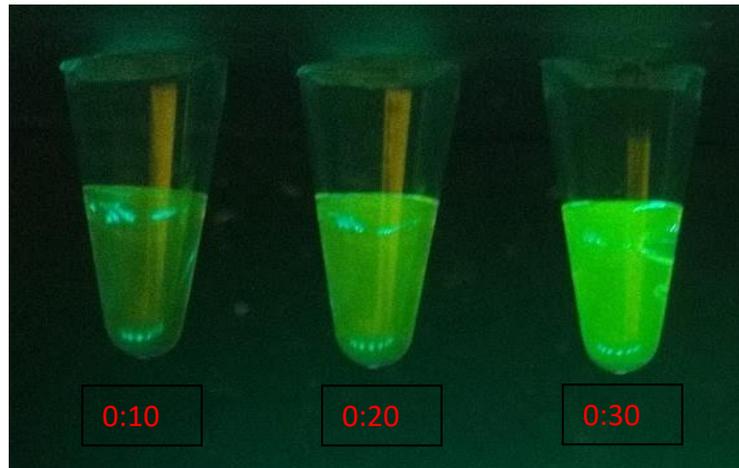
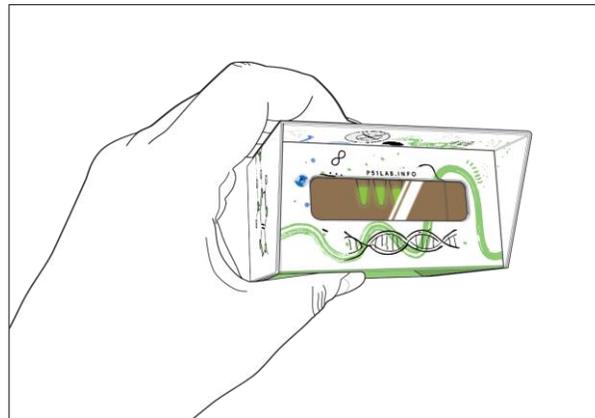


P51 Enzyme Lab: β -Gal Glow™



For use with P51™ Molecular Viewer



(or other blue light illuminator¹)

Student's Guide Contents

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¹ Compatible with blue light transilluminators such as blueGel™, blueBox™ and other 460-480 nm illuminators.

1. Background and significance

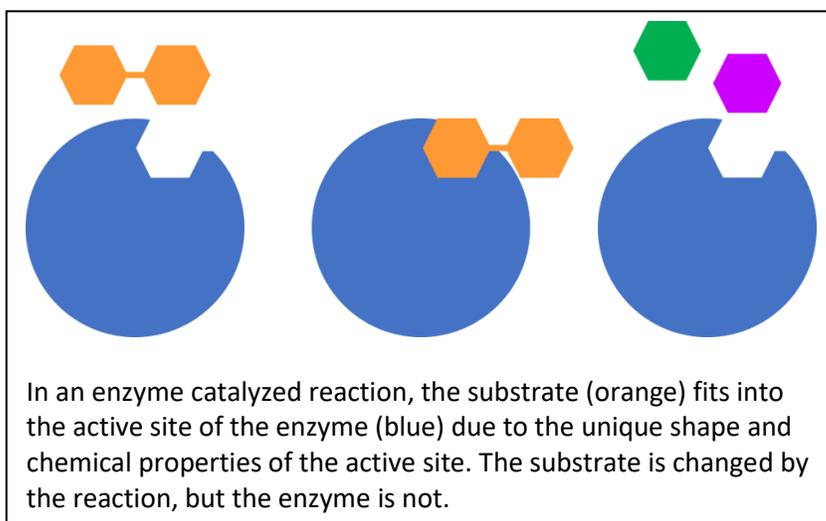
When we think of life, we think of dynamic processes: change, growth, movement. For many of these processes the most central molecules are enzymes — proteins that catalyze (speed up) chemical reactions. Enzymes work in pathways to break down or build up molecules, they digest your food, they generate the movement of the body, they transport molecules in and out of cells, they replicate and transcribe your DNA. If you can think of a process or an action that is actively carried out in the cell, an enzyme is probably central to it. In fact, the primary role of DNA is to carry the instructions for making proteins, chief among them enzymes. Without these molecules, life as we know it would not exist.

Enzymes are proteins, and like all proteins, enzymes are polymers made of amino acid monomers that are folded into precise three-dimensional shapes. Enzymes work by binding very specifically to a certain molecule or molecules, called the *substrate*. The site on the enzyme where the substrate binds is known as the *active site*. When bound to an active site, the substrate is oriented in such a way that a chemical reaction is more likely to occur. As catalysts, enzymes speed up chemical reactions by lowering the amount of energy needed for the reaction to start. Enzymes don't make reactions happen that otherwise wouldn't; they just speed them up—often significantly. Some enzymes can speed to mere milliseconds reactions that may otherwise take millions of years.

In an enzyme catalyzed reaction, substrates are changed by the reaction, but the enzyme is not. This means that the same enzyme can catalyze a reaction repeatedly as long as there is substrate for it to react with. Substrate, however, is permanently changed in the reaction.

