Biochemistry Lab

MDH Plate Reader Real Time Auto Injection Protocol

This protocol will support the small scale 96 well plate real time MDH assay. Each well will have 225 µl final total volume; 150 µl of substrate one and enzyme in buffer and 75 µl of substrate two in buffer. The basic cocktail will include assay buffer, MDH Enzyme, and OAA or NADH. The assay will be initiated by the addition of OAA or NADH depending on the experimental plans.

Stock Solutions: Solutions must be made fresh each day.

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

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

Stock OAA and NADH Solution

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

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

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

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

Working OAA and NADH Solution Volumes enough for two 96-*well plates. Adjust as needed for the number of plates required.*
600 µM OAA. (1.2 ml 20 mM OAA Q.S. to 40 ml with MDH Assay Buffer)

300 µM NADH ((1.2 ml 20 mM OAA Q.S. to 40 ml with MDH Assay Buffer)

Store all of the above compounds *except* MDH Assay Buffer on ICE to prevent breakdown of solutions.

Basic plate reader assay: Each Well Assay will have a final 225 µl total (final) volume:

To measure OAA Km & Vmax values, use a series of enzyme cocktails of Increasing concentrations of OAA each with the same volume and concentration of enzyme. (150 µl OAA/Enz/Buffer). The assay will be initiated with 75 µl of 300 µM NADH. The final concentration of NADH in this condition is 100 µM. Repeat the pattern while varying NADH to determine NADH Km & Vmax values.

Basic single well protocol

- 10 µl enzyme
- 90 µl MDH Assay Buffer
- 100 µl 600 µM OAA.

Pipette 150 µl of this cocktail into each well and incubate at (20°C).

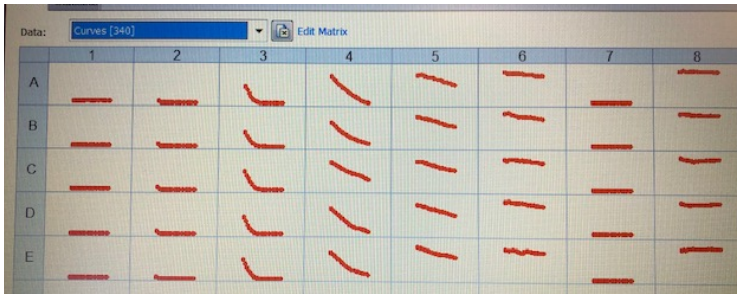
Initiate Assay - Dispense or add OAA or NADH to start assay

- Dispense/Inject 75 µl (600 µM OAA or 300 µM NADH)

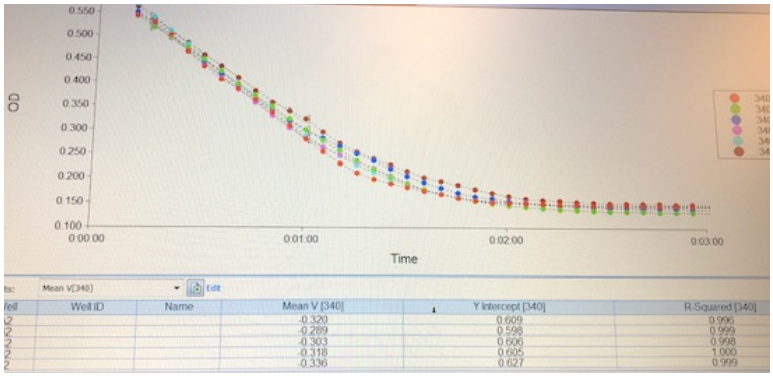
Mix/shake plate and read for 3 min.

Part 1: Range Finder Experiment –determine the dilution of enzyme needed to get a linear reaction for 50-60 seconds using standard enzyme assay concentrations of OAA (200 µM) or NADH (100 µM) to measure rates of MDH preparation at various MDH dilutions.

The key is to get a fast enough rate at the standard conditions (100 µM NADH/200µM OAA) so that the rates achieved with inhibitors or lower concentrations of substrates will give a reasonable and readable rate. A rate which depletes about 0.4 – 0.5 OD over 1-1.5 min will be about right. In our plate reader that is a mean velocity (dOD/min) of about 0.30-0.35. See figure to the right for an example.



As mentioned, the experiment is run in two parts. First, a "range finder" experiment. That is, determine the dilution of your enzyme necessary to achieve the rate indicated above. Using a purified hMDH1 solution of 0.77 mg/ml a series of dilutions from 1:10 to 1:640 were prepared and tested for linearity range. Included is a "blank control" without enzyme to determine the background uncatalyzed rate of NADH oxidation. For comparison purposes, 1:10 is in column 7 next to the blank control in column 8. Notice while both lines are flat indicating no change in absorbance, the blank control has the same absorbance as it started with, while the 1:10 is also flat but is a very low and unchanging rate, indicating the substrate was consumed as soon as the components were mixed together before the plate reader could get the first rate. Column 3 is the 1:80 dilution while the next three are 1:160, 1:320 and 1:640 respectively. The two most dilute enzyme mixes gave a linear rate over 4 min, but the rate was slow enough that in less optimal substrate or inhibitor conditions, the rate would be difficult to detect. Thus in this case between a 1:160 and a 1:300 dilution would work. A 1:200 would be a good choice. BUT depending on your enzyme and mutations, this is ONLY a guide and a full dilution curve MUST be performed.



