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A Guide to Spectroscopic Studies in Biology & Life Sciences

Spectroscopy 101
Biology Edition

Monde Qhobosheane, Ph.D. & Kristopher Blee, Ph.D.

Instruction for ***Ocean Optics' SpectraSuite*** software included

A Guide to Spectroscopic Studies in Biology & Life Sciences

Spectroscopy101 Workshop Manual Using Ocean Optics Spectrometers

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Preface

Students can now study the basic scientific principles on the same world-class equipment used by leading researchers in the university and government labs and more excitingly, NASA. With advances in electro-optics and their continuous impact on the sensing community, high-speed array detectors, inexpensive optical fibers and powerful computers have made optical spectroscopy the sensing technique of choice for many real-world applications.

The development and marketing of scientific instruments and methods have changed in an equally dramatic way; in the past, cutting-edge instrumentation started with expensive research devices that were accessible only to well-funded research and development enterprises, gradually, the technologies filtered into general laboratory use, application-specific instruments and now into the educational settings.

Our knowledge of spectroscopy has been based upon years of experimentation in a wide array of disciplines ranging from art to applied physics. All of the fields have strong roots in education, more specifically teaching and learning the basics of the field. It has been the experience of thousands of science educators that have utilized the Ocean Optics spectrometers with there own specific real world exciting experiments that the students in there classes lives have been enriched and have a greater appreciation for science.

It is important that today's science and engineering students appreciate the capabilities of optical sensing, the fundamental physics of the measurement process, the design trade-offs inherent in selecting and integrating components, and the discipline required to produce quality results. The goal of this lab manual is to provide a vehicle to allow future scientist to study the fundamentals of spectroscopy using modern research/industry instrumentation.

I would like to offer special thanks to the educators who contributed to this lab manual as part of the ongoing Ocean Optics grant program. Also my deepest appreciation to Kristin Detwiler for her ground breaking work in this project.

Note to Educators: If you would like to contribute to future compilations, please send an email to education@oceanoptics.com.

About Kristopher Blee, Ph.D.

Kristopher Blee is Associate Professor of Biology at California State University, Chico. He received his Ph.D. from the Department of Biology at Utah State University in 1997. Dr. Blee has taught university courses in introductory biology, genetics, plant pathology, plant anatomy and development, and molecular biology in England and the United States. His research is on the molecular and cellular biology of plant-microbe interactions. Since his arrival at CSU, Chico, in 2001, he has also worked on changes to the biology curriculum, including raising students to investigator status and providing them the opportunity to test contemporary hypotheses.

Table of Contents

1 Estimation of Bacterial Population Growth Rate

Objectives

- Become familiar with handling liquid bacterial cultures
- Practice pipetting and transferring liquids
- Introduce spectrophotometric analysis
- Develop methods for the estimation of bacterial growth rates
- Plan an experiment for next week focusing on some aspect of bacterial growth that you will be able to analyze statistically

Bacterial cultures are frequently used for population studies because of their rapid growth and small size. Furthermore, their environment can be readily controlled and manipulated. The most frequently used bacterium is *Escherichia coli*, about which enormous amounts of information have been gathered. *E. coli* exists in numerous strains that may differ substantially, so the strain designation should always be recorded.

USE THE FIRST TWO HOURS OF LAB TIME TO MEASURE BACTERIAL POPULATION GROWTH; USE THE LAST HOUR TO PLAN NEXT WEEK'S EXPERIMENT.

Exercise 1: Determination of Bacterial Population Doubling Time

1. Create new groups of no larger than 4 students per group.

2. Move your group to one of the spectrophotometer stations.

3. Turn on your computer and open SpectraSuite. Use the appropriate section of Appendix C to set up the spectrophotometer.

4. Add 1ml overnight culture of *E. coli* to each of the 3 flasks. Note the time. You will take samples every 20 minutes beginning now.

5. Immediately transfer 4 ml from each flask into a cuvette and determine the turbidity of the bacterial culture by reading the **absorbance scale** of the spectrophotometer (the lower scale). Discard the cuvette sample in a waste culture beaker. Rinse the cuvettes with water, then drain inverted on the test tube stand.

6. Sample 4 ml from each flask every 20 minutes. Record your absorbency readings in a raw data table and plot successive absorbance readings on semi-log graph paper (Appendix I) as you collect them.

7. Continue sampling for a total of 100 minutes.

8. Determine the time for each population to double from the graph. This is referred to as the population doubling or generation time. This is done by selecting an absorbence value in the logarithmic phase of growth and noting what the time was. Then select twice that absorbence value and note the time. The doubling time is the time it took the culture to go from the first absorbance value to the second (Figure 1).

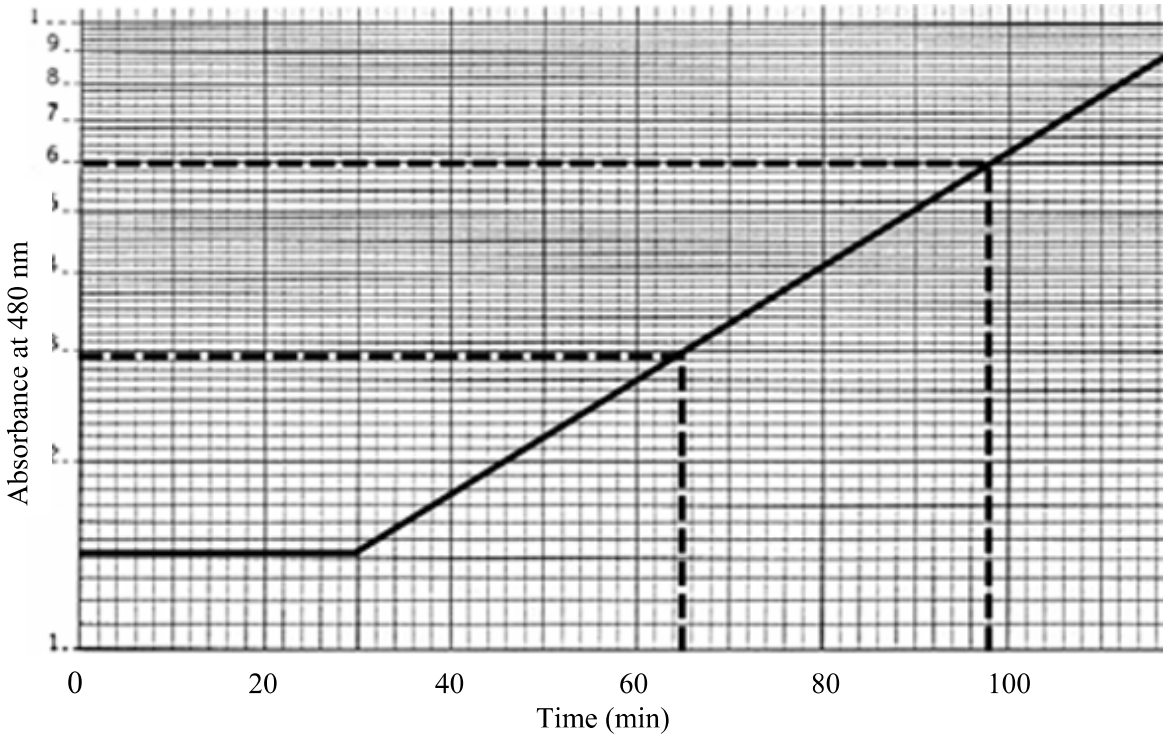


Figure 1. Calculating the doubling time of a population of bacteria. In this example, the population doubled from 0.3 to 0.6 between 65 and 98 minutes, so the doubling/generation time is 98 - 65 = 33 minutes.

9. Determine the standard deviation for generation times from the triplicates, and prepare a summary table of the results.

10. In your notebook clearly state the average doubling time of E. coli under the conditions you tested.

11. What are the conditions for which you determined the doubling time? Hint, what factors that are controlled in your experiment could influence the doubling time? These should be reported along the doubling time in your notebook.

12. In your notebook identify sources of variability among replicates. What do you think are the main sources of error?

Assignment Student-designed bacterial growth experiment

Next week's experiment must be planned today. Discuss several questions to investigate with your group. Materials listed below will be available. Develop a protocol and list of any additional materials you will need. If you do not ask for something you need, you may have no experiment. **Consult with your laboratory instructor about the experiment you plan.**

Materials

Students will work in groups of 4, each class will contain 5 groups (spec stations complete with cuvettes, cuvette racks, tubes, tube holders, serological pipettes, culture discard, disinfectant bottle, water rinse bottle, 70% ethanol rinse bottle, and culture discard).

- Waterbaths set at 35°C
- Spectrophotometers set at 480 nm wavelength (one per group)
- 3 replicate flasks of nutrient broth (50 ml) equilibrated at 35°C (per group)
- 4 ml uninoculated media to blank the spectrophotometer (one per group)
- 5 ml serological pipettes (one can per group with pipettor)
- Spectrophotometer cuvettes (3 per group, with rack or holder)
- Overnight culture of *Escherichia coli* (one per class, or even one per day?)
- Test tubes to hold pipettes (3 per group, with rack or holder)
- Culture discard (one per group)
- Disinfectant squirt bottle (one per group)
- Water squirt rinse bottle (one per group)
- 70% ethanol squirt rinse bottle (one per group)

2 Bacterial Population Growth Rates Period II

Exercise 1: Student Investigation on Bacterial Population Doubling Time

Write this in your lab notebook as a formal investigation including, title, and underlined headings for; observations, question, hypotheses, methods, results (raw and summary), analysis (statistics) and conclusions.

In the results section you must calculate (clearly show all work) and report the final concentration of the compound you added as treatment. In cases where a household product has been added, use the concentration of any substance on the ingredients label for which a concentration is given. You may find the following equation useful for these types of problems.

Concentration initial x Volume initial = Concentration final x Volume final

Fill in known values and solve for the unknown. To use the equation units of all Volumes and of all Concentrations must match and be of the same magnitude.

Use the incubation times between measurements of your experiment to complete Exercise 2 on the following page.

Transparency Assignment Due Next Lab Period

After you perform your analysis prepare a transparency to be presented at the beginning of the next laboratory period when each group will report their findings. Include on your transparency, the question, hypothesis, summary table, calculation for final concentration of substance added, and conclusion including a biochemical / molecular explanation of your findings.

Exercise 2: Micropipetting Skills

Micropipettes allow the precise and accurate transfer of liquids, and good laboratory biologists have well developed micropipetting skills. Performing experiments demands it. And as simplistic as their operation seems, micropipettes are one of the most misused lab instruments among beginning scientists. To avoid damaging the micropipettes you will adhere to the following rules governing their use.

1. Do not dial volumes above or below the maximum and minimum values listed on the micropipette. Any volume between the maximum and the minimum is appropriate for that micropipette.

2. Hold micropipettes like daggers. Notice two thumb buttons. One button is a tip ejector. The other button is for pipetting and has two different resistances to compression by your thumb, easy at first (volumetric measure) and then harder at the end of the stroke (blows the tip clean when dispensing). Learn to recognize these two stages within the plunger stroke.

3. Never tip the micropipette upside down when transferring liquid. This means that the tip should always be lower than the rest of the pipette (you can accomplish this by gripping the pipette as though it were a dagger).

4. Do not touch the tip! To put a tip on the micropipette, press the micropipette onto the tip while it is in the tip box. Eject the tip into a waste container using the ejection plunger on the micropipette.

5. Sometimes you may need to tighten the tip onto the micropipette. To do this, twist the collar of the tip to snug it up. Do not touch the part of the tip that will be in contact with any liquid.

6. Do not insert the pipettor into a reagent bottle beyond the replaceable tip. Tip the reagent bottle so the liquid inside comes close to the opening. Placing the pipette deep into a reagent bottle would allow reagents transfer of the reagents to the outside of the pipette and eventually your hands.

Micropipette Operation

Obtain a micropipettor, box of appropriate tips, a square of parafilm (about 6 cm x 6 cm), and a reagent bottle filled only 1/4 full with water. Practice the following procedure until you are confident in use of the micropipette.

1. Exam the volume range listed on the pipettor you have chosen and the volume ranges listed on your classmates. There are three ranges of pipettes in the lab. What are they?

2. Normally you would select the correct micropipette for the job by noting the volume ranges listed on the pipettor. For this exercise any pipettor will do.

3. What are the units for the volume ranges listed on the pipettors?

4. What range pipettor would be correct to pipette 3 uL?

5. What range pipettor would be correct to pipette 0.3 mL?

6. What would the volume window of this pipette indicate when set correctly to pipette 0.3 mL?

7. Dial an appropriate volume for the range of your pipette.

8. Place a tip on the micropipette without touching the tip. If you do not close the tip box afterwards they are likely to become contaminated.

9. Depress the plunger to the first stop.

10. Place the tip just below the surface of the liquid, while doing so do not insert the micropipettor into the reagent bottle beyond the length of the removable tip. Remember only the removable tip is clean. If you place the micropipettor beyond the length of the removable tip into a reagent bottle you are likely to contaminate the reagent, therefore you must tip the bottle to bring the solution level closer to the top.

11. Slowly release the plunger.

12. Place the tip in contact with the side of the receiving vessel. Normally the receiving vessel would be a tube, however in this exercise use the parafilm square.

13. Slowly depress the plunger past the first stop to the second stop. Watch the fluid leave the tip and collect on the parafilm square. Try to develop the habit of physically keeping track of the droplet. This is especially helpful when pipetting very low volumes as the small droplet sometimes stays stuck to the pipette tip.

14. Remove the tip from the receiving tube (paraffin square) and then let the plunger back up.
15. If you will be immediately continuing to pipette the same reagent and you are sure you did not contaminate the removable tip you may reuse it. Micropipette tips should be treated as sharps, dispose of the tip in the designated used tip container. Do not place used tips in sinks, do not allow them to collect on the bench or floor, and do not place them in the common garbage containers.
16. Repeat steps 9 through 15 several times using the same volume. Are all your droplets the same size?
17. Repeat the above exercise with a different volume pipette.

Developing Micropipetting Skills

Obtain a lab partner, a micropipettor (one that will pipette in the 0.1 mL to 1.0 mL range), a clean tip, a reagent bottle 1/4 filled with water, square of parafilm (about 6 cm x 6 cm), and proceed to a balance.

1. Place your parafilm square on the balance and zero it.

2. Dial an appropriate volume for your pipettor, anything with in its range. Larger volumes may be necessary depending on your balance.

3. Using the procedure above place 10 drops of water on the balance, you're your partner record the weight after each drop in a table in your notebook while you focus your efforts on pipetting.

4. Back at your bench, calculate the weight of each drop, the average, and standard deviation in your notebook.

5. Is your pipettor accurate? Precise?

Materials

Students will work in groups of 4, each class will contain 5 groups (spec stations complete with cuvettes, cuvette racks, tubes, tube holders, serological pipettes, culture discard, disinfectant bottle, water rinse bottle, 70% ethanol rinse bottle, and culture discard)

- Water baths set at 35°C
- Spectrophotometers set at 480 nm wavelength (one per group)
- 4 replicate flasks of nutrient broth (50 ml) equilibrated at 35°C (per group)
- 4 ml uninoculated media to blank the spectrophotometer (one per group)

- 5 ml serological pipettes (one can per group with pipettor)
- Spectrophotometer cuvettes (4 per group, with rack or holder)
- Overnight culture of *Escherichia coli* (one per class, or even one per day?)
- Test tubes to hold pipettes (4 per group, with rack or holder)
- Culture discard (one per group)
- Disinfectant squirt bottle (one per group)
- Water squirt rinse bottle (one per group)
- 70% ethanol squirt rinse bottle (one per group)
- Streptomycin (50 mg/ml) - 50 ul diluted into 50 ml gives final of 50 ug/ml
- Penicillin (200,000 units/ml) - 5 ml diluted into 50 ml gives final of 20,000 units/ml
- 1 M NaOH - 0.9 ml diluted into 50 ml broth gives pH 9
- 1 M HCl - 0.1 ml diluted into 50 ml broth gives pH 6, 1.65 ml into 50 ml broth gives pH 5

Keep these supplies separate to be handed out later in lab period by instructor.

