MCC Summer 2023 In-Person Workshop

Suffolk University, Boston, MA

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SCHEDULE AT A GLANCE

Sunday, July 30
Afternoon - Excursion: Charles River Esplanade, weather permitting
7:00 pm Pizza in Nathan Miller Hall

Monday, July 31
8:30 am Breakfast in Samia Center (20 Somerset St) Rm 214 Conference Room
9:00 am Introductions and orientation, Preparation for experiments
9:30 am Purification: Lab is Rm 713 Samia Ctr

- Cell Lysis, and Centrifugation
- Nickel Affinity Purification

12:00 pm Lunch – in Rm 214. Exact time dependent on workflow (Milk St Café)
1:00 pm Protein Characterization Lab 713, Samia

- Protein Characterization - SDS PAGE
- Gel stain and Destain
- Bradford Protein Assay of Fractions/Pool

4:00 pm Networking With MCC Fellows: Customizing your CURE plan
5:00 pm Break
5:30 pm Travel to Dinner
6:00 pm Dinner at Antico Forno, North End

Tuesday, August 1
8:30 am Breakfast in Samia Center (20 Somerset St) Rm 214 Conference Room
9:00 am. MDH Kinetics – Introduction and instructions
9:30 am MDH Enzyme Assays Lab 713, Samia

- Measuring MDH activity, determine working dilution of protein, with Vernier Specs
- Testing with inhibitors, Determining Km and Vmax, Plate reader assay

12:30 pm Lunch – in Rm 214. Exact time dependent on workflow (Milk St Café)
1:30 pm – in Rm 214. Data Analysis and Calculations
3:00 pm Refining your CURE plan/MCC Startup Projects
3:30 pm Break into Hubs (locations TBA): Refining your CURE plan. All 2023 cohort and other attendees meet with their hub
4:30 PM Break
5:30 PM Dinner TipTap room (walking distance from Samia)

DETAILED SCHEDULE WITH PAGE NUMBERS

Sunday, July 30

Afternoon - Excursion: Charles River Esplanade, weather permitting (details on Slack)
7:00 pm Pizza in Nathan Miller Hall

Monday, July 31

8:30 am Breakfast in Samia Center (20 Somerset St) Rm 214 Conference Room
9:00 am Introductions and orientation to building, campus and plans for the day (Amy, Joe)

- 9:10 Brief intro to Cell Culture and Expression (Amy)
- 9:20 Storage of Bacteria (and Protein). (Joe)

9:30 am Purification: Lab is Rm 713 Samia Ctr

- Cell Lysis, and Centrifugation (Kristin): Page 5
  - 5 minute introduction
  - Groups will divide up and lyse with Bugbuster (Amy) or sonication (Kristin)
- Nickel Affinity Purification (Joe) Page 7
  - Groups will divide up between column format (Page 9) or batch format (Page 10)

12:00 pm Lunch – in Rm 214. Exact time dependent on workflow (Milk St Café)

1:00 pm Protein Characterization Lab 713, Samia

- Protein Characterization - SDS PAGE (Kristin) Page 11
  - We will use pre-cast gels
- Gel stain and Destain (Amy)
  - We will use BioRad Bio-Safe Coomassie stain
- Bradford Protein Assay of Fractions/Pool (start while gels are running) (Joe)
  Standard Curve, test Purification fractions, will use plate reader Page 13

4:00 pm Networking With MCC Fellows (Celeste, Kristin, Kate, Konara) Room 214 Page 4

- Customizing your CURE plan, breakout by CURE type

5:00 pm Break
5:30 pm Travel to Dinner coordinated with Celeste and Amy
6:00 pm Dinner at Antico Forno, North End

Tuesday, August 1

8:30 am Breakfast in Samia Center (20 Somerset St) Rm 214 Conference Room
9:00 am MDH Kinetics - Introduction (Amy)
9:15 am Initial rate determination (talk) (Joe) (same info sheet as above)
9:30 am MDH Enzyme Assays Lab 713, Samia Page 16

- Measuring MDH activity, determine working dilution of protein: 1mL assay Page 17
    - with Vernier Specs

  Then options:
    - Station One: Plate reader assay (Kristin) Page 22 and 25
    - Station Two: Testing with inhibitors (Joe) Page 29
    - Station Three: Determining Km and Vmax (Amy) Page 30

      Attendees can participate in one of these, or try more as time permits

12:30 pm Lunch – in Rm 214. Exact time dependent on workflow (Milk St Café)

1:30 pm – in Rm 214. Data Analysis and Calculations (Amy)
Protein Concentration Page 15
Activity Calculation Page 20
3:00 pm Refining your CURE plan: using MCC Startup Projects (Joe) Page 4
3:30 pm Break into Hubs (locations TBA): Refining your CURE plan, timeline for implementation, getting a mentor. All 2023 cohort and other attendees meet with their hub Page 4
Joe (Ellis- join on Zoom) = Western, Amy = Eastern, Lisa (will join on Zoom)=Central
4:30 PM Break
5:30 PM Dinner TipTap room (walking distance from Samia)

Customizing your CURE plan

Workshop plan

Day 1 4:00 pm Networking: Brainstorm your Syllabus

With MCC Fellows (Celeste, Kristin, Kate, Konara) Room 214

1. Brainstorm a "syllabus" for your CURE. (15 min.)
• What is the overall setup for your hypothesis development? What question are students going to answer with their hypotheses?
• What will you do in lab each week?

2. In small groups discuss questions that came up while you were brainstorming. If you can't answer them all, make a list so that we can address them tomorrow. (30 min.)

3. Refine your "syllabus" based on your discussions (15 min.)

Day 2 3:00 pm Refining your CURE plan

With (Joe) Room 214

1. Refine your "syllabus" and create a timeline for implementing the CURE (15 min.)
    Using MCC Startup Projects as needed
2. Meet in small groups to discuss your plans (15 min.)

Day 2 3:30 pm Break into Hubs

Meet with the members of your HUB (Lisa and Ellis on Zoom; locations TBA)

- Hub resources
- Getting a mentor
- Finding collaborators (for you and/or your students)

MDH Enzyme Workshop Plan

In-Person Workshop Experiments: DAY 1

Experiment: MDH Lysate Preparation

Bacterial Lysis for Recombinant Protein Purification

Background: The MDH that you over-expressed will be in the *E.coli* cytoplasm. Before purifying it, the bacterial cell wall will need to by lysed. Three methods for doing this are below: sonication, bug buster, and autolysis. Note: once you have lysed the cells, keep the high-speed supernatant on ice and proceed directly to purification. Impure protein from lysed cells is degraded by *E.coli* proteases.

Lysis Buffer: It is most convenient to resuspend the bacterial cell pellet in the same buffer that will be used for His-tag protein purification. For bacterial cell lysis by sonication or autolysis, resuspend the bacterial cell pellet from 1L of growth in 40 mL of the basic his-tag protein lysis buffer listed below (if you are working with less than 1L of bacterial growth, scale accordingly). The pellet may now be stored at -70 °C until needed. For bacterial cell lysis by bug busters, it is more convenient to store the *E.coli* cell pellet before resuspending with lysis buffer.

