



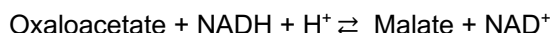
MDH 1.0 ML MDH Assay Protocol

Real Time Enzyme Kinetic Assay

Theory and Introduction

Measuring the kinetic activity of malate dehydrogenase allows us to compare the activities of mutants we construct with that of the wild-type enzyme. The information that we obtain from these assays can be used to determine how the substitution of one amino acid for another alters the activity of the enzyme.

Malate dehydrogenase catalyzes the following reaction:



Although the reaction is reversible and occurs in both directions *in vivo*, depending on the isoform, cellular location, and cellular conditions, the reaction is favored in the direction presented above *in vitro*. Therefore, it is most practical for us to run the reaction in the direction of OAA reduction to malate, with concomitant oxidation of NADH to NAD⁺. The reaction can be monitored spectroscopically because NADH absorbs ultraviolet light at 340 nm, and none of the other components of the reaction absorb light at this wavelength. Thus we can prepare a reaction mixture and then monitor the disappearance of NADH over time by measuring the decrease in absorbance at 340 nm that occurs as NADH is consumed in the reaction.

There are various ways of expressing our data; terms are defined, with units as shown below.

Definition of terms

Enzyme unit: An **enzyme unit** is defined as the rate at which an enzyme catalyzes the conversion of 1 μmole of substrate to product, per minute. In our case, the substrate is NADH, so we will express enzyme units as μmol NADH · min⁻¹.

Enzyme activity: This is defined enzyme units per mL. **Enzyme activity** is therefore expressed in terms of μmol NADH · min⁻¹ · mL⁻¹.

Specific activity: This term accounts for the mass of enzyme protein in the assay and is defined as enzyme units per mg of enzyme.

Specific activity is therefore expressed as μmol NADH · min⁻¹ · mg⁻¹.

Supplies

- A uv-vis spectrophotometer capable of measuring absorbance at 340 nm.
- Stirring rods or plastic transfer pipets for mixing. Fisherbrand (cat. #14-386-22) plastic cuvette stirrers work well.
- Semi-micro cuvettes (1.5 mL) capable of transmitting light at 340 nm. A table of cuvette materials and transmission ranges is shown. Note that plastic cuvettes cannot be used for this assay. Fisherbrand (cat. #314-955-127) polystyrene cuvettes have a wavelength range of 340-750 nm and are an economical choice.

Cuvette material	Transmission range (nm)
Optical glass	340 – 2500
Quartz	190 – 2500
Polystyrene (PS)	340 – 780
Plastic	380 – 850

Solution Preparation

- MDH assay buffer: A standard used buffer is 50 mM sodium phosphate buffer, pH 8.0. This buffer can be prepared ahead of time and stored at room temperature. Keep the assay buffer at room temperature while running the assay.
- 20 mM OAA (equivalent to 2.62 mg/mL), dissolved in MDH assay buffer. Prepare fresh, keep on ice at all times, and use within two hours. The molar mass of OAA is 131 g/mol (equivalent to mg/mmol). Consult the assay protocol (below) and determine the volume of OAA that you will need for your experiment. Prepare this volume, plus an extra 25%.

Note that you could try and precisely weigh out the OAA solid, and then dissolve it in assay buffer. But because it's easier to adjust volume than to adjust mass, especially when weighing out small masses, it's useful to use the equation shown below, in which you enter the mass of OAA that you weighed out (in mg) and calculate the volume of assay buffer (in mL) you should add to it. For example, using the calculation strategy presented below, if you weighed out 10 mg of OAA, you would add 3.8 mL of assay buffer to dissolve the OAA.

$$\text{mass OAA (mg)} \times \frac{\text{mmol OAA}}{131.1 \text{ mg OAA}} \times \frac{\text{L}}{20 \text{ mmol OAA}} \times \frac{1000 \text{ mL}}{\text{L}} = \text{volume of assay buffer to add}$$

i.e. Mass approximately 0.02 – 0.05 g of OAA.

$$\text{Volume of Assay Buffer (ml)} = ((\text{g OAA}/131)/0.02) \times 1000$$

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- 10 mM NADH (equivalent to 7.01 mg/mL), dissolved in assay buffer. Prepare fresh and keep on ice at all times. The molar mass of NADH is 704 g/mol (equivalent to mg/mmol). Use the same strategy as described above: Determine the volume of NADH solution you will need; prepare a 25% excess, weigh out the NADH, and then use the equation below to determine how much assay buffer you will add to it. For example, if you weighed out 10 mg of NADH, you'd dissolve it in 1.4 mL of assay buffer.