- Parafilm and scissors (one roll and scissors for the whole class)
- Balances 50 mg to 0.5 g accuracy, ideally one per group (one per group)
- Micropipettes one each of 0.5 – 20, 20 – 200, and 200 – 1000 and tips (one set of 3 / group)
- Tips do not have to be sterile
- Reagent bottle (100 to 200 mL size) about 1/4 full with nanopure water (one per group)
- Micropipette tip discard collection (one per group)

3 Protein Extraction and Assay

Objectives

- Extract total protein
- Determine protein content in extracts
- Analyze protein content in foods

Many biological experiments are based on the study of cellular constituents *in vitro*. To obtain the cellular constituent of interest investigators often homogenize the tissue in a solvent that the desired cellular constituent is soluble in. Detergents are usually added to dissolve membranes and aid in the release cellular contents. Because of their cell walls homogenization of plants requires vigorous grinding in liquid nitrogen to break open cells and enable cellular contents to mix with the extraction solvent. Centrifugation is commonly used next to remove cellular debris leaving a solution containing the molecules of interest.

The solvent of choice for most proteins (excluding integral membrane proteins) is water. Because protein function is dependent upon structure, it is desirable to isolate proteins in their native 3 – dimensional conformation. Therefore extraction solvents of proteins often contain buffers to maintain pH and protease inhibitors to slow degradation. Protein extraction solutions may also contain detergents to aid in solubilization of hydrophobic proteins. Some proteins are localized within cells through ionic iterations. Addition of salts to the extraction buffer can help to exchange-off these proteins. Solutions for the extraction of proteins are usually individually designed to enable the best possible recovery of a particular protein.

After extraction of protein one of the first tasks that follows is determination of the protein concentration of each sample. Then investigators can determine how much protein extract to use of each sample for an experiment. Evaluation of biochemical experiments using protein extracts is usually based on comparisons among samples, each of which contains the same amount of total protein. There are a variety of methods for the determination of total protein in an extract. The simplest is measuring absorbance of the protein extracts at 260 nm and comparing these absorbance values to the absorbance values for protein solutions of known concentration. Aromatic amino acids contribute to the absorbance of proteins at 260 nm. Plotting absorbance at 260 nm against protein concentration for protein solutions of known concentration creates a Standard Curve. The Standard Curve principle is commonly used in biochemistry to quantify biomolecules. Problems in quantifying particular proteins arise because some proteins may have lots of aromatic amino acids while others very few. There are also molecules within cells other than protein that absorb at 260 nm.

Peptide bonds linking individual amino acids together to form proteins are a more definitive and unique feature of proteins that have been utilized for quantification. This feature of proteins has been exploited commercially for the development of protein assays including the Pierce BCA assay. The first step is the chelation of copper with protein in an alkaline environment to form a blue colored complex (Figure 1). In this reaction, known as the biuret reaction, peptides containing three or more amino acid residues form a colored chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate. During this reaction there is reduction of Cu^{2+} to Cu^{1+} by protein in the alkaline medium. In the second step of the color development reaction, BCA, a highly sensitive and selective colorimetric detection reagent reacts with the cuprous cation (Cu^{1+}) that was formed in step 1. The purple colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations.

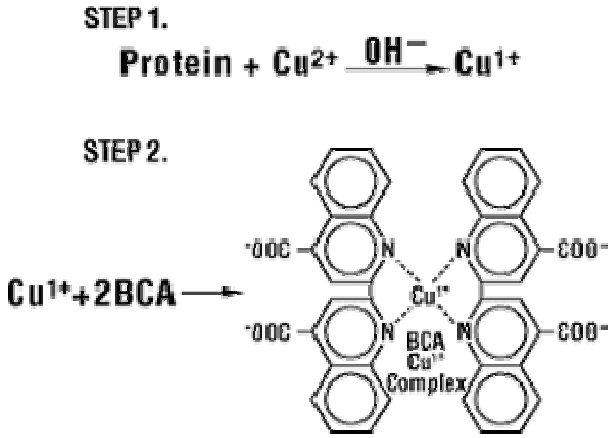


Figure 2. Two step mechanism leading to protein dependent color development of bicinchoninic acid.

Exercise 1: Student Investigation of Protein Content

All organisms are composed of four types of biological macromolecules, carbohydrates, lipids, nucleic acid, and protein. Nucleotides and amino acids are the respective building blocks or monomers cells use to make nucleic acids and proteins. Nucleotides and amino acids both use nitrogen atoms in their structure. Therefore one could say life is dependent on nitrogen. Lucky for us there is abundant nitrogen as N_2 in our atmosphere. However our cells are unable to use gaseous nitrogen. We are dependent on certain bacteria that can reduce gaseous nitrogen to NH3. Still other bacteria partially oxidize the NH_3 to NO_2^- or even further to NO_3^-. Bacteria and plants use these forms of nitrogen in combination with carbon based molecules to create amino acids and nucleic acids. We gain access to reduced nitrogen in the form of amino acids and nucleic acids by eating bacteria or plants directly or by eating other consumers of bacteria and plants.

How much protein do you need? The World Health Organization suggests 56 g of protein per day for a 75 kg man and 48 g per day for a 64 kg woman (454 g equals 1 lb). Using this information you should be able to calculate how much protein your body requires per day. The next problem you face is deciding where to get the required protein. Design an experiment to help you in meeting your protein requirements.

Guidelines for experimentation

1. Generate a question on protein content in two biological materials based on your previous observations.

2. Form hypotheses, including a numerical statement of each hypothesis.

3. Use the protocol below to support your investigation.

Protein extraction

Work in groups of 2 students only.

1. Obtain an ice bucket with ice in which to work. Keep samples on ice at all times during their preparation. Chill a mortar and pestle and the extraction buffer in your ice.

2. Clearly label four 1.5 mL microcentrifuge tubes (one tube for each sample, for example; two replicate samples of one kind or type versus two replicates of another). Chill these tubes on ice.

3. For a single sample collect 0.5 g of material (weigh it on the balance) and immediately transfer it to the pre-chilled mortar. Proceed to the next step quickly.

4. Add 1.5 mL of extraction buffer (a ratio of 3:1 buffer volume to sample weight) and grind vigorously in the mortar using the pestle. Before the sample has a chance to warm up, use a pipette to transfer 500 uL of ground sample to the corresponding labeled and pre-chilled 1.5 mL microcentrifuge tube and place on ice. The actual volume recovered here and placed in the tube is not important, but whatever volume you do use you will use for all samples so that your tubes are all the same weight and can balance each other in the microcentrifuge.

5. Repeat steps 3 and 4 until all samples have been prepared and are incubating on ice. Continue the incubation for 5 minutes on ice after grinding the last sample. If needed use fresh extraction buffer to bring all tubes to the same volume.

6. Spin in microcentrifuge at 10,000 x g for 10 min.

7. Keep these samples on ice at all times. Make sure they are labeled well.

Protein assay

Setting up the standards:

1. Obtain 10 new 1.5 mL microcentrifuge tubes in a rack. Label 5 tubes with the following; 0 uL, 2.5 uL, 5.0 uL, 10 uL, and 20 uL. Label the second set of 5 tubes with the same labels; there are now 2 tubes (duplicates) for each label. See Table 1 below for an example of labeling and overview.

2. In duplicate, pipette the following volumes of a 2 mg/mL BSA solution into correspondingly labeled tubes: 0 uL, 2.5 uL, 5.0 uL, 10 uL, and 20 uL. From a 2 mg/mL BSA solution these volumes are equivalent to the following ug of BSA: 0, 5, 10, 20, 40.

3. Store until samples are prepared.

Setting up the samples:

Obtain and label two new 1.5 mL microcentrifuge tubes for each sample you prepared (that should be 8 new tubes total). In duplicate for each sample, pipette 10 uL of protein extract into correspondingly labeled tubes. The 10 uL volume is a suggested volume that generally works well with plant material.

Performing the assay

1. To each BSA standard and protein extract sample in the microplate add 1 mL of the Pierce BCA solutions A and B assay mixture. Your lab instructor will prepare the Pierce BCA solution A and B mixture for the class (50:1 v/v A to B). Each lab group will need 18 mL.

2. Cover and incubate at 37 C for 30 min.

3. Read absorbance at 562 nm against one of the 0 ug BSA standards.

4. Record your data in a table in your lab notebook. Use Table 1 below as an example.

Table 1. Protein assay raw data table

Tube Label			ug of protein	uL of sample	Absorbance @ 562 nm
BSA standards	0 uL	Measure rep 1	0	0	
		Measure rep 2	0	0	
	2.5 uL	Measure rep 1	5	2.5	
		Measure rep 2	5	2.5	
	5 uL	Measure rep 1	10	5	
		Measure rep 2	10	5	
	10 uL	Measure rep 1	20	10	
		Measure rep 2	20	10	
	20 uL	Measure rep 1	40	20	
		Measure rep 2	40	20	

Tube Label			ug of protein	uL of sample	Absorbance @ 562 nm
Sample 1	Sample rep 1	Measure rep 1	?	10	
		Measure rep 2	?	10	
	Sample rep 2	Measure rep 1	?	10	
		Measure rep 2	?	10	
Sample 2	Sample rep 1	Measure rep 1	?	10	
		Measure rep 2	?	10	
	Sample rep 2	Measure rep 1	?	10	
		Measure rep 2	?	10	

Materials for Protein Extraction and Assay Lab

Students will work in pairs; plan for 10 groups per lab section.

Materials

Ice buckets with ice (one per group)
Fine tip marking pen (one per group)
Mortar and pestle (one per group)
1.5 mL microcentrifuge tubes (22 per group)
Microcentrifuge (2 if possible for class, could these be set up in cold room?)
1.5 mL microcentrifuge tube rack (two per group)
Micropipetors and tips (one 0.5 - 10 uL and 20 - 200 uL and 200 - 1000 uL for each group)
500 mL graduated cylinder (one for class)
500 mL beaker (one for class)
Pierce BCA assay solutions A and B, Pierce #23225
(instructor mixed, each group needs 18 mL)
50 mL beakers (one per group to obtain mixed BCA assay from instructor)
37 ºC water baths, lots of 1.5 mL tube floats, 18 tubes/group. Racks and walk-in?
Plastic disposable cuvettes (3 per group)
Spectrophotometers (1 per group)

Solutions

1. Extraction buffer (6 mL per group). Provide an aliquot per group? Or one bottle and small beaker for each group to pour some out.

	For 100 mL
50 mM MOPS	1.05 g
100 mM NaCl	0.58 g
10 mM EDTA	0.37 g
0.1 % Triton X-100	0.1 g
0.1 % sodium lauryl sarcosine	0.1 g

pH to 7.0

2. 2 mg / mL BSA solution as protein assay standard (might be part of Pierce BCA assay). (Each group will need 150 uL). Provide an aliquot per group?

	For 10 mL
Water	10 mL
BSA	0.2 g

Other

Grocery items to support student investigations on protein content, for example:

Variety of meats or other animal products
Variety of vegetables
Variety of fruits
Variety of beverages

4 Protein Standard Curve and Concentration of Sample Extracts

Objectives

- To create and manipulate spreadsheets in Excel
- To graph, perform trendline analysis, and install custom error bars in Excel.
- To complete investigation on protein content of samples.

Exercise 1: Student Investigation of Protein Content

Creation of the standard curve

1. Working in groups no larger than 2 students.

2. Open a new spreadsheet in Excel. Name it (something you like) and save it to your memory stick. Save often.

3. Use Figure 1 below as a guide to enter your data in your spreadsheet. Columns B and C have been titled X and Y respectively to indicate graph axes when the standard curve is created (steps below). Column B should contain your results for the BSA standards you assayed.

	A	B	C	D	E
1					
2	BSA data				
3		X	Y		
4		Absorbance	ug BSA	uL 2 mg / mL BSA	
5		0.000	0.0	0.0	
6		0.000	0.0	0.0	
7		0.299	5.0	2.5	
8		0.230	5.0	2.5	
9		1.025	20.0	10.0	
10		1.040	20.0	10.0	
11		1.993	40.0	20.0	
12		1.993	40.0	20.0	
13					

Figure 3. Example entries into an Excel worksheet.

4. Click on the "Chart Wizard" in the Excel upper tool bar. In the new window that pops up select the "X-Y Scatter Chart Sub-Type" and click on "Next".

5. After the pop up window refreshes click on the "Series" tab at the top of the window. Then click on "Add" under the "Series" selector.

6. After the pop up window refreshes, click in the box to the right of "Name" to get a curser, and then type a name such as "BSA standards" in the box. Next click on the little red arrow to the right of the "Values" box. The pop window will downsize, at this time use the mouse to highlight just the Y values for your standard curve (these are the ug BSA values on your spreadsheet. Once the Y values for your graph have been selected click on the little red arrow of the downsized pop up window. After the pop up window refreshes click on the little red arrow right of the "Category X axis labels" box. After the pop up window downsizes use the mouse to highlight the values under the X labeled column of your spreadsheet. Then click on the little red arrow of the downsized window.

7. After the pop up window refreshes click on "Next". The window will refresh again and here you will type in labels for the X and Y axes of your graph. Remember axes labels include units if appropriate. For example your Y axis label should be "ug BSA". After you type in your labels click on "Next".

8. After the pop up window refreshes check to see the "save in sheet" radio button is selected then click on "Finish".

9. Lets draw a trend line to see how well you did pipetting. I am not sure what you have clicked on last, so that we are all at the same starting point click on some empty cell of the spreadsheet. Now using the mouse right click on a data point on your graph. A new pop up window will appear and click on "Add Trendline". Then click on "Linear", then (before clicking on OK) click on the options tab and select "Display equation on chart" and "Display R-squared value on chart", and finally click "OK". Hopefully your R-squared value is close to 1. If not, your pipetting was rather poor.