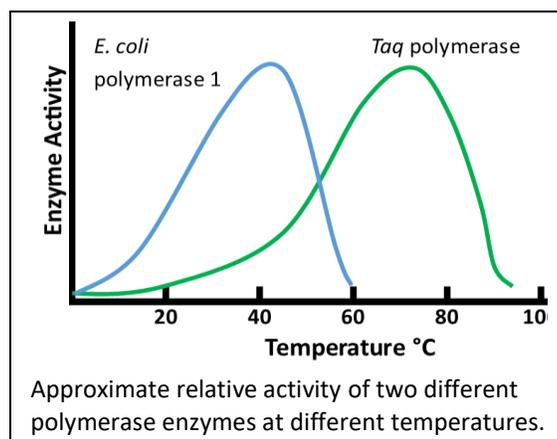
Enzymes typically work best in the very specific physiological environments where they carry out

their biological roles. This is because evolution has shaped each type of enzyme to function optimally in the conditions under which they are typically found, and enzymes tend to be highly susceptible to changes in these conditions. Factors such as pH, temperature, concentration, and other molecules present in solution can have profound effects on an enzyme's efficiency. The effect of each is discussed below.



Temperature

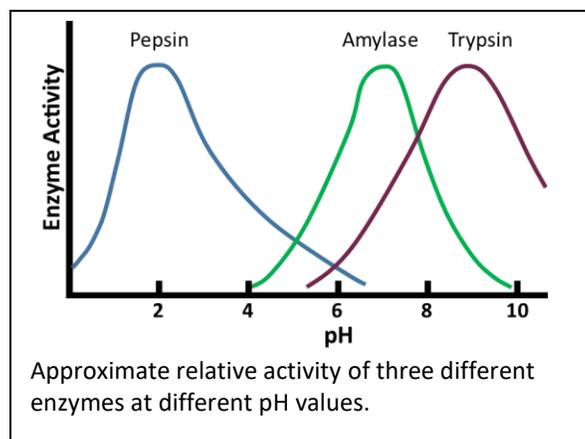
Most enzymes function best in a relatively narrow temperature range. As a general rule, when a system is too cold, the lack of energy in the system will slow reactions down. As more heat is added to a system, the general increase in energy will cause reactions to occur more quickly. Adding too much heat to the system, however, can destabilize and change the shape of the enzyme. If an enzyme is too hot for too long, it may permanently change shape or *denature*, breaking its three-dimensional conformation, thus permanently impairing its ability to catalyze reactions. Depending on the organism that the enzyme normally operates in, the amount of heat energy needed to denature an enzyme can vary greatly. For example, *E. coli* DNA polymerase 1 and *Thermus aquaticus* (*Taq*) DNA polymerase are both DNA polymerases that are found in bacteria. *E. coli* generally live in the guts of other animals, and for this reason, *E. coli* enzymes tend to work well at around 37-40° C, about body temperature. *Thermus aquaticus* is a thermophilic organism that lives in hot springs, and therefore *Taq* polymerase functions best at temperatures above 70° C—hot enough to burn you.



pH

pH is a measure of the relative amount of positively charged hydrogen (H⁺) ions and negatively charged hydroxide (OH⁻) ions in solution. Changes in the concentration of these two ions can influence enzyme catalyzed reactions in two ways.

First, the active site of an enzyme contains amino acids that will form temporary bonds with the substrate. An increase or decrease in H⁺ ions in solution can interfere with the ionization state of the amino acids in the active site, altering their charge. Such a change will greatly affect the ability of the substrate to bind within the active site, reducing or eliminating the ability for the enzyme to catalyze the reaction.



Second, the specific three-dimensional structure of an enzyme must be maintained for the enzyme to function properly. This structure relies on hydrogen bonds between amino acids to maintain the stability. Hydrogen bonds are relatively weak interactions between polar amino acids. Charged H⁺ or OH⁻ ions can bind to the polar regions of amino acids, interfering with their ability to bond with other regions of the enzyme. Affecting hydrogen bonds in this way can result in the three-dimensional structure of the

enzyme becoming unstable and losing its catalytic ability. Changing pH by too much can lead to the enzyme denaturing, permanently altering its shape and ability as a catalyst.

Like temperature, though, different enzymes have evolved to function optimally in different pH conditions. Pepsin, for example, is an enzyme that breaks the peptide bonds of proteins. Pepsin is found in your stomach, and so is optimized to function at extremely low (acidic) pH values (high H⁺ concentration). In contrast, amylase is an enzyme produced in your salivary glands and pancreas and operates best at more typical neutral pH values found in the body (close to pH 7), breaking down starches into simple sugars. Trypsin, another enzyme that breaks down proteins, is active in the small intestine and functions best at a pH greater than 8.

Enzyme and substrate concentration

Individual enzymes catalyze single reactions at a time, so how quickly a reaction progresses can depend heavily on both the amount of enzyme and substrate in solution.

