# Biochemistry Lab

***MDH Enzyme Continuous "real time" Assay Protocol***

This is the basic protocol for running a 1.0 ml, continuous enzyme assay (AKA real time) measuring the reduction of $NAD^+$ at 340 nm. One unit oxidizes one µmole of NADH per minute at 25°C and pH 7.4 under the specified conditions. See the introduction to enzyme assay handout for a more complete description of the background of and the performing an enzymatic assay.

Malate + $NAD^+$ <-> Oxaloacetate + NADH + $H^+$

Stock Solutions: Solutions must be made fresh each day. Prepare 25 % more than you calculate you will need. If only measuring one or two samples, one ml of OAA and NADH should suffice. For more complicated assays such as specific activity or other kinetic assays, predict your volume of OAA and NADH needed and prepare 25% more of each solution.

- ***For all powders stored at –20°C allow bottle to equilibrate at room temperature 10 min*** before opening as not to let water condense on the material.
- Use commercial MDH or MDH frozen in glycerol to test your enzyme assay cocktail.

50 mM Na Phosphate buffer (MDH Assay Buffer) pH 8.0 Can be made and stored at RT

20 mM OAA (MW= 131.1 amu) in MDH Assay Buffer. Must be made fresh each day (2.62 mg/ml of buffer)

- Mass approximately 0.02-0.05 g OAA. OAA vol (ml) =( (g massed/131)/0.02)x1000

10 mM NADH (MW = 704 amu) in MDH Assay Buffer. Must be made fresh each day (7.01 mg/ ml of buffer)

- Mass approximately 0.02-0.05 g NADH. NADH vol (ml) = ((g massed/704)/0.01)x1000
- With a spectrophotometer zeroed at 340 nm, mix 10 µl of NADH and 990 µl of MDH Assay Buffer. The OD should be close to 0.6 AU. IF higher, dilute appropriately. If to low, add a small amount of NADH needed to achieve the correct OD.

Store all of the above compounds *except* Pi buffer in ICE to prevent breakdown of solutions.

**Procedure/Important notes:**

- Allow the temperature to equilibrate prior to addition of enzyme.
- Zero spectrophotometer at 340 nm with MDH Assay Buffer.
- Use semi-micro 1.5 ml, plastic, polystyrene cuvettes
- After all components are added, the final volume should be 1.0 ml
- **The initial absorbance** should 0.6 AU (substituting buffer for enzyme). If not, adjust NADH vol.
- Remember that a flat line means either there is no activity or all of the substrate is gone. In the first case, the absorbance will remain the same as before enzyme addition; in the latter, the absorbance should be significantly decreased (much more than half of the initial absorbance). Always be aware of the magnitude of the OD@340 nm

| | Volume of Stock | Final Concentration |
|---|---|---|
| MDH Assay Buffer | 970 µl or QS to final vol of 1.0 ml | |
| OAA (20 mM) | 10 µl | 200 µM |
| NADH (10 mM) | 10 µl | 100 µM |
| Enzyme *** | 10 µl *** initiate with enzyme in spec! | 0.005-0.01 µg/ml* |

** for active wt enzyme 0.5µg/ml to 0.1 mg/ml starting protein conc…*

**Initiate Assay by addition of enzyme.** Be careful to mix well with stir rods or with disposable pipette. The assay should be linear for at least 30-60 sec. Ignore the first few seconds as those times are due to mixing. If the ΔOD/min is not linear, dilute enzyme with MDH Assay Buffer as necessary and re-assay.

- A non-linear – too fast of a rate *indicates that rate is not in first order kinetics and not following requirements to use MM equation!*

**Determining Km/Vmax**: For NADH range from 10-200µM (the high end absorbance may be too high depending on instrument) and OAA range from 10-2000µM.

## Calculating enzyme units

- Enzyme Activity (aka enzyme unit) is defined as the rate at which an enzyme catalyzes the conversion of 1 µmole of substrate to product/minute.
- Specific Activity (SA: a slightly different value) takes in account the activity per mass of protein. Thus SA = 1 µmole of substrate to product/min/µg of enzyme. *This calculation is performed when the concentration of the pure protein or in a purification to indicate changes in SA. To calculate SA, first start with the Enzyme Units then divide by µg of protein added to the assay.*

To calculate the units in any spectrophotometric-based assay, Beer's Law is used.

$$A = \varepsilon C l$$

Where A = absorbance ($M^{-1}$ $cm^{-1}$),
l = pathlength of the cell ( 1 cm for a 1ml cuvette and 0.585 for 225 µl in a 96 plate well),
C = conc of the absorbing species (M)
ε = the molar ext coefficient ($M^{-1}cm^{-1}$).

When assaying enzyme activity we measure the slope of the enzyme reaction. Change in absorbance over time (seconds or minutes). Using minutes, this means we are measuring ΔA/min (change in absorbance per time).

Thus: $\Delta A = \varepsilon l (\Delta C)$ - as the concentration of chromophore changes so will the absorbance.
Beer's law can be arranged to determine chance in concentration per time (minute) as a function of absorbance:

$\Delta$ A/min = $\varepsilon$ l ($\Delta$ C/min) adds in the time factor
$\Delta$ C/min = ($\Delta$A/min)/($\varepsilon$ x l) rearrange factors

***Units of Enz using NADH in a 1ml assay:***
*NADH has an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$*

$\Delta$C/min = ($\Delta$A/min)/ ($\varepsilon$ x l)
$\Delta$C/min = ($\Delta$A/min)/ (*6.22 $mM^{-1}cm^{-1}$* x 1.0 cm)
$\Delta$C/min = $\Delta$A/min x (0.161 *$mM^{-1}$*)
$\Delta$C/min = change in absorbance (per min) x (0.161 *$mM^{-1}$*) = mM/min substrate converted

***This is the change in mM of NADH/minute, not the final unit of enzyme activity but close… We want moles not Molarity!***

***Next convert mM/min to mmole/min, then to µmole of NADH converted/min***

Using the molarity equation: Molarity = moles/liter ; mole = liter x Molarity = mmol = ml x mM
mmole of NADH = vol of reaction (ml) x mM NADH converted in the reaction (per min)

- For a 1.0 ml total assay: mmol of NADH converted / min = 1 ml x mM NADH/ min.

Finally convert mmol/min to µmol/min (multiply by 1000) for the final enzyme unit of activity of µM substrate per min.

***Final Conversion Equation from absorbance/time to units of enzyme activity (in min)***

1) IF units are in ΔAbs /second, convert to ΔAbs/min by dividing by 60.
2) ΔAbs/min x 0.161 *$mM^{-1}$* x 1000 = µmol NADH converted / min