

Isocitrate Dehydrogenase

Parameters of Enzyme Activity

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Introduction

Four Introductory Biology laboratory meetings are devoted to student research on properties of a model enzyme, isocitrate dehydrogenase. During the first laboratory meeting (not presented here), students learn how to use micropipets, make solutions, spectrophotometry, absorption spectra, and how to establish and use standard curves. During the second and third laboratory meetings, students perform experiments to test hypotheses, analyze their data, propose new hypotheses and design research protocols to test these new hypotheses. Three-hour laboratory sessions allow adequate time for discussion, independent thought, repeating experiments, and even doing additional experiments, if students so desire. A computer graphing program is used to organize their data for oral presentation of their experiments and interpretations to their peers, during the fourth laboratory meeting.

Equipment

96-Well-Plate Readers (1-2 for four groups of students)

We use BioRad Model 3550UV 96-well plate readers, which have been replaced with a newer model. There are multiple sources of comparable equipment and demonstrations should be requested from several suppliers.

Printers attached to the 96-well plate readers.

We “salvaged” IBM ProPrinters that were “too slow” for other purposes.

Microcentrifuge

To spin down tissue debris in homogenates in Lab 3.

Computers

We use older MacIntosh machines that were recycled from faculty offices.

Graphing program

We use CA Cricket Graph, but many of our students know and use other programs.

Water bath and thermometer

Set at 37°C.

P-20, P-200, P-1000 micropipets

8-Channel Multi-Pipets (one at each plate reader)

Supplies

Yellow Tips

Buffer Troughs

Sharpies

Blue Tips

1.5 ml Microfuge Tubes

Trash Receptacles

96-Well Plates

Microfuge Tube Racks

Solutions

Assay Buffer	Pig Heart IDH	NADP ⁺ Solutions
Isocitrate Solutions	EDTA Solution	Metal Ion Solutions
pH Buffers		

Instructors Notes

An excellent assignment is to have students verify molarities of these various solutions. Students will have had some exposure to molar concentrations but, for most, this will be the first time they are expected to determine the concentrations of solutions used in their research projects.

Enzyme and NADP⁺ solution should be prepared the day of use; enzyme solutions should be kept on ice at all times. All other solutions are stable for months. We prepare stock solutions and provide samples for each group of students. This practice avoids problems of crowding and waiting around a “reagents bench” and contamination of stock solutions.

Assay Buffer, pH 8.5 (0.2 M Tris, 1 mM MgCl₂)

Tris-HCl	7.32 g
Tris Base	18.6 g
MgCl ₂	203 mg
Distilled water to	1 liter

Enzyme Solution

Pig heart NADP⁺-IDH in 50% glycerol (Sigma # I-2002)
 Dilute 60 µl in 1 ml of Assay Buffer (above)
 Test for appropriate levels of activity and adjust as needed
 Keep on ice at all times

Isocitrate Stock Solution (4.6 mM)

11.9 mg of DL-isocitrate (Sigma # I-1252) in 10 ml of Assay Buffer (above)

Concentrations of Isocitrate for Experiment 3 (1 ml each; about 100 assays)

Tube	mM*	µl of Stock Isocitrate Sol	µl of Assay Buffer
1	0.23	1000	0
2	0.058	252	748
3	0.029	125	875
4	0.019	83	917
5	0.01	43	957

*Final concentration when 10 µl is diluted in 200 µl of final volume.

NADP⁺ Stock solution (2.88 mM)

11 mg of NADP⁺ (Sigma # N-0505) in 5 ml of Assay Buffer

Dilute Concentrations of NADP⁺ (1 ml each; about 100 assays)

Tube	mM*	μ l of NADP ⁺ Stock	μ l of Assay Buffer
1	0.144	1000	0
2	0.072	500	500
3	0.036	250	750
4	0.0216	150	850
5	0.0144	100	900

* Final concentration when 10 μ l is diluted in 200 μ l of final volume.

Tris-HCl Buffers for pH Experiment

Stock Solutions A: 0.2 M Tris Base 24.2 g in one liter
 B: 0.2 M HCl 16.7 ml of 12 N HCl in one liter
 C: 1.0 mM MgCl₂ 2.03 g in 10 ml

Mix 50 ml of A + X ml of B + distilled water to 200 ml final volume

Add 20 μ l of C to each 200 ml solution

X	Approx pH*	Determine Actual pH
5.0	9.0	
13.3	8.5	
26.8	8.0	
39.5	7.5	
47.0	7.0	

Divalent Metal Ion Stock Solutions (100 mM)

Metal*	Sigma Cat. #	mg / ml
MgCl ₂	M-9272	20.3
MnCl ₂	M-3634	19.8
CuCl ₂	C-6641	13.5
ZnCl ₂	Z-3500	13.6

* Final concentration when 10 μ l is diluted in 200 μ l of final volume: 5 mM.

EDTA Stock Solution (100 mM)

38 mg EDTA (Tetrasodium salt; Sigma # ED4S) in 1 ml of water

Final concentration when 10 μ l is diluted in 200 μ l of final volume: 5 mM.