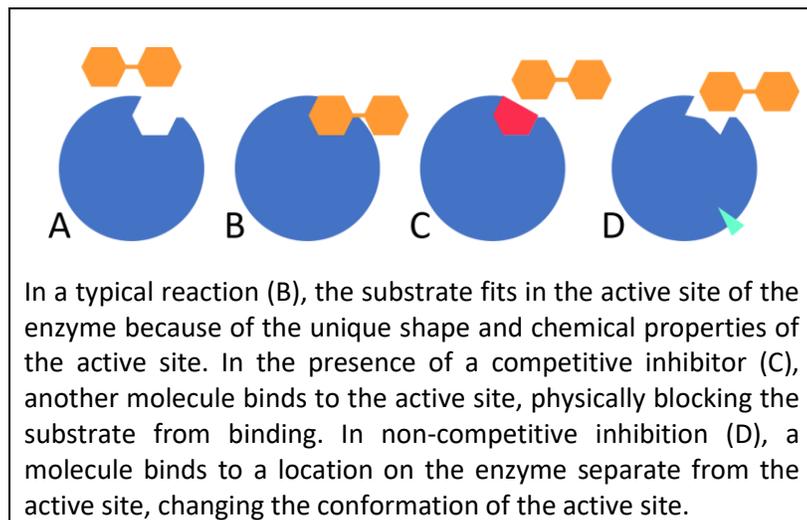
Enzymes and substrates interact when they come in contact with each other in the correct conformation. But enzymes and substrates typically only come in contact through random movement of molecules in solution, so-called Brownian motion. The more molecules of an enzyme that are present in solution, the more frequently an enzyme and substrate will come in contact and react. The fewer molecules of enzyme in solution, the less often that will happen. For this reason, as a rule, for a given amount of substrate, adding more molecules of enzyme in solution will cause a reaction to proceed more quickly. Fewer molecules of enzyme in solution lead to a reaction proceeding more slowly.

The same is true for substrate. As more substrate is added to solution enzymes will encounter the substrates at a greater rate, increasing the rate of the reaction. Adding enough substrate, however, will result in enzymes becoming saturated. This occurs when there is so much substrate that all available enzyme molecules in solution are bound to a substrate, and as soon as an enzyme and substrate react, a new molecule of substrate binds to the enzyme. Under these conditions adding more substrate will not increase the reaction rate. Lowering substrate concentration below the saturation point will reduce the speed of the reaction, as at lower concentrations enzyme and substrate will again be less likely to meet in solution.

When a reaction is proceeding, the concentration of substrate will decrease over time due to it being processed by the enzyme. Therefore, the rate of an enzymatic reaction is expected to slow as it progresses. For this reason, when measuring reaction rates scientists usually measure the initial reaction rate, the rate of the reaction when enzyme and substrate are first combined.

Inhibition

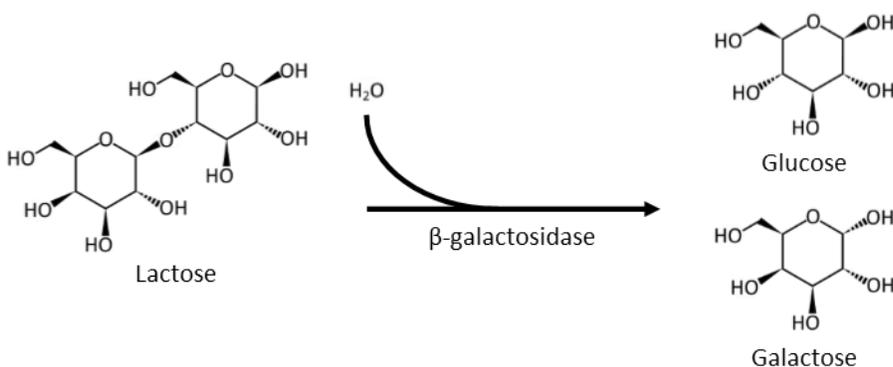
Molecules that can interfere with enzymatic activity are called *inhibitors*. *Competitive inhibitors* work by occupying the active site of the enzyme, physically blocking the substrate from binding. *Non-competitive inhibitors* bind to a region of the enzyme that is separate from the active site, usually changing the conformation of the enzyme slightly, thereby impairing the enzyme's ability to catalyze the reaction. Because competitive inhibitors physically block the active site, but do not change the structure of the enzyme, they can slow reactions, but generally do not stop the enzyme from catalyzing the reaction altogether. And if a substrate is in much higher concentration than a competitive inhibitor, the inhibitor will have little effect because the active site is more likely to encounter substrate than inhibitor. If the inhibitor is in higher concentration than the substrate, it will have more of an effect, because the enzyme is more likely to come in contact with and bind to the inhibitor than it is to the substrate.



Non-competitive inhibitors are not affected by the concentration of substrate. Because the substrate and inhibitor bind to different regions of the enzyme, changing concentration of the substrate does not affect the inhibitor binding to the enzyme. With a non-competitive inhibitor, even with very large concentrations of substrate, the reaction will still be inhibited.