<u>Basic His-Tag Protein Lysis Buffer</u>

- 50 mM Tris-Cl (pH 8.0)
- 0-1 mM Imidazole (1 mM imidazole will help reduce non-specific protein binding to Ni column, but for some mutants it may be better not to include imidazole in the buffer)
- 100 mM NaCl
- 0.1 mM EDTA

Standard Bacterial Cell Lysis by Sonication:

- Thaw the resuspended bacterial cell pellet at room temperature. When thawed, place in a 50 mL glass beaker on ice. Add the following:
  - DNAse A (optional and for use with >500 ml cultures). Add 100 µl of 5 mg/ml DNAseA (Follow directions from vendor- may need to add 5mM MgCl2 and 130 µM Ca^{+2} for optimal activity.
  - **EDTA free** protease inhibitor (critical to be EDTA free or will interfere with His-tag purification) as per vendor's instructions. Once dissolved, the half-life is quick so do not add until ready. Alternatively, 1 mM PMSF may be used in place of protease inhibitors.
  - 10 mM ᛋ (2-) mercaptoethanol (optional; note: pure ᛋ ME is 14 M)
  - Lysozyme (optional-final conc of 1.0-0.5 mg/ml and incubate on ice for 30 min while rocking / resuspending manually).
- Pre-chill (4 °C) the centrifuge rotor that you will be using to pellet the bacterial cell debris after sonication.
- Sonicator settings: microtip, power: ~40%, sonicate for 1 minute total using a 15 second on, 10 second off cycle.
  - Position sonicator tip into the beaker of cell pellet (on ice) so that it is centered in the beaker and approximately half- way down in the solution.
  - Put ear protection on and close the door to the room with the sonicator.
  - Sonicate for 1 minute total, with a 15 second on, 10 second off cycle to minimize heating. When the sonicator tip is positioned properly, you will see gentle movement of the sample on the surface. If you hear a high pitch screeching (different from the usual sonicator sound) and/or you see sample being sprayed from the sonicator tip, stop the sonicator and reposition the tip).
- Save 50 µL of this crude cell lysate for SDS-PAGE (to ensure that protein was over-expressed).
- Place the sonicated sample into centrifuge tubes. Balance tubes that will be across from each other in the rotor.
- Centrifuge at 4 °C, 12,000 rpm, 20-30 minutes.
- Gently pour out the supernatant and store on ice until ready for purification.
- Save 50 µL of this high-speed supernatant for SDS-PAGE (to ensure protein is soluble).

Standard Bacterial Cell Lysis by Bug Buster: **(**Bug Buster protein extraction reagent is available from Millipore/Sigma (70584-3))

- In the steps below, you work with the crude bacterial cell pellet. If you have already resuspended it in lysis buffer, recentrifuged and isolate the pellet.
- After centrifugation, weigh the pellet to determine the wet mass of your cells.
- Add 5 mL of bug buster/gram of cell pellet. Add 1µL of DNase for each mL of bug buster. Add 1µL of EDTA free protease inhibitor cocktail per 20 mg of cells. Re-suspend the cells immediately by gently pipetting up and down.
- Incubate at room temperature for 15 minutes on a slow shaking platform.
- Centrifuge at 4 °C, 17,000 X g for 10 minutes.
- Gently pour out the supernatant and store on ice until ready for purification.
- Save 50 µL of this high-speed supernatant for SDS-PAGE (to ensure protein is soluble).

Bacterial Cell Lysis by AutoLysis: XJ Autolysis™ *E. coli* strains are an alternative for bacterial transformation and lysis from Zymoresearch. These strains are efficiently lysed following arabinose-induced expression of the bacteriophage λ endolysin protein, coupled to a single freeze-thaw cycle. XLa Autolysis cells are JM109 cells with the gamma lysozyme gene under an arabinose promotor. XJb Autolysis cells are BL21 cells with the gamma lysozyme gene under the arabinose promotor. Both XJa and XJb come with or without DE3. XJb is ideal for recombinant protein expression. For this method to work, 3 mM L-arabinose and 1 mM $MgCl_2$ must be included when inducing protein with IPTG. After growth is complete, and cells have been centrifuged, the bacterial cell pellet may be stored at -70 °C in the basic his-tag protein lysis buffer listed above.

- When ready for purification, thaw the resuspended bacterial cell pellet at room temperature and add:
  - DNAse A (optional and for use with >500 ml cultures). Add 100 µl of 5 mg/ml DNAseA (Follow directions from vendor- may need to add 5mM MgCl2 and 130 µM Ca^{+2} for optimal activity.
  - **EDTA free** protease inhibitor (critical to be EDTA free or will interfere with His-tag purification) as per vendor's instructions. Once dissolved, the half-life is quick so do not add until ready. Alternatively, 1 mM PMSF may be used in place of protease inhibitors.
  - 10 mM ᛋ (2-) mercaptoethanol (optional; note: pure ᛋ ME is 14 M) 0.5% triton X-100
- Freeze resuspended pellet on ice or isopropanol/dry ice bath. Thaw, resuspend and freeze a second time.
- Fully resuspend the pellet.
- Centrifuge at 4 °C, 8,000 x g for 20 minutes.
- Gently pour out the supernatant and store on ice until ready for purification.
- Save 50 µL of this high-speed supernatant for SDS-PAGE (to ensure protein is soluble).

MDH PURIFICATION -Workshop Plan

Overall Goal: Learn two approaches for purifying recombinant, His-tagged MDH.

Each group will have lysed 50 ml of induced culture containing MDH. Using the clarified lysate, the group will divide into two options: column chromatography and batch chromatography.

For today's workshop we will be using the BioRad Profinity IMAC Resin, Ni-charged. The resin is supplied as a 50% slurry of beads. The binding capacity is well in excess of 15 mg/ml. 50 ml of high expressing MDH should produce 3-10 mg of protein. Other preparations of Ni-agarose columns will work in place of the BioRad beads.

Option One – Gravity Flow, Column Chromatography:

- Set up a Econo-Pac 20 ml column column, with lure lock and tubing.
- Prepare ~15 x 1.5 ml microfuge tubes and 50 ml of 1X protein assay in microfuge tube rack.
- Gather two 100 ml beakers or flasks to collect flow through and wash fractions.
- Prepare 15 microfuge tubes to collect elution fractions
- Prepare a two ml column in the Econo-Pac 20 ml column using a 4 ml 50% slurry. Two ml of bed volume will be sufficient for 50 -100 ml of lysate.
- Add 10ml of Elution Buffer followed by 2x10 ml binding buffer to prepare the column for binding lysate.
- Follow the instructions on the standard chromatography – adjusting from the 1 liter instruction to the 50 ml lysate used in today's workshop.
- Collect 0.5 ml fractions.
- One designated group will reserve 50 µl of each fraction for plate reader assays.

Option Two – Batch Purification:

- Prepare ~15 x 1.5 ml microfuge tubes and 50 ml of 1X protein assay in microfuge tube rack.
- Prepare 15 microfuge tubes to collect elution fractions
- Use a column to prepare 2 ml of Ni-charged beads as described for the gravity flow chromatography.
- Using binding buffer, transfer the washed beads to a 15 ml falcon tube.
- Follow the protocol with the following changes:
    - Adjust the volumes for a 2 ml bed volume.
    - Rotate the beads for 10-15 min.
    - Centrifuge for 500-100 x g for 5 min each spin.
    - Elution buffer exposure can be decreased to 5 min. (1 min may be more than enough)

Experiment: MDH Purification

His Tag-Recombinant Protein Purification

Theory and Introduction: **Ni-Affinity Chromatography** uses the ability of His to bind nickel. Six histidine amino acids at the end of a protein (either N or C terminus) is known as a 6X His tag. Nickel is bound to an agarose bead by chelation using nitriloacetic acid (NTA) beads. Several companies produce these beads as His Tagged proteins are some of the most used affinity tags in today's market. The general method is to absorb the protein onto the beads, where low concentrations of imidazole are used to remove weakly bound proteins. If needed, the imidazole can be increased to 20 mM before most His tagged proteins will begin to elute.
Finally, higher concentrations of imidazole are used to elute the His tagged protein from the NTA beads. There are three options described here: column, batch, and spin chromatography.

Important Points to Consider for Ni-Affinity Chromatography:

- Preparation of resin: The NTA-agarose beads are very expensive and need to be saved and recycled. As long as the beads have a light blue tint to them, there is still nickel bound to the beads. The blue is due to Ni^{2+}. If the are no longer blue, the beads can be recharged with Ni^{2+} (see manufacturers recommendations).
- Average binding capacity of Ni Beads is about 5-10 mg of His tagged protein per mL of beads. Some companies claim their media can bind up to 100 mg of protein per mL of beads. Protein expression can yield 2-100 mg of His tagged protein per liter of medium.
- Expression level: The actual yield of His-tagged MDH depends on conditions and actual clones. Most of the MDH clones are in pET28 vectors which are strong promotor/expression systems. wgMDH is in a pQE vector (also a strong expression system). wgMDH, hMDH1, hMDH2 are all high to medium high expressors with 20-100 mg of purified protein from a 1L culture.
- Column and Sample Preparation: For this purification you should use the plastic column. No pump is necessary; simply allow gravity to draw the buffer and sample through the column.
- Increasing the pH of the equilibration buffer from 7 to 8 will increase the binding of your protein to the NTA beads, but will also increase non-specific binding of other proteins. Start using a pH 7.0 buffer unless you are having a difficult time getting the protein to bind to the beads. Lowering pH to 6.8 or adding 10-50 mM imidazole can reduce binding of endogenous proteins but reduce binding capacity.
- Adding imidazole to buffers will change the pH of the solution. Double check the pH of the solution after adding imidazole.
- If there is a high level of contaminant in the eluted fractions, the concentration of imidazole in the equilibration buffer may be increased to 50 – 75 mM in the wash step.
- Regeneration of the resin: The beads can be regenerated with 10 column volumes of the following: 1) MES Buffer wash at pH 5.0, 2) wash with water, 3) 20% EtOH. Store the beads in 20% EtOH.