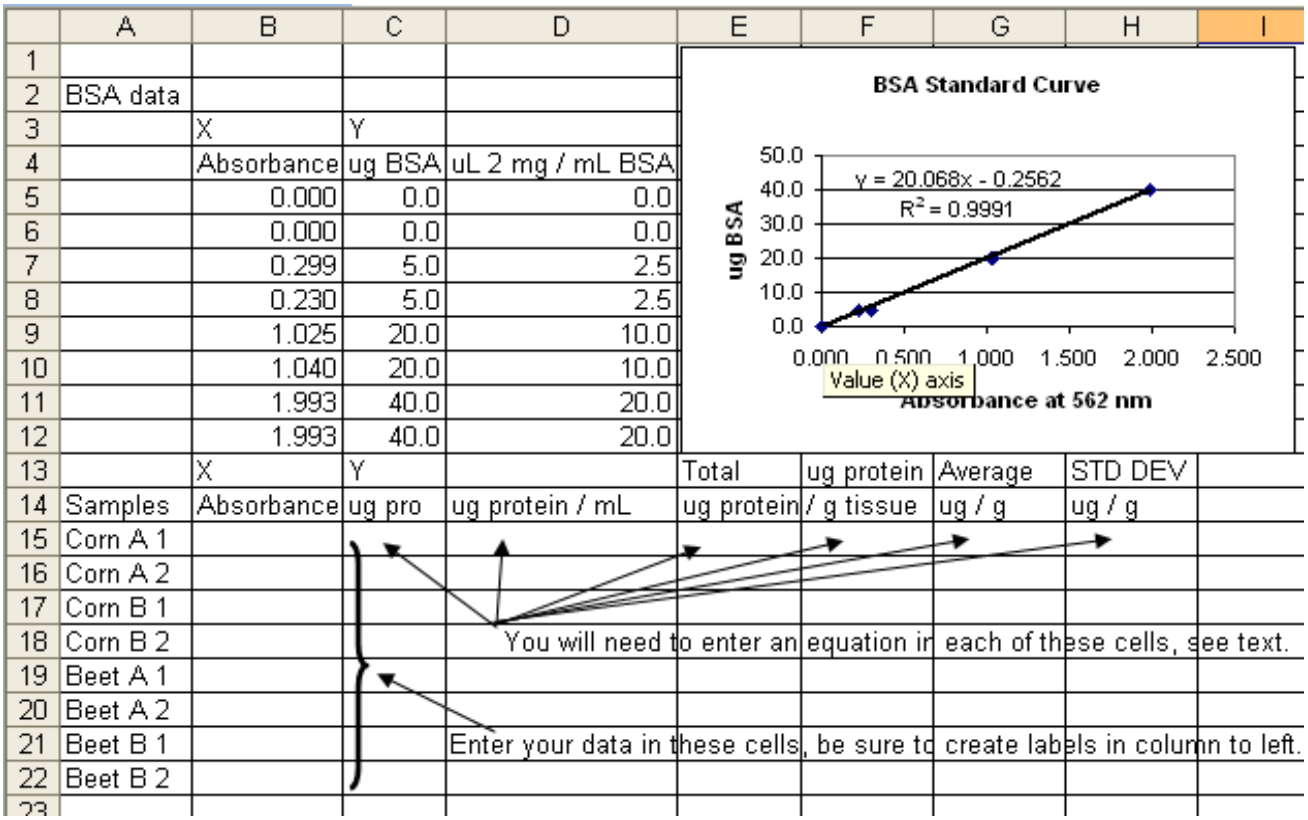
Using the standard curve to determine protein concentrations

1. Use the equation of your standard curve to calculate protein content in your samples. To do this first enter the absorbance values for your samples in your spreadsheet as a continuation of column "X" (see Figure 2 for example). Be sure to add labels as to sample identity in the column immediately left.

2. Next click on the cell to the right of your upper most sample absorbance value. With the cell selected from clicking on it type "=" followed by the rest of the equation of your line for calculating Y, then hit the return / enter key. To apply this same calculation to all your sample absorbance data, left click once on the cell, then left click and hold down the button on the cross that appears at the bottom right hand corner of the cell as you drag the cross down to automatically fill in the calculation for all rows with data. The numbers generated equal the ug amount of protein present in the volume of sample you assayed.

3. Calculate the concentration of protein in each of your samples. To do this all you have to do is divide the amount of protein by the volume it was present in. Click in the upper most cell to the right of your sample ug protein column. Once this cell is highlighted type "=" followed by (if your cells are in the exact position as those in Figure 2) "C3/10" (this also assumes you assayed 10 uL of the extracts you prepared.

4. Use your new skills in typing in equations to calculate average and standard deviation of protein concentrations for your experiment.



	A	B	C	D	E	F	G	H	I
1									
2	BSA data								
3		X	Y						
4		Absorbance	ug BSA	uL 2 mg / mL BSA					
5		0.000	0.0	0.0					
6		0.000	0.0	0.0					
7		0.299	5.0	2.5					
8		0.230	5.0	2.5					
9		1.025	20.0	10.0					
10		1.040	20.0	10.0					
11		1.993	40.0	20.0					
12		1.993	40.0	20.0					
13		X	Y		Total	ug protein	Average	STD DEV	
14	Samples	Absorbance	ug pro	ug protein / mL	ug protein	/ g tissue	ug / g	ug / g	
15	Corn A 1								
16	Corn A 2								
17	Corn B 1								
18	Corn B 2								
19	Beet A 1								
20	Beet A 2								
21	Beet B 1								
22	Beet B 2								

Figure 4. Example of additional columns for calculation of protein concentration (Column D), total protein extracted (Column E), protein recovered per gram of tissue (Column F).

Calculating the amount of protein in a gram of starting material

1. In the next row to the right, calculate the total protein extracted from each sample. You will be on your own here to come up with the equation and apply it. If you are stuck recall that when you multiply total sample volume x sample concentration the units of volume cancel out and you are left with units of mass. But just exactly what was your total sample volume. The protocol suggested you recover 0.5 mL of fluid from the mortar and pestle after grinding, so you recovered only a portion of the original sample. The protocol suggested you grind 0.5 g of tissue with 1.5 mL of extraction buffer. Assuming biological tissues are mostly water by weight your total sample volume was closer to 2 mL.

2 From what mass of starting material did you extract this amount of protein? In a new column to the right calculate the amount of protein you would expect to find in 1 gram of starting material.

3. Add two more columns to support summarizing your data, one column where you calculate average protein content and one calculating standard deviation of protein content.

Summarizing your results

1. Fix a copy of your completed spreadsheet in your notebook.

2. Graph your summary data (In Excel with custom error bars!!). Use the "Chart Wizard" in Excel as you did before but choose "Column" chart sub-type instead of the scatter plot we used previously. Enter your data as before. With the chart complete right click on the bars in the chart and a pop up menu should appear with "Format data series" at the top of the list. Click on "Format data series". Now click on the "Y error bars tab" and then select "Plus" under "Display" and select "Custom" under "Error amount". Now use the little red arrows to the right of the boxes to get the standard deviations you calculated as error bars.

3. Prepare a table of your summary data.

4. Perform a t-test.

5. Write your conclusion.

6. Prepare a transparency of your experiment. Present your question, hypotheses, your standard curve (so everyone can see how well you pipette), a summary table or graph, t-test results, and conclusion.

Materials for Protein Extraction and Assay Lab

Students will work in pairs, plan for 10 groups per lab section.

Computer with Excel (one per two students)
Memory stick (supplied by student)

5 Measuring Enzyme Activity

Objectives

- To gain a more complete understanding of enzyme catalysis
- To gain a better understanding of saturation kinetics
- To gain an understanding quantitative colorimetric assays
- To be able to use extinction coefficients
- To be able to calculate concentrations of components within an assay
- To be able to calculate specific activity for an enzyme
- To develop a better understanding of competitive and allosteric regulation
- To design and carryout an experiment addressing regulation of the citric acid cycle
- To be able to identify modes of inhibition from kinetic data

Citrate synthase catalyzes the first reaction of the citric acid cycle (Figure 1), the condensation of the acetate group from acetyl-CoA with oxaloacetate to form citrate (Figure 5). During the catalysis citryl CoA is formed as an intermediate at the active site. Citryl CoA is a high energy thioester, and hydrolysis of the thioester liberates a considerable amount of energy driving the reaction forward. The mammalian citrate synthase is a dimer of two identical subunits. Each subunit is a single polypeptide with two domains, one large and rigid, and one small and flexible. The active site resides between these two domains. During catalysis the enzyme displays ordered sequential binding of substrates. First, the large domain of the enzyme binds oxaloacetate, which induces a large conformational change in the flexible domain (Figure 7 and Table 2, from Hayward 2004), creating a site for the second substrate acetyl-CoA. Amino acid residues of the large domain, hinge region, and small domain have been identified (Table 3, from Hayward 2004). One hypothesis regarding forces driving movements of the hinge includes interaction of the carbonyl group of His274 with the negatively charged oxaloacetate (Roccatano *et al.*, 2001). After bending is complete the closed conformation is at least in part stabilized by the side-chain interaction of His274 with oxaloacetate and also by oxaloacetate salt-bridging to Arg329 and by hydrogen bonding to His320 (Hayward 2004).

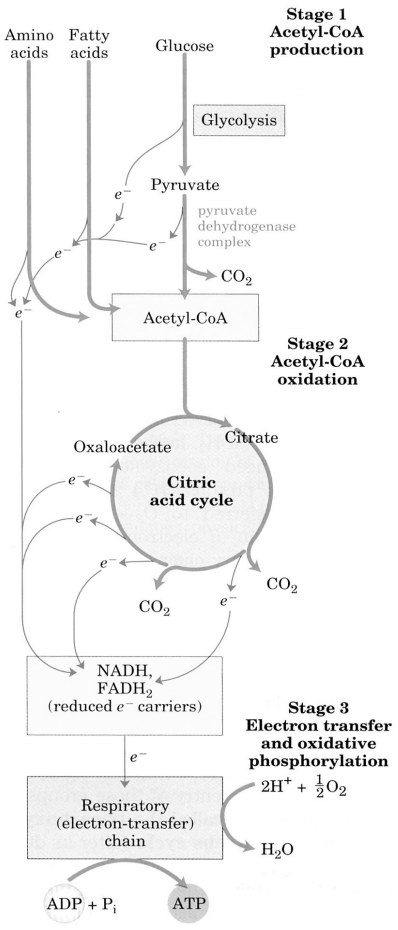


Figure 5: Central Metabolism

Because of the roles they play in bending His274, Arg239,

and His320 are designated closure-inducing residues. The binding site for acetyl-CoA is formed after flexing of the hinge region by regions of the both the large and small domains and acetyl-CoA can only bind after the oxaloacetate induced movements. Once the citryl CoA intermediate has formed another conformational change occurs to hydrolyze the thioester and release CoA-SH. Citrate then leaves the active site and the enzyme returns to its initial conformation ready for the next catalytic cycle.

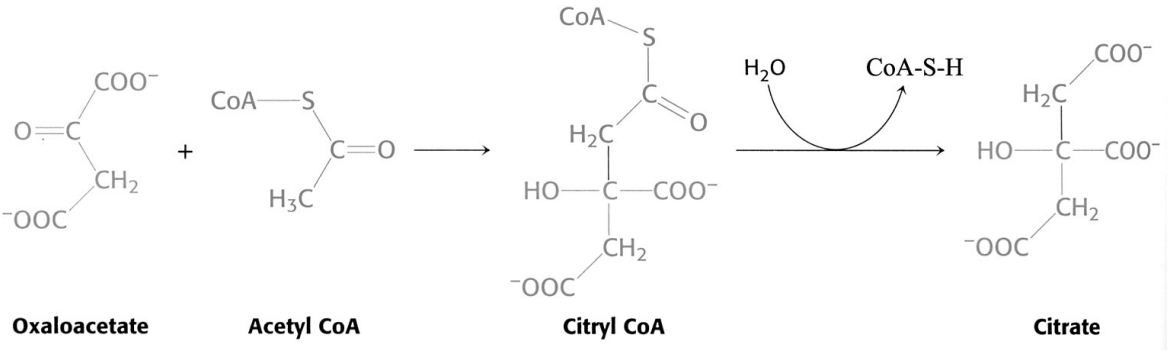


Figure 6: The reaction catalyzed by Citrate Synthase

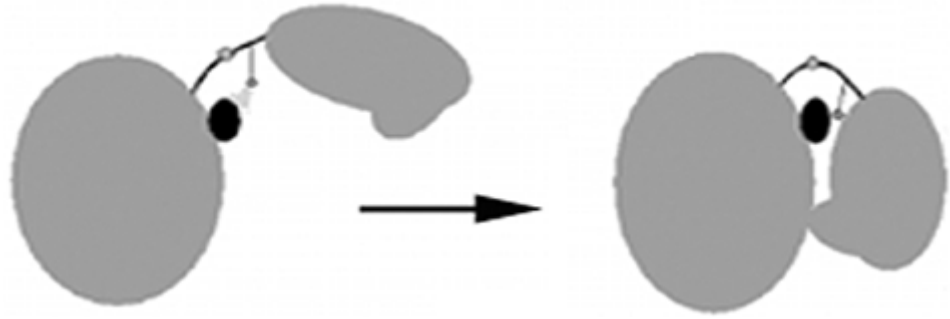


Figure 7: Ligand-induced closure. Ligand binding to the large domain followed by intersection with the side chain of the hinge produces torque about a hinge point causing the closing domain to close upon the ligand and binding domain.

Table 2: Closure-inducing ligand and angle of rotation upon closure

Enzyme	Closure-inducing ligand(s)	Angle of rotation (deg.)	PDB accession codes and chain identifiers for pair of conformers studied	
			Closed[a]	Open
Adenylate kinase	AMP, AMPPNP	42, 51	1ANK_B	4AKE_A
Aspartate aminotransferease	α-Methylaspartate, AMA	15	1AMA	9AAT_A
Citrate synthase	Oxaloacetate, OAA	19	5CTS	5CSC_A
Liver alcohol dehydrogenase	NAD	10	2OHX_A	8ADH
cAMP-dependent protein kinase (catalytic subunit)	ATP	15	1ATP_E	1CTP_E

[a] Contains structure of closure-inducing ligand.

Table 3. Definitions of domains and bending regions and contacting residues in aspartate aminotransferase, citrate synthase, liver alcohol dehydrogenase and the catalytic subunit of cAMP-dependent protein kinase, all two-domain proteins

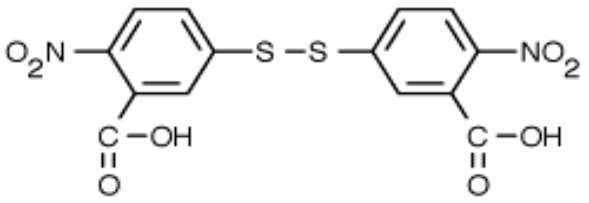
Enzyme	Large domain	Bending between large and small domain[a]	Small domain
Aspartate aminotransferase			
Residue ranges	42–322	34–41, *323–329*, *357–361*	15–33, 330–356, 362–410
AMA contacting residues in closed[b]	107–109, 112, 140, 194, 225, 258, 266, PLP[c]	37, 38, 360	17,386
Citrate synthase			
Residue ranges	3–55, 66–272, 330–335, 382–433	*56–57*, *64–65*, 273–283, *328–329*, 336–337, *379–381*	58–63, 284–327, 338–378
OAA contacting residues in closed[b]	238, 242, 397, 401	274, 329	320
Liver alcohol dehydrogenase			
Residue ranges	1–175, 318–374	*176–177*, 290–301, *315–317*	178–290, 302–314
NAD contacting residues in closed[b]	46–48, 51, 174, 318, 319, 362, 369	292–294, 317	178, 199–203, 223–225, 228, 268–271
cAMP-dependent protein kinase			
Residue ranges	15–28, 94–104, 123–315	29–30, *92–93*, *105–106*, *121–122*, 316–330	31–91, 107–120, 331–350
ATP contacting residues in closed	104, 123, 127, 166, 168, 170, 171, 173, 183, 184	121, 122, 327	49–55, 70, 72, 120

Numbering is from the closed structure PDB file (see Table 1).
[a] Italics indicate that the bending region is a mechanical hinge, i.e. contains a residue with its C^{α} within 5.5 Å of the interdomain screw axis.[13]
[b] Only residues in the subunit to which the ligand is bound are considered.
[c] PLP is the cofactor of AspAT.

Exercise 1: Determination of Enzyme Activity in an Extract

Enzymes are generally obtained through the homogenization of cells, tissues, organs, even whole organisms. Homogenization protocols liberate enzymes from cells by breaking cells open in the presence of an extraction buffer, in which the enzyme of interest is soluble in. After centrifugation of the homogenate to pellet insoluble material, cellular debris, and unbroken cells, a crude extract is obtained. The catalytic activity of the enzyme(s) of interest may be studied directly, or after purification of the enzyme from other cytosolic components present in the extract that might otherwise alter its activity. You will be studying citrate synthase purified from porcine heart.

Biochemists track the activity of citrate synthase with 5,5'-dithiobis(2-nitrobenzoic acid (DTNB or Ellman's reagent, Figure 4, upper panel) in a quantitative colorimetric assay. DTNB in the presence of CoA-SH, forms TNB which is yellow and absorbs strongly at 412 nm, and CoA-S-S-TNB (Figure 8, equation or lower panel).



$$CoA\text{-}SH + DTNB \rightarrow TNB + CoA\text{-}S\text{-}S\text{-}TNB$$

Figure 8. DTNB (upper) and formation of the mercaptide ion (lower).