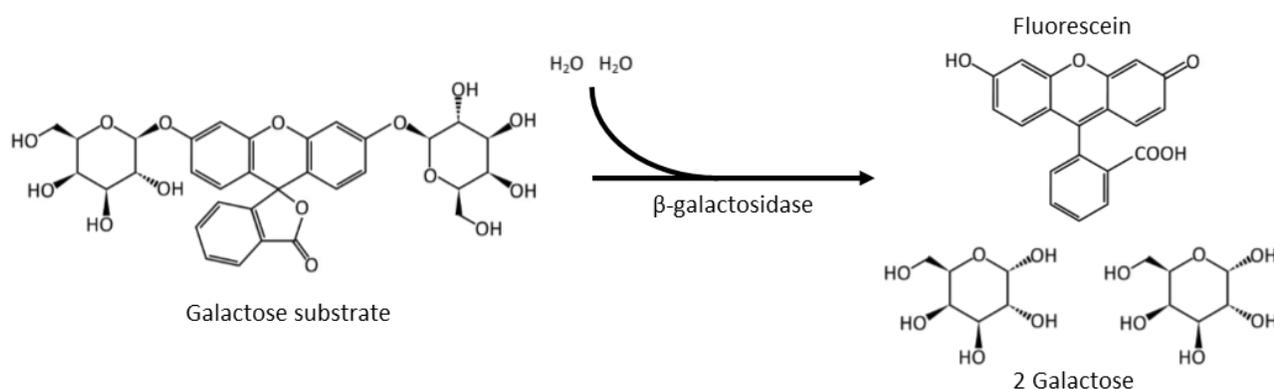
Today's lab

You will be investigating the function of β -galactosidase. β -galactosidase is a type of enzyme that is found in many organisms from bacteria to humans. It catalyzes the hydrolysis of the glycosidic bond between the monosaccharide galactose and other carbohydrates. It is a specific form of β -galactosidase, lactase, that gives mammals the ability to digest milk. Inside you, lactase breaks the glycosidic bond in the primary sugar found in



milk, lactose. Lactose consists of a galactose and a glucose monomer, linked to form a disaccharide. β -galactosidase will break that bond, but it will also readily break any glycosidic bond between galactose and another molecule. This is because the active site of the β -galactosidase enzyme only binds to the galactose portion of the substrate and is not dependent on the structure of other components of the molecule.

In this lab, you will be using the enzyme β -galactosidase to catalyze the breakdown of a molecule that contains two galactose monomers bound to a fluorescein molecule. Fluorescein is a molecule that will appear bright fluorescent green when exposed to blue light and is commonly used in biological applications. But fluorescein will not fluoresce when bound to the two galactose monomers in the substrate; only when the galactose monomers are no longer bound to the fluorescein will fluorescence be observed.



To measure the progression of your reactions, you will be comparing the brightness of fluorescence in your reaction as a measure of how much fluorescein has been produced. To quantify brightness, you will first create a *standard curve* using known concentrations of fluorescein. By comparing the brightness in your reaction to a standard curve of fluorescein, you will be able to measure the relative progression of the reaction over time and obtain an estimate of reaction rate.

Useful vocabulary:

Enzyme: A protein which catalyzes (speeds up) a chemical reaction.

Substrate: The molecule or molecules that react through binding to the enzymes active site during an enzyme catalyzed reaction.

Product: The molecules that are formed from the rearrangement of bonds in the substrate.

Active site: The location on an enzyme where it interacts with the substrate to catalyze a reaction.

Inhibitor: A molecule that slows down or prevents an enzyme catalyzed reaction.

Non-competitive inhibitor: An inhibitor that binds to an enzyme at a location other than the active site, typically changing the conformation of the enzyme and preventing it from functioning properly.

Competitive inhibitor: An inhibitor that binds to an enzyme's active site, physically blocking access to the active site by the substrate.

Denaturation: The process by which a complex organic molecule loses its three-dimensional structure. When an enzyme denatures it is no longer functional.

2. Laboratory guide

1. Observing the reaction

a. Creating a standard curve

1. To measure your reaction, you will need a standardized dilution series to compare your data to.
 - Label five tubes 1 through 5
 - To tube 5, add 20 μl of 40 μM fluorescein
 - To tube 4, add 20 μl of 40 μM fluorescein
 - Add 20 μl of Dilution Buffer to tubes 1-3.
 - Add 20 μl of Dilution Buffer to tube 4. Mix well by pipetting up and down.
 - Take 20 μl from tube 4 and add it to tube 3. Mix well by pipetting up and down.
 - Take 20 μl from tube 3 and add it to tube 2. Mix well by pipetting up and down.
 - Take 20 μl from tube 2 and add it to tube 1. Mix well by pipetting up and down.
 - Remove 20 μl from tube 1 and discard.
 - All tubes should now have 20 μl of fluorescein solution.

2. Place the five tubes in P51™ or other blue light illuminator.
 - Place the tubes in P51 in numerical order, with tube 1 on the edge and tube 5 closer to center.
 - There should be three open slots in P51 for additional tubes; these will be needed later.
 - Tubes 1 through 5 can remain in the illuminator for the rest of the experiment.
 - Note: tubes may photobleach some over time. Refrain from exposing to blue light excessively/unnecessarily to preserve brightness. Enough fluorescein is provided to each group to remake this dilution series if necessary later in the lab.