Hint – if your reaction is fairly fast as seen in a single well assay or for concentrated wild-type MDH enzymes, you may want to only initiate one or two columns at a time. You can pipette all of part 1 components into all the wells. Depending on the speed of your plate reader, you might only want to initiate a few wells (one or two columns) to get enough reads. 3-8 seconds between each read for a vigorous reaction (one that is complete in about a min) will need about this many reads to get a line you can trust.

Make sure your pipetting is rock solid, if using a multichannel pipette ensure its accuracy and precision and practice with it!!!

ENZYME DILUTION: Prepare a series of enzyme dilutions. 8 total dilutions PLUS a blank control tube for 9 total dilution tubes/plate columns.

- Enzyme Dilution Tube 1: If the protein conc is 0.5 mg/ml or so, then Make 400 µl of a 1:10 dilution (40 µl enzyme mixed with 280 µl assay buffer). If the concentration is less OR if using a mutant of unknown or low activity, start with undiluted enzyme. If the concentration is ~1mg/ml or higher, start with a 1:20 dilution.
- Enzyme Dilution Tubes 2-8:
  - Place 200 µl of assay buffer in tubes 2-8.
  - Perform 1:2 serial dilutions transferring 200µl of tube one into tube 2.
  - Mix and transfer 200 µl into the next tube and continue.
  - Tube 8 will contain 400 µl when you are done.
- Blank Control – Tube 9. Add 200 µl of assay buffer into tube 9. Add no enzyme to this tube.

<u>In your notebook indicate the protein conc starting concentration and all dilutions you've prepared.</u>

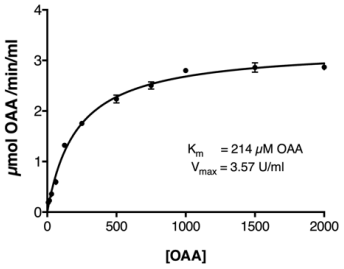
- Using these dilutions prepare the following cocktail for *<u>each</u>* enz dilution and blank control tube. 9 total tubes
  - 60 µl enzyme from dilution tube (or blank control)
  - 540 µl MDH Assay buffer
  - 600 µl OAA (600 µM)
  - Mix well.

- Using columns for each dilution, pipette 150 µl of enzyme cocktail into 5 wells (A1-E9) This is sufficient for our purposes. There should be 9 columns. A1-E1, A2-E2…
- Follow the instructions below to load, wash and prime the appropriate injector with 300 µM NADH. You will be injecting 75 µl of NADH for a final concentration of 100 µM in each well.
- Use the MDH Range Finder protocol to initiate your sample. CHECK the settings to see they are correct for your experiment, don't assume it will be correct as someone ALWAYS changes things.
  - Note that the instrument will dispense in a few columns of well at a time. This is to allow as many reads possible to get enough data points to determine the rate. Carefully analyze the "MDH Range Finder Protocol to understand how the reader will assay sets of columns at a time.
- Inject/dispense NADH solution (75 µl) into each well, shake and measure activity for 3 min.
- Determine the dilution needed to achieve a linear rate for 1 min. See explanation and guide on page one.
- THIS MUST BE DONE FOR EACH ENZYME – they all are at different concentrations and depending on isoform and mutation will need different dilutions.
- KEEP GREAT RECORDS of starting enzyme dates and preparation, starting enzyme concentrations and all dilutions. You will need this for the final calculations.

PART TWO- KINETIC DETERMINATION OAA and NADH Km & Vmax:

Each wild-type and mutant will be analyzed for both OAA and NADH kinetics.

- NADH conc: 200, 175, 150, 125, 100, 75, 50, 25, 12.5, 6.25 and 0.0 µM
- OAA conc: 2000, 1500, 1000, 750, 500, 250, 125, 62.5, 31.25, 15.63, 7.8 and 0.0



This range of MDH substrates will produce a curve similar to the attached figure to measure the Km of MDH for OAA. Notice the distribution to allow a good spread of concentrations to provide a good result.

Enzyme dilution preparation. 12 substrate concentrations x 10 wells x 10 µl of enzyme x 2 MM substrate plots (NADH and OAA) = 2000 µl. SO prepare ***2.5 ml*** of diluted enzyme to assay against both substrates. Store on ice. Odds are this will be a 1:40 or greater dilution, thus you will need much less than 100 µl of purified enzyme to make the dilution.

Assay cocktail preparation. Each well will contain 150 µl of the following: enzyme (diluted to give linear curve in std conditions for 2-3 min), MDH Assay Buffer, and either OAA or NADH (the substrate being changed). Total volume of assay is 225 µl. For OAA substrate change - 150 µl mixture will consist of OAA (75µl) enzyme (7.5µl) and buffer (67.5µl).