NaCl Solution (5 M)

Dissolve 0.293 g in 1 ml of assay buffer

Survey of IDH in Different Organisms

Obtain fresh materials

Homogenize 3-5 g in equal volume of Assay Buffer

Freeze (preferably at -70°C)

Thaw before use

Centrifuge to remove cellular debris; use supernatant as IDH source

Student Handouts

Introduction

Enzymes are biological catalysts with remarkable power, increasing reaction rates by at least a million-fold. They do this by lowering activation energies, allowing chemical reactions to proceed under physiological conditions. Enzymes are highly specific as to substrates and reactions catalyzed. They are usually proteins, although some are other types of biological molecules. Enzymes function best in dilute aqueous solutions under limited conditions of temperature, pH and salt concentration. Some enzymes require one or more non-protein components called “coenzymes” and “cofactors”; a coenzyme is an organic molecule, while a cofactor may be a metal ion. Some enzymes simultaneously require both a cofactor and a coenzyme. Isocitrate dehydrogenase [IDH] is one of these, requiring both NADP^+ as a coenzyme and a divalent metal cofactor, Mg^{2+} or Mn^{2+} .

IDH is a ubiquitous enzyme, being found in all living organisms and has two catalytic activities [Figure 1]. As its name implies, IDH removes hydrogens from its substrate, isocitrate; in addition, it is a decarboxylase, removing a CO_2 from the six-carbon substrate to generate a five-carbon product, α -ketoglutarate.

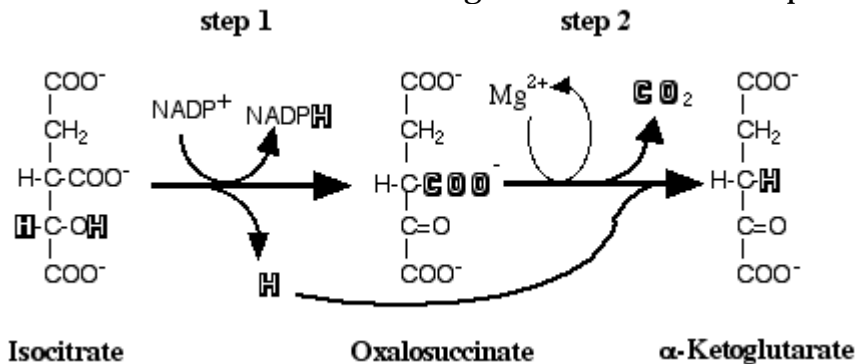


Figure 1. IDH catalyzes the sequential dehydrogenation and decarboxylation of isocitrate to α -ketoglutarate. A coenzyme, NADP^+ , and a cofactor, Mg^{2+} (or Mn^{2+}), are required for activity.

Two distinct forms of IDH are found in higher organisms. They differ in their distribution within the cell and in coenzyme requirements. The soluble form of IDH requires NADP^+ as its coenzyme [Figure 2].

The NADP^+ -dependent form of IDH is considered to be the only IDH in bacteria and cyanobacteria and is the most prevalent form of IDH in most plants and animals. In higher organisms, this form appears to be found in all organs and tissues. The NAD^+ -dependent form of IDH is limited to eukaryotic organisms and is localized in mitochondria. You may already know this form of IDH from previous study of the Krebs cycle. Both forms of IDH require a divalent metal ion.

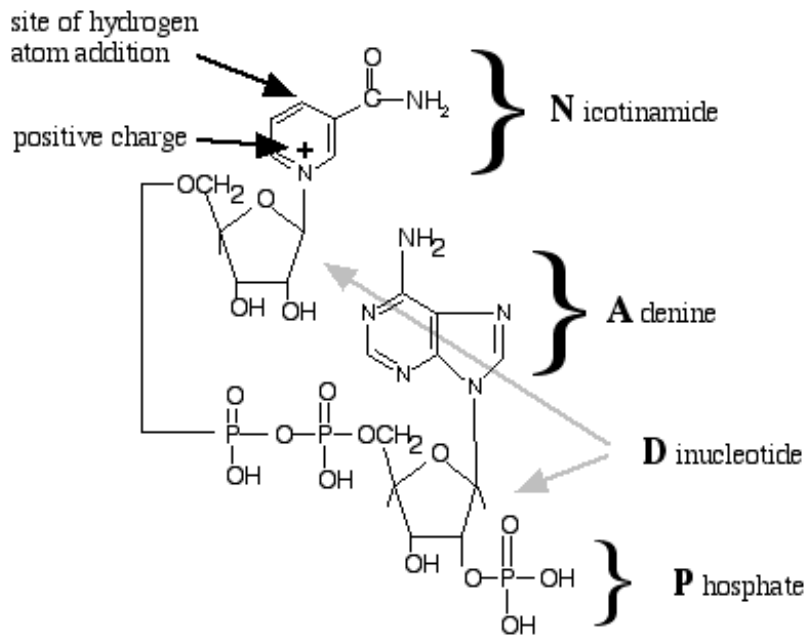


Figure 2. The molecular structure of NADP⁺/NAD⁺. The active site is the same in both compounds. In NAD⁺, the phosphate group is replaced with an H⁺. This diagram illustrates what the letters N-A-D-P represent.