3. Calculate the concentration in each tube of your standard curve.

Tube	1	2	3	4	5
Concentration					40 μM

Tubes 1 through 5 represent your standard curve. The fluorescein provided to you was given in a 40 μM concentration. The reaction you will observe will produce fluorescein as a product. You will use your standard curve to measure the amount of fluorescein produced over time.

b. Observing the reaction

1. **Label a single 200 μ l PCR tube "S".**
 - Use a fine tip permanent marker to write on the side wall of the tube.
2. **Add 10 μ l of 2X Substrate to the tube.**
 - Use a micropipette and be sure that the substrate is at the bottom of the tube and free of bubbles.
 - Keep the cap of the tube open.
3. **Add 10 μ l of Dilution Buffer to create a 1X Substrate solution.**
4. **Turn ON P51™ and place the tube inside.**
 - Place the tube in the next open position after your standard curve (tubes 1-5)
 - Be sure that the light inside the P51™ Viewer is on.
5. **Add enzyme and observe the reaction.**
 - While the Substrate tube is still in P51™ **add 5 μ l of 1X Enzyme** directly into the substrate solution, pipetting up and down 3-4 times to mix.
 - Close the lid of P51™ and watch the reaction closely.

Based on what you observed, describe what occurred in the tube. Underline the terms *enzyme*, *substrate*, and *product* in your answer.

c. Measure the rate of the reaction (Reference Reaction)

Now that you are familiar with the reaction, we will measure its progression over time.

1. **Repeat steps 1-5 from part B using a new tube of Substrate.**
2. **Record the progression of the reaction.**
 - This time, as soon as you add the enzyme to the substrate tube, have one group member start a stopwatch/timer. Make sure the P51 light is ON.
 - Every 10 seconds, estimate the brightness of the reaction by comparing it to the five tubes in your fluorescein dilution series.
 - Record your brightness scores in the *Data Table* (page 23)
3. **You may wish to try timing the reaction one more time until you are comfortable with the procedure.**

d. Changing one variable in your reaction – Substrate concentration (optional)

In this lab you will design experiments to see how different variables affect enzyme rate. We will do one trial with substrate concentration as an example. This is presented as a way for students to become more familiar with the basic experimental design before embarking on the inquiry portion of the activity. Your teacher may ask you to skip this step if they feel it is not necessary.

- 1. Label a single 200 µl PCR tube 0.5X.**
 - Use a fine tip permanent marker to write on the side wall of the tube.
- 2. Add 5 µl of 2X Substrate to the tube.**
 - Use a micropipette and be sure that the substrate is at the bottom of the tube and free of bubbles.
 - Keep the cap of the tube open.
- 3. Add 15 µl of Dilution Buffer to create a .5X Substrate solution.**
 - Pipette up and down 3-4 times to mix.
 - Be sure that the substrate is at the bottom of the tube and free of bubbles.
 - Keep the cap of the tube open.
- 4. Turn ON P51™ and place the tube inside.**
 - Be sure that the light inside the P51™ Viewer is on.
- 5. Add enzyme and observe the reaction.**
 - While the substrate tube is still in P51™ add 5 µl of 1X Enzyme directly into the substrate solution, briefly pipetting up and down to mix.
 - Close the lid of P51™ and watch the reaction closely.
- 6. Record the progression of the reaction.**
 - When you add the enzyme to your reaction have one group member start a stopwatch.
 - Every 10 seconds, estimate the brightness of the reaction by comparing it to the five tubes in your fluorescein dilution series.
 - Record your data in the *Data Table*.
- 7. Compare your data to the Reference Reaction (point C.)**

Explain your observations in the space below.

Based on what you observed, describe what occurred in the tube. Compare these observations to the Reference Reaction.

2. Inquiry investigation

You will now choose to pursue one of the four investigations in the table at the bottom of this page.

General instructions

- For each investigation use the *CER Table* and *Data Table* to plan what you will do, record your results, and say what you learned. If you do more than one investigation, you will need more than one CER table. Do not begin an investigation until you have filled in the top half of your CER table.
- Use the reactions that you have already completed as a guide for designing your experiments.
- Whatever substrate or enzyme concentration you decide to use, make sure to use the same volume for each reaction. When changing the concentrations of any solution, use the Dilution Buffer to equalize the final volume of your reactions. A standard reaction of 25 μl , consisting of 20 μl 1X substrate solution plus 5 μl 1X enzyme solution, is a recommended as a starting point for planning your investigations.
- Observe reactions one at a time. Do not try to run multiple reactions simultaneously.



Substrate solution is provided to you in a 2X concentration so that you may add other solutions to the reaction. Make sure that your final concentration is what you want it to be for your investigation.

The following is a list of reagents and materials that your teacher will make available to you for each investigation.