1) Label 12 tubes and add the indicated volume of MDH Assay Buffer
2) Add the indicated OAA and volume to each tube Mix well between each addition
3) Prime and fill the appropriate injector with 300 µM NADH

OAA Well Cocktail Solution Calculations for 10 assays (wells) MIX MIX MIX

OAA Tube	Assay Buffer	Initial OAA (µM)	µl of OAA added to dilution buffer		Final OAA (µM)	Notes
1	1,050 µl	6,000	450 µl of 20 mM		2,000	
2	1,162 µl	4,500	338 µl of 20 mM		1,500	
3	750 µl	3,000	750 µl of 6,000 µM		1,000	
4	750 µl	2,250	750 µl of 4,500 µM		750	
5	750 µl	1,500	750 µl of 3,000 µM		500	
6	750 µl	750	750 µl of 1,500 µM		250	
7	750 µl	375	750 µl of 750 µM		125	
8	750 µl	187.5	750 µl of 375µM		62.5	
9	750 µl	93.75	750 µl of 187 µM		31.25	
10	750 µl	46.9	750 µl of 93.75 µM		15.63	
11	750 µl	23.4	750 µl of 46.9 µM		7.8	*Remove 750 µl after dilution
12	750 µl	0.0	0.0 ml		0.0	

- When done, each OAA tube should have 0.75 ml.
- To **each** tube, add 675 µl assay buffer
- To **each** tube, add 75 µl of diluted enzyme.

Each OAA dilution tube will serve to assay n=6 replicates (wells in a column). For each dilution of OAA, pipette 150 µl of well cocktail solution (OAA, Buffer and Enzyme) to **6 wells** in a column.

Initiate assay with **75 µl** of 300 µM NADH by injection, shake plate for **10 sec** at highest setting, then record absorbance at 340 nm for **3 min**. Determine linear rates for each well and record dOD/sec

1) Label 12 tubes and add the indicated volume of MDH Assay Buffer
2) Add the indicated NADH and volume to each tube Mix well between each addition
3) Prime and fill the appropriate injector with 600 µM OAA

NADH Well Cocktail Solution Calculations for 10 assays (wells)

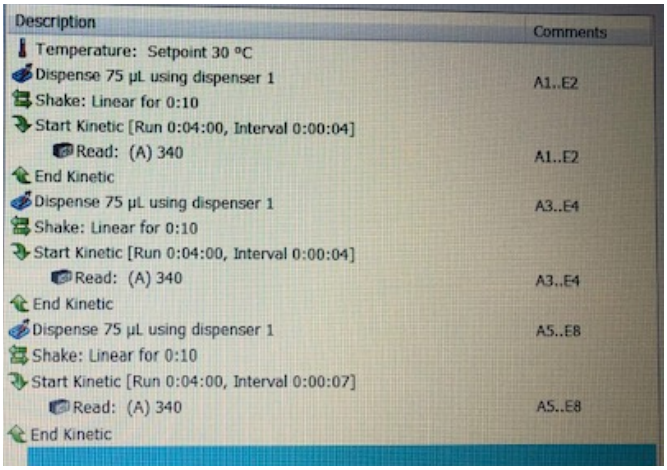
Tube	Assay Buffer	Initial NADH (µM)	µl of NADH added to dilution buffer		Final NADH (µM)	Notes
1	1,410 µl	600	90 µl of 10 mM		200	
2	710 µl	525	40 µl of 10 mM		175	
3	1,432 µl	450	67.5 µl of 10 mM		150	
4	722 µl	375	28 µl of 10 mM		125	
5	750 µl	300	750 µl of 600µM		100	
6	750 µl *	225	750 µl of 450 µM		75	*Remove 750 µl after dilution
7	750 µl	150	1750 µl of 300 µM		50	
8	750 µl	75	750 µl of 150 µM		25	
9	750 µl	37.5	750 µl of 75 µM		12.5	
10	750 µl	18.75	750 µl of 37.5 µM		6.25	
11	750 µl *	9.37	750 µl of 18.7 µM		3.12	*Remove 750 µl after dilution
12	750 µl	0.0	0.0 ml		0.0	

- When done, each NADH tube should have 0.75 ml.
- To **each** tube, add 675 µl assay buffer
- To **each** tube, add 75 µl of diluted enzyme.

For each dilution of NADH, pipette **150 µl** of well cocktail solution (NADH, Buffer and Enzyme) to **6 wells** in a column. Initiate assay with **75 µl** of 600 µM OAA, shake plate for **10 sec** at highest setting, then record absorbance at 340 nm for **3 min**. Determine linear rates for each well and record dOD/sec.

GENERAL NOTES:

- After adding all enzyme cocktails to a plate, incubate in the pre-warmed plate reader for 5 min prior to starting assay. This is enough time to bring your sample to 30°C.
- Because there are so many wells and the reaction for the fastest mixtures (typically the highest 3-5 concentrations of substrate) will be over faster than latter, the plate reader is designed to start the reaction (via injection of either NADH or OAA) in a few columns, shake and then read those columns for 3 min. Once those columns are finished, the reader will automatically move to start the next set of wells. This allows more reads per minute and will give you a rate you can trust.



- Pipetting – air bubbles are not your friend. The injection and shaking should remove most of these, but avoid if you can.
- Use the electronic 1 ml pipette set at 150 µl. You can do one fill and 6 dispenses accurately and quickly. Keep the tip just on the side of the well. Don't "shoot" into the bottom. The multi-channel pipettes we own are not accurate enough. Don't use them.
- If you like there is a LED device to help guide you where to pipette. It is easy to get mixed up when pipetting in a 96 well plate.
- MIX all samples after you make them. MIX MIX MIX.
- Sloppy pipetting will result in sloppy results. Don't be that way!
- NAD+ Km reported 42 and 60 µM for mito and cyto. Need to figure out range higher than 5 mM
- Malate Km reported at 1,500/770 µM need to start at 10mm or higher.

