

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.

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 spectroscopy 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 will 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 have strong UV absorbances, but at 340 nm, NADH has a much higher absorbance than NAD⁺. Therefore, the enzyme's 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 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 ΔOD/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 in the time it took you to add the components together, mix and close the lid of the spec, the assay was already completed.

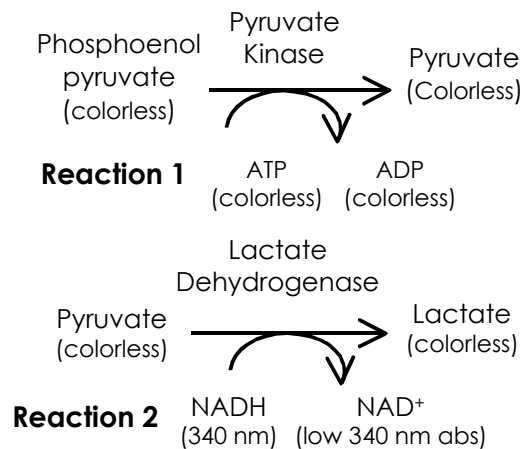
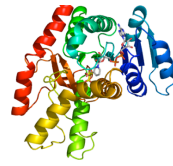


Figure 1. Example of a coupled assay. The pyruvate kinase reaction is measured indirectly by the loss of absorbance at 340nm.



Proper Rates: This depends on each enzyme. For MDH, a rate of 0.05 to 0.4 Δ OD/min is good enough. If the reaction 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 has exhausted the 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 to mix the next set of tubes while assaying one set. The 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, there is more than enough solution to conduct many assays.

Calculating enzyme units: 1 Unit of enzyme catalyzes the conversion of 1 μ mole of substrate to product per minute. To calculate the units in any spectrophotometric based assay, Beer's law is used: $A = \epsilon l C$
Where A = absorbance ($M^{-1} \text{ cm}^{-1}$), 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 $\Delta A / \text{min}$ (change in absorbance per time). So $\Delta A = \epsilon l (\Delta C)$ - as the concentration of chromophore changes so will the absorbance. **FOR PLATE READERS, ADJUST THE CALCULATIONS FOR THE PROPER PATHLENGTH.**

$$\Delta A / \text{min} = \epsilon l (\Delta C / \text{min}) \quad \text{adds in the time factor}$$

$$\Delta C / \text{min} = (\Delta A / \text{min}) / \epsilon l \quad \text{rearrange factors}$$

$$\Delta C / \text{min} = (\Delta A / \text{min}) / (6.2 \times 10^3 \times 1)$$

Example for enzymes that use NADH in a standard 1 cm pathlength cell. NADH has an extinction coefficient of $6.2 \times 10^3 M^{-1} \text{ cm}^{-1}$

$$\Delta C / \text{min} = (\Delta A / \text{min} \times 0.161 \times 10^{-3}) M / \text{min}$$

inverse of the denominator

$$\Delta C / \text{min} = (\Delta A / \text{min} \times 0.161 \times 10^{-3}) (\text{moles/liter}) / \text{min}$$

convert M to mole/liter

$$\Delta C / \text{min} = (\Delta A / \text{min} \times 0.161 \times 10^3) (\mu\text{moles/liter}) / \text{min}$$

convert to μ mole

$$\Delta C / \text{min} = (\Delta A / \text{min} \times 0.161) (\mu\text{moles/ml}) / \text{min}$$

convert to ml

This is the Units /ml of enzyme in the assay itself. But you only measure a few μ l of actual enzyme from the test tube.....

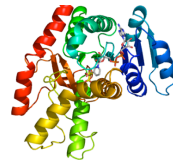
Suppose: X ml of enzyme in a final volume of Y ml gives a $\Delta A / \text{min}$.

Then: Units of enzyme / ml of the enzyme in your test tube. Using a 0.5 ml total assay volume with 0.020 ml of enzyme sample: $\Delta A / \text{min} \times 0.161 \times (Y/X)$

$$\Delta A / \text{min} \times 0.161 \times (0.5/0.01)$$

then 8.05 x $\Delta A / \text{min}$ is the U/ml in your assay cuvet

Note: This will be different for an assay that uses a different chromophore with a different molar extinction coefficient.



Malate Dehydrogenase Enzyme Assay Protocol



The reaction velocity is determined by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH. One unit oxidizes one μmole of NADH per minute at 25°C and pH 7.4 under the specified conditions.

Stock Solutions: For solutions that need to be made fresh each day make 50% more than you calculate you will need. For all powders that are stored at -20°C allow to equilibrate at room temperature 10 min before opening so as not to let water condense on the material.

Base Buffer Solutions: 1.00 M K_2HPO_4 1.00 M NaH_2PO_4

Assay Buffer: 10 mM K/Na Phosphate Buffer, pH 7.4 (1L)

Combine 8.02 ml 1.00 M K_2HPO_4 with 1.98 ml 1.00 M NaH_2PO_4

Adjust to pH 7.4 and QS to 1 liter with milliQ water

250 ml Stop Solution: 1 M Na_2CO_3 dissolved in water, pH should be around 12

20 mM OAA dissolved in assay buffer; Formula weight – 131.10. Make fresh each day.

2 mM NADH dissolved in assay buffer; Disodium Salt Formula weight – 709.40

Positive Control Enzyme Solution 50 U/ml in Assay Buffer, Porcine heart in glycerol stock

Real Time/Continuous Assay: Incubate all solutions at 25°C prior to mixing.

- 700 μl assay buffer
- 100 μl NADH
- 100 μl Enzyme solution
- 100 μl 20 mM OAA * Do not add OAA until ready to start the reaction!

- Zero spectrophotometer at 340 nm with assay buffer, use polystyrene cuvetts.
- Prepare one mixture without enzyme, replace with assay buffer to ensure constant cuvet volume. Ensure the absorbance is more than 1.0 but less than 2.5. This is the absorbance of your non-reacting sample (neg control).
- Start reaction with addition of OAA. Mix well.
- Read for 5-10 min. Observe oxidation of NADH at 340 nm.
- The assay should be linear for at least 1 min. If not, dilute as necessary and re-assay. The change in absorbance per minute should be from 0.05 to 0.5. A rate less than 0.05 $\Delta\text{OD}/\text{min}$ is generally considered no activity (0.0 $\Delta\text{OD}/\text{min}$) and a rate greater than 0.5 is not likely to be linear, meaning it is not in first order kinetics. Dilute enzyme and re-assay.

Stop Time Assay: Combine the following components except OAA into microfuge tubes, cap and vortex. Incubate all solutions at 25°C 5-10 min prior to starting reaction.

- 700 μl assay buffer
- 100 μl NADH
- 100 μl Enzyme solution
- 100 μl 20 mM OAA * Do not add OAA until ready to start the reaction!

- Once the reaction has started (mix well by vortexing and incubate in 25°C waterbath)
- Stop the reaction by adding 100 μl of stop reagent, vortex and immediately place on ice to ensure enzyme reaction is stopped.
- Transfer 300 μl into a 96 well plate. Read absorbance at 340 nm.
- Include samples without NADH (zero control) and another sample with NADH but without MDH (no reaction control).
- Ensure reaction is linear over the time of the assay by performing a series of different times and dilution of enzyme.
- Calculate $\Delta\text{OD} = \text{starting absorbance (no reaction control)} - \text{the absorbance of the sample}$
- $\Delta\text{OD} / \text{min} = \Delta\text{OD}$ divided by the total time of reaction. Use this value to calculate Units of Enzyme Activity.