By combining the events from equations in Figures 6 and 8, the amount of TNB formed should be equivalent to either the acetyl-CoA or the oxaloacetate used by citrate synthase during catalysis. Using the molar extinction coefficient for TNB (13600 / M cm) biochemists can calculate the amount of product formed or substrate consumed in moles in the assay.

If:

Absorbance at 412 nm / 1 cm light = (13600 / M cm)(product concentration in reaction)(1 cm light)

Then:

(Absorbance at 412 nm / 1 cm light)(M cm / 13600)(1 cm light) = product concentration in reaction

Since the volume of the reaction (the assay volume) is known it is easy to calculate the moles of product formed. Remember the units on concentration are Molar (always a big M). Molarity = moles (always spelled out moles) per liter. Multiply by the reaction volume and the volume units cancel out leaving moles.

(moles / liter) (liter) = moles

While studying an enzyme it is useful if the reaction rate can be adjusted so that catalysis occurs in a convenient time frame for measurement. Not too fast and not too slow. This requires knowing how much citrate synthase activity is present in the supplied preparation. Since we do not know the amount citrate synthase activity in the supplied preparation you will have to determine the amount of activity experimentally. You will do this by creating several different dilutions of the enzyme in the hopes that one dilution or possibly all of them will result in a reaction rate that we can accurately measure. It should make sense that with less enzyme around, the rate of the reaction we are monitoring will slow. If not, this experiment will demonstrate this principle. The results of this experimentation will also help you choose reaction conditions for future experiments.

Working in groups no larger than 2 students:

1. Obtain an ice bucket filled with ice in which to work.

2. In labeled 1.5 mL tubes kept on ice, dissolve the pre-weighed aliquot of DTNB in 2 mL of 1 M Tris-HCl pH 8. Store at room temperature.

3. In labeled 1.5 mL tubes kept on ice, use 50 mM Tris-HCl pH 8 to prepare the dilutions of the citrate synthase listed in the table. But first you will have to prepare the 50 mM Tris-HCl ph 8 from a 1 M stock solution. **Keep the citrate synthase and any dilution you make of it on ice at all times.**

Table 4. Preparing dilutions of citrate synthase

Dilution	1x CS enzyme solution (uL)	50 mM Tris-HCl pH 8.0 (uL)
1 :5	20	80
1 :5	20	80
1 :10	20	180
1 :50	20 (of the 1:5)	180
1 :100	20 (of the 1:10)	180
1 :500	20 (of the 1:50)	180

4. Make sure your spectrophotometer is on and warmed up.

5. Boil one of the tubes of 1:5 diluted enzyme for 5 min, then quickly place it on ice to cool, and continue on. Spin 10 seconds in a balanced configuration in a microcentrifuge before using.

6. Set up reactions as indicated in Table 5 below one at a time. Begin with reaction 6 as outlined in Table 5 below by assembling the all reaction components in a cuvette except Acetyl-CoA in the order they appear from left to right across the table. Go to step 7.

Table 5. Contents of cuvettes to be examined for citrate synthase activity

Reaction or Cuvette / CS dilution	uL of 10 mM Oxaloacetate	uL of Water	uL of 1 mM DTNB	uL of Enzyme	uL of 2.5 mM Acetyl-CoA
1. 1:5	43	777	100	20 (boiled)	60
2. 1:5	43	777	100	20	60
3. 1:10	43	777	100	20	60
4. 1:50	43	777	100	20	60
5. 1:100	43	777	100	20	60
6. 1:500	43	777	100	20	60

7. Set the spectrophotometer at 412 nm. Refer to Appendix B or C for operation of the spectrophotometer. Add Acetyl-CoA only to the prepared cuvette 6, cover with parafilm, **mix by inversion**, then insert the cuvette into the spectrophotometer and if using Ocean Optics USB4000 begin recording data by clicking on the "Restart Selected Acquisition" button (green arrowhead, be sure to have read the instructions for spectrophotometer operation in the Appendix for considerations on how to retrieve your data). This cuvette contains all the same components as the others. At 412 nm you are monitoring the mercaptide ion (Figure 8). None of the other starting materials absorb at this wavelength.

8. Continue reading the 1:500 reaction.

9. While you collect data, have your lab partners use Step 6 above to begin setting up cuvette 5.

10. When your reaction is done, your lab partners can add acetyl-CoA (do not forget to mix by inversion, better use a clean piece of parafilm for each cuvette) to cuvette 5, place it in the spectrophotometer, quickly zero the absorbance with the right hand knob while starting a timer, and record the absorbance values at 2-minute intervals and record the values in a raw data table in your notebook.

11. You should begin setting up cuvette 4.

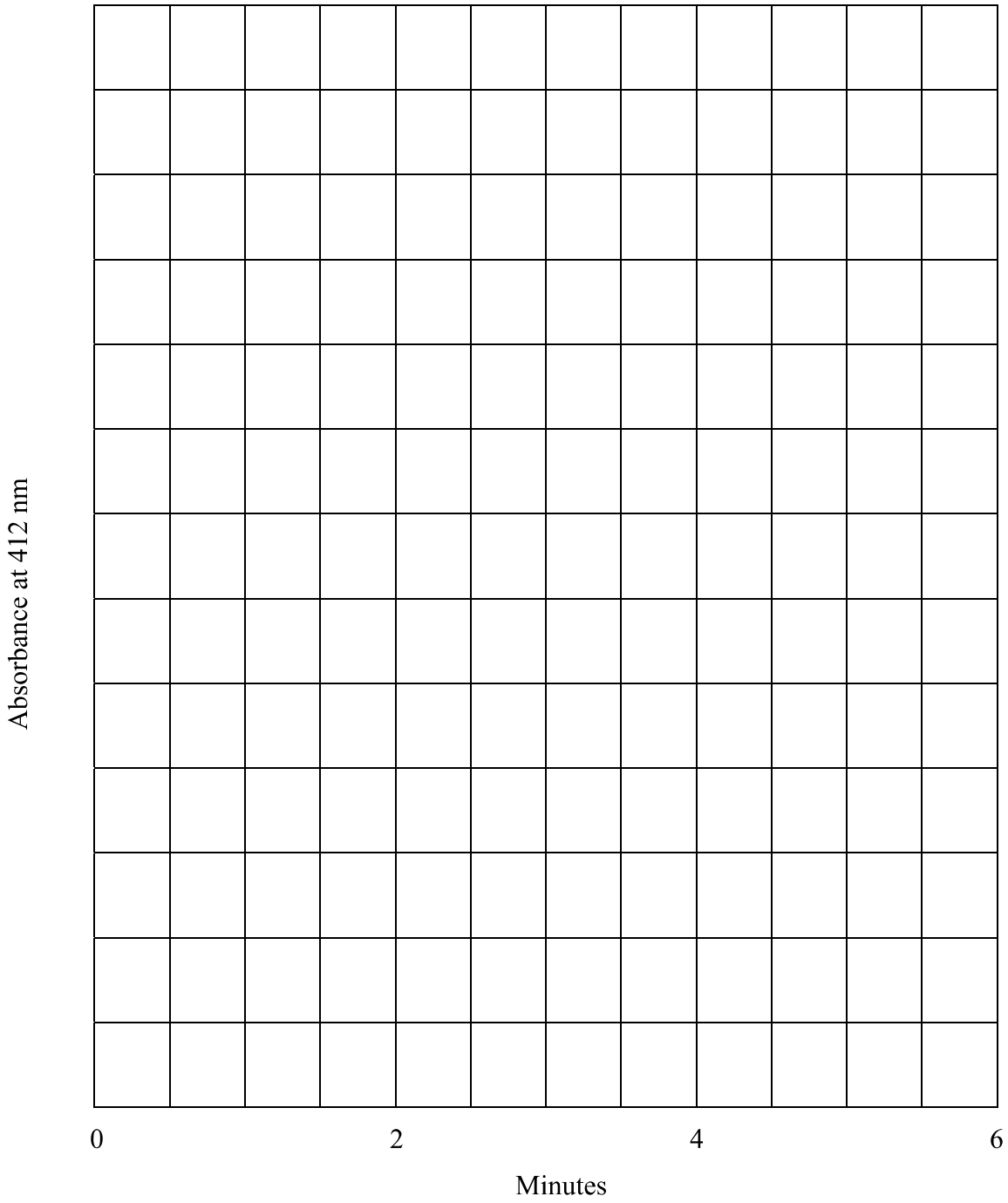
12. Continue carrying out the assays, up the citrate synthase dilution series.

13. The last reaction is with citrate synthase that has been previously boiled for 10 minutes and then cooled on ice. There should be little or no activity from this sample. Why?

Table 6. Absorbance at 412 nm at room temperature for citrate synthase assays

Minutes	Cuvette 1	Cuvette 2	Cuvette 3	Cuvette 4	Cuvette 5	Cuvette 6
0						
2						
4						
6						

14. On a single graph in your notebook, plot your results for each cuvette with time in minutes on the x-axis and absorbance at 412 nm on the y-axis.



15. Look at the curves for each cuvette in your graph. Do they all have the same profile? Why or why not?

16. What does the slope of the lines on your graph represent?

17. What dilution would be the most useful in determining the affects of substrate concentration or inhibitors on citrate synthase catalysis? Why?

18. Which reaction maintained linearity (for reaction rate) for the greatest amount of time?

Exercise 2: Student Investigation on the Regulation of Citrate Synthase

In living cells the rate of the citric acid cycle is adjusted to meet needs for ATP. When ATP levels are low the cycle needs to be speed up, when ATP levels are high it makes sense to slow catalytic rates of enzymes in the cycle. For many pathways, enzymes at the beginning, or end, or those that catalyze steps with large changes in free energy serve as control points. These control point enzymes respond via allosteric sites to certain molecules that indicate energy status. What molecules might be indicators of low energy? What molecules might be indicators of high energy?

Look again at Figures 5 and 6. Now examine the structures of the substrate molecules (Figure 9). From the molecules in Figure 9, which ones might compete for the active site of citrate synthase? Which ones might be indicators of cellular energy levels? Are there any molecules that might do both, bind to allosteric sites and compete for the active site of citrate synthase?

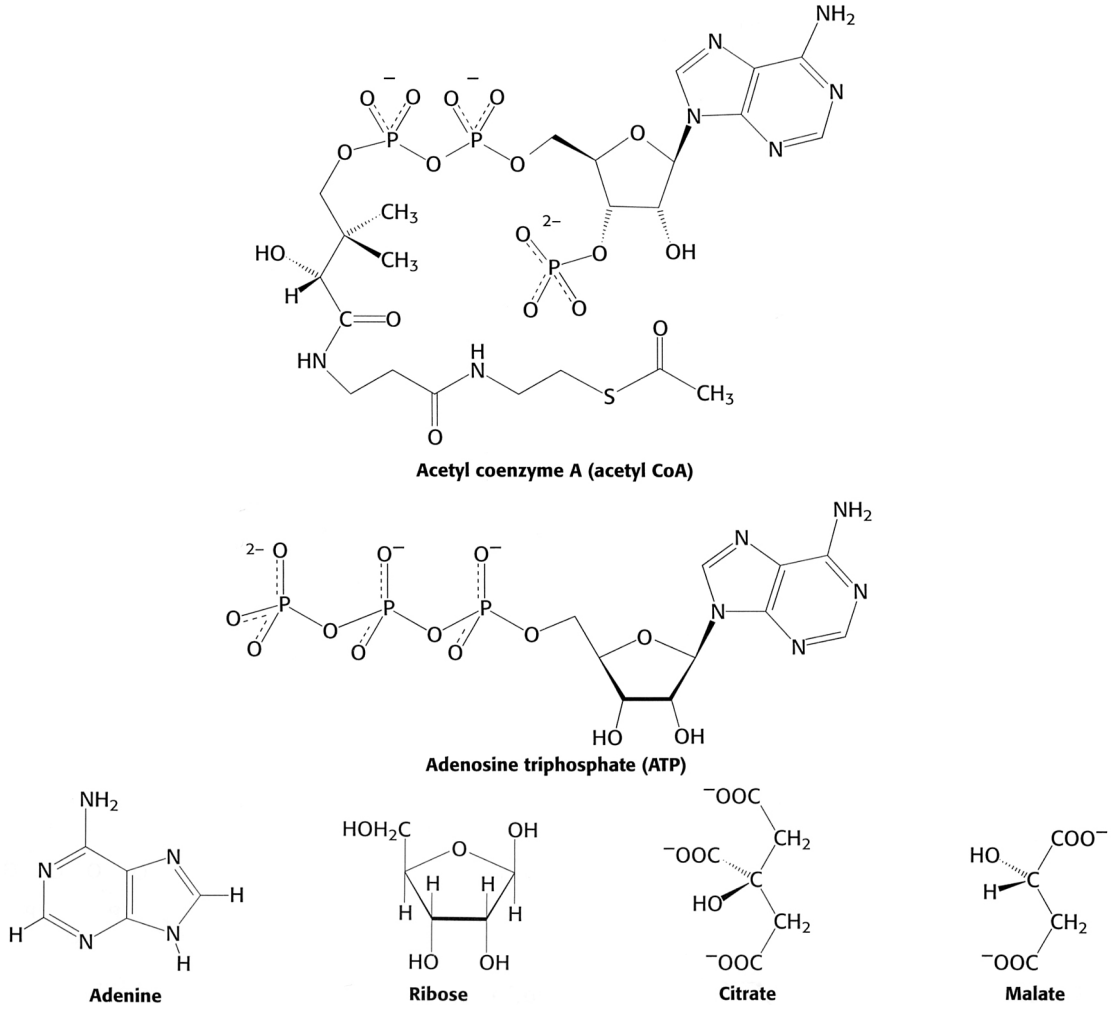


Figure 9. Molecules and their structures

To help you envision what substrate molecules look like when bound to the active site of citrate synthase we will use the Cn3D software provided by NCBI to view files containing 3-D models generated from x-ray crystallography data of citrate synthase at various stages during catalysis. Examine the structural models paying careful attention to functional groups of substrates that appear in close proximity to the functional groups of amino acids within the active site. These functional groups are likely to be important in molecular recognition.

Now reread the introductory material to this lab and examine the structures of the native substrate in Figures 6 and the structures of possible regulatory molecules in Figure 9. Based on structural similarities, answer the following:

1. What molecules might be competitive inhibitors of citrate synthase? Why?

2. What molecules might be allosteric regulators of citrate synthase? Why?

3. Within your group identify one molecule from the structures provided that you suspect may alter the activity of citrate synthase, formulate a question and hypotheses to test next week.

4. Write the name of the molecule on a sheet of paper and give it to your instructor before leaving.

Period I Questions (must be answered in your notebook)

1. Describe the reaction catalyzed by citrate synthase.

2. Why is the addition of DTNB necessary for quantification of the reaction?

3. What would you predict would happen if you added Co-A to a reaction?

4. Describe the relationship between the formation of the TNB and CoA. Be quantitative.

5. Describe the relationship between rate of catalysis by citrate synthase for the assay you performed and the amount of enzyme present. Be quantitative.

Transparency Assignment Due Next Period

Prepare a transparency to be presented at the beginning of the next laboratory period when each group will report their question and hypothesis regarding their inhibitor investigation. The presentations should include ample discussion on the molecular structure of the potential inhibitor chosen and whether you expect a competitive or allosteric mechanism and why?