Calculating Enzyme Activity (µmol of substrate converted to product/min/ml)

- The pathlength for light traveling through 225 µl of a 96 well plate is calculated using the dimeter of 7 mm. The pathlength will be 0.585 cm. Convert to absorbance/minutes if saved in absorbance/seconds

$$\text{mM NADH/min} = \Delta C/\text{min} = (\Delta A/\text{min}) / (\varepsilon \times l)$$

$$\Delta C/\text{min} = (\Delta A/\text{min})/ (6.22 mM^{-1}cm^{-1} \times 0.585 \text{ cm})$$

$$\Delta C/\text{min} = \Delta A/\text{min} \times 0.275 \text{ mM}$$

$$\text{Enzyme Unit} = \text{vol (ml)} \times (\Delta A/\text{min} \times 0.275)$$

$$\text{Enzyme Unit} = \Delta A/\text{min} \times 0.0619 \text{ µmol NADH converted / min}$$

This value divided by ml of enzyme added (7.5 µl) is the enzyme activity

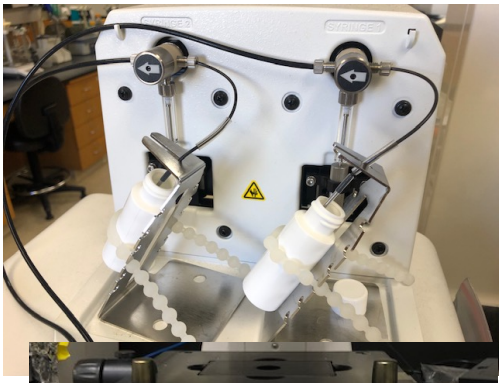
Enzyme activity = ΔA/minl x 8.25 (µmol NADH converted per min per ml)

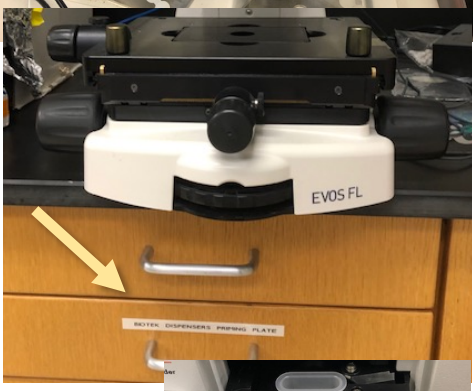
- To convert from enzyme activity to specific activity, determine the mg of protein in the 7.5 µl added to the well and divide Enzyme activity by that value
- To do this first calculate the mg/ml of your diluted enzyme added to the cocktail. Multiply that value in mg/ml by 0.0075 ml to determine the mg of protein added to each well.

Biochemistry Lab

MDH Plate Reader Real Time Auto Injection Protocol

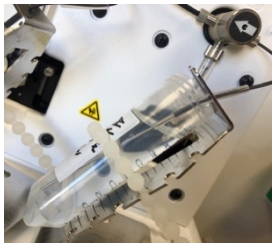
Plate Reader Preparation and Using Data.



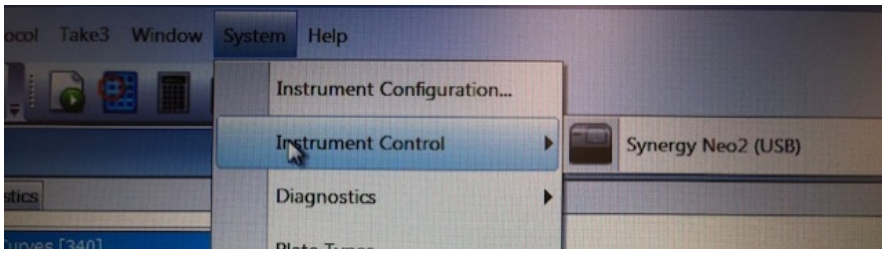


- ***Turn on*** the plate reader and allow the instrument to fully go through its startup functions BEFORE turning on the laptop

- ***Prepare the injectors***. The injectors should be purged then primed in water using the white bottles after your last step and should be in this condition when you start. It doesn't hurt to purge and prime in fresh water in the white bottles just to be sure the tubes and pumps are cleaned from other solutions.
  - PURGE MODE – reverses the fluid from the tubing into the bottle.
  - PRIME MODE – washes the fluid from the injector bottle through the tubing into the white priming plate. The plate reader MUST have the white Biotek Dispenser "Priming Plate" located in the drawer under the microscope. PLEASE clean with miliQ water and replace when you are finished.