Cell Lysate Preparation: Follow bacterial cell lysis protocol.

Column Basics:

- Purification of MDH from 1 liter of bacterial culture will require 20 mL of beads.
- A rule of thumb is to wash with 10-20 column volumes (volume of packed beads) for the wash buffer.
- Elute in a total of 5-10 column volumes of elution buffer.
- Divide the elution into 8-12 total fractions which will be viewed by SDS-PAGE.

His-Bind Buffers (for all 3 His-Bind purification options):

His-Binding and Lysis Buffer:	*His-Wash Buffer:*	*His-Elution Buffer:*
• 50 mM Tris-Cl (pH8.0) • 1 mM Imidazole* • 100 mM NaCl • 0.1 mM EDTA • ** include 1 mM PMSF made fresh	• 50 mM Tris-Cl (pH8.0) • 300 mM NaCl • 10 mM Imidazole* • 0.1 mM EDTA • include 1 mM PMSF made fresh	• 50 mM Tris-Cl (pH8.0) • 50 mM NaCl • 300 mM Imidazole • 0.1 mM EDTA • include 1 mM PMSF made fresh

NOTES:

- Some use 50 mM sodium phosphate buffer in place of Tris buffer.
- *Depending on individual protein binding, imidazole in binding/lysis buffer and His-Wash Buffer can range from 0.1-10 mM.
- ** Add a broad range protease inhibitor if budget allows or protein degradation requires. In a pinch, inhibitors are only used in binding/lysis buffer.

OPTION 1: COLUMN CHROMATOGRAPHY *Purification Instructions (per 1L culture):*

Preparation of Ni-Agarose Beads/Resin:

- Prepare 20 mL of beads by transferring 40 mL of a 50% slurry of beads into a clean column. If the beads have never been used, wash by running 100 mL of water followed by 200 mL of His Binding/Lysis Buffer through the column. If the beads have been used, wash by running 100 mL of His Elution Buffer followed by 200 mL of His Binding/Lysis Buffer through the column. This can be done ahead of time. Prepared beads should be stored at 4 °C with a few mL of His Binding buffer at the top of the column (do not let the column run dry).

Charging (Protein Binding to) the Resin:

- Load the Column: Add clarified lysate (high speed supernatant following centrifugation after bacterial cell lysis) to the top of the prepared column. Be careful not to disturb the beads for better resolution during elution. If the lysate is clumpy, cloudy, or precipitated material is present re-centrifuge and keep the supernatant. If the solution is viscous the DNA will slow the column down – sonicate or treat with DNAse as described in lysis protocol.
  - Save the eluant (flow-through) for analysis by SDS-PAGE.
  - If there is a concern with binding efficiency the flow-through may be reapplied before elution.
- Wash I**:** Wash the column with 50 mL of His Binding and Lysis Buffer. Save this wash for analysis by SDS-PAGE.
- Wash II: Wash the column with 50 mL of His Wash Buffer. This step will remove some of the weakly binding protein. Continue with the His-Wash buffer until no detectible protein is found in eluant*. Save this wash for analysis by SDS-PAGE.
- Elution: Elute the protein with 10 x 1.5 mL of His Elution Buffer additions. Save ALL fractions. Stop eluting only when protein is no longer detected in the eluant.*
- Check each fraction for total protein (Bradford assay) and determine purity by SDS-PAGE with Coomassie staining

*** Important Note: A quick check of wash and elution fractions with a Bradford assay (20 μL sample mixed with ~1 mL of 1X Bradford dye; see Bradford protocol) will inform you of the relative amount of protein in the fraction.**

OPTION 2: BATCH CHROMATOGRAPHY *Purification Instructions (per 1L culture):*

Preparation of Ni-Agarose Beads/Resin:

- Prepare 20 mL of beads by transferring 40 mL of a 50% slurry of beads into a clean column. If the beads have never been used, wash by running 100 mL of water followed by 200 mL of His Binding/Lysis Buffer through the column. If the beads have been used, wash by running 100 mL of His Elution Buffer followed by 200 mL of His Binding/Lysis Buffer through the column. This can be done ahead of time. Prepared beads should be stored at 4 °C with a few mL of His Binding buffer at the top of the column (do not let the column run dry).

Charging (Protein Binding) The Resin:

- Add the 20 mL of prepared Ni-NTA resin to the clarified supernatant (high speed supernatant following centrifugation after bacterial cell lysis) and keep mixed by gently rotating for a minimum of 2 hours at 4°C (this step is conveniently done overnight in the cold room). This step is easily done in a 50 mL falcon tube.

Batch Purification:

1) Allow charged resin to settle at room temperature. This step can be aided by centrifugation for 1 minute at 1000g.
2) Carefully remove as much supernatant as possible with a pipette. Save this "flow thru" supernatant for analysis by SDS- PAGE.
3) Add 40 mL of Lysis/Binding Buffer to the retained beads, resuspend with gentle mixing for 1-2 minutes, and allow resin to settle as step 1. Remove supernatant as step 2.
4) Add 50mL of Wash Buffer to retained beads, resuspend with gentle mixing for 1-2 minutes, and repeat step 1.
5) Remove supernatant as in step 2. Save this supernatant as "wash" for analysis by SDS-PAGE.
   - Repeat step 4 and 5. Complete three total times including the first wash of step 4.
   - After each wash, check removed supernatant for protein using quick Bradford test* If no protein is present, proceed to step 6. If protein is present in the supernatant, repeat steps 4-5.

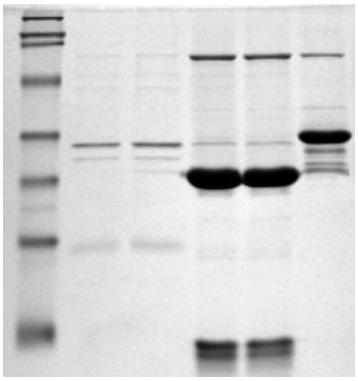
   This His-tagged MDH bound to beads can be stored at 4°C for elution at a later date.

6) Elution. Add 10 mL of Wash Buffer to the beads. Allow resin to settle (can be aided by centrifugation for 1 min at 1000g)
7) Carefully remove supernatant from resin (this should contain no protein- check using quick Bradford test*)
8) Add 10 mL of Elution Buffer and gently resuspend. Keep in suspension for 15 minutes.
   - Allow resin to settle (this can be aided by centrifugation for 1 minute at 1000g).
   - Carefully remove supernatant and save in a tube labeled elution 1 for analysis by SDS-PAGE.
9) Repeat step 8 for 4-10 additional elution fractions.
10) Check each fraction for protein using quick Bradford test*.

*** Important Note: A quick check of wash and elution fractions with a Bradford assay (20 µL sample mixed with ~1 mL of 1X Bradford dye; see Bradford protocol) will inform you of the relative amount of protein in the fraction.**

Experiment: Protein Characterization: SDS PAGE

Background



When an electrical field is applied across a solution, the movement of the charged particles (proteins) is influenced not only by the charge but also the voltage, distance between electrodes, the size and shape of the molecule, temperature, and time. Polyacrylamide gels are polymerized products of acrylamide and bisacrylamide (n,n'-methylene bisacrylamide). When ammonium persulfate (APS) [-03S-0-0-S03-] is added to water it breaks down, forming unstable O4S-. free radicals, which can then initiate the polymerization reaction. TEMED (tetra methyl ethylene diamine) is a tertiary amine that reacts with these radicals to form TEMED free radicals, which in turn react with acrylamide to induce polymerization. The addition of bisacrylamide forms crosslinking of the long acrylamide molecules creating an average size pore. The size of the pore can be regulated by the concentration of acrylamide and bisacrylamide. Relatively small proteins will migrate faster through SDS-PAGE gels than larger proteins. Gels with lower percent bisacrylamide are more useful for larger proteins. In this case the smaller proteins will not be retarded by the size of the pores and will have a similar mobility. That is, they will run very close to each other at the bottom of the gel, separating the larger proteins more distinctly.

