MDH Assay Enzyme Hints & Tips:

Simple notes on enzyme assays and kinetics

***Enzyme assays**: Just a few simple notes and helpful hints to guide your way along the fun world of enzyme kinetics. This can be a time where you generate a ton of interesting fun data or where you generate more than your fair share of frustration. Most of the problems with assays are due to simple mistakes that are often* due to a lack of attention to detail. Enzyme assays need lots of concentration and attention to detail.

The purification, characterization and quantitation of proteins depends upon the accurate determination of both the enzyme activity and the protein concentration. These are related in the term specific activity. Protein activityranges from enzymatic activity to binding to structural roles in cells and can be assayed in a variety of ways including continuous or discontinuous (stop time) enzyme assays.

Assay Basics - Each type of assay presents particular problems that must be considered and taken into account to obtain accurate activity measurements. Similarly, there are a number of ways to determine the concentration of the protein in a given solution, and again each type of assay has its own particular limitations and assumptions. Thevarious types of assays and some examples of using such data to characterize a pure protein and to quantitate active protein are discussed below.

Chromophores: The concept of enzyme assays relies on measuring the loss of a substrate or the increase of a product. If either is readily identifiable by UV/VIS spec then your world just got easier. You can simply create the conditions necessary for the analysis of your chromophore, and you are ready to go. If not then there are a number of other means to measure your substrate or product and it is beyond the scope of this page. For MDH assays, NADH and NAD^+ absorb at two different wavelengths. You can look for changes of NADH at 340 nm. ***REMEMBER that an increase in absorbance corresponds to an increase in the concentration of NADH in the cuvette***. Look for the conversion from absorbance per min to units per ml on the MDH assay.

***Detection Method**: The study of an enzymatic reaction or assay is to follow either the loss of the substrate (a reactant) or the formation of one or more of the products. There are two main ways to measure an enzyme's reaction, coupled or direct. If the substrate or product has a characteristic absorbance or spectral "fingerprint," the changes in concentration can be directly measured. This is the case for many of the dehydrogenase enzymes. Both NAD^+ and NADH strongly absorb have strong UV absorbances, but at 340 nm, NADH has a much higher absorbance than NAD^+. Therefore, the enzymes activity can be directly measured.*

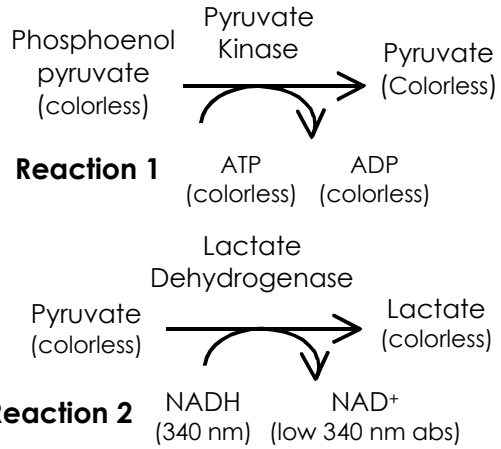

Figure 1. Example of a coupled assay. *The pyruvate kinase reaction is measured indirectly by the loss of absorbance at 340nm.*

A coupled reaction (Fig 1) uses one of the products as a reactant for an additional enzyme. That enzyme is typically easy to measure. There are lots of considerations with this type of assay. There must be enough of the second enzyme present, so that it isn't limiting the rate. The reactants for the second reaction also must be in excess, so the rate is limited only by the production of the reactant for the second enzyme.

Assay method: There are two common methods of determining the activity of an enzyme: stop time assay and a real-time assay, also called a continuous assay. A stop time assay is just that; start the

reaction and stop or read the results at a given time. This is the easiest way to do many assays at one time, **BUT** there are two things that need to be considered before doing this form of the assay. First, is the assay linear? In other words, in the time that I am running the assay,, is the product being produced (or substrate converted) at a linear rate? If the conditions of the assay tube are such that the reactants (substrate) are depleted or the products are inhibiting the enzyme, then you **CANNOT** use this assay. Second, is the compound you are measuring stable enough to wait to read and are the conditions used to stop the enzyme, i.e. acid or base too harsh to maintain the structure of the readout? We will be doing both stop time and real time/continuous assays. Meaning the change in absorbance (also known as optical density – OD) vs. time. From this graph (done on the spectrophotometer), you will select a region that is reasonably linear and determine the dOD/min and then convert it to Units of enzyme activity per ml.

Absorbance: Most specs can only read between 0.01 and 3.0 abs units. At either end of this range there will be too much noise.

Always run a control assay – This is an assay that does not contain enzyme. It will tell you any drift in the baseline absorbance. If you get an appreciable amount of drift, you will have to subtract this ΔOD/min from your enzyme assay tube. If it is about zero, then baseline corrections are not needed. The control assay will also tell you what the starting absorbance is. Remember, if after an assay your results show the opposite absorbance but no change in absorbance per min. If your enzyme is too concentrated, that is if E>>S. it is likely that the time it took you to add the components together, mix and close the lid of the spec, the assay was already completed.

Proper Rates: This depends on each enzyme. For MDH, a rate of 0.05 to 0.4 ΔOD/min is good enough. If the rate is over too fast (*see above*,) then dilute the enzyme. If you are not certain how much to dilute the enzyme, do a 1:2 or 1:5. I have included notes in the MDH assay for our favorite expressed enzyme.