$$\text{mass NADH (mg)} \times \frac{\text{mmol NADH}}{704 \text{ mg OAA}} \times \frac{\text{L}}{10 \text{ mmol NADH}} \times \frac{1000 \text{ mL}}{\text{L}} = \text{volume of assay buffer to add}$$

i.e. Mass approximately 0.04 – 0.08 g of NADH.

$$\text{Volume of Assay Buffer (ml)} = ((\text{g NADH}/707)/0.01) \times 1000$$

- Enzyme solution to be assayed. Active wild-type enzyme should have a concentration between 0.5 and 100 $\mu\text{g/mL}$. MDH mutants, especially if their activities are low compared to wild type, may require a higher concentration.

Enzyme Assay Procedure

- Turn on the spectrophotometer, warm up the lamp and calibrate it with a water sample. Set the wavelength at 340 nm.
- Add 970 μL of assay buffer to the cuvette, wipe the outside of the cuvette carefully with a Kimwipe, and place in the spectrometer. Zero the spectrophotometer using assay buffer as a blank.
- With the cuvette seated in the spectrophotometer, add 10 μL of 10 mM NADH to the cuvette and mix well, using either a stirring rod or a disposable transfer pipet. An optimal starting point is 0.6 absorbance units at 340 nm.
- When you are ready to begin the assay, add 10 μL of 20 mM OAA to the cuvette and mix well.
- Immediately after the addition of OAA, initiate the reaction by adding 10 μL of enzyme solution. Mix very quickly and very well with a stirring rod or a disposable transfer pipet. Monitor the decrease in absorbance at 340 nm for 60 to 200 seconds. Ideally, the decrease in absorbance over this time period should be linear, although the first few seconds may not be, because of mixing.

Assay component	Stock solution volume	Final concentration in assay
MDH assay buffer	970 μL (or quantity sufficient to final volume of 1.0 mL)	
OAA (20 mM)	10 μL	200 μM
NADH (10 mM)	10 μL	100 μM
Enzyme	10 μL	

- If the absorbance drops very rapidly from the initial absorbance to less than half of the initial absorbance, or even close to zero, this indicates that the enzyme is highly active and was able to convert all available substrate to product. Under these conditions, the kinetics of the initial rate are not first order.
 - If this is the result, dilute the enzyme with assay buffer and run the assay again with diluted enzyme.
 - Continue to dilute the enzyme until the absorbance change ranges from 0.001 – 0.005 absorbance units per second. This range should produce linear plots between 15 and 40 seconds of assay time.
 - If, after the addition of enzyme, the absorbance remains unchanged from the initial absorbance, the enzyme may have a low activity.
 - If this is the result, increase the volume of enzyme used, and decrease the amount of MDH assay buffer used accordingly, so that the final assay volume is maintained at 1.0 mL.
- In your notebook, record the velocity of your reaction in terms of absorbance units per minute. If you recorded your reaction velocities in units of $\Delta A \cdot \text{s}^{-1}$, convert to $\Delta A \cdot \text{min}^{-1}$ by dividing by 60.

Data Analysis

Calculating Enzyme Units

Enzyme unit: An **enzyme unit** is defined for malate dehydrogenase as the conversion of 1 μmole of NADH, per minute.

When assaying enzyme activity we measured the decrease in absorbance over time and generated a plot with a slope of $\Delta A \cdot \text{min}^{-1}$. This represents the change (in our case, a decrease, which is why the slope is negative) in absorbance of NADH per minute. In our data analysis, the first step in converting our assay data to units of enzyme units involves using Beer's Law to convert our reaction velocities from $\Delta A \cdot \text{min}^{-1}$ to $\text{M} \cdot \text{min}^{-1}$. The Beer's Law equation is presented below. Note that the absorbance (of NADH, at 340 nm) is directly proportional to the NADH concentration, so as the concentration of NADH decreases as it is consumed in the assay, the absorbance decreases as well.

$$A = \epsilon c \ell \quad \text{Equation 1}$$

where...