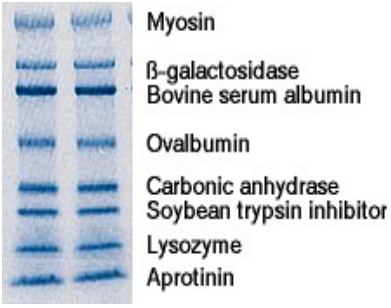
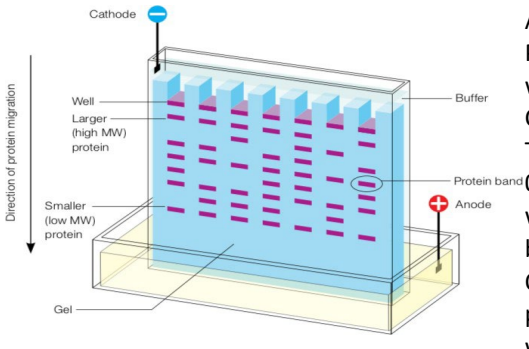


Fig 2 Prestained Molecular Weight Standards. Prestained standards are used in SDS-PAGE and Western Blotting applications. They provide a quick and easy way to assess blotting efficiency and allow continuous monitoring of protein separations during electrophoresis. Pre-stained standards can be used as a control for repetitive blotting experiments or in locating proteins for excision from unstained preparative gels.

Traditional SDS PAGE - The gels often used in labs are discontinuous gels. These are gels that contain both a stacking gel and a resolving gel. When glycine from the upper reservoir enters the low pH of the stacking gel, it will principally be in the neutral form. This prevents glycine from being an effective carrier of electrical current. The Cl- ions now carry the current and migrate toward the anode. During this step the Cl- ion concentration becomes lower at the top of the gel and higher at the bottom of the stacking gel. As electrophoresis continues, protein molecules (which are negatively charged due to the SDS) will become greatly retarded, allowing the trailing protein molecules to catch up. The stacking gel is very low in percent to ensure there is little separation based on the pore size. This will ensure all of the proteins enter the resolving gel at the same time to get small tight bands.



After running a 10-12% SDS-PAGE gel, the gel is stained with Coomassie blue. Coomassie blue is a dye that will bind to the acidic amino acids in proteins. This method of staining will stain most proteins in the gel with as little as 0.1 µg of protein in a single band. Washing of the gel with destain solution will remove the unbound dye from the gel but not the protein, leaving behind several nice blue bands that show each protein.

Once the gel is destained, we can measure the relative mobility (Rf) of the proteins and use molecular weight standards to determine the molecular weight of an unknown protein. The Rf is calculated by dividing the distance the protein migrates by the distance the tracking dye migrates.

The tracking dye (bromophenol blue) is a small colored molecule that runs much faster than proteins and approximates the movements of small ions in the electric field. Therefore, it is important not to run the dye off the bottom of the gel. A plot of the relative mobility for each standard protein vs. the log of the molecular weight of the standard proteins will be linear, and the molecular weight of the unknown protein can be determined from its position on the plot. To determine the Rf, measure the distance the protein has moved from the top of the resolving gel to the center mass of the band of protein. Divide this value by the distance the dye traveled. This is the relative distance.

Pre-Cast Gels – There are many options to purchase pre-cast gels. While they are convenient, the cost is 15 times that of a "homemade" gel. Follow specific manufacturer's instructions for buffers and preparation of pre-cast gels.

Sample Preparation

- Prepare each of the following samples in a 500 µL eppendorf tube, heat at 100 °C for 10 minutes before loading into the gel
    1. For the crude cell lysate (CCL) and high-speed supernatant (HSS): 8 µL sample, 2 µL 4X Blue Juice gel loading buffer (BJ), 1 µL βMeSH (beta-mercaptoethanol). Note βMeSH is a thiol and does not smell good. I use in the hood.
    2. For all other purification samples: 20 µL sample, 5 µL 4X Blue Juice gel loading buffer (BJ), 4 µL βMeSH

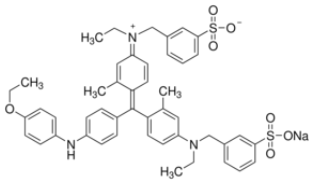
Gel Set-up, Load and Run

- Open the purchased, precast 12% SDS gel (or pour a 12% SDS gel) and remove the outside strip
- Place two of these gels in the Biorad Mini-PROTEAN gel holder assembly, one on each side. Orient the gels so that the shorter front plate is facing back towards the rubber gasket.
- Place the gel holder assembly into the tank, fill with 1X Tris-glycine SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) so that buffer covers the wells of each gel.
- Load up to 20 µL of each sample (gel loading tips help), or 10 µL of the markers. Tips to consider when loading samples: make sure you have loaded samples in a way that you can tell which is lane 1 vs. lane 10 after the gel comes out of stain/destain (for example, don't put samples in lanes 1 and 10 that would be hard to distinguish between). If you are going to stain/destain 2 gels in the same container, make sure you can tell which gel is which (for example, markers in lane 1 for one of the gels and markers in lane 2 for the other gel would work).
- Place the top on the tank and plug into the power supply.
- Run the gel at ~100V until the blue tracking dye reaches the bottom of the plate.
- Gently remove the gel from between the plates and stain to visualize the protein. We will use biosafe coomassie to quickly stain/destain the gels.

Experiment: Bradford Protein Assay

Bradford Reagent Micro-Assay (96 well plate) version

Theory and Introduction: Protein Assay – There are many reasons to conduct a protein assay. During the purification of a protein, you determine sample purity by measuring the amount of enzymatic activity, taking into account the protein concentration. To compare samples on an SDS-PAGE gel or a Western blot, you need to add the same amount of protein from various preparations to obtain consistent results. Many protein assays take advantage of a reaction between a reagent dye and the protein of interest that results in a shift or increase the absorbance at a particular wavelength. There are a few regularly used methods to determine protein concentration. The Lowery/Bicinchoninic acid assay (BCA) utilizes a copper-protein chelate where the absorbance of a reduced copper solution is measured to detect protein. The intrinsic UV absorbance of Phe, Tyr and Trp residues within a protein contribute to the extinction coefficient of a protein which is used in the Warburg-Christian method. The individual amino acid composition varies widely from protein to protein; thus, knowledge of the protein sequence is required. Some proteins contain few if any Trp residues, while the number of Tyr and Phe residues can change several fold from protein to protein. Therefore, contaminating proteins may contribute unequally to the UV absorbance of a sample, making this method unsuitable for determining concentration of all but the purest of protein samples.



Structure of CBB G-250

Bradford reagent: The Bradford protein assay is a colorimetric protein assay originally described by Marion Bradford (Anal Biochem 72:248-54, 1976). This method uses a disulfonated triphenylmethane compound called Coomassie Brilliant Blue G-250 (CBB G-250). In the absence of protein and in acidic solution, the dye is red-brown. Under acidic conditions, the protein binds to mostly basic amino acids such as arginine, lysine, and other amino acids including histidine. These dye-amino acid interactions result in a shift in pKa of the dye leading to a color change that ranges from green/brown tint to blue. The more protein, the more intense the blue color. The absorbance of the protein-dye complex is measured at 595 nm. Amino acid composition and post-translational modification of proteins can lead to variation of dye intensity.
Bradford dye is easy to use, is fast and sensitive, but several compounds can interfere with the assay (conduct a search or look on the Biorad website for Bradford reagent interfering compounds). Finally, care must also be taken that the dye is not the limiting reagent in the assay as this will result in a non-linear standard curve.

