Biochemistry Lab
MDH Assay Experiment

Purpose: The overall goal of this set of experiments is to determine the linearity of a stopped time assay. You will first perform a continuous / real time assay to observe and relate the rate of the MDH enzyme assay over time. You will then utilize this information to design your own experiments where you will conduct a stop time assay of MDH.

Safety: The reagents are not hazardous and can be disposed in the sink. Ensure your 96 well plate and cuvets are cleaned and well rinsed with miliQ water.

Procedure: Use the information below and the information from the enzyme assay handout to design an experiment as described below. Work with your research team for the experiment. Your homework assignment is described at the end of this document.

Step 1 Continuous Assay: Using the enzyme assay handout, measure the MDH enzyme activity of a purified, 50U/ml enzyme sample. Pay attention to the time the reaction is linear. Using the spectrophotometer’s software, determine and record the change in OD / min and calculate the enzyme activity in the sample. If possible, record the enzyme assay curve using your camera phone. Print out and place the image in your laboratory notebook.

Step 2 Stop Time Assay: Time Dependence. The goal for this step is to design an experiment using your observations from the continuous assay to determine the linearity of the stop time assay. Design an experiment using standard OAA and NADH concentrations (calculate and record the final concentration in your lab book). In triplicate, measure the MDH activity for up to 10 min at several timed intervals. Include several incubation times around 15, 30 and 60 seconds. Consult with your instructor on the incubation times.
- In your lab notebook, create a table of start and stop times for each enzyme assay.
- Record the absorbance of each sample including the controls described in the handout.
- To allow reasonable handling, allow a 15 second delay between each sample.
- Depending on how your group plans the experiment, you may want to perform the assay in sets or groups of incubation times.
- Calculate the Units of MDH activity as described in the handout.
- Graph the Enzyme Activity vs time to determine the optimal time to measure MDH enzyme activity of a 50 U/ml sample.

Step 3 Stop Time Assay: Enzyme Dilution. The goal for this step is to demonstrate the importance of enzyme concentration on measuring the MDH activity in a sample. You will start with a 500 U/ml sample and create a series of dilutions (500 µl of each dilution) six to ten dilutions including the initial enzyme solution. Using the optimal time determined in Step 2, design and perform an enzyme assay with each of the dilutions using the same total enzyme reaction/incubation time. Be certain to include each of the controls.
- Graph the Units of MDH activity vs Units / ml
- Determine and comment on the importance of having an appropriate enzyme concentration to measure MDH activity in a sample. Consider what happens both when the enzyme solution is concentrated and very dilute.

Step 4 Stop Time Assay: Determine the affinity constant (Km) of OAA. The goal of this step is to become familiar with the requirements of determining affinity constants of MDH. We will do this by determining the Km for one of the substrates of MDH, OAA while holding the second substrate NADH constant.
- Prepare, in triplicate, a series of tubes to measure MDH activity with the following volumes of stock OAA:
  - 200, 150, 100, 75, 50, 25, 15, 10, and 5 µl of OAA.
  - Calculate the final concentration of OAA in the assay.
  - Adjust the volume of assay buffer to ensure the final volume is 1.00 ml after all components are added.
  - Allow the enzyme to react as determined in the earlier steps.
- Calculate the MDH activity for each OAA concentration.
- Graph the S vs V, Lineweaver-Burk and the Eadie-Hofstee plots to determine the Km for OAA (you may need to refer to your textbook or find an appropriate website for the graphs).
Step 5 Stop Time Assay: Determine the specific activity in bacterial lysate. The goal of this step is to measure the activity of MDH in un-induced and induced bacterial lysate. Uninduced lysate will still contain a significant amount of MDH activity from the bacterial enzyme, while the induced sample has been treated to express watermelon MDH at a much higher level than the control, uninduced lysate. Specific activity is the measure of Units of enzyme divided by the total protein. An enzyme with a high specific activity will have relatively more activity per protein as does a sample with less enzyme. We can use this constant to determine if MDH was induced in the bacterial lysate.

- For each lysate, create a series of dilutions of sample (500 µl).
- Determine the activity for each dilution. Depending on the concentration of MDH in the lysate, some of the samples will be too concentrated while other samples may be diluted past the threshold of detection. Consult with your instructor while designing your dilutions and experiment.
- Calculate the enzyme activity for those samples within the linear range of your experiment.
- Using the protein concentration of the lysates determined earlier this semester, determine the specific activity of each sample.
- From this data, can you determine if the induced sample expressed MDH over the endogenous level of MDH?

HOMEWORK ASSIGNMENT – Clearly answer each of the questions from Steps 1-5 (5 points each). Re-print each of the required graphs. Ensure each graph is properly labeled and prepared as described in the notebook format and lab math handout (last page).