<i>Temperature sensitivity</i>		<i>pH sensitivity</i>	
<input type="checkbox"/> 2X Substrate solution	150 μl	<input type="checkbox"/> 2X Substrate solution	150 μl
<input type="checkbox"/> 1X Enzyme solution	80 μl	<input type="checkbox"/> 1X Enzyme solution	80 μl
<input type="checkbox"/> Dilution buffer	150 μl	<input type="checkbox"/> Dilution buffer	150 μl
<input type="checkbox"/> Ice bath		<input type="checkbox"/> 100 mM NaOH	50 μl
<input type="checkbox"/> miniPCR machine		<input type="checkbox"/> pH paper (optional)	
OR		<i>Note: pH values lower than six cannot be investigated using this lab, as fluorescein does not fluoresce at low pH</i>	
<input type="checkbox"/> Warm water bath (40-55°C) and			
Hot water bath (Above 75°C)			
<i>Concentration dependence</i>		<i>Competitive inhibition</i>	
<input type="checkbox"/> 2X Substrate solution	150 μl	<input type="checkbox"/> 2X Substrate solution	150 μl
<input type="checkbox"/> 1X Enzyme solution	80 μl	<input type="checkbox"/> 1X Enzyme solution	80 μl
<input type="checkbox"/> 4X Enzyme solution	10 μl	<input type="checkbox"/> Dilution buffer	150 μl
<input type="checkbox"/> Dilution buffer	150 μl	<input type="checkbox"/> 500 mM Sucrose solution	50 μl
		<input type="checkbox"/> 500 mM Lactose solution	50 μl

CER Table

Fill in the table based on your results from the lab. Use the attached rubric to help guide your answers.

Pre-lab

Question:	
Hypothesis:	
Evidence from text or other sources that supports your hypothesis:	I will test this by (overview of experimental design):

Post-lab

Claim:
Evidence from observations:
Reasoning (Be sure to integrate evidence from both text and observations):

CER Table

Fill in the table based on your results from the lab. Use the attached rubric to help guide your answers.

Pre-lab

Question:	
Hypothesis:	
Evidence from text or other sources that supports your hypothesis:	I will test this by (overview of experimental design):

Post-lab

Claim:
Evidence from observations:
Reasoning (Be sure to integrate evidence from both text and observations):

	4	3	2	1
Hypothesis	Statement is a clear and general prediction that addresses the question. The statement is clearly testable and falsifiable.	Statement is a general prediction that addresses the question. The statement could be tested.	Statement does not directly address the question. It is not clear that the statement could be tested or falsified.	Statement is not a prediction or is not related to the question. The statement is not testable or falsifiable.
Evidence from text	The evidence provided is clear, relevant and accurate. The evidence clearly supports the student's hypothesis.	The evidence provided is relevant and generally accurate. The evidence supports the student's hypothesis	The evidence provided is generally related to the hypothesis and mostly accurate. The evidence may not fully support the hypothesis	The evidence provided is not related to the hypothesis or is generally inaccurate. The evidence may not support the hypothesis.
I will test this by	The experimental design is clearly relevant and directly addresses the student's hypothesis. It is clear from the description how different results will support or refute the hypothesis.	The experimental design is relevant and addresses the student's hypothesis. It can be determined from the description how different results will support or refute the hypothesis.	The experimental design generally addresses the student's hypothesis, but may not be clearly defined. It is unclear how results will support or refute the hypothesis.	The experimental design does not address the hypothesis or is difficult to understand and follow. It is unclear how results will support or refute the hypothesis.
Claim	The claim is clear accurate and complete. It states whether the hypothesis is supported or refuted.	The claim is accurate and complete. It states whether the hypothesis is supported or refuted.	The claim is mostly accurate. It may or may not state whether the hypothesis is supported or refuted.	The claim is inaccurate. It may not state whether the hypothesis is supported or refuted.
Evidence from observations	The evidence is clear, relevant and sufficient to support the claim.	The evidence is relevant and supports the claim.	The evidence is mostly relevant to the claim. The evidence is not sufficient to support the claim on its own.	The evidence is not relevant. The evidence does not support the claim.
Reasoning	Evidence from both the text and observations are used in a way that clearly link the claim to scientific principles.	Evidence from both the text and observations are used in a way that link the claim to scientific principles.	Evidence is used to link the claim to scientific principles, but evidence from one source may be lacking. There may be some minor misconceptions on how scientific principles are related to the claim.	Evidence is not used or is used incorrectly to link the claim to scientific principles. There are some clear misconceptions on how scientific principles are related to the claim.

We recommend that you use the following scale when assessing this assignment using the rubric.

Teachers should feel free to adjust this scale to their expectations.

Rubric Score	6	7	8	9	10	11	12	13	14	15	17	18	19	20	21	22	23	24
Equivalent Grade	57.5	60	62.5	65	67.5	70	72.5	75	77.5	80	82.5	85	87.5	90	92.5	95	97.5	100

Data Table

Question:

Independent Variable:

Dependent Variable:

DATA:

Record the relative brightness of your reaction over time. For each time reading score the brightness of your reaction 1-5 based on which tube of fluorescein it appears closest to in terms of overall brightness.

		TIME POINT	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Condition	Reference Reaction 20 μ l Subs. + 5 μ l Enz.	0																

Note:

We recommend scoring each reaction every 10 seconds until a score of 5 is reached or until at least 2 minutes has passed. You may wish to alter this depending on the condition you are testing.