Materials for Enzymology Lab Periods I and III

From Sigma (prices 2006)

Citrate synthase	C3260	1000 units	70.70	
DTNB	D8130	5g		45.10
Acetyl-CoA	A2056	100mg		599.00
Oxaloacetic acid	O4126	5g		33.50
ATP	A2383	1g		24.40
ADP	A2754	1g		35.30
Citric acid	C0759	500g		18.80
D-ribose	R7500	5g		8.80
Adenine	A8626	5g		15.90
Malic acid	M1125	5g		21.90

For the class

- I, III Parafilm and scissors
- I Boiling water baths with floating 1.5 mL tube holders
- I, III 1.5 mL tube centrifuges
- I Balances 50 mg to 0.5 g accuracy, ideally one per group (a total of 6)
- I, III Ice

For each group (students working in pairs so try to set up for 10 groups)

- I Micropipettes one each of the 20 – 200, and 200 – 1000 and tips
- III Micropipettes one each of the 0.5 – 20, 20 – 200, and 200 – 1000 and tips
- I, III Reagent bottle (100 to 200 mL size) about 1/4 full with nanopure water
- I, III Micropipette tip discard collection
- I, III 1.5 mL microcentrifuge tubes (one source of tubes per student group)
- I, III Spec 20 and 1 mL disposable cuvette adapters
- I, III 6 Spec 20 1 mL cuvettes
- I, III Ice bucket (one per student group)
- I, III Fine tip permanent marker
- I, III Water squirt bottle for rinsing cuvettes
- I, III Timer
- I, III Reagent bottle with 50 mL 1 M Tris-HCl pH 8.0
  1 M Tris-HCl pH 8.0 also for making some of the solutions below.
  121 g Tris-base (FW 121) in 800 mL of water, pH to 8.0, bring up to 1 L.

Solutions to be made, stored, and provided in the -20 C (bag for each section)

1 mM 5,5'-dithiobis(2-nitrobenzoic acid) or DTNB

3.9 mg of DTNB (free acid sigma #D8130, FW 396.4) in 1 mL of 1 M Tris-HCl pH 8.1 (Must be made fresh each day). For period I weigh out 8 mg aliquots in 1.5 mL tubes and let students add 2 mL of 1 M Tris-HCl just before use. For period III 8 mg aliquots are needed to which the students will add 2 mL of buffer.

2.5 mM acetyl-CoA

2.02 mg of Acetyl-CoA (sigma #A2056, FW 809.6, mM extinction coefficient of 15.4) per 1 mL water (can be frozen -20 C). Hopefully 5 uL of this solution in 995 uL of water will give an absorbance at 260 nm of 0.1925. Each group requires exactly 360 uL for period I, and 360 uL for period III.

10 mM oxaloacetate

1.32 mg oxaloacetate (sigma #O4126, FW 132.1) in 1 mL of 0.1 M Tris-HCl pH 8.1 (can be frozen -20 C). Each group requires exactly 258 uL for period I, and exactly 430 uL for period III.

0.1 units / uL citrate synthase

Porcine heart citrate synthase (sigma #C3620, usually shipped as 0.3 mL, at 9.3 mg / mL, aka 374 units / mg). One unit will form 1.0 umole of citrate from oxaloacetate and acetyl-CoA per min at pH 8.0 at 37 C (can be frozen -20 C). To make, CS is shipped as an ammonia sulfate precipitant. Flick the bottle to resuspend the flocculent precipitant and quickly draw off all fluid (or as much as desired, concentration is on the bottle) and put in to a 1.5 mL tube and spin. Remove supernatant with pipette. Add 50 mM Tris-HCl pH 8.0 to final concentration of 0.1 units / 1 uL. Using the info on the reagent bottle calculate the protein concentration and report it to the lab instructors. Assuming 374 units per mg, 40 uL of this solution in 560 uL of water should give an absorbance at 280 of ~0.033. Each group requires exactly 60 uL for period I, and 20 uL for period III. (We used it at 0.267 mg / mL spring of 2006 and at 0.206 mg / mL fall of 2006.

Additional reagents for Period III only, prep only reagents requested by instructors.

10 mM ATP

5.51 mg of ATP (sigma # A2383, FW 551.1, mM extinction coefficient of 15.4) per 1 mL of 100 mM Tris-HCl pH 8.0 (can be frozen -20 C). Hopefully 1 uL of this solution in 999 uL of water will give an absorbance at 260 nm of 0.1540. One group requires exactly 140 uL.

10 mM ADP

4.27 mg (sigma #A2754, FW 427.2) per 1 mL of 100 mM Tris-HCl pH 8.0 (can be frozen -20 C). One group requires exactly 140 uL.

10 mM Citric acid

1.92 mg (sigma #C0759, FW 192.1) per 1 mL of 100 mM Tris-HCl pH 8.0 (can be frozen -20 C). One group requires exactly 140 uL.

10 mM D-ribose

1.50 mg (sigma #R7500, FW 150.1) per 1 mL of 100 mM Tris-HCl pH 8.0 (can be frozen -20 C). One group requires exactly 140 uL.

10 mM Adenine

1.35 mg (sigma #A8626, FW 135.1) per 1 mL of 100 mM Tris-HCl pH 8.0 (can be frozen -20 C). One group requires exactly 140 uL.

10 mM Malic acid

1.56 mg (sigma #M1125, FW 156.1) per 1 mL of 100 mM Tris-HCl pH 8.0 (can be frozen -20 C). One group requires exactly 140 uL.

6 Calculating Specific Activity

Calculating Enzyme Specific Activity and Graphing

Calculating Specific Activity for an Enzyme in Microsoft Excel

How is the catalysis of an enzyme quantified? We could report the number of catalyzed events per enzyme molecule per unit time for defined conditions of temperature, pH, ionic concentrations. However, in quantifying enzyme activity we do not usually express activity per actual number of enzyme molecules. Additionally the number of catalyzed events can be reported as either the number of moles of product formed or the number of moles of substrate consumed. Therefore scientists more commonly report enzyme activity as the number of moles of product formed per mg protein per minute, the units = moles product / mg protein / 1 minute. Expressed in this fashion the catalysis by an enzyme in a given time period, is called specific activity. When comparing catalytic capabilities of enzymes, it is the specific activities that are compared.

You have taken the first step in calculating the specific activity for the porcine heart citrate synthase when last week you collected kinetics data on the rate of catalysis for various dilutions of the enzyme. Today your instructor will guide you through an exercise on calculation of specific activity and a graphing exercise that will help you in the analysis of future data you collect on citrate synthase catalysis.

Exercise 1: Specific Activity

1. In Excel label nine columns with the column headings shown in Table 7 below. You are essentially reproducing the upper portion of the spreadsheet in Appendix C. Now complete the first two columns exactly as they appear in the Appendix.

2. Looking at your data in Table 6 from last week or the graph you created from this data what dilution of enzyme produced a linear reaction rate for a long period of time? Enter the data from a linear 6 minute period of the reaction chosen above (see your graph of Table 6) in the next two columns of your spreadsheet, then in the fifth column calculate the change in absorbance per the 6 minute period. For this fifth column you will not be entering any data, instead enter code for an equation that will perform calculation all the entries in this column (the equation is shown below). The equation is also displayed in the corresponding position of Appendix C displaying exact text that must be typed for the equation to work, except that it must be preceded with "=". To do this click on the top "cell" in the fifth column to "highlight" it. Then, for example, in the "equation box" type =(f9-e9)/6 and then hit "enter" (f9 and e9 are the coordinate locations, 6 the time frame in minutes). Notice the next cell down in the column is now highlighted, click back on the cell above and then click and hold on the lower right corner of the box and drag the highlight box down the column to highlight as many cells vertically as you have data for. This enters the calculation for all cells in this column for which you have initial data.

   Change in absorbance / unit time = (Abs 6 min - Abs 0 min) / (6 minute period)

3. The equation under the sixth column heading of the Appendix C spreadsheet will convert your reaction rate from change in absorbance per 1 minute, to moles of CoA produced per 1 minute using the millimolar extinction coefficient for TNB of 13.600 / 1 mM 1 cm, recall that you measured absorbance of the reaction in a 1 cm cuvette. For this column you will not be entering any numbers, instead you will enter code for an equation that will be used to calculate all the entries in this column (see equation below for this column).

Activity = (change in absorbance / cm * 1 min) (1 mM * 1 cm / 13.600) (assay volume, in L)

To do this click on the top "cell" in the sixth column to "highlight" it. Then, for example, in the "equation box" type =g9/13.6*0.001 and then enter (g9 is the coordinate location of the initial data, 13.6 from the mM extinction coefficient, and 0.001 the assay volume in liters for this experiment only). Notice the next cell down in the column is now highlighted, click back on the cell above and then click and hold on the lower right corner of the box and drag the highlight box down the column to highlight as many cells vertically as you have data for. This enters the calculation for all cells in this column for which you have initial data. In the second column we now have activity expressed as millimoles product formed in 1 minute by the amount of enzyme we added to the assay. But how much enzyme did we add?

4. Recall that the amount of citrate synthase added to your reaction was 20 uL. Therefore the activity just calculated was from some finite number of enzyme molecules in the volume of enzyme added to the reaction. Though the volume of enzyme added to each reaction (Table 6 above) was the same, each reaction received a different number of enzyme molecules since dilutions were prepared (dilutions were reported in the second column of your spreadsheet). Remember, in quantifying enzyme activity we do not usually express activity per number of enzyme molecules. Instead activity units are usually expressed per mg of protein. Enzymes are proteins and more protein means more of the enzyme.

   This final step in quantifying enzymatic activity reflects the rate at which product is formed per mg protein. The result, moles product formed / per unit time / per unit protein is known as the specific activity of the enzyme preparation. You will need to know how concentrated in terms of protein, the citrate synthase preparation is. Your instructor will perform a protein assay on the enzyme preparation for you and report to you the protein concentration of the citrate synthase preparation. We try to provide the enzyme at 0.267 mg / 1 mL, use this value in absence of data from a protein determination on the enzyme.

   Determine the mg of citrate synthase (protein) in your reaction and enter it in the appropriate cell of column four of your table. To do this you need to know the starting protein concentration of the citrate synthase enzyme, how much you diluted it for the reaction, and how much you added to the reaction (see the equation below).

(XX mg / mL citrate synthase) (xx mL of citrate synthase) (dilution) = ? mg citrate synthase

Therefore the equation you would type in to the seventh column should look something like =0.267*0.02/d9 (d9 represents the coordinates for the dilution that must be corrected for, this will be different for each reaction). Use the click and drag method to apply your equation to the rest of the cells in the column.

5. Now that you know how much protein was in each assay, calculate the specific activity of all your citrate synthase reactions (see equation below). Click on the top cell in the eighth column of your worksheet labled "millimoles CoA produced /1 min / 1 mg protein" to highlight it, then for example, in the equation box enter "=h9/i9" and then enter (h9 is the coordinate location of millimoles CoA / 1 min and i9 the location of mg citrate synthase in the reaction). Use the spreadsheet skills you learned above to apply this calculation to all cells in this column.

Specific activity = (millimoles CoA / 1 min) / (mg protein)

Table 7. Calculation of citrate synthase specific activity (millimoles CoA produced / 1 min / 1 mg protein)

Rxn	Fold dilution	Abs 0 min	Abs 6 min	Change in abs/ 10 min	millimoles CoA produced / 1 min	mg citrate synthase in reaction	Millimoles CoA produced /1 min / 1 mg protein	Micromoles CoA produced / 1 min / 1 mg protein
1	5							
2	5							
3	10							
4	50							
5	100							
6	500							

6. Notice the size of the numbers in column eight of your table in the Excel worksheet, they are likely to be at or around 1. When possible for graphing purposes it is nice to utilize whole numbers, not decimals or fractions. In the last column report your specific activities as micromoles (micromoles = umoles) CoA produced instead of millimoles (millimoles = mmoles). Can you generate and apply the correct formula for the conversion?

Exercise 2: Template for Next Week's Data

Use your new spreadsheet skills in Excel to develop a template for calculations to be performed on next week's data. Examine Table 9 of next weeks lab exercise. Remember reactions 1-5 are without a potential inhibitor and reactions 6-10 contain the molecule of your choice.

1. In Excel open the spreadsheet you completed above. Hopefully it looks something like the upper portion of the example in Appendix C. Now add the lower portion of Appendix C to your spreadsheet in preparation for next week's data. Use your new skills, next weeks protocol, lab mates, and your instructor develop and enter the equations needed to summarize next weeks data. Everyone in the class should come up with the same equations.

2. Use the first four columns of the lower portion addition to your spreadsheet to indicate the volume of acetyl-CoA added (first column), the volume of any potential regulator of citrate synthase activity added (second column), the reaction number (third column), and the fold dilution of the citrate synthase preparation you used (forth column).

3. In the fifth and sixth columns you will be entering the change in absorbance for a 6 minute period you monitored for each reaction. No entries here this week.

4. In the seventh column you will be calculating change in absorbance per 1 minute (equation shown below). Notice the equation is identical to that used in the upper portion of the spreadsheet (provided you set yours up exactly like the Appendix C example). Simply click and drag from the last cell of the upper portion of your spreadsheet to apply the calculation to the cells in the lower section.

   Change in absorbance / unit time = (Abs 6 min - Abs 0 min) / (6 minute period)

5. In the 8th column develop (or just click and drag as above to apply the equation to the lower cells) an equation to convert your reaction rates from change in absorbance per 1 minute, to micromoles of acetyl-CoA produced per minute using the millimolar extinction coefficient for DTNB of 13.600 / mM cm.

   Activity = (change in absorbance / 1 cm * 1 min) (1 mM *1 cm / 13.600) (assay volume, L)

6. Now in the ninth column calculate (or click and drag from above as before) the mg of citrate synthase (protein) in each of your reactions, it will be the same for all the reactions in this experiment. To do this you need to know the starting protein concentration of the citrate synthase enzyme, how much you diluted it, and how much you added to each reaction (see equation below). Use the protein assay result from last week.

(XX mg / mL citrate synthase) (xx mL of citrate synthase) (dilution) = ? mg citrate synthase

7. In the tenth column develop (or click and drag as before) the equation to calculate the specific activity of citrate synthase in each of your reactions.

   Specific activity = (millimoles acetyl-CoA / 1 min) / (mg protein)

8. In the eleventh column report your specific activities as micromoles (micromoles = umoles) CoA produced instead of millimoles (millimoles = mmoles). Can you generate and apply the correct formula for the conversion?

9. Test your spreadsheets' performance by entering the absorbance values for your 500x diluted or 100x diluted (which ever one is appropriate) reaction in the appropriate row for the assay with no potential regulatory molecule added.

10. Correct your equations if needed, remembering to reapply any corrections to an equation to all the cells of that column by highlighting the first cell and then clicking on that highlighted cell and dragging the lower right corner of the box across all cells of the column.

11. Add a twelveth column to your Excel spreadsheet and title it uM Acetyl-CoA present. You will develop and enter an equation to calculate the concentration of Acetyl-CoA for each reaction in this column. Recall that in setting up these reactions you used a known volume of a solution of known concentration in assembling the assay. The final volume of the

assay can be determined by adding up the volumes of all the components. A convenient formula to remember when working with solutions is

Concentration initial x Volume initial = Concentration final x Volume final

When using this equation ***initial refers to the solution or reagent used*** and ***final refers to the solution or assay you are making***. You must keep track of units while using this equation and all units for concentration and for volume must be the same on both sides of the equation. Fill in what you know and solve for the concentration of acetyl-CoA. Remember that in terms of Acetyl-CoA concentrations reactions 6-10 are repeats of 1-5. So you have 5 calculations to perform, final units in uM acety-CoA.

12. Add a thirteenth column and using the same logic as above in step 11, develop and add an equation to calculate the concentration in uM of the molecule you will be testing in reactions 6 through 10.

13. Save your spreadsheet on your memory stick.

Exercise 3: Graphing

Use the calculations on the sample data above to generate a graph summarizing the response of porcine heart citrate synthase to varying concentrations of acetyl-CoA in the presence of a potential inhibitor.