Points of Interest: (i) NADP⁺-dependent IDH activity is especially high in cardiac tissue and is often monitored in the blood of heart attack patients. Detectable IDH activity in the arterial blood suggests severe tissue damage with leakage of the soluble (cytosolic) IDH into the blood system. (ii) In 1996, a team of researchers found a species of vole, living in the area contaminated by the Chernobyl nuclear disaster, that is resistant to mutations caused by radiation. They found that the voles had elevated levels of IDH, which they believe is protecting them from radiation-induced mutations (*Science*, Vol 273, 19 July, 1996).

Protocols

IDH activity is routinely measured using a spectrophotometer to monitor the reduction of NADP^+ to NADPH. While performing assays, the spectrophotometer is set at 340 nm, the absorption maximum of NADPH (Appendix A). Assays are performed at a standard temperature, usually 25 or 30°C.

Before a scientist begins an experiment, she must first define a problem and suggest possible explanations based upon previous knowledge or observations. In other words, she must develop an hypothesis, which might be considered an “educated guess” or a tentative explanation as to the cause and effects relating to that problem. A good hypothesis is one that is testable and fosters predictions that consider one variable at a time. The hypothesis may turn out to be incorrect, but it is a good hypothesis if it can be tested. In fact, an hypothesis that cannot be tested is useless to science - it may be good philosophy, but not good science. Hypotheses can not be proven to be correct - they may be tested extensively and rigorously and they may be proven to be incorrect, but an hypothesis can never be proven to be true.

Our scientist must first define a problem and then develop her hypothesis. She must next devise predictions that will hold, or will not hold, if the hypothesis were true. These predictions lead to experiments. Many experiments may be possible, and may all be tried eventually; however, it is important to perform one discrete experiment at a time. After designing an experiment, our scientist must outline a series of logical procedures to be completed in the laboratory or in the field. This written sequence of steps is called a **protocol**. A well planned protocol will include the following elements:

1. An outline of the sequence of detailed procedures.
2. Calculations of volumes, concentrations, *etc.*, of all reagents to be used.
3. Tables constructed for recording data.
4. Procedures for testing and organizing data for presentation

LAB #2

Objectives

With this laboratory, we begin a three week study and discussion of some of the properties of NADP⁺-dependent isocitrate dehydrogenase [IDH]. The objectives of these laboratory sessions are:

1. Learn spectrophotometric analyses of enzyme activity.
2. Determine how the amount of enzyme in the assay affects the rate of activity.
3. Determine how the amount of substrate in the assay mixture affects rates of activity of an enzyme.
4. Determine the effects of environmental conditions on enzyme activity.
5. Learn how to organize our data into tabular and graphic form.

Experiment 1. How To Perform IDH Assays

Hypothesis 1: A successful assay for IDH activity simultaneously requires enzyme, isocitrate and NADP⁺.

Hypothesis 2: Under ideal conditions, IDH activity will be linear for at least three minutes.

To test your hypotheses, you will need to set up assays as in **Table 1**. You should ask yourself “What is the purpose of each assay?” You should also ask why Assays 5 - 7 are identical.

Table 1: How to perform IDH assays.

Wells	Buffer	NADP ⁺	IDH	Isocitrate
A 1	190	10	0	0
A 2	180	10	0	10
A 3	180	10	10	0
A 4	180	0	10	10
A 5	170	10	10	10
A 6	170	10	10	10
A 7	170	10	10	10

All volumes are in μl . In this experiment, you will initiate the reactions by adding 10 μl of substrate solution as the last step. You will use a multi-tip pipet, at the plate reader, to add substrate to all wells.

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the indicated wells.
2. Use the P-20 micropipet to add 10 μl of NADP⁺ to all wells, except A 4.
3. Use the P-20 micropipet to add 10 μl of IDH to all wells, except A 1 and A 2.
4. Place the microplate in chamber of the plate reader.
5. Use the Multi-8 micropipet to add 10 μl of Isocitrate to all wells, except A 1 and A 3.
6. Activate the plate reader.
7. After printing, remove your plate from the plate reader.
8. Retrieve your data from the printer.
9. Return to your station and organize your data in the **Table 1a**.
10. Prepare a graph of your data.

Table 1a: Data from triplicate IDH assays.

Time, min	Well A 5	Well A 6	Well A 7
0			
0.5			
1.0			
1.5			
2.0			
2.5			
3.0			

Considerations - Experiment 1

Compare your data from Wells A 1 through A 7. Was there activity in Wells A 1 - A 4? Was there activity in Wells A 5 - A 7? Was activity the same in Wells A 5 - A 7? Was activity linear for three minutes? If not, explain your observations. Do your data support your hypotheses? If not, how will you change the protocol?

Determine the “corrected” reading for each assay by subtracting the reading from the “control”, Well A 1. (Would Well A 2, A 3 or A 4 provide better “control” data?)

Construct a graph that visually portrays your data from Wells A 5 - A 7 by plotting absorbance as a function of time (in minutes). The initial rate of a reaction may be determined from the slope of the line joining each successive point.

Experiment 2. Effects Of Varying Enzyme Concentration

Problem: What is the relationship between the rate of a reaction and the amount of enzyme in the assay solution when substrate and coenzyme are abundant (non-limiting)? This question might become “In subsequent experiments, how much enzyme solution should I use in each assay?”