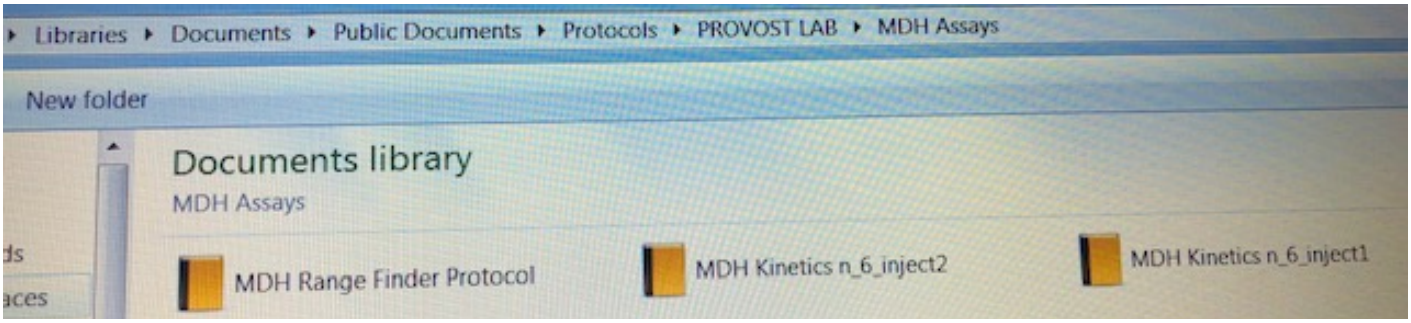
- Find the purge/prime window through Systems->Instrument control -> Synergy Neo2(USB).



- With the white bottle in place purge the tubes into the white bottle. It takes 2.0 ml to flush out and fully prime the injectors and tubing.

- AFTER purging and priming with water, replace the white injector bottle with a 15 or 50 ml conical tube. Ensure the tip is as far down as possible. Then prime the dispenser with 2000µl of NADH for dispenser 1 (on the right as you face the instrument). Repeat purge and prime for OAA in dispenser 2. Rinse out the white injector bottle between uses and refill with milliQ water.

Programing the plate reader: For the MDH assays, there are three pre-programed protocols. In the figure below, you can see the protocols under libraries -> documents -> Protocols -> PROVOST LAB -> MDH Assays. Do not change these but always double check that they weren't altered.

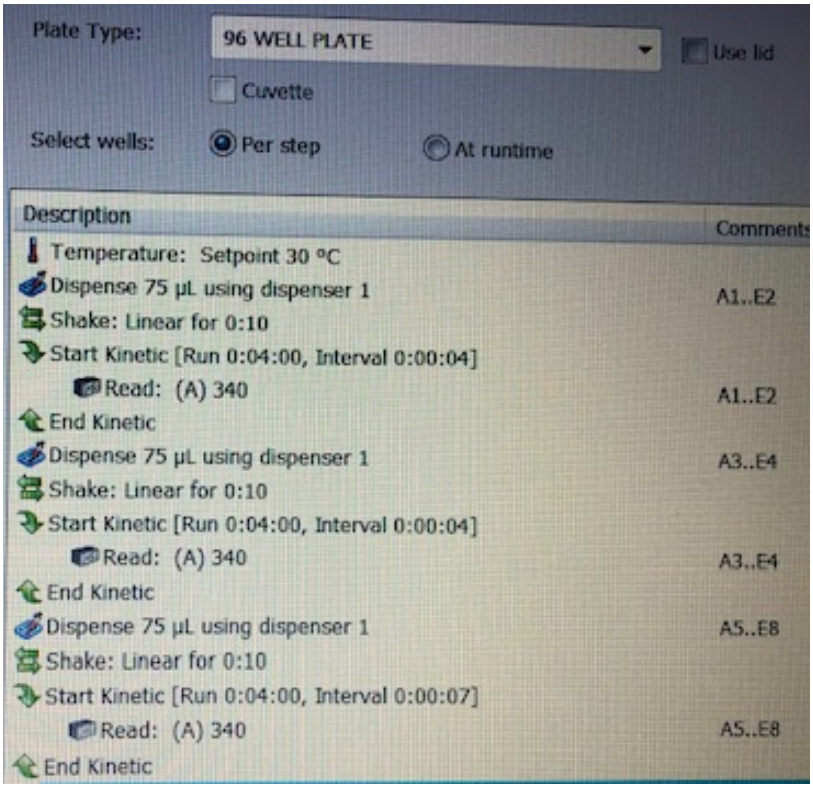


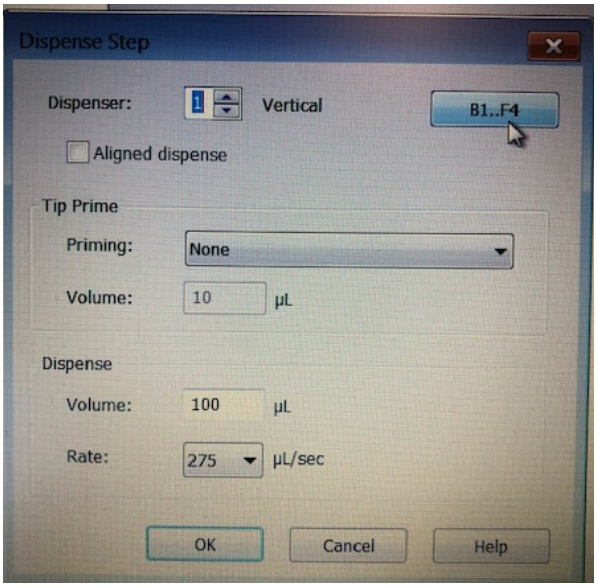
Start with the Range Finder Protocol. This is the protocol to define the dilution of Enzyme as described in Part I earlier in this document.

Range Finder Protocol.