1. In Excel with your spreadsheet open, look on the toolbar and click on the "chart wizard" and select the "X Y scatter", use the default "subtype" and click on "next".

2. Look at the tabs in the upper left of the window and click on "series".

3. Examine the series window and notice there are boxes to designate X and Y data. Remember in graphing the independent variable is always on the horizontal or x-axis. To select data for the X series click on the little red icon to the right of the X series box. Once that opens a new little window highlight the cells on your spreadsheet that contain your intended X series (the concentrations of acetyl-CoA for just reactions 6-10, then click on the red icon of that little window.

4. Now enter the corresponding Y series data the same way, but just the data for reactions 6-10.

5. In the name box you might type in "XX mM inhibitor" since reactions 6-10 contained an inhibitor, use the value you just calculated above in place of the XX.

6. With the name in place, now click on the "add" button in the lower left. This will add a new series of X and Y and name for reactions 1-5, and allow you to graph a second line on this same axis.

7. Click "next" and in the next window under the "title tab" enter appropriate titles. Remember figures always have a title and explanatory text under them, so it may be a good idea to leave out a figure title here. But do name the axes with units. Then click next.

8. In this last graphing window you might want to make the graph "chart 1" it will be easier for you to print when it comes time. On the other hand if imbedded in the spread sheet you can watch the graph while you make changes without going to another page (chart 1). Yes that's right, once we click finish (click on it), if we go back and change an equation or a type-O in the data it automatically gets carried through to the graph you just made. Check it out. Cool huh?

9. Look at your figure and right click on one of the data points, a window should open with an "add trendline" option, go there.

10. In the new window that opens click on the "options" tab and select "display R squared" and "display equation". Next in this window click on the "type" tab and assign one of the types listed (try logarithmic) to each line (using the lower scroll bar) on your graph before clicking "OK".

11. Go back to your spreadsheet, creating two new additional columns,

    - one titled; 1 / uM acetyl-CoA

    - one titled; 1 / umoles acetyl-CoA / 1 min / 1 mg protein

    Taking 1 and dividing by a value generates the reciprocal of that value. Reciprocals have considerable value in graphing enzyme kinetics data. Develop and enter the equation for the cells under these new column titles.

12. Now make a new graph using the reciprocals, since both the X and Y values are reciprocals graphs such as these are called double reciprocal plots. Follow the same graphing steps as before but when adding a trendline choose a linear function. Also while in "trendline" under the options investigate the forecast "forward" and "backward" options. They will become valuable in analyzing future data.

Save changes on your memory stick. You are now ready for next week's data.

Materials

Students will be working in pairs. Set up for 10 groups.

Computers with Microsoft Excel (one per student group).

7 Enzyme Kinetics and Inhibitors

Citrate Synthase and Regulation of the Citric Acid Cycle

Introduction to Enzyme Kinetics

Kinetic parameters of enzymes are often calculated to help describe a particular enzyme being studied, to differentiate it from enzymes from other tissues or organisms which catalyze the same reaction, and / or to characterize an enzyme's behavior in the presence of drugs. Many enzymes display saturation kinetics (Michaelis-Menten kinetics) in which the initial velocity, v, of the reaction steadily increases with increasing substrate before leveling off at still higher substrate concentrations as all the active sites become saturated (Figure 10). The maximum velocity of the reaction (Vmax) is reached when every enzyme molecule contains substrate at its active site, in other words, when the enzyme is saturated. Complete saturation requires an abundance of substrate and can be difficult to achieve in the lab. Therefore Vmax is usually estimated from rates at close to saturation levels by a horizontal asymptote (Figure 10, dotted line). The Michaelis constant (Km) is the substrate concentration at ½ of Vmax (Figure 1). Vmax under these conditions is a function of the number of enzyme or substrate molecules present. Increasing enzyme abundance will increase Vmax as will increasing the substrate. Km is a unique characteristic of the enzyme. Km can be interpreted as an enzyme's affinity for a substrate. For example, an enzyme with a Km of 50 mM for Acetyl-CoA would be considered to have a five-fold lower affinity than an enzyme with a Km of 10 mM. This is because the enzyme with a Km of 50 mM requires a 5 times greater substrate concentration to achieve the same velocity, ½ Vmax. The physiological conditions in cells dictates that the Km's of many enzymes be in the uM to mM range.

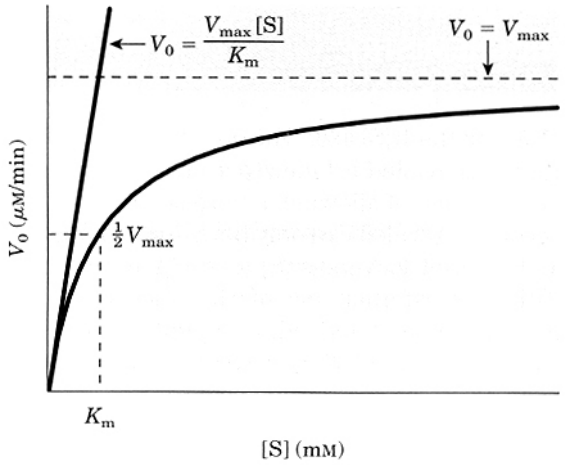


Figure 10. Determining Km and Vmax from kinetic data.

Plotting the double reciprocal of the data above yields a curve where the Y-intercept equals 1/Vmax and the X-intercept equals -1/Km (Figure 11). With good data that include several data points approaching saturation, or at saturation, double reciprocal plots allow easy, mathematical determination of Vmax and Km.

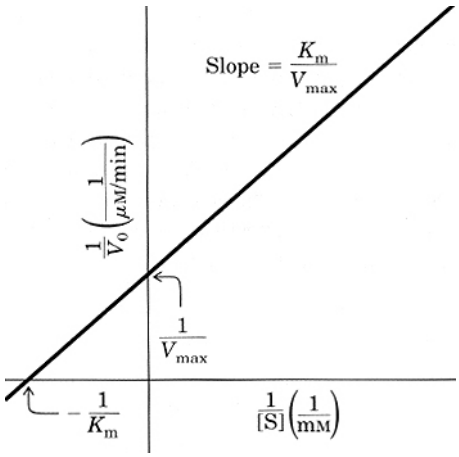


Figure 11. Double reciprocal plot of kinetic data to establish Km and Vmax.

The Michaelis-Menton model is based on an enzyme (E) which combines with a substrate (E) to first form an enzyme-substrate complex (ES) which goes on to form a product (P) and the free enzyme (E).

$$E + S \leftrightarrow ES \rightarrow E + P$$

Enzymes speed up chemical reactions by lowering the energy of activation, or stated another way they stabilize the transition state. During this time the induced fit of enzyme to substrate provides tighter binding of the enzyme to the substrate resulting in stabilization of the transition state. As a consequence molecules that mimic transition-state compounds are potent and specific inhibitors of enzymes. Citrate synthase sequentially binds two substrates during which time the acetyl group from Acetyl-CoA (second substrate S2) is transferred to oxaloacetate (first substrate S1).

$$E + S1 + S2 \leftrightarrow ES1 + S2 \leftrightarrow ES1S2 \rightarrow E + P1 + P2$$

The activity of an enzyme can be reduced by the effects of inhibitors. Enzyme inhibitors are classified as irreversible and reversible. Irreversible inhibitors bind covalently or so tightly to the enzyme that they generally destroy the catalytic capability of the enzyme. In contrast reversible inhibitors are in equilibrium with the enzyme. They compete with substrate molecules for the active site. Competitive inhibitors reduce the reaction velocity by reducing the number of enzyme molecules that can bind substrate. Noncompetitive inhibition reduces the turnover number.

Competitive inhibitors can be differentiated from noncompetitive by performing experiments to determine if the inhibition by a molecule can be overcome by the addition of substrate. If it can, then the inhibition is competitive. For example, a series of assays on citrate synthase with increasing amounts of acetyl-CoA, and another series of assays based on increasing amounts of acetyl-CoA but included in each of this series is a fixed amount of inhibitor. From these two series of assays two curves can be generated on a double-reciprocal plot, one from each set of assays. Examination of the two curves' relationship to one another on the double reciprocal plot identifies the kinetic behavior of the inhibitor on the enzyme (Figure 12). Notice that for competitive inhibitors do not alter Vmax while noncompetitive inhibitors do not alter Km.

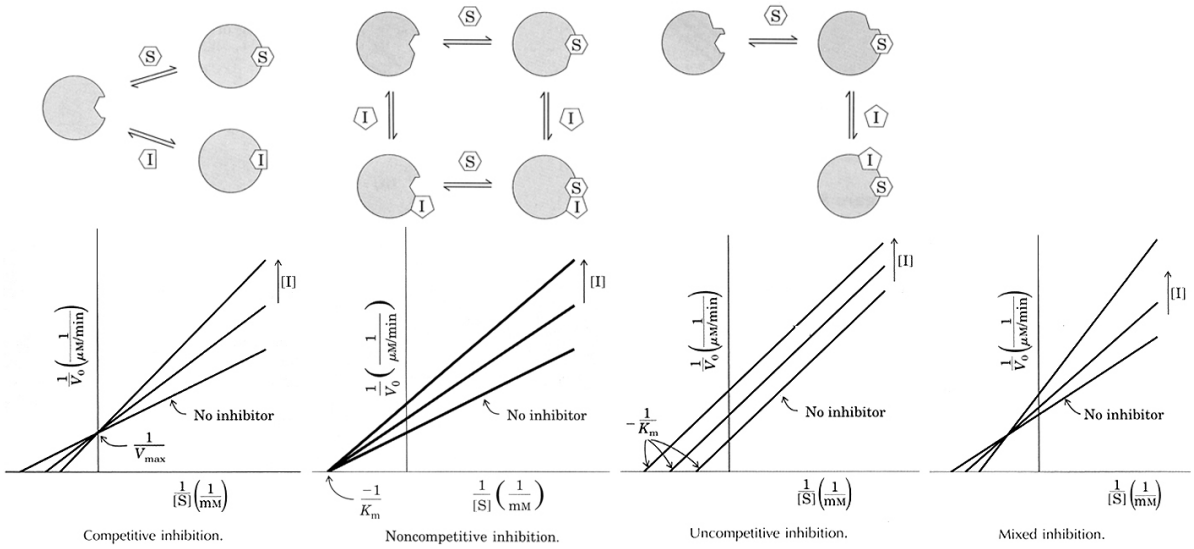


Figure 12. Using double reciprocal plots to characterize mode of inhibition.

Upper panel summarizes interaction of inhibitor molecules with enzyme (grey ball) for different types of reversible inhibition. In competitive inhibition binding of the inhibitor at the active site prevents substrate binding. In noncompetitive inhibition since the inhibitor binding site is separate from the substrate site, order in which the inhibitor binds is unimportant. Uncompetitive inhibitors bind elsewhere on the enzyme but are only effective once the substrate has bound. Lower panel displays double reciprocal plots characteristic of each type of inhibition.

Student Investigation: Regulation of Citrate Synthase and the Citric Acid Cycle

To determine if citrate synthase activity is affected by a particular molecule, rates for two series of reactions must be performed. The two series of reactions are similar in that both utilizes' varying amounts of one reactant. However, one series is performed with the molecule of interest, and the other without. Varying the amount of one substrate must be done carefully to provide a range of reaction conditions that include enzyme saturated with substrate and enzyme that is not. Therefore, successful determination of the correct dilution of enzyme from last week is essential for your success this week. Your results from today's lab will enable you to calculate the Vmax, Km, and determine what effect the molecule you have chosen to investigate has on citrate synthase and the TCA cycle.

1. Obtain an ice bucket filled with ice in which to work.

2. Make sure your spec is on and warmed up.

3. Using 1.5 mL microcentrifuge tubes, resuspend the aliquot of dry DTNB in a total of 4 mL of 1 M Tris-HCl pH 8. Store at room temperature.

4. In a labeled 1.5 mL tube kept on ice, prepare 1 mL of diluted citrate synthase. You are trying to recreate the dilution you determined last week that gave linear results in the citrate synthase assay. Use Table 4 from last week as a guide, but be warned you get only 20 uL

of citrate synthase to start with today. Remember you must first make the 50 mM Tris-HCl pH 8 from a 1 M stock solution and be sure to keep citrate synthase and any dilutions of it on ice at all times.

5. One reaction at a time, starting with cuvette 5, use Table 8 as a guide to set up all 10 reactions listed. For cuvette 5, add the components for the reactions in the order they appear in the table from left to right **except for the Acetyl-CoA**. Perform the reactions in Table 5 in this order, after 5 is done move to cuvette 10, then 4, then 9, then 3, then 8, then 2, then 7, then 1, and finally 6. **Do not incubate pre-prepared cuvettes containing reaction components on ice.**

Table 8. Citrate synthase reactions for kinetics determinations

Cuvette	uL of 10 mM Oxaloacetate	uL of Water	uL of 1 mM DTNB	uL of Enzyme	uL of 10 mM	uL of 2.5 mM Acetyl-CoA
1	43	737	100	20	-	100
2	43	787	100	20	-	50
3	43	813	100	20	-	24
4	43	825	100	20	-	12
5	43	831	100	20	-	6
6	43	723	100	20	14	100
7	43	773	100	20	14	50
8	43	799	100	20	14	24
9	43	811	100	20	14	12
10	43	817	100	20	14	6

6. Use Table 9 below a guide to create a table in your notebook to record the results of your assays, collect data at 2 minute intervals but only out to 6 minutes.

Table 9. Absorbance at 412 nm at room temperature for citrate synthase assays.

Min.	Cuvette 1	Cuvette 2	Cuvette 3	Cuvette 4	Cuvette 5	Cuvette 6	Cuvette 7	Cuvette 8	Cuvette 9	Cuvette 10
0										
2										
4										
6										

7. Set the spectrophotometer at 412 nm. Refer to the Appendix for operation of the spectrophotometer. Add Acetyl-CoA only to the prepared cuvette 5, cover with parafilm, **mix by inversion**, then insert the cuvette into the spectrophotometer if using Ocean Optics USB4000 begin recording data by clicking on the "Restart Selected Acquisition" button (green arrowhead). Be sure to have read the instructions for spec operation in Appendix C for considerations on how to retrieve your data.

8. Set up the remaining cuvettes in the order from step 5, (do not forget to mix by inversion, you better use a clean piece of parafilm for each cuvette.

9. Read the absorbance values at 2-minute intervals and record them in your notebook.

10. Calculate the specific activity of citrate synthase in each reaction. You will want to use Excel and your saved spreadsheet from last week to do this work.

Assignment Due Next Period (Individually)

Hans Lineweaver and Dean Burk developed a simple and more accurate way of estimating both Vmax and Km from data sets similar to yours. They simply plotted the reciprocal of activity (1 / citrate synthase specific activity) on the y-axis versus the reciprocal of acetyl-CoA concentration (1 / acetyl-CoA concentration) on the x-axis. After plotting their data they drew a straight best fit line through their data points and extended it to cross both the y and the x axes. The y-intercept is = 1 / Vmax and the x-intercept is = -1 / Km.

1. Using Excel or other spreadsheet software and your kinetics data, generate both a non-reciprocal plot and a double reciprocal plot, complete with labels and figure legends.

2. Look at your two styles (graphs) of presenting kinetics data carefully. Estimate the Vmax for citrate synthase in the absence and in the presence of the molecule you have chosen from each graph? Record these values in your notebook.

3. Refering to your graphs again, estimate the Km for acetyl-CoA in the absence and in the presence of the molecule you have chosen? Record these values in your notebook.