Hypothesis: IDH activity will vary directly with the amount of enzyme in each assay.

To test this hypothesis, you will need to follow a protocol that holds all conditions constant except the amount of enzyme added to each assay. All tests should be run more than once; routinely, enzyme assays are run “in triplicate”. For example, Wells B 1, B 2 and B 3 in Table 2 are triplicate assays containing 5 μl of IDH. Set up reactions as per Table 2.

Table 2: The effects of varying enzyme amounts.

Wells	Buffer	NADP ⁺	IDH	Isocitrate
B 1-3	175	10	5	10
B 4-6	170	10	10	10
C 1-3	165	10	15	10
C 4-6	160	10	20	10
D 1-3	180	10	0	10

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the indicated wells.
2. Use the P-20 micropipet to add 10 μl of NADP⁺ to all wells.
3. Use the P-20 micropipet to add the indicated volume of IDH to each well.
4. Place the microplate in chamber of the plate reader.
5. Use the Multi-8 micropipet to add 10 μl of Isocitrate to all wells.
6. Activate the plate reader.
7. After printing, remove your plate from the plate reader.
8. Retrieve your data from the printer.
9. Return to your station and organize your data in Table 2a.
10. Prepare a graph of your data.

Table 2a: Data from varied enzyme amounts.

Time, min	5 µl of IDH	10 µl of IDH	15 µl of IDH	20 µl of IDH
0				
0.5				
1				
1.5				
2				
2.5				
3				

Considerations - Experiment 2

Compare the data from Wells B 1 through C 6. Was there activity in all wells? Did activity vary with the amount of enzyme in each assay? Was activity the same in the three wells with the same amount of enzyme? Was activity linear for the first three minutes for each volume of enzyme? If not, explain your observations. Do your data support your hypothesis?

Determine the mean activity for each set of triplicate assays. Construct a graph to portray your data. Compare activity with the volume of enzyme in the assay solution. [Hint - take advantage of the formula: $y = mx + b$.]

Construct another graph that compares volume of enzyme versus absorbance at a specific time, *eg.*, the three-minute reading, or the slope of the three lines from your previous graph. What conclusions can you reach from your results?

Experiment 3. Effects Of Varying Isocitrate Concentration

Problem: What is the relationship between the rate of a reaction and the amount of isocitrate in the assay solution when the amounts of IDH and NADP⁺ in the assay are held constant? Before you start this experiment, develop an hypothesis and sketch a graph predicting the relationship of activity vs isocitrate concentration.

Procedure: To test your hypothesis, you will need to follow a protocol that holds all conditions constant except the amount of isocitrate added to each assay.

Table 3 outlines such a protocol using five concentrations of isocitrate. Each concentration is tested in triplicate. Add reagents to your wells as listed from left to right.

Table 3: The effects of varying isocitrate concentration.

Wells	Buffer	Isocitrate		IDH	NADP ⁺
		Sol*	Vol		
E 1-3	170	1	10	10	10
E 4-6	170	2	10	10	10
F 1-3	170	3	10	10	10
F 4-6	170	4	10	10	10
G 1-3	170	5	10	10	10
G 4-6	180	Blank		10	10

*The concentration of these isocitrate solutions will be provided by the Instructor. The second number refers to the volume [μ l] to be used. NOTE: In these assays we will initiate reactions with NADP⁺. Do not add NADP⁺ until your plate is in the plate reader.

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the indicated wells.
2. Use the P-20 micropipet to add 10 μ l of Isocitrate to all wells, as indicated.
3. Use the P-20 micropipet to add 10 μ l of IDH to all wells.
4. Place the microplate in chamber of the plate reader.
5. Use the Multi-8 micropipet to add 10 μ l of NADP⁺ to all wells.
6. Activate the plate reader.
7. After printing, remove your plate from the plate reader.
8. Retrieve your data from the printer.
9. Return to your station and organize your data in **Table 2a**.
10. Prepare a graph of your data.

Table 3a: Data from varied isocitrate concentrations.

Time, min	0.23 mM	0.058 mM	0.029 mM	0.019 mM	0.01 mM
0					
0.5					
1					
1.5					
2					
2.5					
3					

Considerations - Experiment 3

Compare the data from your experiment. Determine the mean activity for each concentration of isocitrate. Construct a graph that compares activity as a function of isocitrate concentration. Do your data support your hypothesis? Is the relationship between activity and concentration of substrate linear? Explain this relationship, referring to Figure 1.

Preparation For Next Week's Lab:

In next week's lab, you will study the effects of environmental conditions on enzyme activity. Each team of students will design an experimental protocol to study one of the following:

1. What are the effects of 37°C on the stability of IDH?
2. What are the effects of pH of the assay solution?
3. What are the effects of NADP⁺ concentration?
4. What are the effects of different divalent metal ions?
5. What are the effects of varying salt concentrations?
6. Which species or tissues have the most activity?

Before leaving lab today, each group will complete the following:

1. Develop a clear, concise and simple hypothesis about the effects of one of the above environmental conditions upon enzyme activity.
2. Design an experiment to test that hypothesis.
3. Prepare a protocol to carry out that experiment.