First click on the dispense protocol. Ensure the following options are selected:

- "Use Lid" should NOT be checked.
- Select wells should be "per step"
- As described earlier, it is not appropriate to read the entire plate at one time, so the assays will be placed in plate but initiated via the dispenser in groups of wells. Make sure the plate is going to dispense the same set of wells as will be read in the following kinetic section. The comments section has this information in the protocol description.



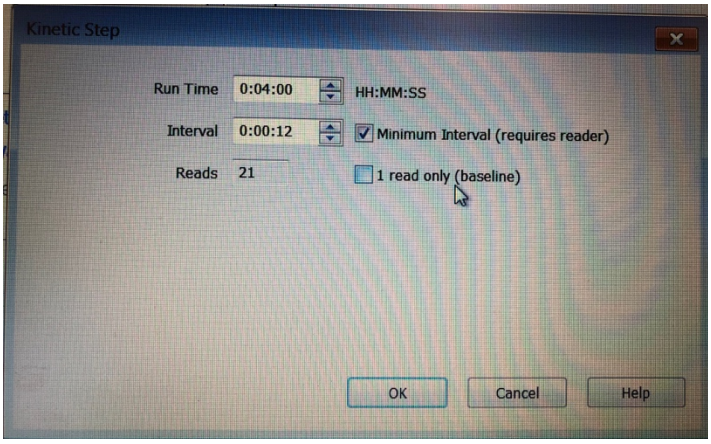


Clicking on the Dispense in any step will bring up the dialogue box shown here. Make certain the instrument is programed to dispense in the correct wells (see upper right corner where the arrow is in the figure). For this assay, do not prime and leave "aligned dispense" unchecked.

The CORRECT volume for our assays is 75 µl and use 275 µl/sec injection rate.

Notice the dispenser option in the top left of the dialogue box. This is where to program dispenser 1 (the right injection tube: NADH for our use) or dispenser 2 (the left and OAA injection tube).

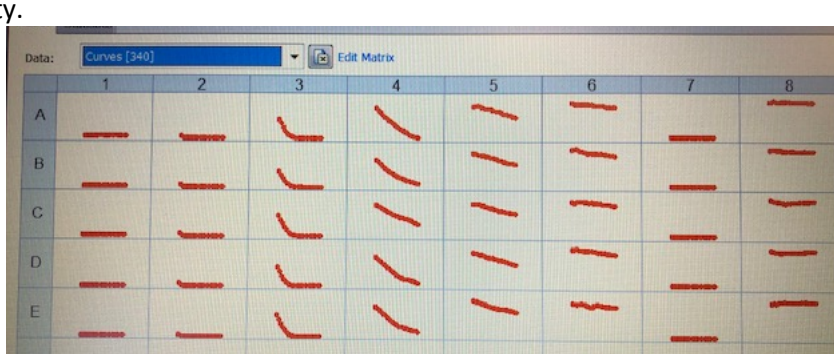
The kinetics step (click on "start kinetic" in the protocol dialogue box), will open the box shown on the right. Ensure the run time is 3 min. When you click on "minimum interval" option, ignore the actual value, after "validating" the program (last step), the program connects with the reader and will determine the least time between reads for each well. If you do not select this and the value is too small for the number of wells, the instrument can't move through all of the wells in time, you will receive an error message in the validation. If the interval time is longer, you will not get a warning message, but the number of reads per interval could be longer than optimal for your assay. Thus it is good practice to chose the "minimal interval" option each time.



