

Enzyme Assay Help Guide

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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. So to help things along I have included the Bene Gesserit Littainy against Fear. Frank Herbert, Pg 19 of Dune

*I must not fear.
Fear is the mind-killer.
Fear is the little-death that brings total obliteration.
I will face my fear.
I will permit it to pass over me and through me.
And when it has gone past I will turn the inner eye to see its path.
Where the fear has gone there will be nothing.
Only I will remain. (or my ending, only the data will remain)*

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. REMEMBER that an increase in absorbance corresponds to an increase in the concentration of NADH in the cuvette. Each assay that we will be doing here (MDH or GST) has a section to help you convert the absorbance per min to units per ml.

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. NAD⁺ and NADH both strongly absorb have strong UV absorbances, but at 340 NADH has a much higher absorbance than NAD⁺. Therefore the enzymes activity can be directly measured.

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 to the production of the reactant for the second enzyme.

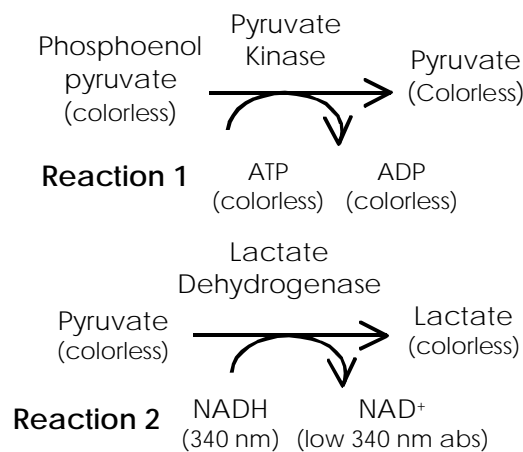


Figure 1. Example of a coupled assay. The pyruvate kinase reaction is measured indirectly by the loss of absorbance at 340nm.

Assay method: There are two common methods of determining the activity of an enzyme. A stop time assay and a real-time 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 can NOT 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 real time 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 OD/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 OD/min from your enzyme assay tube. If it is about zero then skip it. The control assay will also tell you what the starting absorbance is. Remember this 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 $E \gg S$, it is likely that the time it took you to add the components together, mix and close the lid of the spec, the assay is dead.

Proper Rates: This depends on each enzyme. For MDH, a rate of 0.05 to 0.4 OD/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 control: Use a sample that you know has the enzyme. Often this can be from an extract or some purified protein already prepared. For MDH, there is plenty of it frozen in aliquots in the Hagen Hall Freezer.

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 Pipeting: 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 there is more than enough solution to conduct many assays. As the saying goes, when the data is working don't quit, don't sleep keep going.