Standard curve: A good protein assay requires two main components: 1) a standard curve using protein of known concentration and 2) the samples of unknown concentration. The concentration of the unknown protein is determined using data from the standard curve, which is a plot of absorbance of proteins samples of known concentration vs. the concentration of those samples. Depending on the method, proteins generally vary in the degree of reaction with the color dye in the different protein assays. Therefore, it is important to note which specific control or reference protein was used to make the standard curve. Two common proteins used for standard curves are bovine serum albumin (BSA) and an immunoglobin (IgG). These two proteins have a different amino acid composition, which leads to a different absorbances at the same protein concentration on the standard curve. These differences

lead to slight discrepancies in the final determination of the unknown protein concentration. Furthermore, the color development is dependent on the amino acid composition of the protein or the presence of a prosthetic groups (especially carbohydrates). A purified sample of the target protein being assayed or a closely related protein is a preferred standard, if available.

Be linear: Once you have performed the assay, the results are graphed to generate the standard curve. Before calculating the concentration of your unknown protein from the standard curve, you must be certain that the absorbance is a linear function of standard concentration. A common reason for a lack of linearity is the depletion of one of the reagents necessary for color production. Another reason for error is that the absorbance is too high for the detector in the spectrophotometer. Remember that absorbance is 1/transmittance, so a high absorbance means very little light is making it through the sample. The spectrophotometer reads how much light goes through the sample and low light levels lead to a decrease in accuracy of measurement. This decrease in measurement accuracy manifests as a plateau in the standard curve and you should not use the points with the high absorbance. High absorbance values are between 1.2-
1.5 for most plate readers and 1.7 – 2.5 for most spectrophotometers. If multiple measurements have high absorbance values, then the accuracy of the standard curve will significantly decrease. Thus, readings should always be taken in the region where all reagents are in excess of the standard or sample protein concentration so that the curve of absorbance vs. standard concentration is linear.

Unknown Sample Preparation*:* In parallel with the measurement of the standard proteins, you will measure the absorbance of your sample protein. When determining the protein concentration of an unknown sample, several dilutions should be used to ensure the protein concentration is within the range of the assay where the Bradford reagent is in excess of the sample. Usually several 10-fold dilutions are used to get the unknowns within the absorbance range of the standard curve. It is important that the sample absorbance values are between the minimum and maximum absorbance values of the standard curve. When you don't know the concentration, this is hard to predict, so several dilutions are used to try to get as many measurements within the standard curve range as possible. Without the additional dilutions, you will have to start over again. Don't forget that the dilutions values must be considered when calculating the final concentration of the protein. Finally, as with every assay, the absorbance of a "blank" must be included as a control. The blank or the well without a standard protein contains the same buffer as the samples. This way the effect of buffer on the protein assay reagent can be determined and the absorbance of each sample can be corrected for this buffer or solvent absorbance. The "blank" is used to set the instrument to the 100% transmittance or 0 absorbance.

Protocol – *NOTE*: *USE A CLEAR 96 WELL PLATE.*

FIRST ADD PROTEIN SAMPLES (EITHER STANDARDS OR UNKNOWNS) OR THE BLANK SOLUTION TO THE WELL ***THEN*** *ADD BRADFORD REAGENT TO THE WELLS. Calculate your additions in your lab book in table format.*

Prepare 0.5 ml of each known BSA protein standard. Freeze unused standards for your next assay!

- The stock BSA concentration is 1.00 mg/ml. Use water to dilute. Adjust if the concentration is different.
- Protein standards = 0.0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml.

Determine how many wells you will need for your assay.

- Run EACH standard and sample in triplicate. (If checking column fractions, n=1 will be fine.)

3 - Add 250 µl of 1X Bradford reagent in each well.

4 - Add 10 µl of EITHER BSA standard OR your unknown sample to the Bradford Reagent

5 - Determine the absorbance at 595 nm using the plate reader

- Use methods or protocols to find the Bradford assay file and measure your protein.
- Record your data and include it in your notebook.

6 - Wash your plate and if needed a rinse with methanol followed by water to remove residual blue dye.

Calculations –

1. Prepare a graph of the standards (the standard curve) with the dependent variable (mg/ml) on the X axis and the independent variable (abs 595 nm) on the Y axis.

- Calculate the protein concentration of your knowns using C1V1=C2V2. *See #3 below*.
- Plot the data as a scatter plot and do not "connect the dots" of the standard curve. Instead perform a linear regression to your data.
- Show the regression "r" value in the box of your graph. The closer to 1.0 the number the more in line your data are. 0.9 to 1.0 are reasonable values. *Look at the line, if the last one or two points indicate that the absorbance does NOT increase in a linear fashion with added protein, exclude these values from your curve. Think about why this might be the case.*
- You do NOT know the protein concentration of your experimental/unknown samples. Therefore, the unknown samples/fractions/experimental assays can NOT be graphed on your standard curve.

2. Use the linear regression to calculate the concentration in your unknown samples.

- The equation from the standards has the form of Abs = slope x [concentration] + constant
- If you diluted your sample, don't forget to multiply the concentration by your dilution factor.

3. When performing dilution calculations for the standard protein and the sample protein, ignore the volume of the Bradford reagent. You need to know the concentration of protein that you added to the cuvette!

- Look at the consistency of the concentration measurement for your sample protein. Think about whether the concentration from each dilution should be the same or different after you correct for the dilution.

MDH Enzyme Assay Plan

Part One - Range Finder and Specific Activity:

Beginning experiments using recombinant MDH should be first assessed to determine the dilution needed to achieve a first-order rate that is linear for 15-30 seconds. Typically, this rate will have a change in absorbance of 0.002 to 0.005 dOD/second.

- Using the protocol for a 1 ml real-type (continuous) MDH assay (Page 17), measure the rate for an undiluted sample of MDH. Remember to run the spec for 5-10 seconds before adding MDH and mixing.
- Record the screen and interpret the results as if you were teaching a student new to enzyme assays. Consider the starting absorbance, what a reaction would look like if the enzyme had no activity and interpret the resulting curve from your undiluted sample of MDH.
- Depending on the linearity of the rate, now determine the appropriate dilution to achieve a linear rate. To do this prepare 3-4 tubes of diluted purified enzyme using assay buffer as a dilutant. Start with a 1:50 dilution. Use the following input for the rest of the dilutions.:

  A 1mg/ml solution of wild-type MDH of most species will require a 1:200-1:400 to achieve a linear, first order reaction.
- Measure the activity of these dilutions and identify the appropriate dilution.
- Using the appropriate dilution, measure the rate of the enzyme 3-4 times. It is permissible to terminate the reaction after the reaction is no longer linear to save time.
- Perform a Grubb's test (use the online Prism/GraphPad webpage) to look for outlier data. Later we will use this information to convert dOD/sec to units of enzyme activity and specific activity.

Part Two – Three Enzyme Assay stations: Workshop participants will choose which station to participate. If time allows, two or three station experiences may be achieved.

Station ONE – Plate Reader Assay: Determine MDH activity using stop time and/or continuous MDH assays. Page 22, 25

Station TWO - Impact of Inhibitor on MDH: Determine the effect of MDH by small molecule. Page 29

Station THREE – K_m/V_{max}: Determine the Michaelis constant (~affinity) and maximum velocity for MDH with OAA. Page 30

PART ONE: MDH 1 mL Enzyme Assay Protocol

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:

Oxaloacetate + NADH + H+ ⇄ Malate + NAD+

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 in enzyme units per mL. **MDH activity** is 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. Ex: Fisherbrand (cat. #14-386-22) plastic cuvette stirrers.
• 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

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

i.e. Mass approximately 0.02 – 0.05 g of OAA.
Volume of Assay Buffer (ml) = ((g OAA/131)/0.02) X 1000

- 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. Volume of Assay Buffer (ml) = ((g NADH/704)/0.01) X 1000

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

Enzyme Assay Procedure

1. Turn on the spectrophotometer, warm up the lamp and calibrate it with a water sample. Set the wavelength at 340 nm.
2. 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.
3. 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.
4. When you are ready to begin the assay, add 10 µL of 20 mM OAA to the cuvette and mix well.
5. 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.