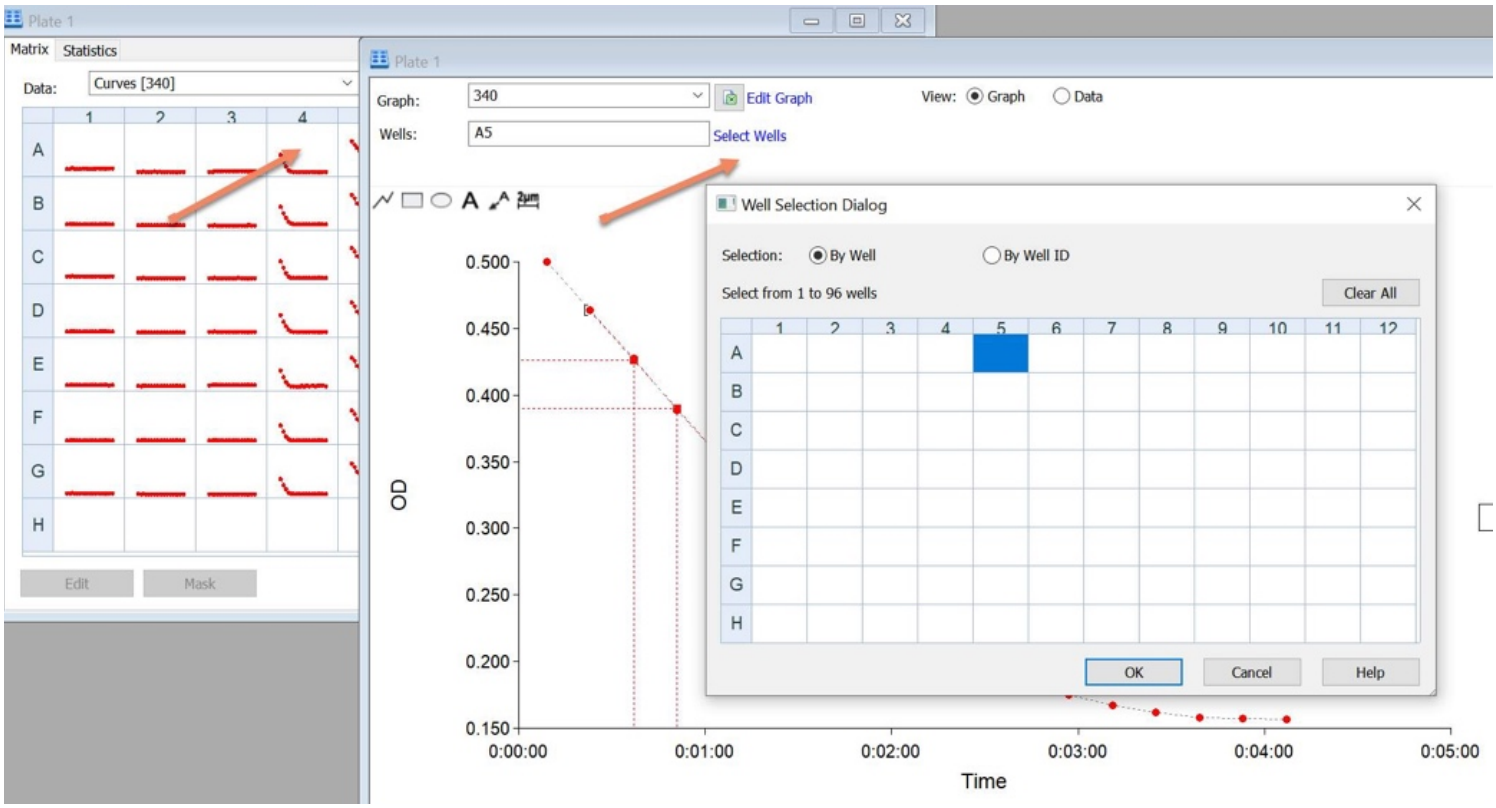
Shake – select linear, 10 seconds at max intensity.

Data Processing.

When the program is running, you will see something like the results shown to the right…

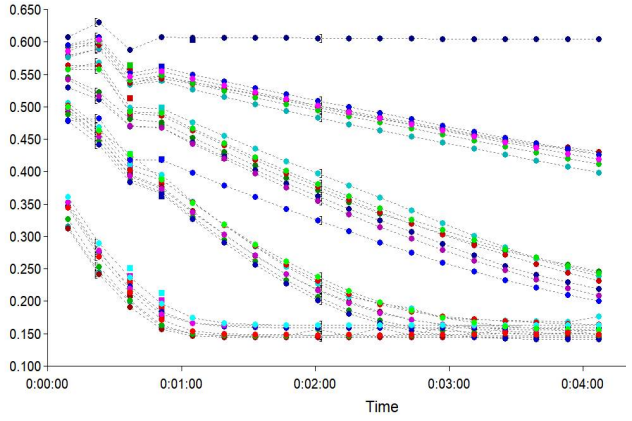


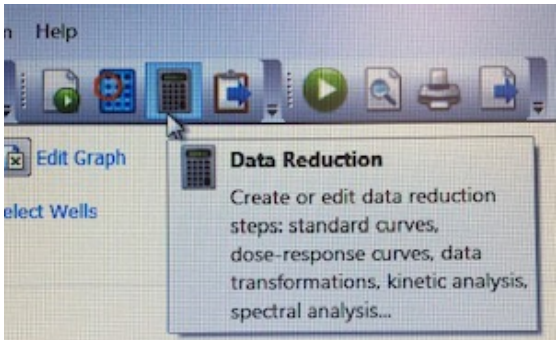
To get an expanded version of the assay, you can select one well by clicking on that well.



To show more than one assay in a larger display, click on one of the wells you wish to expand. Then click on "select wells", followed by dragging/clicking on the wells you want to display in a single window.

You can "mask" points that are clearly off due to mixing or something by clicking the cursor on the section.