Run a positive and negative control: Always include a sample that has every component except NADH. The absorbance from these samples represent the absorbance when no/little NADH is left and the reaction is exhausted substrate. ALSO include a sample with NADH but NO enzyme. This is the starting concentration/absorbance. Samples that have the same absorbance did not have an active enzyme or enough enzyme to accurately be measured (below threshold of detection). ALWAYS keep the total volume the same; thus replace your NADH or MDH with an equivalent volume of enzyme assay buffer.

Run a positive enzyme assay control: Use a sample that you know has the enzyme. Often this can be from an extract or some purified protein already prepared.

Temperature: Bring all solutions to room temp before starting assays. The easiest way to do this is mix the next set of tubes while assaying one set. Enzyme should always be on ice before adding to the enzyme cocktail or it will denature. 10°C can bring about a 2 fold change in kinetics. Be consistent.

Measuring and Pipetting: This is another problem area. Day to day variations or even batch to batch changes in how you make up your enzyme or substrate solutions will cause a lot of error. For the MDH assay, always prepare at lest 25% more reagents than you need.

Mixing: Pipetting the enzyme up and down a few times with your micropipet DOES NOT EFFECTIVELY MIXTHE REACTION. You will get irreproducible data. With the cuvette in the spectrophotometer, hold the Pasteurpipette, air expelled in one hand and the micropipettor with the enzyme in the other. As soon as you pipet the enzyme into the reaction mix, gently suck up the reaction mix into the Pasteur

pipet and expel back into the cuvette without introducing bubbles. Start recording A340nm versus time. This effectively mixes the enzyme and reaction mix. You should get reproducible data with this method. Because of the "deadtime" phenomenon discussed early, you should develop a consistent pattern of these steps before starting the recording at A340nm.

Calculating enzyme units: 1 Unit of enzyme catalyzes the conversion of 1 µmole of substrate to product per minute. To calculate the units in any spectrophotometric based assay, Beer's law is used: $A = \varepsilon l C$

Where A = absorbance (M^{-1} cm^{-1}), b = pathlength of the cell (1 cm), C = concentration of the absorbing species (M) and ε = the molar extinction coefficient.

When assaying enzyme activity we use Δ A/min (change in absorbance per time).
So $\Delta A = \varepsilon l (\Delta C)$ - as the concentration of chromaphore changes so will the absorbance.

Δ A/min = εl (Δ C/min) *adds in the time factor*
$\Delta C / min = (\Delta A/min)/ \varepsilon l$ *rearrange factors*
$\Delta C / min = (\Delta A/min)/ (6.2 \times 10^3 \times 1)$

Example of enzymes that use *NADH* in a standard 1 mm pathlength cell.

- NADH extinction coefficient = 6.2 x 10^3 $m^{-1}cm^{-1}$

Δ C/min = (Δ A/min x 0.161 x10^{-3})	M/min	*inverse of the denominator*
Δ C/min = (Δ A/min x 0.161 x10^{-3})	(mole/liter)/min	*convert M to mole/liter*
Δ C/min = (Δ A/min x 0.161 x10^{3})	(µmole/liter)/min	*convert to µmole*
Δ C/min = (Δ A/min x 0.161)	(µmole/ml)/min	*convert to ml*

* µmole/ml is the same as mM thus mM/ml

This is the mM/ml of enzyme in the assay itself. Units are expresssed in µmole/min not µmola**r** But you only measure a few µl of actual enzyme from a test tube that are placed into an enzyme assay cuvette, the concentrations must be accounted for… use the ratio of total assay volume X to enzyme added vol Y

- *Δ A/min x 0.*061 ((µmole/ml)/min) x total assay vol/enzyme added volume

Then: Units of enzyme / ml of the enzyme in your test tube. Using a 1.0 ml total assay volume with 0.01 ml of enzyme sample: Δ A/min x 0.161 x (Y/X) Δ A/min x 0.161 x (1.0/0.01)

Δ A/min x 16.1 is the U/ml in your assay cuvet with a pathway of 1.0 ml and a 10 µl enzyme addition

What is Specific Activity?

In conjunction with the determination of the activity of an enzyme solution, you can use the protein concentration and activity measurement to determine a parameter known as the "specific activity" of an enzymecontaining solution:

Specific Activity = Enzymatic Activity/Protein Concentration

As discussed above, the specific activity of a pure protein is a characteristic of that protein just as is its molecularweight or amino acid sequence and can be utilized to follow the purification of the protein.

What are the units of specific activity? The answer is complicated. If enzyme activity is measured in change of absorbance of the substrate as it is converted to product (often the case with Malate Dehydrogenase), the units of enzyme activity could be D Absorbance/minute. If the protein concentration in the cuvette is in mg/mL, then the units of specific activity would be:

(Δ Absorbance/minute)/(mg/mL)

What is the Turnover Number?

The turnover number of an enzyme (also known as Kcat or catalytic rate constant) is the maximal number of molecules of substrate converted to product per active site per unit of time. This is calculated as:

k_{cat} = Vmax / $[E_T]$ where E_T is the enzyme concentration used to determine Vmax.

k_{cat} is also \ expressed as molecules converted per enzyme molecule active site (equivalent to moles per mole) per sec, whereas the specific activity of a pure enzyme is in units (µmoles per min) per mg enzyme