6. 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 $\Delta A \cdot s^{-1}$, convert to $\Delta A \cdot min^{-1}$ 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 $\Delta A \cdot min^{-1}$. 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 $\Delta A \cdot min^{-1}$ to $M \cdot min^{-1}$. 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.

$$A = \varepsilon c \ell \qquad \text{Equation 1}$$

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 $M^{-1} \cdot cm^{-1}$)

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

$$c = \frac{A}{\varepsilon \ell} \qquad \text{Equation 2}$$

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

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

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

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

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

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

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

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

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

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 µmoles $\cdot$ min^{-1}.

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

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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 μmol NADH · min^{-1} · 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

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

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

$$\text{mass (mg)} = \frac{\text{mg} \cdot \text{mL}^{-1}}{\text{dilution factor}} \times \text{assay volume (mL)}$$ Equation 11

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

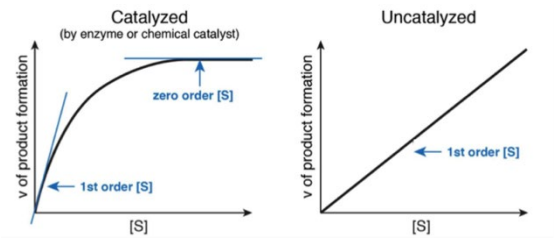
$$\text{Specific activity} = \frac{\text{Enzyme units}}{\text{Enzyme mass in assay}}$$

$$\text{Specific activity} = \frac{\frac{\Delta A}{\text{min}} \times 0.161\ \mu\text{mol}}{\frac{\text{enzyme conc (mg} \cdot \text{mL}^{-1})}{\text{dilution factor}} \times \text{volume of enzyme added to cuvet (mL)}}$$ Equation 12

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

PART TWO: Measuring an enzyme's behavior... Kinetics

Enzyme kinetics is the study of the rates and mechanism of enzyme-catalyzed reactions. It involves the measurement and analysis of the reaction rates, substrate and product concentrations, and other factors that influence the enzyme-catalyzed reaction. Enzymes are catalysts, and like any other catalyst, an enzyme decreases the energy barrier of action. As biological catalysts, enzyme kinetics is the study of the rates of the reaction and the affinity (how well a substrate binds to the enzyme) in a reaction. One model used to study enzyme kinetics is the Michaelis-Menten equation. The Michaelis-Menten equation describes the relationship between the rate of an enzyme-catalyzed reaction and the concentration of substrate. The equation assumes that the substrate binds to the enzyme quickly and is followed by a slower step, where the complex of substrate and enzyme breaks down to form product and free enzyme. The equation is a mathematical way to predict and describe the relationship between the rate of an enzyme reaction and the concentration of substrate. The equation can be used to determine the maximal rate of the reaction (Vmax), the binding affinity (Km) and the enzyme's catalytic efficiency (kcat/Km). Enzyme kinetics can be used to study the effects of various factors on enzyme activity, including pH, temperature, substrate concentration and enzyme concentration. Kinetics can also be used to study the inhibition of an enzyme's activity by various inhibitors, including competitive, noncompetitive and uncompetitive inhibitors. Enzymes have an order or reaction. First order, second-order, and zero-order enzyme kinetics refer to different types of enzyme-catalyzed reactions, based on the dependence of the reaction rate on the concentration of the substrate.



In first-order enzyme kinetics, the reaction rate is proportional to the concentration of the substrate. This means that as the substrate concentration increases, the rate of the reaction also increases linearly. The Michaelis-Menten equation is an example of a first-order kinetics model, where the reaction rate (v) is proportional to the substrate concentration ([S]), and can be described by the equation where Vmax is the maximum velocity of the reaction and Km is the Michaelis constant, which reflects the affinity of the enzyme for the substrate.

$$v = Vmax[S]/(Km + [S]),$$

In second-order enzyme kinetics, the reaction rate is proportional to the square of the substrate concentration. This type of kinetics is often observed in enzymes that catalyze bimolecular reactions, such as enzyme-catalyzed reactions that involve the binding of two substrate molecules. An example of a second-order kinetics model is the Briggs-Haldane equation, which describes the reaction rate (v) as proportional to the product of two substrate concentrations ([S1][S2]), and can be described by the equation:

$$v = (Vmax[S1][S2])/([S1]Km1 + [S2]Km2 + [S1][S2]),$$

where Km1 and Km2 are the Michaelis constants for each substrate.

In zero-order enzyme kinetics, the reaction rate is independent of the substrate concentration. This occurs when the enzyme is saturated with substrate and the reaction rate is limited by the rate of the catalytic process. The reaction rate is constant at this point, and cannot be increased further by increasing the substrate concentration. This type of kinetics is often observed in enzymes with high substrate affinity or when the substrate concentration is very high. An example of zero-order kinetics is the reaction rate of alcohol dehydrogenase, which catalyzes the conversion of ethanol to acetaldehyde, but is saturated with ethanol at physiological concentrations, resulting in a constant reaction rate.

Station One-A: Plate Reader Stop-Time MDH Assay

For plate readers @ 340 nm absorbance detection

The characterization of enzymes depends upon the accurate determination of enzymatic activity. This is important to our study of mutants, in which the enzyme activity of a mutant enzyme is compared to that of the wild type enzyme. These data provide insights into the enzyme mechanism and structure/function relationships. In other studies, we may be interested in how various ligands or interactions with other proteins affect the activity of the enzyme of interest. In this document, we address the various aspects of the enzyme assay procedure required to produce high quality data.

Continuous versus discontinuous assays

There are two common methods of determining the activity of an enzyme: (1) continuous, or "real-time" assays, or (2) discontinuous or "stop-time" assays. In a stop-time assay, a reaction mixture containing all of the required components, except one, is prepared and equilibrated, and the reaction is initiated by adding the missing component (usually, but not always, the enzyme). The reaction is then halted after a given time, usually by denaturing the enzyme in some way by adding acid or base, a metal ion chelator (for metalloenzymes) or detergent. For stop-time assays it is important to verify that the rate of the reaction (as determined by either the disappearance of substrate or the formation of product) is linear over the time period of the reaction. Reaction rates are not linear if substrates are depleted over the course of the reaction, or if the product acts as an inhibitor of enzyme activity. If either of these conditions apply, a stop-time assay cannot be used. A second consideration is that it must be verified that the conditions used to denature the enzyme to stop the reaction do not interfere with the absorbance measurement of the chromophore.

Controls

The controls described below should be run, prior to beginning your kinetic studies.

- *A no-enzyme control*: This reaction mixture contains all components except for the enzyme. Monitoring the absorbance of this reaction mixture at 340 nm over the same time period as your experimental assays will reveal any drift in the baseline absorbance. If there is appreciable drift, you should determine the slope of the plot in units of $\Delta A \cdot min^{-1}$ and subtract this value from the $\Delta A \cdot min^{-1}$ data obtained for assays containing enzyme. Ideally, the absorbance at 340 nm will remain constant because in the presence of NADH, and in the absence of enzyme, the concentration of NADH does not change.
- *A no-NADH control:* Without NADH, the reaction cannot proceed, and the absorbance of the reaction mixture should be zero or close to zero. This is the same situation that occurs when the reaction occurs very rapidly and the NADH is completely consumed. Running this control will allow you to recognize this situation when it occurs.
- *A positive control*: If you are assaying a mutant enzyme for the first time, and you don't observe any activity in the assay, it can be difficult to know if your mutant protein is inactive, or if there is something wrong with one of your assay solutions, the instrument settings, or your protocol design. Running a positive control reaction, with wild-type enzyme or a commercial preparation of MDH, or perhaps another enzyme prepared in your laboratory with known activity, can assist in trouble-shooting your experimental design.

Assay conditions

Finally, there are other considerations described below which you will need to keep in mind when running your assays.

Absorbance: Most spectrometers can only read between 0.01 and 3.0 absorbance units. At either end of this range, absorbances cannot be measured accurately.

Temperature: Bring all solutions to room temperature before starting your assays. The easiest way to do this is mix the next reaction mixture while assaying another. The enzyme should be on ice at all times when running assays, but the volume added is small enough that the final temperature of the reaction mixture should not change appreciably.