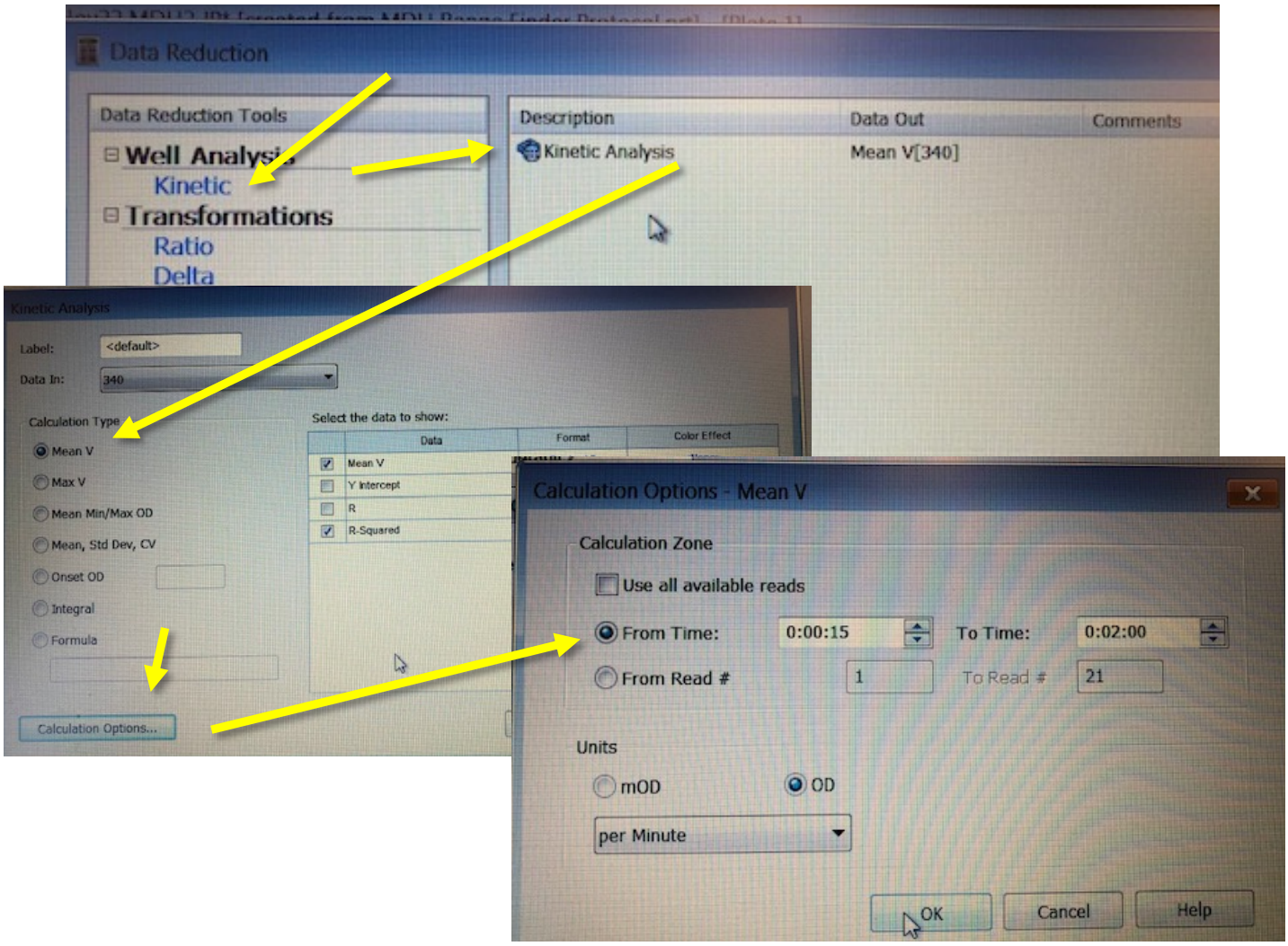


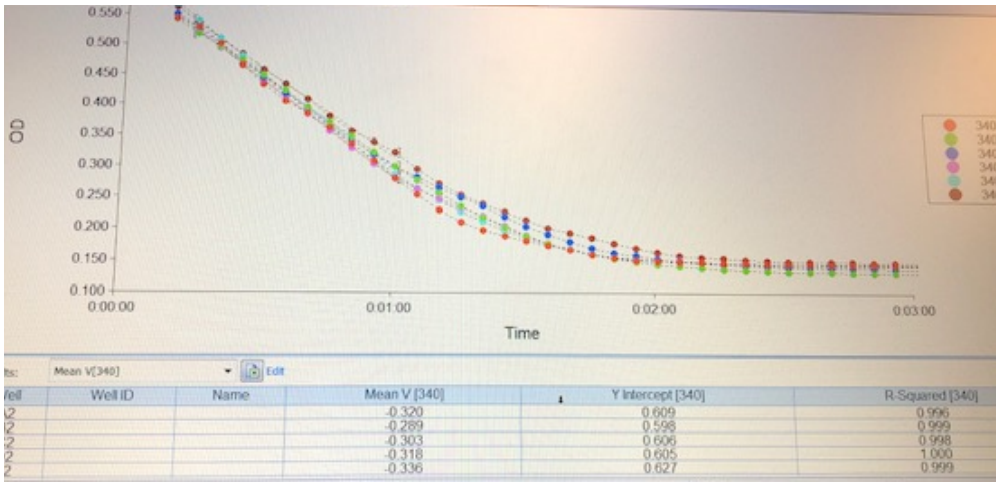
To calculate the rate without exporting and re-graphing in another program, you need to choose the "data reduction" option.

Select Kinetic -> kinetic analysis Then choose "Mean V" this is the average velocity or rate for the assay.

To choose the portion of your reaction that is linear, click on the "calculation options" box.

- Select the start and stop time you wish to calculate the rate for. You may have to do this for sets of data rather than all the wells based on each graph.
- Ensure OD and not "mOD" is selected and the units in "per minute".





Well	Well ID	Name	Mean V [340]	Y Intercept [340]	R-Squared [340]
2			-0.320	0.609	0.996
2			-0.289	0.598	0.999
2			-0.303	0.606	0.998
2			-0.318	0.605	1.000
2			-0.336	0.627	0.999

The Mean V (velocity) NOT MAX!!!! will then be displayed in the grouped graph. See the brackets to ensure you are selecting linear portions of the reaction.

Export in a number of ways. Save on your own file.