Biochemistry Lab

Kinetics Experiment Instructions

This is a multi-part experiment. Combined, you will measure several kinetic parameters of your MDH preparation. Regardless of which wild-type MDH you've purified, you will be expected to determine each of the following kinetic measurements on each wild-type (wgMDH, MDH1, and MDH2) as well as your mutant MDH.

You will perform two sets of measurements: 1) specific activity performed in a one ml cuvette assay in the Vernier spectrophotometer and 2) Km and Vmax performed in a 96 well plate using the plate reader. EACH of the two experiments will have their own separate experiment in lab archives.

Supporting Information:

- Background for enzyme assays, assay troubleshooting and kinetics background will be found on the Biochem Lab Enzyme Assay Background handout. READ the first two pages of the background to understand the key issues of conducting a successful enzyme assay. The remaining pages are for background as needed.
- Don't forget your biochem textbook will have lots of good information and formulas for you to use in answering questions on kinetics and calculating the specific values.
- Specific instructions to perform assays using the Vernier spectrophotometer are found in the "Vernier Spec Instructions" Handout. After connecting and calibrating the spectrophotometer, scroll down to the "Absorbance versus time – Kinetics" to perform your measurements.
- Specific instructions for a 1ml continuous, real-time MDH assay is found on the "MDH Real Time Assay Protocol" handout.
- Specific instructions to perform a 96 well assay using a plate reader is found on the "96 Well Plate Reader Real Time Assay Protocol" handout.

Experiment 1: Determination of specific activity using a 1 ml cuvette MDH assay

Each student group should have access to three or four Vernier Spectrophotometers. Each student will conduct their own assay of each enzyme conducted with at least 4-6 replicates. Data from each acceptable replicate will be pooled and averaged for the team. This means each student will be measuring the enzymatic activity of MDH in all four protein assays 4-6 times then combining the data for statistical analysis.

Enzyme cocktail: This refers to all of the components of an enzyme assay minus one reagent needed to start the assay.

Before you start, determine and *record* the temperature of the assay buffer. Temperature impacts any kinetic measurement and must be recorded in your lab book. The MDH Assay Buffer should be at room temp. The OAA, NADH and enzyme (your purified protein) must be on ice. The small additions of colder solutions to the larger room temp assay buffer will not significantly impact/change the temperature.

OAA and NADH are prepared as described in the Real Time Assay Protocol. *Record* the stock concentrations, the volumes of each added into your cuvette for the standard assay and the final concentrations of OAA and NADH in the cuvette. Use this information for your calculations. Remember the final volume will always need to be 1.0 ml for this assay. *Record* deviations and adjustments to the MDH Assay Buffer volumes in your lab book for each enzyme assay recording.

Warning - Pipetting is CRITICAL for success and repeatability for these experiments.

Part 1A: Range Finder. Depending on the concentration and activity of an enzyme preparation the overall level of activity must first be determined. Prepare enough enzyme cocktail (buffer, OAA and NADH) for 6 assays and place in a clean tube leave at room temp. Blank the spectrophotometer and check the absorbance at 340 nm ensuring it is ~0.6 OD. Start the spectrophotometer and allow it to capture 10 sec or so of initial absorbance without enzyme. Then add 10 µl of undiluted purified MDH, mix well and observe the

change in absorbance over time. If the rate is to fast or slow, prepare the appropriate dilution or add more enzyme to the assay to achieve a reasonable rate that is linear for more than 30 sec. Use Assay Buffer for enzyme dilutions. Repeat until you are convinced you have a good dilution ratio. Do this for EACH enzyme preparation. Record the results of each run in your lab archives.

Part 1B: Specific Activity.

- Share dilutions needed for each enzyme preparation with your laboratory partners. Determine the dilution or volume needed for each enzyme so each person can share the results of Part 1B.
- Prepare a fresh set of enzyme dilutions for at least ten assays (i.e. make at least 100 µl of diluted enzyme.
- Prepare 10 assays worth of enzyme cocktail. Record the dilution or if low activity/dilute enzyme, adjusted volume greater than 10 µl needed for the assay.
- Conduct and record >5-6 replicates with good agreement on the rate. Record the slope and the R value for each assay. Ensure you pay attention to the units (min or sec).
- Capture the raw data for one of your runs for each enzyme to replicate on an excel graph later in the calculations section.
- Screen capture and save the screen showing the slope/rate calculation in your note book.
- Repeat for each enzyme preparation.

Calculations:

1. Using excel, prepare a graph of example Abs vs time for each enzyme solution using the raw data saved.
2. For each enzyme preparation, prepare a table of slope, R2 value, enzyme activity and specific activity. Enzyme Activity and Specific Activity derivation and conversion factors are on the second page of the Real Time Assay handout.
3. Determine the average and standard error of the mean of specific activity for each enzyme preparation for YOUR data and the group.

Experiment 2: Determination of Km and Vmax using a 96 well plate MDH assay

The second experiment is similar to the one ml real-time assay but using a 96 well plate to allow 12 concentrations of OAA and 12 concentrations of NADH to be measured with an n=5 in a relatively short amount of time. That adds up to 130 assays for a single enzyme, 520 for all four of your purified MDH assays!

This experiment will be done as a group. There are lots of dilutions and step. Assign one person to lead and another to be responsible for quality control. Let the best pipettor do the individual pipetting. This will require lots of coordination within your group and the other groups to finish all of the work.

As in the 1 ml assay, follow the plate reader assay protocol to first perform a range finder experiment for each enzyme, then create the dilutions as indicated to measure the Km and Vmax for each MDH preparation.

Once finished Capture the rates and graph the rate (specific activity) vs concentration of OAA or NADH. Use these values to generate a Lineweaver Burk plot to calculate the Km and Vmax. Alternatively, we can use a software package such as Prism GraphPad to analyze our data.

Once the Km and Vmax for each enzyme has been calculated, Each group will determine the Kcat (also known as turnover number) and catalytic efficiency.

A final graph of Enzyme Specific Activity and all other kinetic constants for each wild-type and mutants in a single table in your lab book.