Ionic strength: Enzyme activity can be affected by ionic strength, so be consistent in your use of the assay buffer.

pH: Enzyme activity can vary greatly at different pH values. This is shown in Figure 3. While most of the kinetic data for MDH reported in the literature was collected at pH 8.0, it's important to remember that intracellular and mitochondrial pH values are typically lower than this. Keep this in mind when collecting your data, and ensure that you collect your data at a consistent pH, whichever pH value you choose.

Substrate stock solutions: Solutions of OAA and NADH must be made up fresh just prior to use. Determine how much of each you will need for your laboratory period and prepare at least 25% in excess of what you think that you will need. Batch to batch variations in how enzyme and substrate solutions are made can contribute to significant sources of error.

96 well plates: Be sure that you are using polystyrene plates that transmit light at 340 nm. Glass and plastic cuvettes typically absorb light themselves at 340 nm and cannot be used for these assays; check the manufacturer's specifications to be certain.

$$\text{Malate} + NAD^+ \text{ <-> } \text{Oxaloacetate} + \text{NADH} + H^+$$

The reaction velocity is determined by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH. One unit of MDH 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. ***OAA degrades and should NOT be used after ~2 hours.***

Materials: ***Assay Buffer***: 50 mM Na^+ Phosphate Buffer, pH 7.4 (1L)
Stop Solution: 1 M Na_2CO_3 dissolved in water, pH should be around 12
5 mM OAA dissolved in assay buffer; Formula weight – 131.10. Make fresh each day.
Positive Control Enzyme Solution: 50 U/ml in assay buffer (from Porcine heart or recombinant MDH)
100 mM NADH stock: Use to prepare ***2 mM NADH*** in assay buffer for use. Make fresh each day.

Stop Time Assay: For a single reaction, combine the following components *except enzyme* into a microfuge tube, cap and **vortex**. Incubate all solutions at room temperature for 5-10 min prior to starting reaction.

- 700 µl assay buffer
- 100 µl 2 mM NADH
- 100 µl 5 mM OAA
- 100 µl Enzyme solution **Do not add enzyme until ready to start the reaction!

- Once the reaction is started, mix well by vortexing and incubate at room temperature.
- Stop the reaction by adding 100 µl of stop reagent at the determined time and vortex immediately.
- Transfer 300 µl of each sample into a 96 well plate. Read absorbance at 340 nm.
- Include samples without NADH (negative 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 $\phi\lambda A$ = starting absorbance (ie. the no reaction control) – the absorbance of the sample
- $\phi\lambda A$ / min = $\phi\lambda A$ divided by the total time of reaction. Use this value to calculate Units of Enzyme Activity as described.

Calculating enzyme units: 1 Unit of enzyme catalyzes the conversion of 1 µmole of substrate to product per minute. To calculate the units in any spectrophotometric based assay, Beer's law is used: $A = \varepsilon l c$
Where A = absorbance, l = pathlength of the cell (1 cm for a cuvette, or height of liquid in 96 well plate), c = concentration of the absorbing species (M) and ε = the molar extinction coefficient for the absorbing species at a defined wavelength (M^{-1} cm^{-1}).

When assaying enzyme activity, we use $\phi\lambda$ A / min (change in absorbance per time). So $\phi\lambda A = \varepsilon l (\phi\lambda c)$ - as the concentration of the chromophore changes, so will the absorbance.

For the plate reader, adjust the calculations for the correct pathlength. HINT – calculate the height of the cylinder when filled with the total volume for the enzyme assay – look up the diameter of a single well of a 96 well from the web.

$\phi\lambda$ A/min $= \varepsilon l$ ($\phi\lambda$ C/min) *add in the time factor*

$\phi\lambda$ C/min $= (\phi\lambda$ A/min)/ εl *solve for $\phi\lambda$ C/ min*

$\phi\lambda$ C/min $= (\phi\lambda$ A/min)/ $(6.22 \times 10^3 \times 1)$ *substitute values for e (6.22×10^3 M^{-1} cm^{-1}) & l (1 cm)*

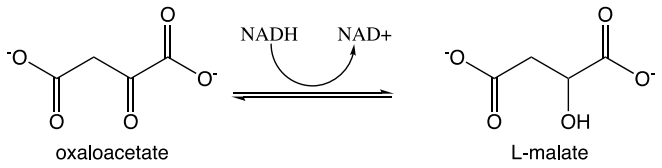
Example for enzymes that use NADH in a standard 1 cm pathlength cell:
NADH has a molar extinction coefficient of 6.2×10^3 M^{-1} cm^{-1}

$\phi\lambda$ C / min $= (\phi\lambda$ A/min x 0.161×10^{-3}) M/min *inverse of the denominator*
$\phi\lambda$ C / min $= (\phi\lambda$ A/min x 0.161×10^{-3}) (moles/liter)/min *convert M to mole/liter*
$\phi\lambda$ C / min $= (\phi\lambda$ A/min x 0.161×10^{3}) (µmoles/liter)/min *convert to µmole*
$\phi\lambda$ C / min $= (\phi\lambda$ A/min x 0.161) (µmoles/mL)/min *convert to mL*

Recall Unit = µmol substrate or product/min therefore this is units / ml of enzyme in the assay itself. But you only measure a few µl of actual enzyme from the test tube.....

Station One-B: Plate Reader Continuous MDH Assay

Overall Goal: Semi-Quantitative MDH Assay for Column Fractions



Experimental Workflow:
Read quickly through the entire procedure before getting started so that you understand what we are doing. Failure to do so will cause you to take much more time in lab.

Assay your elutions and the crude sample using the procedure below. I have included some starting points for the dilutions below. Do the dilutions with Equilibration Buffer, pH 8 not water. Diluting with water will lead to loss of activity.

Dilute the crude 1:10 and 1:100.
Dilute E1-E6 1:10
Dilute the sample with the highest protein concentration 1:20

Obtain the following stock solutions:

0.5 M sodium phosphate, pH 8.0
5 mM oxaloacetate, pH 8.0
1 mM NADH
Keep these at **room temperature**.

For each sample, you will need the following components in each microplate well:

20 µL	0.5 M sodium phosphate, pH 8
20 µL	5 mM oxaloacetate
20 µL	1mM NADH
120 µL	deionized water
180 µL	**Total Volume without enzyme**

Because once you add the enzyme, you need to measure the data right way, we **will not add enzyme** to our wells until step #5.

1. Prepare enough solution to make two wells for each sample* you are going to analyze. Mix well by pipetting up and down. Avoid bubbles!

*This can be done more efficiently by preparing a master mix. Figure out the total number of samples you want to analyze, then double it. Let's say you want to do 10 samples, then you start with 20 as your number. Multiply each volume in the table above by 20 (you get 400 µL NaPi, 400 µL OAA, 400 µL NADH, 2400 µL DI). Combine these amounts in one larger tube. Mix well on the vortex. Add 180 µL of this master mix to each well in the microplate.

2. Note that accurate pipetting is very important for reliable data.
3. Pipet 180 µL into each of two wells for each sample (including a blank).

4. Bring your microplate (at room temperature) and the enzyme solution *on ice* to the microplate reader. Set up the microplate reader for the **MDH Assay** method. Make sure that you are reading the correct plate area(s).
5. Organize all your samples so that you can access them quickly. Put them in the order you want to add them and record your plan in your notebook.
Students get the best results if they analyze no more than four samples each time, so only add enzyme to four sample wells.

Add 20 µL of each elution (E1, E2, etc.) into a separate well relatively quickly so that the start time for each well is not that different.
Mix gently by pipetting up and down without blowing out the tip.
Try to get all the samples in within a minute.

Place the microplate reader onto the drawer and press the **Drawer** button on the instrument.

Press the green **Read** button at the top of the window.
It will monitor the absorbance at 340 nm in each well every 10 sec. for 90 sec.

6. Examine the shape of the curve on the screen when the scan is finished.
If the curve is not linear for the entire time, repeat the experiment.