4. What type of inhibition does your molecule display?

5. What affect will the appearance of this molecule in the mitochondrial matrix have on the citric acid cycle?

6. Type the answers to the above questions along with your Vmax and Km calculations and turn them in with your plot

Transparency Assignment Due Next Period

After you perform your analysis prepare a transparency to be presented at the beginning of the next laboratory period when each group will report their findings. Include on your transparency, the question, hypothesis, summary figure (double reciprocal plot), and conclusions including an explanation at the molecular level of your findings (your answers to questions 1 through 3 above. Also be ready to share your citrate synthase specific activities in the absence of inhibitor with the class in an additional graphing exercise.

Materials

Materials for this lab are listed at the back of the first lab of the enzymology series.

8 Mechanisms of Enzyme Inhibition

Citrate Synthase and Regulation of the Citric Acid Cycle

Exercise 1: Group Presentations of student Investigations

During the presentations have each group post citrate synthase specific activities for last weeks reactions 1-5 in a table on the board.

1. What molecules are inhibitors? What mechanisms are employed by each?

2. What were the Vmax values reported for porcine heart citrate synthase in the absence of potential inhibitors by the different groups?

3. What were the Km values reported for porcine heart citrate synthase in the absence of potential inhibitors by the different groups?

Exercise 2: Calculation of Vmax and Km using Replicates

In this exercise you will use data from all the groups in the class as replicates to calculate the Vmax and Km of porcine heart citrate synthase in the absence of any potential inhibitors. While working in your investigative group your goal for the remainder of this period is to use the class data on the board to produce a double reciprocal plot with error bars, labeled axes, title, legend, Vmax, and Km. Turn in the printed document next period with names of all those who participated in its creation.

Some helpful hints.

- You will want to start by putting each groups data in a separate column, with assays of the same substrate concentration all in the same row.
- You will want a new column with average specific activity of each row in it.
- You will want a new column with standard deviation of specific activity of each row in it (for error bars).
- You may want a new column with 2 x the standard deviation of each row in it (for error bars). Why 2?
- You will want a column for acetyl-CoA concentrations with the correct concentrations in the rows as to match the assay data in other columns.
- You will want columns with reciprocals of items 2 through 5.
- You will want to use the “forecast” option within “trendline”.
- To add error bars right click on a data value within your figure, then select “format data series”. Once there use the “Y error bars” tab and highlight the “custom” radio button and use the little red icon to get your reciprocals of 2 x standard deviation.
- Print it and turn it in with labeled axes, title, legend, Vmax, and Km.
- Enter these (axes labels, title, legend, Vmax, and Km in your lab notebook too.

Materials

Students will be working in pairs. Set up for 10 groups.

Computers with Microsoft Excel (one per student group).

9 Isolation of DNA and PCR Detection of Genetically Modified Maize in Foods

Objectives

- To learn techniques of DNA extraction
- To learn PCR

The genetic blueprint for all life forms is carried in the deoxyribonucleic acid (DNA). Scientists are unraveling complex phenomena of development, gene regulation, and evolution by studying the base sequence of the DNA from a variety of organisms. The entire genetic sequence has been determined for over 100 bacteria and several plants and animals (including humans). Isolation of DNA is a necessary process in modern biological research. Following isolation, a specific segment of the DNA may be targeted for amplification. From a single linear segment of double stranded DNA amplification by PCR can yield enough of the targeted segment to allow visualization. Therefore PCR is commonly used in assays to detect specific sequences in DNA, for example, the presence or absence of a particular gene. To actually visualize the amplified segment, PCR tube contents are often loaded onto an agarose gel, electrophoresed, stained with ethidium bromide, and visualized using UV light.

DNA isolation protocols range from simply breaking open cells and their nuclei in a protective buffer, to a series of complex steps designed to eliminate all other biomolecules (carbohydrates, lipids, and proteins) by taking advantage of the biochemical characteristics of each. For example after cell disruption, extraction with organic solvents such as phenol or chloroform or both may follow. Under these conditions, lipids partition to the organic phase while proteins denature and partition to the organic-aqueous interphase. Carbohydrates are often separated from the remaining DNA by precipitation of the DNA using a salt such as sodium chloride and an alcohol such as ethanol. Precipitated genomic DNA can be removed from other nucleic acids (mRNA, tRNA, rRNA) by spooling it out. Genomic DNA is quite long if left unsheared during the isolation and creates white fluffy masses in the precipitation that can be collected. This relatively pure DNA is then resuspended in a small volume of water or a protective buffer for further analysis.

The polymerase chain reaction or PCR is a series of three steps that are repeated over and over for as many as 30 to 40 times. Each set of three steps, more commonly referred to as a cycle, results in the replication of a segment of DNA. The steps making up a single cycle are denaturation, annealing, and synthesis. During denaturation the temperature rises to around 94 C, high enough to disrupt the hydrogen bonding holding the two strands of a DNA molecule together. During annealing the temperature cools to the 40 to 60 ºC range to allow short deoxyoligonucleotides (primers) to anneal by hydrogen bonding to complimentary sites on the denatured DNA. The exact temperature required for annealing is defined by the base composition of the primer because that will determine the strength of the hydrogen bonding. During synthesis the temperature rises to 72 °C, the optimal temperature for the DNA polymerase from many thermal vent bacteria.

DNA polymerase requires a double stranded segment to start synthesis, which the annealed primers provide. DNA polymerase then reads the DNA strand opposite the primer (template strand) in the 3' to 5' direction while synthesizing a complementary molecule. Primer pairs for PCR are designed to utilize the direction of replication by DNA polymerase and ultimately result in the semi-conservative replication of the region between the two primers. Each cycle of the PCR doubles the number of template copies, thus PCR can amplify specific regions of DNA millions of times.

During electrophoresis, DNA will move towards the positive electrode due to the negative charge from each of the phosphodiester linkages between nucleotides. In a gel matrix such as agarose the smaller (shorter) molecules move faster and larger (longer) molecules move slower. After electrophoresis the gel may be stained with ethidium bromide. This molecule is plainer and inserts itself in the space between two-pairs of paired bases (between the steps of the ladder). This arrangement absorbs UV light then reemits (fluoresces) visible red light back.

This investigation requires two lab periods to complete. In the first period you will isolate DNA and carryout PCR targeting two genes. In second period you will perform the electrophoresis and examine your results.

Exercise 1: Student Designed Investigation

In 2003, 40% of all corn grown in the U.S. was genetically engineered, and many of the corn-based foods you eat contain genetically engineered corn. One gene (there are others) used in the genetic modification of corn is *cry*. The *cry* gene, originally from the bacterium *Bacillus thuringiensis* and encodes delta-endotoxin. This protein is effective in controlling some Lepidoptera larvae (caterpillars), especially those of the European corn borer (bores into the stalks). Not all species of Lepidoptera share the same level of sensitivity to the toxin.

To kill a susceptible insect, a part of the plant that contains the delta-endotoxin protein (not all parts of the plant necessarily contain the protein in equal concentrations) must be eaten. Within minutes, the protein binds to the gut wall and the insect stops feeding. Within hours, the gut wall breaks down and normal gut bacteria invade the body cavity. The insect dies of septicaemia as bacteria multiply in the blood.

The protein is very selective, generally not harming insects of other orders, such as beetles, flies, bees and wasps. Therefore genetically modified organisms that have the *cry* gene are compatible with biological control programs because they harm insect predators and parasitoids much less than broad-spectrum insecticides. The *B. thuringiensis* delta-endotoxin is considered safe for humans, other mammals, fish, birds, and the environment because of its selectivity. The bacterium *B. thuringiensis* has been available as a commercial microbial insecticide since the 1960s and is sold under many trade names. These products have an excellent safety record and can be used on many crops until the day of harvest.

Transgenic crops that express insect toxins must pass 2 separate reviews of environmental safety before they can be sold commercially in the US. Approval for human consumption is yet another separate process. In evaluating environmental safety first, the U.S. Environmental Protection Agency (EPA) reviews laboratory studies assessing a crop's effects on particular nontarget organisms, including pollinators, predatory insects, and often times soil invertebrates. Second, after a crop's developer has collected sufficient field data examining a crop's performance and safety, the developer may petition the U.S. Department of Agriculture (USDA) to allow commercial-scale cultivation. By July 2000, the USDA had approved 50 petitions. Of those 50, 14 petitions were for approval of crops with insect-resistant traits; all of those 14 were utilizing

the *B. thuringiensis* toxin. As of 2001, the approved *Bt*-crop species were corn, cotton, potato, and tomato.

StarLink corn is a variety of genetically modified corn developed by Aventis Corporation to include two new traits. Each trait is encoded by a single gene: 1) *Bt* based resistance to Lepidopteran caterpillar pests by a *cry* gene, and 2) tolerance to glyfosinate herbicides. StarLink is unlike other *Bt* toxin corn varieties because its developers engineered it with a modified version of the *Bt* toxin protein known as Cry9C, an Aventis proprietary technology.

If a genetically modified organism produces a new protein, there may be some risk that the new protein could be an allergen to humans. Therefore the FDA suggests that genetically modified food developer examine potential allergenicity. Most known allergenic proteins have several chemical features in common, small size, resistance to heat, acid, and stomach enzyme digestion. Current methods for allergen assessment of unknown proteins require examination of the unknown protein for these same characteristics. *Bt* toxins are quickly degraded in the stomach and have not been considered an allergy risk. However, heat stability tests submitted to the EPA by Aventis indicated that their proprietary Cry9C version of the Bt toxin may be more heat stable and digestion resistant than other members of the *Bt* toxin family. For this reason the EPA would not allow StarLink in human food.

After detection of StarLink contamination in Taco Bell brand taco shells 24 people came forward claiming that they had had severe allergic reactions after eating corn products containing StarLink corn. Of those 24, 17 allowed their blood to be drawn and tested by the USDA and the Centers for Disease Control and Prevention (CDC). The CDC released a report (July 13, 2001) concluding that the claimants did appear to have had severe allergic reactions to something, but their blood tests demonstrated that they (all 17) were not allergic to StarLink. On July 19, 2001 the EPA's scientific advisory panel determined that although the blood tests demonstrated that those individuals were not allergic to StarLink, the test data should not be used to conclude that no one could be allergic to StarLink. Therefore the EPA on July 27, 2001 ruled that it would not accept a petition by Aventis to allow traces of StarLink to remain in the food supply, and that this policy of zero tolerance would continue. To date, there have been no documented cases of allergic reactions to StarLink corn.

In this lab, you will examine corn-based foods for the presence of the *cry* gene. The assay you will use is PCR based and will produce a 170 bp product if the *cry* gene is present. The PCR assay will also contain a second pair of primers that are targeted at an invertase gene (*ivr*) of corn. The presence of a 226 bp product will allow you to establish that corn DNA is present in the food product. Each student will examine one corn based food product. Therefore to carry out an investigation several students may assemble to maximize their investigative power and increase the number of different corn products that can be examined in an investigation.

Materials

- 1-3 g of several corn-based food product (cereals, meals, chips, etc.)
- Mortars and pestles and liquid nitrogen
- Spatulas
- 15 mL disposable tubes, chloroform compatible
- 5 mL serological pipettes and bulbs, chloroform compatible
- 1.5 ml microcentrifuge tubes
- positive and negative control samples

- CTAB Buffer (5 mL per isolation) Stocks For 5 mL

100 mM Tris-Cl, Ph 8.0	2 M	250 uL
1.4 M NaCl	5 M	1.4 mL
20 mM EDTA	0.5 M	200 uL
2% CTAB	5 %	2 mL
4% PVP	dry chemical	0.2 g
water		1.15 mL

- 2-mercaptoethanol
  100 uL per isolation
- Chloroform:isoamyl alcohol (24:1)
  10 mL per isolation
- Isopopanol
  5 mL per isolation
- DNA wash solution (6 mL per student)

	Stocks	For 6 mL
76 % ethanol	100%	4.56 mL
10 mM ammonium acetate	7.5 M	13 uL
Water		330 uL

- Clinical centrifuge for spinning 15 mL disposable tubes
- Chloroform waste
- Microcentrifuges
- 55 C water bath with 15 mL tube floats or rack
- 100 uL micropipettes and tips
- 1-10 uL micropipettes and tips
- Ice bucket & centrifuge racks
- Fine point sharpies
- nitrile gloves
- PCR primer / loading buffer mix with primers (each PCR/student needs 8.0 uL, instructor will make and aliquot into PCR tubes)

Final concentration	Stock solution	for (1) rxn	for (30) rxn
0.4 uM Cry1A4-5' primer	25 uM	0.16 uL	4.8 uL
0.4 uM Cry1A4-3' primer	25 uM	0.16 uL	4.8 uL
0.4 uM Ivr1A primer	25 uM	0.16 uL	4.8 uL
0.4 uM Ivr1B primer	25 uM	0.16 uL	4.8 uL
1x PCR GoTaq Green (includes buffer, $MgCl_2$, Taq polymerase, dNTPs)	2x	5.0 uL	150 uL
water	water	2.36 uL	71 uL

- 0.2 mL PCR tubes (96 well thermocycler plate with sealable lid)
- Thermocycler

Procedure: Isolation of DNA and Amplification

You will work in pairs, each pair will begin with step 2 below and eventually set up one PCR tube using their extracted DNA.

1. Add 4.9 mL of CTAB buffer to extraction tubes and heat to 55 ºC.

2. Using a mortar and pestle, grind 1 g of fresh plant tissue in liquid nitrogen and add powder quickly to hot CTAB buffer, mix and proceed to the next step. In the absence of liquid nitrogen plant samples could be ground directly in the CTAB extraction buffer.

3. Add 100 uL of mercaptoethanol, mix, incubate 25 minutes at 55 ºC.

4. Add 5 mL of chloroform:isoamyl alcohol (24:1), mix, BURP, incubate 15 to 20 min with occasional mixing.

5. Spin at maximum in clinical centrifuge for 10 min, collect supernatant in a new tube, discard old tube containing the lower phase (chloroform) in the chloroform collection container.

6. Repeat steps 4 through 5.

7. Add 1 volume of ice-cold isopropanol and mix gently, incubate at –20 if desired.

8. Spool out DNA with a flame sealed glass Pasteur pipette, or suck up DNA with a micropipettor, or spin to collect DNA as pellet. Transfer the DNA to a 1.5 mL microcentrifuge tube containing DNA wash solution.

9. Rinse DNA 3x (1 mL each rinse, invert to mix, then spin to pellet DNA, pour off wash solution, repeat) in DNA wash solution. Allow at least 15 min in last wash.

10. Air dry pellet, resuspend in 250 uL of sterile distilled water.

11. Setting up the PCR. Transfer 2 uL of the resultant supernatant to a 0.2 mL PCR tube on ice. These tubes are in your instructors ice bucket, eight tubes to a strip. Leave the tubes assembled as strips. Try to use strips adjacent to other members of your investigative team. Notice only the sides of the first and last tube of each strip is labeled. Record the number of the tube you placed your sample in, this will be the only record of your sample identity.

12. Add to your sample in the PCR tube (keep on ice); 8 uL of 2x REDExtract Ready Mix with primers.

    This mix contains a buffer, magnesium, dNTPs, DNA polymerase, and two pairs of primers.

*Cry*1A4-5’	5’ GGACAACAACCCMAACATCAAC 3’	(M = A or C)
*Cry*1A4-3’	5’ GCACGAACTCGCTSAGCAG 3’	(S = C or G)
*Ivr*1A	5’ CCGCTGTATCACAAGGGCTGGTACC 3’	
*Ivr*1B	5’ GGAGCCCGTGTAGAGCATGACGAYC 3’	(Y = C or T)

    (The Cry1A4 and the Ivr1B primers are *degenerate*: they contain mixtures of different primers).

13. Place your tubes in the thermocycler and start the amplification. The thermocycler will expose the samples to an initial denaturation of 3 min at 94 C, followed by 40 cycles of: 45 sec at 94 C, 45 sec at 61 ºC, and 30 sec at 72 ºC.