LAB #3

Environmental Parameters of Enzyme Activity

I. Introduction

Last week you learned:

1. How to perform isocitrate dehydrogenase assays.
2. The relationship between activity and amount of enzyme in an assay.
3. The relationship between activity and substrate concentration.
4. How to present experimental data in graphic form.

In this week's lab you will determine the effects of environmental perturbations of our standard assay conditions. Each group of students will have:

- i. Chosen one of the following experiments to complete.
- ii. Designed an experimental protocol for that experiment.
- iii. Reviewed the protocol with the Instructor.
- iv.

II. Methods and Materials

You will use the same general methods that you used in Lab #2. All equipment, solutions and supplies required to carry out your experiments have been prepared and ready for your use. You may wish to review your protocol again and assign specific tasks before you start your experiments.

Experiment 4: The Effects of pH on IDH Activity

Hypothesis: pH of the assay buffer will have no effect on IDH activity.

To test this hypothesis, you will need to follow a protocol that holds all conditions constant except the pH of the assay buffer.

Table 4: The effects of varying pH of the assay buffer.

Wells	pH	Buffer	NADP⁺	IDH	Isocitrate
A 1-3	7.0	170	10	10	10
A 4-6	7.5	170	10	10	10
B 1-3	8.0	170	10	10	10
B 4-6	8.5	170	10	10	10
C 1-3	9	170	10	10	10
C 4-6	Blank	180	10	0	10

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the indicated wells.
2. Use the P-20 micropipet to add 10 μl of NADP⁺ to all wells.
3. Use the P-20 micropipet to add 10 μl of IDH to all wells, except C 4-6.
4. Place the microplate in chamber of the plate reader.
5. Use the Multi-8 micropipet to add 10 μl of Isocitrate to all wells.
6. Activate the plate reader.
7. After printing, remove your plate from the plate reader.
8. Retrieve your data from the printer.
9. Return to your station and organize your data in the table below.
10. Prepare a graph of your data.

Table 4a: Data from varying pH of the assay buffer.

Time	pH 7	pH 7.5	pH 8	pH 8.5	pH 9
0					
0.5					
1					
1.5					
2					
2.5					
3					

Considerations - Experiment 4

Does IDH activity vary when the pH of the assay mixture varies, or are levels of activity the same regardless of pH? Do you need to test activity other pH values? Explain how the pH of the assay mixture might affect activity of an enzyme.

Experiment 5: Does IDH Have a Metal Ion Requirement?

Hypothesis: IDH activity does not require a divalent metal in the assay solution.

To test this hypothesis, you will need to follow a protocol that holds all conditions constant except the presence or absence of divalent metal ions in the assay solution.

Table 5: Does IDH Have a Metal Ion Requirement?

Wells	Metal, μl	Buffer	NADP ⁺	IDH	Isocitrate
D 1-3	None	170	10	10	10
D 4-6	EDTA 10	160	10	10	10
E 1-3	Mg ²⁺ 10	160	10	10	10
E 4-6	Mn ²⁺ 10	160	10	10	10
F 1-3	Cu ²⁺ 10	160	10	10	10
F 4-6	Zn ²⁺ 10	160	10	10	10
G 1-3	Blank	180	10	0	10

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the wells, as indicated.
2. Use the P-20 micropipet to add metal solutions to wells, as indicated.
3. Use the P-20 micropipet to add 10 μl of NADP⁺ to all wells.
4. Use the P-20 micropipet to add IDH to all wells, except G 1-3.
5. Place the microplate in chamber of the plate reader.
6. Use the Multi-8 micropipet to add 10 μl of Isocitrate to all wells.
7. Activate the plate reader.
8. After printing, remove your plate from the plate reader.
9. Retrieve your data from the printer.
10. Return to your station and organize your data in the table below.
[Subtract the mean control value (G 1-3) from other mean values.]
11. Prepare a graph of your data.

Table 5a: Data from metal ion experiment.

Time	None	EDTA	Mg ²⁺	Mn ²⁺	Cu ²⁺	Zn ²⁺
0						
0.5						
1						
1.5						
2						
2.5						
3						

Considerations - Experiment 5

Does IDH require a divalent metal ion for activity? Does additional Mg²⁺ added to the standard assay buffer increase activity? What does this observation mean? Does the addition of Mn²⁺ added to the standard assay buffer increase activity? What does this observation mean? How do you explain the effects of Cu²⁺ and Zn²⁺ on IDH activity?

Experiment 6: The Effects of Temperature on IDH Stability

Hypothesis: Exposure to 37°C will have no effect on IDH stability.

To test this hypothesis, you will need to follow a protocol that holds all conditions constant except temperature. You will incubate samples of IDH at 37°C for 0, 2, 4, 6, 8, 10 min prior to assaying activity. Old all samples on ice until incubations are complete and assay at the same time.

Heat treatment

1. Label six 1.5 ml microfuge tubes: 0, 2, 4, 6, 8, 10. Place on ice.
2. Use the P-200 micropipet to add 100 µl of IDH to each tube.
3. Place Tubes 2 - 10 in the water bath @ 37°C.
4. Remove each tube at the appropriate time and place on ice.
5. Assay IDH activity as in **Table 6** (all volumes are microliters):
- 6.