A = absorbance (no units)

ℓ = pathlength (typically 1.0 cm for a 1.5 mL semi-microcuvette)

c = concentration of the absorbing species (in units of molarity, or M)

ϵ = the molar absorptivity (or extinction coefficient—in the older literature—expressed in units of $\text{M}^{-1} \cdot \text{cm}^{-1}$)

We can rearrange Beer's Law, Equation 1, to solve for concentration:

$$c = \frac{A}{\epsilon \ell} \quad \text{Equation 2}$$

But because we measured the decrease in absorbance over time, if we enter the reaction velocity in units of $\Delta A \cdot \text{min}^{-1}$, we obtain units of concentration per minute.

$$c = \frac{A \cdot \text{min}^{-1}}{\epsilon \ell} \quad \text{Equation 3}$$

Next we'll substitute in the value of ϵ for NADH, which is $6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$; and the pathlength, ℓ , which is 1.0 cm for a semi-micro cuvette, into Equation 3:

$$c = \frac{\Delta A}{\text{min}} \times \frac{1}{6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1} \times 1.0 \text{ cm}} \quad \text{Equation 4}$$

Since ϵ and ℓ are constants, we can simplify Equation 4:

$$c = \frac{\Delta A}{\text{min}} \times 1.61 \times 10^{-4} \text{ M} \quad \text{Equation 5}$$

Because enzyme units are expressed as $\mu\text{mol NADH} \cdot \text{min}^{-1}$, we need to convert M to μmoles . First convert from M to μM :

$$c = \frac{\Delta A}{\text{min}} \times 1.61 \times 10^{-4} \text{ M} \times \frac{10^6 \mu\text{M}}{\text{M}} \quad \text{Equation 6}$$

$$c = \frac{\Delta A}{\text{min}} \times 161 \mu\text{M}$$

Finally, we account for the volume of the assay mixture (1.0 mL) to convert μM to μmoles , and we obtain our enzyme units, expressed as $\mu\text{moles} \cdot \text{min}^{-1}$.

$$\text{Enzyme units} = \frac{\Delta A}{\text{min}} \times \frac{161 \mu\text{mol}}{\text{L}} \times \frac{\text{L}}{1000 \text{ mL}} \times 1.0 \text{ mL} \quad \text{Equation 7}$$

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$$\text{Enzyme units} = \frac{\Delta A}{\text{min}} \times 0.161 \mu\text{mol}$$

Equation 8

Calculating Enzyme Activity

Enzyme activity is defined enzyme units per mL. **Enzyme activity** for malate dehydrogenase is expressed in terms of $\mu\text{mol NADH} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$. Once we've calculated enzyme units, we can easily calculate **enzyme activity**.

$$\text{Enzyme activity} = \frac{\text{Enzyme units } (\mu\text{mol} \cdot \text{min}^{-1})}{\text{Enzyme volume (mL)}}$$

Enzyme activity is determined by taking the enzyme units (as determined using Equation 8) and dividing by the volume of enzyme used in the assay (in this protocol, we used 10 μL , which is equivalent to 0.010 mL):

$$\text{Enzyme activity} = \frac{\Delta A \cdot \text{min}^{-1} \times 0.161 \mu\text{mol}}{0.010 \text{ mL}}$$

Equation 9

$$\text{Enzyme activity} = \Delta A \cdot \text{min}^{-1} \times 16.1 \mu\text{mol} \cdot \text{mL}^{-1}$$

Equation 10

Calculating Specific Activity

The term specific activity accounts for the mass of enzyme protein in the assay and is defined as enzyme units per mg of protein. **Specific activity** is expressed as $\mu\text{mol NADH} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. Once we've calculated enzyme units, we can easily calculate **specific activity**.

Note that the enzyme concentration is determined by a protein assay, such as the Bradford assay, in a separate experiment. Calculate the mass of protein in the assay by multiplying the volume used in the assay by the concentration of the enzyme solution. Note that if you diluted the enzyme, you must account for this in your calculation of protein mass. For example, if you diluted your enzyme by a factor of 100, you'll need to divide the protein concentration by 100.

$$\text{Mass of protein in assay} = \frac{\text{protein concentration}}{\text{dilution factor}} \times \text{volume in assay}$$

Equation 11

$$\text{mass (mg)} = \frac{\text{mg} \cdot \text{mL}^{-1}}{\text{dilution factor}} \times \text{assay volume (mL)}$$

To calculate specific activity, divide the enzyme units (Equation 8) by the mass of protein in the assay (Equation 11).

$$\text{Specific activity} = \frac{\text{Enzyme units}}{\text{Enzyme mass in assay}}$$

Specific activity

$$= \frac{\frac{\Delta A}{\text{min}} \times 0.161 \mu\text{mol}}{\frac{\text{enzyme conc (mg} \cdot \text{mL}^{-1})}{\text{dilution factor}} \times \text{volume of enzyme added to cuvet (mL)}}$$

Equation 12

It is important to use the volume of enzyme added and not the final volume in the cuvet!