14. After amplification your samples will be placed in a freezer for storage.

Questions

1. Design a pair of primers to the sequences below that should work to amplify the region bracketed by the sequenced sites. Though primers tend to be 20 or more bases long, for simplicity each primer you design is to be 9 bases long. Dashes in the diagram below simply represent unknown bases.

	5' sequenced site for forward primer	3' sequenced site for reverse primer	
5′	----------GCTCCGCGG----------	TATTTTGTA----------	3′
3′	----------CGAGGCGCC----------	ATAAAACAT----------	5′

   a. 5' or forward primer sequence (write it 5' to 3')

   b. 3' or reverse primer sequence (again write it 5' to 3')

2. If each dash in the diagram above represents a single base, how long would you predict the PCR product to be? Your answer should include units.

3. Which of the two primers would you expect to be able to withstand the highest annealing temperature? Why?

4. When designing a primer what factors would limit how short a primer can actually be?

10 Agarose Gel and Spectral Analysis of DNA Detection of Genetically Modified Maize in Foods

Objectives

- To learn how to perform a wavelength scan and analyze DNA spectrophotometrically
- To learn gel electrophoresis
- To learn how to analyze DNA bands on gels to give information about genes
- To consider the impact of genetically modified crops on food production

A variety of methods are available to analyze nucleic acids. In today's lab you will focus on spectral methods used routinely in the quantification and analysis of purity of DNA and RNA and on agarose gel electrophoresis which allows the investigator to measure how large the nucleic acid polymers are. These methods are often the first steps in an experiment using DNA or RNA. Spectral analyses tell how much DNA the extraction yielded and how clean it is. This enables the scientist to make decisions on whether additional purification or concentration of the DNA is needed in order to perform the remaining experiments. Spectral analysis however does not readily identify the size of nucleic acids, or their state of polymerization. A long polymer of DNA would give roughly the same spectral analysis result as a collection of nucleotides that could generate a polymer of equal sequence and length. Therefore agarose gel electrophoretic analysis of DNA often follows the spectral analysis in an evaluation of the quality of the DNA isolated.

Materials

- Metaphor high-resolution agarose (0.6 g pre-weighed; 3/class)
- 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA)

Component	for 1 L of 50X stock
Tris	242 g
Glacial acetic acid	57.1 mL
EDTA (disodium salt)	18.6 g

- 25 mL graduated cylinder
- microwave
- gel boxes and combs
- 250 mL graduated cylinder
- EtBr solution (1 mg/L) in tray for gel staining
- Water in tray for gel destaining
- Rapid agarose gel electrophoresis rigs and power supplies (3 per class)
- 10-well combs (3 per class)

- DNA size markers in loading buffer, in aliquots
- Transilluminator
- Ethidium stained gel waste
- Latex gloves
- 1000 uL micropipettes and tips
- 1-10 uL micropipettes and tips
- UV-VIS spectrophotometer
- UV compatible cuvettes
- 1.5 mL microcentrifuge tubes
- Sterile distilled water

Procedure: Agarose Gel Electrophoresis, Staining, and Observation

1. 3 students will prepare agarose gels for the class. Add 20 ml 1X TAE to the agarose, swirl and cover with a plastic beaker. Microwave carefully just until completely melted, do not boil over, try 15 sec at medium power and repeat until completely melted. Let cool 5 min. and then pour into the casting tray. Add the 10-well comb as shown. Let cool.

2. Obtain and thaw your food product PCR samples.

3. After the gel has polymerized assemble the electrophoresis station as demonstrated by your instructor. Use 250 mL of 1x TAE in the lower chamber, place the gel in, and add 250 mL of water for cooling. Once the cooling water has been poured over the gels, you should load your samples quickly, as the TAE buffer will start to leach out of the gel eventually.

4. 8 student PCRs will be loaded on each gel. Skip lanes 1 and 10, as these will be used for ladder and negative control. Load 10 uL of your sample to one lane on a gel. Use a separate pipette tip for each sample, and load carefully to avoid air bubbles.

5. After all 8 student samples are loaded, load 5 ul of the ladder in lanes 1 and 10. One of the gels should have 10 ul of the negative and positive control samples.

6. Draw a map of the gel in your notebook with the lanes labeled.

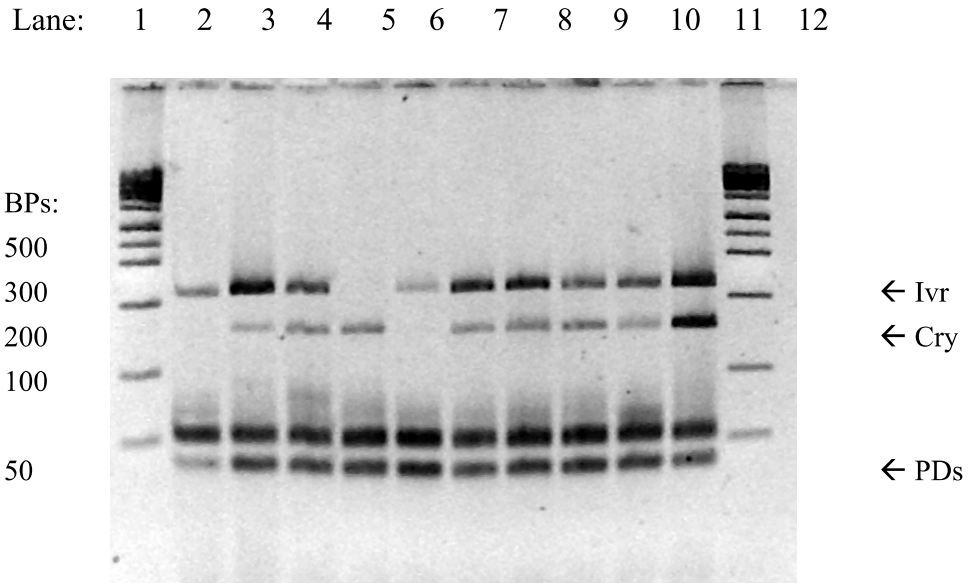
7. Place the cover on the gel box, connect the electrophoresis station to a power supply, and run the electrophoresis at 210 V for 9 min or until the dye has move about 2/3 of the way down the gel. Do not run the dye off the gel!

8. Stain the gel for 3 min (no longer) in 1 mg/L ethidium bromide solution, and observe or destain in water for 10 min before observing. Used gloved hands to handle the gel during staining and at all times afterwards.

9. Place the gel tray on the transilluminator and view the results. Create a figure of your gel in your notebook complete with a descriptive figure title.

10. Using gloves, dispose of gel in the ethidium waste.

Here is an example gel. Lanes 2 and 6 negative for Cry. Lane 11 is Monsanto Yieldguard corn. Lane 5 appears to be negative for Ivr.



Procedure: Spectral Analysis of DNA

1. In a new 1.5 mL microcentrifuge tube add 990 uL of sterile distilled water followd by 10 uL of your resuspended DNA, mix by inversion. Your DNA in this tube has now been diluted 100x.

2. Open SpectraSuite on the computer and follow the directions in Appendix C for completing a spectral scan then proceed with the protocol below.

3. Transfer your diluted DNA solution into a UV compatible cuvette, and perform the spectral analysis. Record your solutions absorbance at 260 and 280 below.

Sample ID:	
Sample dilution:	
Absorbance at 260 nm	Absorbance at 280 nm
Concentration (see calculation below):	
260 / 280 ratio (see calculation below):	

4. Look at the spectrum. Spectrum of nucleic acid in water gives a single relatively symmetrical peak at approximately 260 nm. The actual peak for DNA is close to 256 nm and around 258 for RNA. Any deviation from this could mean very little DNA is present or there is contamination by some other bio-molecule or even by a component of the extraction reagents. This could jeopardize any attempts on our part to manipulate the DNA in the future with enzymes.

5. Quantification of your DNA. Use the following equation to calculate the concentration of your DNA extract. It is based on the extinction coefficient for double stranded DNA of 50 ug per mL per 1 cm light path. Fill it in the table above.

ug / mL of DNA = (Abs @ 260 nM) x (50 ug / mL) x (100, the dilution)

6. Purity of your DNA. Use the 260 and 280 nm absorbance values in your table above to calculate the 260 / 280 ratio. Enter it in the table above. DNA purified from other bio-molecules such as protein and RNA typically gives a 260 / 280 ratio of 1.8. With contaminating RNA present the ratio increases and with contaminating protein the ratio decreases.

Questions

1. On your gel figure:
   a. Label the sizes of the DNA size markers.
   b. Identify and label the band(s) that are likely to be a result of amplification of a portion of the maize *ivr* gene? For what reason did you select the band you did? What mechanism of the PCR amplification allows you to pick this band with confidence?
   c. Identify and label the band(s) that are likely to be a result of amplification of the *cry* gene?
2. What does the lack of bands in your negative control (PCR with no DNA template) tell you about your experiment?
3. What would you conclude about your experiment if your negative control lane contained a 226 bp product?
4. Create a table in your notebook listing the foods you analyzed for the presence *inv* and *cry*. Using additional columns in your table identify which foods contained PCR products for inv and *cry*.
5. Soybean and cotton have also been engineered to express *cry*. Could the amplification of cry in some of the foods be due to use of soybean or cotton to make the food product? Why or why not?
6. Using your results table conclude in your notebook which foods:
   a. contained corn
   b. which foods were engineered to produce the *Bt* toxin
   c. which foods contained genetically engineered corn.

7. Would you expect the delta-endotoxin in the corn food products to still be effective against Lepidoptera larvae? Why or why not? What experiments could be done to investigate this hypothesis?

Assignment Due Next Period

Prepare a figure from you gel photo. To do this down load the file from the course website. Once you have the figure prepared (remember figures are stand alone documents, all that is needed to understand them is present in the figure) revisit Lab 10 Exercise 2 for tips on writing the text that accompanies figures in a "Results" section. Then write a text paragraph that would guide the reader through your professionally prepared gel figure. Turn both the figure and the text in on one typed page next week.

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Appendix Ocean Optics USB4000 Spectrometer

Instructions for taking single absorbance or OD readings at a single wavelength and/or spectral analysis.

Example uses: Monitoring bacterial growth
Protein assays
Spectral analysis of nucleic acids
Other endpoint assays

1. **Match computer and spec.** Make sure the identification numbers on your computer and Ocean Optics USB4000 spectrophotometer match.

2. **Open software.** Find and open the software application "SpectraSuite" that allows operation of the Ocean Optics USB4000.

3. **Adjust spectrophotometer signal to avoid saturation.** When the application opens with no cuvette in the instrument: a, check the "Strobe/Lamp Enable" box; b, set the "integration time" to 9 milliseconds; c, set the "scans to average" to 10; d, set the "boxcar width" to 40. Notice the red line representing "intensity (counts)" at various "wavelengths (nm)". Intensity represented by this line should not exceed 4000 counts for any one wavelength. If it does lower the "Integration time".

4. **Take reference spectra.** Assemble or otherwise obtain a solution to be used as a reference or blank (all solutions except the sample). Place a cuvette containing the reference into the instrument and click on the "Store reference spectrum" icon (the yellow light bulb icon on the toolbar).

5. **Take dark spectra.** Remove cuvette from instrument, uncheck the "Strobe/Lamb Enable" box, and click on the "Store dark spectrum" icon on the toolbar (the grey light bulb just left of the yellow one). Then remember to recheck the "Strobe/Lamp Enable" box before continuing.

6. **Switch to absorbance mode.** Click on the "Absorbance" icon in the toolbar (the blue A). The label on the y-axis should now read "Absorbance (OD)". The red line on the graph represents real-time absorbance the instrument is currently measuring. It the movement of the red line is bothersome to you click on the "Pause Selected Acquisition" button.

7. **Obtain a cursor and select a wavelength**. Using the mouse, guide the cursor to anywhere on the x-axis and then left click. You should get a "cursor", a vertical green line at a "wavelength (nm)" that is indicated in a box below. Use the up and down arrows to select the wavelength you will be performing measurements at. As you do so notice the red number to the right. This number is the absorbance at the wavelength you just dialed in. You will be recording this number after making future measurements.

8. **Read samples.** Place a sample in a cuvette, insert it into the instrument, and click on the "Perform Single Acquisition" button (green arrowhead followed by two black-vertical-parallel lines). Now record the absorbance value in a data table in your notebook.

Instructions for taking multiple absorbance readings at a single wavelength over time.

Example uses: Kinetic assays

1. Match computer and spec. Make sure the identification numbers on your computer and Ocean Optics USB4000 spectrophotometer match.

2. Open software. Find and open the software application "SpectraSuite" that allows operation of the Ocean Optics USB4000.

3. Adjust spectrophotometer signal to avoid saturation. When the application opens with no cuvette in the instrument: a, check the "Strobe/Lamp Enable" box; b, set the "integration time" to 9 milliseconds; c, set the "scans to average" to 10; d, set the "boxcar width" to 40. Notice the red line representing "intensity (counts)" at various "wavelengths (nm)". Intensity represented by this line should not exceed 4000 counts for any one wavelength. If it does lower the "Integration time".

4. Take reference spectra. Assemble or otherwise obtain a solution to be used as a reference or blank (all solutions except the sample). Place a cuvette containing the reference into the instrument and click on the "Store reference spectrum" icon (the yellow light bulb icon on the toolbar).

5. Take dark spectra. Remove cuvette from instrument, uncheck the "Strobe/Lamb Enable" box, and click on the "Store dark spectrum" icon on the toolbar (the grey light bulb just left of the yellow one). Then remember to recheck the "Strobe/Lamp Enable" box before continuing.

6. Switch to absorbance mode. Click on the "Absorbance" icon in the toolbar (the blue A). The label on the y-axis should now read "Absorbance (OD)". The red line on the graph represents real-time absorbance the instrument is currently measuring. It the movement of the red line is bothersome to you click on the "Pause Selected Acquisition" button.

7. Shift to strip chart mode. Click on the "Strip Chart" icon in the upper right corner of the toolbar. In the window that opens under "Range Selection" set the desired wavelength. Next under the "Auto-Save" tab "Enable Trend Auto Save" by checking the box (optional read steps 9, 10, and 11 below) and to the right of "Save to Directory" click on the … box and plug in and select your memory stick as a destination for saved files (use option 2 below to recover data). Before leaving, give these files a "Base Filename" and change the "Save after every" to 10 minutes (it is going to save a new file every ten minutes). Proceed by clicking on "Accept". On the next pop up window that appears click "close".

8. Zoom out. Click on the "Zoom out to maximum" icon. It is the icon at the left end of the strip chart tool bar (looks like four little red arrows pointing inward to a small black circle). This will put the timeline at a more appropriate scale for your assays.

9. Read samples. Place a sample in a cuvette, insert it into the instrument, and click on the "Restart Selected Acquisition" button (green arrowhead). After just a few minutes click on the "Pause Selected Acquisition" button. You should in your notebook record the identity of the assay just performed and the time listed by the software since you will need these to identify the data (automatically being saved to your memory stick. Before proceeding with the rest of your assays consider the two options below on how you will recover your data.

10. Option 1. Obtain a cursor and select times to read absorbance (favored because you can readily choose data from linear portions of the assay). Using the mouse, guide the cursor to anywhere on the x-axis and then left click. You should get a "cursor", a vertical green line at a "time (s)" that is indicated in a box below. Use the up and down arrows to select the time you will be collecting measurements at. As you do so, notice the red number to the right. This number is the absorbance at the time you just dialed in. You should record absorbance values at required times in a table in your notebook. Record times only from portions of your assay that were linear over time.

11. Option 2. Using the times you recorded from the software during performance of the assays search through the data files automatically stored on your memory stick. You should record absorbance values at required times in a table in your notebook.

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