Table 6: Does exposure to 37°C affect IDH stability?

Wells	Min, 37°	Buffer	NADP ⁺	IDH	Isocitrate
D 1-3	0	170	10	10	10
D 4-6	2	170	10	10	10
E 1-3	4	170	10	10	10
E 3-4	6	170	10	10	10
F 1-3	8	170	10	10	10
F 4-6	10	170	10	10	10
G 1-3	Blank	180	10	0	10

Procedure

1. Use the P-200 micropipet to add Assay Buffer to wells, as indicated.
2. Use the P-20 micropipet to add 10 µl of NADP⁺ to all wells.
3. Use the P-20 micropipet to add 10 µl of IDH to all wells, except G 1-3.
4. Place the microplate in chamber of the plate reader.
5. Use the Multi-8 micropipet to add 10 µl of Isocitrate to all wells.
6. Activate the plate reader.
7. After printing, remove your plate from the plate reader.
8. Retrieve your data from the printer.
9. Return to your station and organize your data in the table below.
[Subtract the mean control value (G 1-3) from other mean values.]
10. Prepare a graph of your data.

Table 6a: Data from temperature experiment.

Time, min	0 Min	2 Min	4 Min	6 Min	8 Min	10 Min
0						
0.5						
1						
1.5						
2						
2.5						
3						

Considerations - Experiment 6

Is IDH stable at 37°C? How can your results be explained? How would you design this experiment to test your hypothesis further?

Experiment 7: Does enzyme activity vary with concentration of NADP⁺?

Problem: What is the relationship between the rate of a reaction and the amount of coenzyme in the assay solution when the amount of enzyme is held constant? Before you start this experiment, develop an hypothesis and sketch a graph predicting the relationship of activity vs substrate concentration.

Procedure: To test your hypothesis, you will need to follow a protocol that holds all conditions constant except the amount of coenzyme added to each assay.

Table 7 outlines such a protocol using six concentrations of NADP⁺. Each concentration is tested in triplicate. Add reagents to your wells as listed from left to right.

Table 7: The effects of varying the concentration of NADP⁺.

Wells	Buffer	NADP⁺		IDH	Isocitrate
		Sol*	Vol		
D 1-3	170	1	10	10	10
D 4-6	170	2	10	10	10
E 1-3	170	3	10	10	10
E 4-6	170	4	10	10	10
F 1-3	170	5	10	10	10
F 4-6	180	Blank		10	10

†The concentration of these NADP⁺ solutions will be provided by the Instructor. The second number refers to the volume [μl] to be used. NOTE: In these assays we will initiate reactions with isocitrate. Do not add isocitrate until your plate is in the plate reader.

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the wells, as indicated.
2. Use the P-20 micropipet to add 10 μl of NADP^+ solutions to indicated wells.
3. Use the P-20 micropipet to add IDH to all wells.
4. Place the microplate in chamber of the plate reader.
5. Use the Multi-8 micropipet to add 10 μl of Isocitrate to all wells.
6. Activate the plate reader.
7. After printing, remove your plate from the plate reader.
8. Retrieve your data from the printer.
9. Return to your station and organize your data in the table below.
[Subtract the mean control value (F 4 - 6) from other mean values.]
10. Prepare a graph of your data.
- 11.

Table 7a: Data from varying the concentration of NADP^+ .

Time, min	0.144 mM	0.072 mM	0.036 mM	0.0216 mM	0.0144 mM
0					
0.5					
1					
1.5					
2					
2.5					
3					

Considerations - Experiment 7

Organize the data from your experiment. Determine the mean activity for each concentration of NADP^+ . Construct a graph that compares activity as a function of NADP^+ concentration. Do your data support your hypothesis? Is the relationship between activity and concentration of NADP^+ linear? Explain this relationship, referring to the model in Figure 1.

Experiment 8: The Effects of NaCl on IDH Activity

Hypothesis: Sodium chloride will have no effect on IDH activity.

To test this hypothesis, you will need to follow a protocol that holds all conditions constant except concentration of NaCl in the assay solution.

Table 8: The effects of NaCl on IDH activity.

Wells	[NaCl] M	Buffer	5M NaCl	NADP^+	IDH	Isocitrate
D 1-3	0.0	170	0	10	10	10
D 4-6	0.1	166	4	10	10	10
E 1-3	0.2	162	8	10	10	10
E 4-6	0.3	158	12	10	10	10
F 1-3	0.4	154	16	10	10	10
F 4-6	0.5	150	20	10	10	10
G 1-3	Blank	180	0	10	0	10

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the wells, as indicated.
2. Use the P-20 micropipet to add NaCl to the wells, as indicated.
3. Use the P-20 micropipet to add 10 μl of NADP⁺ to all wells.
4. Use the P-20 micropipet to add IDH to all wells, except G 1-3.
5. Place the microplate in chamber of the plate reader.
6. Use the Multi-8 micropipet to add 10 μl of Isocitrate to all wells.
7. Activate the plate reader.
8. After printing, remove your plate from the plate reader.
9. Retrieve your data from the printer.
10. Return to your station and organize your data in the table below.
11. Prepare a graph of your data.

Table 8a: Data from the effects of NaCl on IDH activity.