- Lack of linearity is most likely due to the fact that your concentration of enzyme is either too low or too high.
    - If your sample is too dilute, there is not much you can do. This sample has a low concentration of enzyme.
    - If it is too concentrated, dilute an aliquot of your original sample with the wash buffer and repeat the assay.
        - Be sure to take no less than 10 µL of your sample when doing dilutions, as measuring very small volumes cannot be done with great accuracy.
        - If you need to do more than a 1:10 dilution, add more elution buffer, not less sample.

If the following are all true:

- The change in absorbance is linear with time
- the rate is in the range of -15 mAU/min to -80 mAU/min
- the data are not noisy

save the file, then record the rate values along with the dilution you used
7. Continue this process until all samples produce linear responses during the assay. The maximum possible volume of enzyme sample in the assay is 20 µL. **Record the values of the rate and the dilution used for each sample**
8. Calculate the enzymatic activity of your samples following the directions on above

Quantitative MDH Assay for Determining Kinetic Constants

We will use the kinetics program on the microplate reader to monitor the decrease in absorbance at 340 nm (velocity of the reaction) due to the conversion of NADH to NAD^+ at a variety of [oxaloacetate] (the substrate). This will allow us to plot velocity vs. [S] as in the Michaelis-Menten equation.

Stock Solutions:
0.5 M sodium phosphate, pH 8.0
1 mM oxaloacetate, pH 8.0
1 mM NADH

A. Determine the concentration of enzyme to use in the kinetic assay

We are using different concentrations of oxaloacetate in the kinetic assay than we did in the activity assay. Therefore, we need to do a pilot experiment to decide which dilution of the enzyme is going to give us the most reliable data for the kinetic assay.

1. Prepare 1 mL of 50 mM phosphate buffer, pH 8.0 by diluting the stock solution.
2. Choose the most active elution sample that also has a significant concentration of protein. Discuss with your instructor before you finalize your choice.
3. Dilute aliquots of the elution sample you have chosen in 50 mM phosphate buffer, pH 8.0 as indicated below:
   1:100, 1:200 and 1:400
   Make about 100 µL of each dilution. Keep them on ice.
4. Prepare assay mix in a microfuge tube as indicated in the table below. Store the mix at room temperature.

Note that <u>accurate pipetting</u> is very important for reliable data.

- According to the table, add the NADH, phosphate and water first. Mix well by pipetting up and down. Avoid bubbles!
  Don't add the oxaloacetate yet...it isn't stable when mixed with NADH.

Volume 1 mM NADH (µL)	Volume 0.5 M sodium phosphate, pH 8 (µL)	Volume DI water (µL)	Volume 1 mM oxaloacetate (µL)
120	120	610	50

5. Bring your microplate (at room temperature) and the enzyme solutions *on ice* to the microplate reader. Set up the microplate reader for the **MDH Kinetics** method. <u>Make sure that you are reading the correct plate area(s).</u>
6. Once this is all set up, go ahead and add the oxaloacetate to your microfuge tube, mix well and aliquot 180 µL into each well - one well for each dilution.
7. For each dilution you will perform the following procedure:
   Add 20 µL of enzyme solution to the appropriate well of the microplate and mix gently by pipetting up and down. Avoid bubbles!
   Place the microplate reader onto the drawer and press the **Drawer** button on the instrument.
   Press the green **Read** button at the top of the window.
   It will monitor the absorbance at 340 nm in each well every 10 sec. for 90 sec.

8. Look at your data and make sure they make sense. If the change in absorbance is linear with time, the rate is at least -10 mAu/min, and the data are not noisy then print it out. <u>If there are questions or problems, discuss them with your instructor before continuing</u>.
9. Use this data to determine which dilution of enzyme you should use for the kinetic assay. <u>Record this value in your lab notebook.</u> Note that the [oxaloacetate] will be varied in the assay and the concentration you used in this step is in the middle of the range.
10. When you have determined the correct dilution, make a larger amount of that dilution by diluting an aliquot of the purified enzyme/elution sample with 50 mM phosphate buffer, pH 8.0. If you have enough enzyme to make 500 µL of the dilution, that is great. If not, discuss with the instructor.

B. Running the kinetic assay

1. Prepare assay mix in microfuge tubes as indicated in the table below. Store the mix at room temperature.

Note that <u>accurate pipetting</u> is very important for reliable data.

- According to the table, add NADH, phosphate and water first. Mix well by pipetting up and down. Avoid bubbles!
  Don't add the oxaloacetate yet...it isn't stable when mixed with NADH.

Tube Label	Volume 1 mM NADH (µL)	Volume 0.5 M Na phosphate, pH 8 (µL)	Volume DI water (µL)	Volume 1 mM oxaloacetate(µL)
A	60	60	420	0
B	60	60	414	6
C	60	60	408	12
D	60	60	396	24
E	60	60	384	36
F	60	60	360	60
G	60	60	300	120
H	60	60	240	180

*Note that the volumes in this table give you enough reagent mix to perform 3 assays.

2. When it is your turn to use the plate reader bring the microplate (at room temperature), the oxaloacetate and the enzyme solution *on ice* to the microplate reader. Set up the microplate reader for the **MDH Kinetics** method. Make sure that you are reading the correct plate area(s).

3. Now, add the oxaloacetate to tubes **A-D** (only) according to the table above. Mix well, then transfer 180 µL of the solution in each of the microfuge tubes into each of two adjacent wells of the microplate. When finished you should have two adjacent columns of wells containing assay mix in your plate.

4. Add 20 µL of enzyme solution to each well in the microplate containing assay mix and mix gently by pipetting up and down. Avoid bubbles!

Place the microplate reader onto the drawer and press the **Drawer** button on the instrument so it can equilibrate at the temperature of the instrument.

Wait 15 seconds before measuring data.

After 15s, press the green **Read** button at the top of the window.

It will monitor the absorbance at 340 nm in each well every 10 sec. for 90 sec.

5. Look at your data and make sure they make sense. If the change in absorbance is linear with time and the data are not noisy then record the values for the rates in your notebook. If there are questions or problems, discuss them with your instructor before continuing to the second column.

6. Once the data from the first set (**A-D**) look OK, repeat steps 3-5 for tubes **E-H.** When all your data are collected, analyze your data on Worksheet 4.

Station Two: Effect of inhibitors on MDH activity

Overall Goal: Become familiar with the details of an MDH assay.

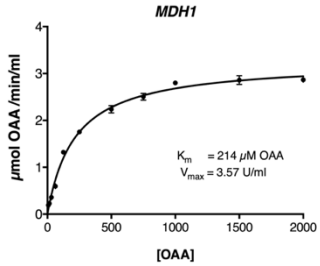
- Using the dilution that results in a linear rate from part one, measure the activity of MDH in the presence of 1mM and 10 mM inhibitor.

  - Potential inhibitors include: Citrate, a keto-glutarate, ATP, ADP, aspartate
  - Each inhibitor is provided as a 100 mM stock in assay buffer
  - Final volume of assay cocktail = 1.0 ml. Maintain OAA and NADH at 10 µl each (200 and 100 µM)
  - For 1 mM inhibitor final concentration, add 10 µl stock inhibitor – adjust assay buffer
  - For 10 mM inhibitor final concentration, add 100 µl stock inhibitor

- Measure and record the rate without and with inhibitor at least three times.
- Determine the specific activity for the wild type enzyme without and with the inhibitor.
- Determine the average specific activity of the wild-type enzyme. Use this to determine the % inhibition of each wild-type and inhibited sample. Prepare a graph of % inhibition for all three conditions.

Station Three: K_m/V_{max}: Determining Michaelis constant and maximum velocity for MDH with OAA.

Using the 1 mL Assay



- Prepare 1 ml of diluted enzyme (in assay buffer) as determined in the first experiment.
- Create a table to prepare MDH assays with the final concentrations: 0, 50, 100, 250, 500, 750, and 1000 µM OAA.
    - Use the 20 mM stock OAA for your calculations.
    - Adjust the final assay buffer to ensure the enzyme assay is 1000 µl final volume.

- If time allows, measure the activity of three or more replicates. To speed up the work, prepare an "enzyme cocktail" aka "master mix" of assay buffer, OAA and NADH for 5 reactions. The cocktail should be mixed within a few min of use and kept at room temperature.

Graph the [S] vs Vo results and estimate the Vmax and Km.