Use as many rows or columns as you need to accomplish your experimental design. You may add extra rows or columns if you find it necessary. Groups may find it easier to create their own data tables.

If doing more than one investigation, groups will need more than one copy of this page.

Data Table

Question:

Independent Variable:

Dependent Variable:

DATA:

Record the relative brightness of your reaction over time. For each time reading score the brightness of your reaction 1-5 based on which tube of fluorescein it appears closest to in terms of overall brightness.

		TIME POINT	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Condition	Reference Reaction 20 μ l Subs. + 5 μ l Enz.	0																

Note:

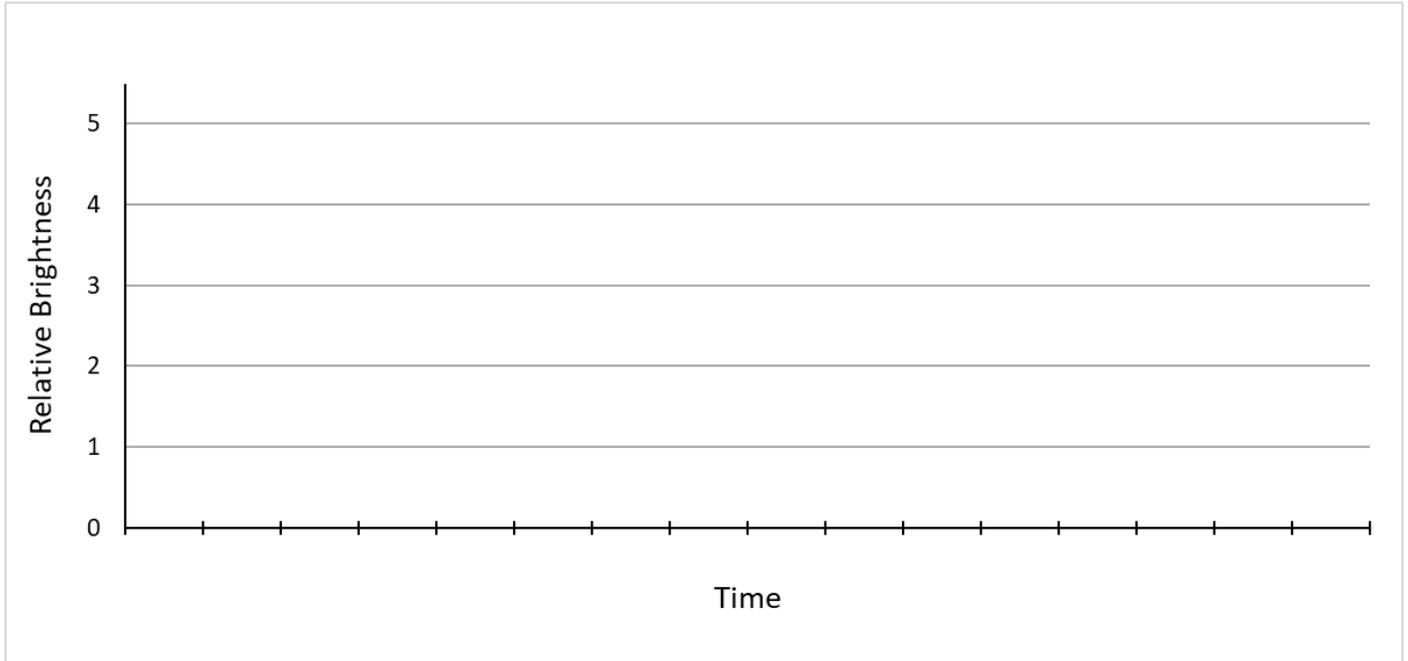
We recommend scoring each reaction every 10 seconds until a score of 5 is reached or until at least 2 minutes has passed. You may wish to alter this depending on the condition you are testing.

Use as many rows or columns as you need to accomplish your experimental design. You may add extra rows or columns if you find it necessary. Groups may find it easier to create their own data tables.