Time, min	0 M	0.1 M	0.2 M	0.3 M	0.4 M	0.5 M
0						
0.5						
1						
1.5						
2						
2.5						
3						

Considerations - Experiment 8

Did you observe differences in activity between the treatments? What was the relationship between the concentration of NaCl and activity? Explain how salt might affect enzyme activity.

Experiment 9: Does IDH activity vary among different organisms or tissues?

This is an “open ended” experiment; you may design a single, additional experiment, comparable to the ones listed above, or expand these topics into a research project of wider magnitude.

1. You may chose to survey IDH activity:
 - i. In a wide variety of related species [think salad bar].
 - ii. In different tissues of a single species [think chicken giblets].
2. Homogenize your samples in cold Assay Buffer, using a kitchen blender.
3. Filter the homogenate through two layers of cheesecloth into a small beaker that is on ice.
4. Transfer 1 ml samples to 1.5 ml microfuge tubes, spin for 5 minutes.
5. Transfer the supernatant to clean 1.5 ml microfuge tubes, on ice.
6. Use standard conditions to assay IDH activity.

Before You Leave Lab

1. Be certain that you have collected all of the data you need to make your experiment complete.
2. Be certain that each member of the group fully understands what was done and has a copy of all of your data.
3. Schedule a meeting of your group [why not now?] to analyze your results and prepare slides for your group's oral presentation.

Appendix A

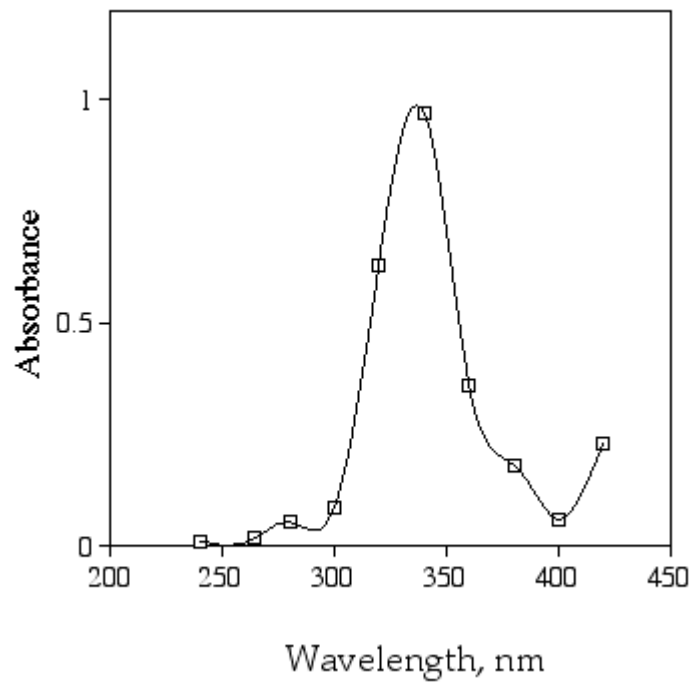


Figure A-1. An absorption profile for NADPH.

Appendix B

Representative Results from Lab #2

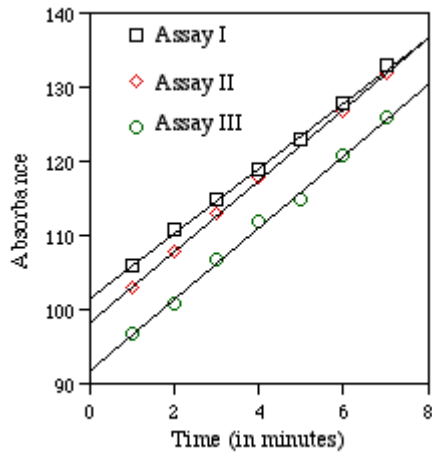


Figure B-1. IDH assays are repeatable and are linear for at least three minutes.

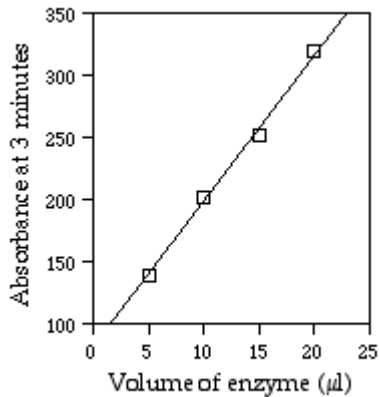


Figure B-2. IDH activity is directly proportional to the amount of enzyme added to the reaction mixture.

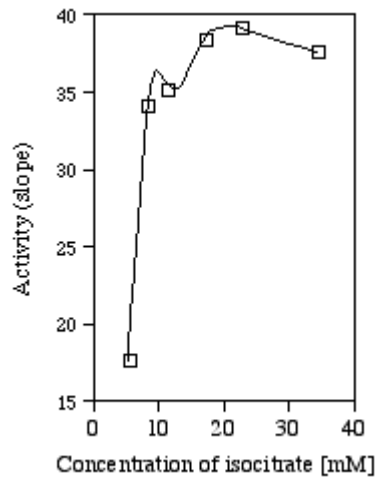


Figure B-3. IDH activity is not directly proportional to the amount of substrate added to the reaction mixture.

