.0162
20	0.0223	0.0202	0.0196

Use these data to plot the Michaelis-Menten curve for the case with no inhibitor. If you run into anything strange with your numbers or plots, it could be the old Excel favorite, the comma/decimal-point problem, which can be fixed as described here: https://support.office.com/en-us/article/change-the-character-used-to-separate-thousands-or-decimals-c093b545-71cb-4903-b205-aebb9837bd1e

(Note that the Michaelis-Menten plot has a similar **SHAPE** to the plot you got your velocity estimates from, but **IT IS NOT THE SAME THING!)** Label your axes clearly with ΔA/min and mM! (I recommend that you keep your values in mM and ΔA/min until the end, since these are the "units" that you will actually be "seeing" in the lab.)

Show the Lineweaver-Burke plot for the case with no inhibitor, and use it to calculate k_{cat} and K_m. Again, label axes clearly, and state the units at each stage. Do not report to many "significant figures", e.g. 6.3 mM is enough!

Calculations for kcat seem to be particularly difficult for people, so I'll go through that here. You'll get a value for Vmax in units of ΔA/min. To convert it to units of M and sec-

1, you will need to divide by 72 / cm-M (i.e. using Beer's law with the epsilon for ribulose 5-phosphate, to convert absorbance change to concentration change) and by 60 (to get from minutes to seconds).

Since kcat = Vmax/[E], you now need to calculate the enzyme concentration in the reaction mixture. In this example, you took 5 ul of a 0.32 mg/ml solution of RpiB, and diluted it to 1 ml (1000 ul) in the reaction:

-> 0.32 mg/ml x 5 ul / 1000 ul = 0.0016 mg/ml = 0.0016 g/l

If the molecular weight of the RpiB protein is 17,300 Da, there are 17,300 g/mol:

➔ 0.0016 g/l / (17,300 g/mol) = 9.2 x 10^{-8} mol/l = 92.4 nM

Now, you should be able to calculate kcat.

Show the Hanes-Wolff plot ([S]/v versus [S]) for the same case, and determine k_{cat} and K_m from this. (Here, the x-intercept is $-K_m$ and the slope is $1/V_{max}$.)

Do you get the same values from the Lineweaver-Burke and Hanes-Wolff plots?

Now determine the apparent k_{cat} and K_m at different concentrations of 4PEH using Hanes-Wolff plots. Plot the apparent K_m for each case versus [inhibitor]. The slope of the line is K_m/K_i. Is this a competitive, non-competitive, or uncompetitive inhibitor? Will the method for determining the K_i be the same for all types of inhibitor?

Please send the Excel file (possibly accompanied by a Word file, if that is convenient for you as your summary) at the time indicated on the schedule.