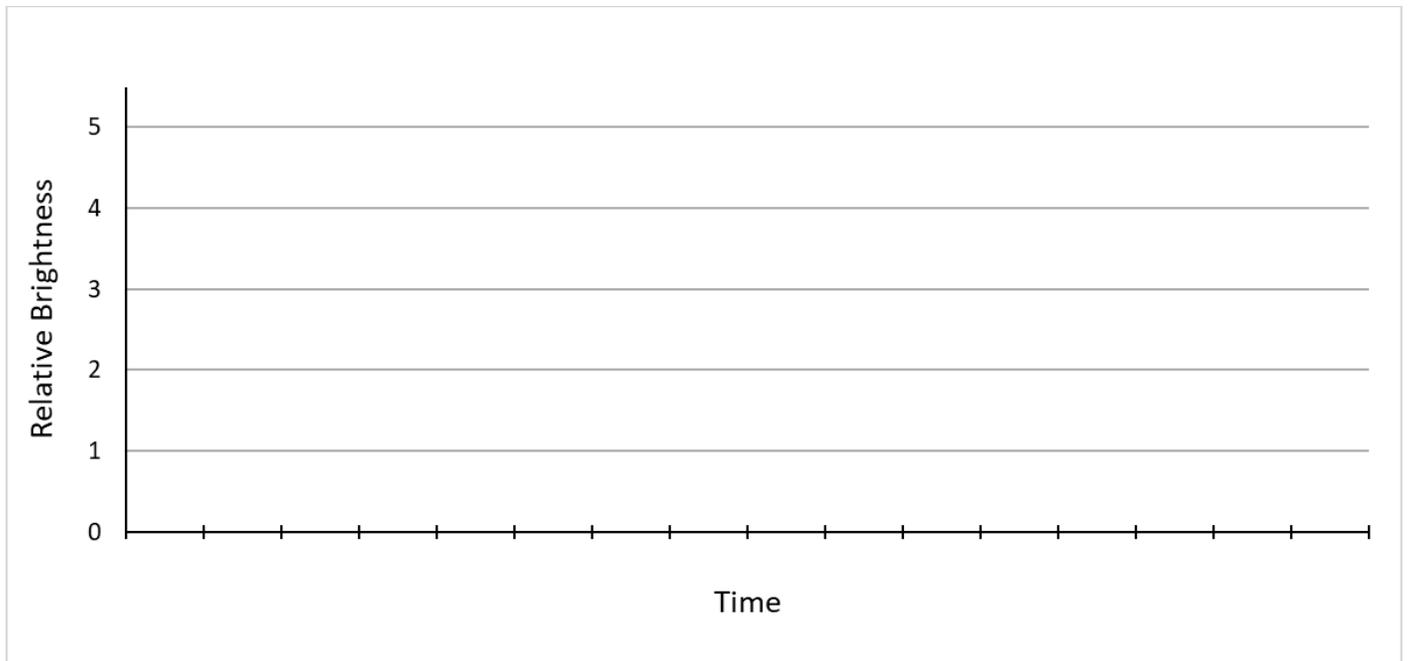
If doing more than one investigation, groups will need more than one copy of this page.

Data analysis

Investigation: _____



Investigation: _____



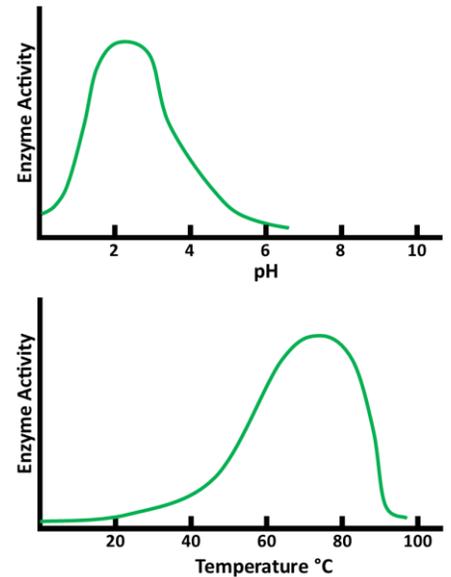
4. Study questions

Questions for before experimental set-up

1. In an enzyme catalyzed reaction, how are the reactants related to the products?
2. Which scenario would result in the most amount of total product produced, regardless of time?
 - a. A lot of enzyme and a little substrate.
 - b. A lot of substrate and a little enzyme.Justify your answer.
3. If enzymes only work well in a very narrow range of conditions, why is that *not* a problem for the enzymes that function in your body?
4. Describe the importance of the active site of an enzyme.
5. What happens when an enzyme denatures, and how does that affect the reaction rate?

6. Look at the two graphs to the right of the page. Knowing only this information about an unknown enzyme, which description about the organism that the enzyme comes from sounds most likely?
- A sea urchin that lives under the sea ice on the shores of Antarctica.
 - A species of bacteria isolated from an acidic hot spring.
 - A desert adapted plant that lives at the edge of salt flats.
 - Helicobacter pylori*, a species of bacteria adapted to live in the stomach of some organisms.

Justify your answer.



7. An enzyme catalyzed reaction can be stopped by both cooling it enough and by heating it enough. Which of these processes is more likely to be reversible? Justify your answer.
8. Sometimes changing pH will slow or stop an enzyme catalyzed reaction, but changing the pH back will allow it to proceed. Other times, the change is irreversible. Can you think what might be occurring differently in those two cases?
9. If you wanted a reaction to proceed extremely fast, would adding more substrate or more enzyme be more effective? Justify your answer.
10. In some reactions, the products of the reaction can also be competitive inhibitors. Using your knowledge of enzymes, substrates and active sites, why do you think this might be so?

Questions for Concentration

6. Which seemed to have a greater effect on reaction rate, changing the substrate concentration or changing the enzyme concentration? Explain why you think this is so.

7. Which seemed to have a greater effect on the total amount of product produced in the reaction? Explain why you think this is so.

Questions for Competitive Inhibition

8. Which molecule seemed to have a greater effect on the reaction rate, sucrose or lactose?

Why do you think this is so?

9. Imagine that instead of lactose, we added a different molecule that contained galactose but this new molecule could not be broken down by β -galactosidase. Do you think this would have a greater effect or lesser effect on the rate of the reaction? Justify your answer.