Appendix C Representative Results from Lab #3

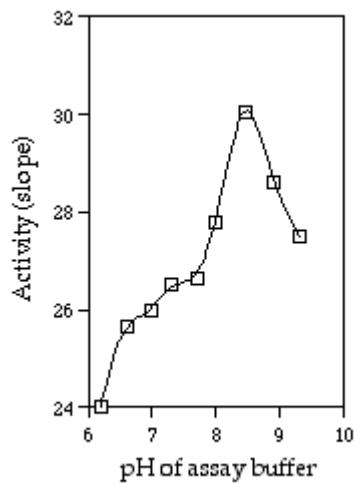


Figure C-1. Maximum IDH activity depends on the pH of the assay mixture.

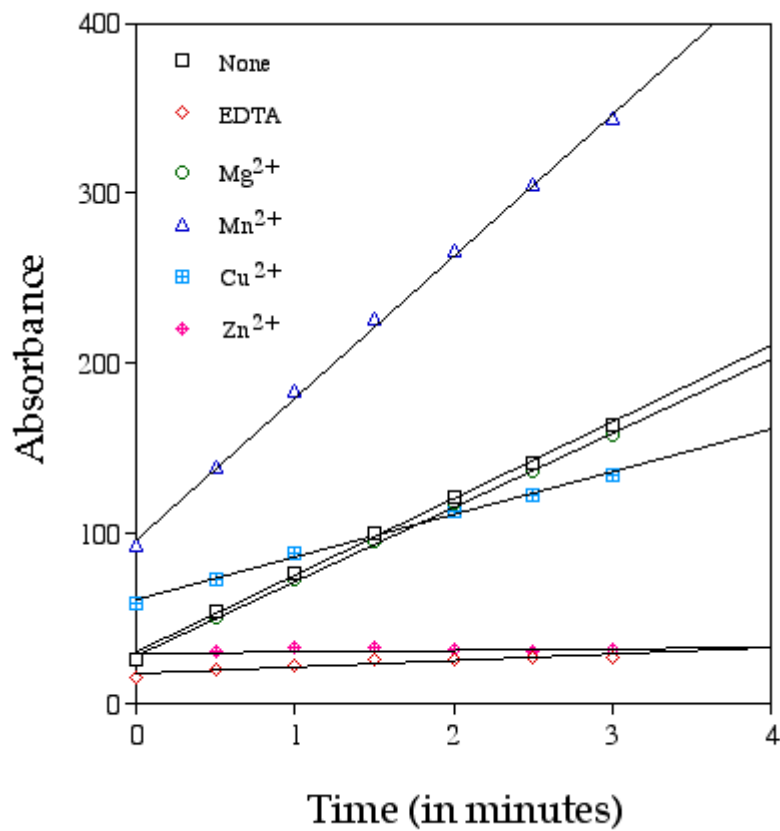


Figure C-2. IDH activity is dependent upon the presence of a divalent metal ion. However, some metal ions are toxic to IDH activity.

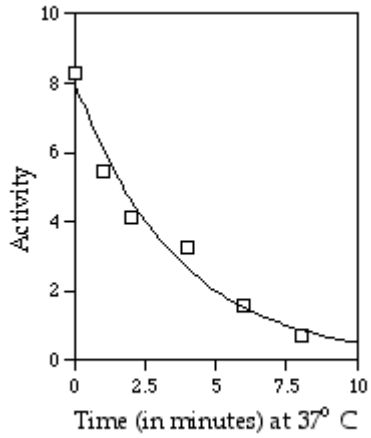


Figure C-3. IDH activity decreases when the enzyme is incubated at 37°C.

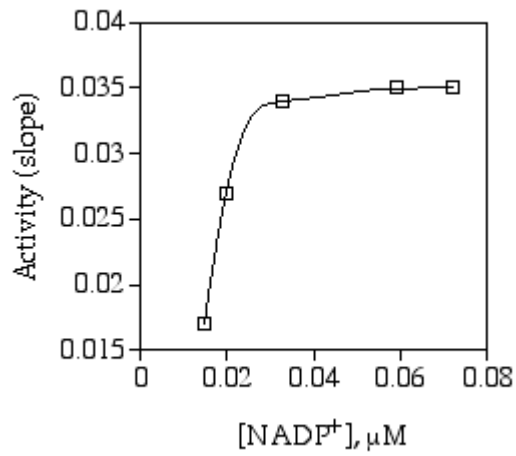


Figure C-4. IDH activity is not directly proportional to the amount of coenzyme added to the reaction mixture.

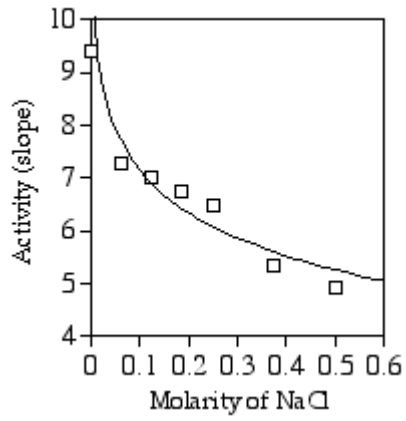


Figure C-5. IDH activity decreases as the concentration of NaCl in the assay mixture increases.

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