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The rAmylase Project by Ellyn Daugherty

Determining the Concentration of Amylase in a Solution

(Lab 7d)

(Cat. # BTNM-7G)

Developed in partnership with

The rAmylase Project by Ellyn Daugherty

Determining the Concentration of Amylase in a Solution (Lab 7d)

Teacher's Guide

The following laboratory activity is adapted from "Laboratory 7d: Determining the Concentration of Amylase in Solution" from *Biotechnology: Laboratory Manua*l by Ellyn Daugherty. For more information about the program, please visit www.emcp.com/biotechnology.

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About Ellyn Daugherty: Ellyn Daugherty is a veteran biotechnology educator and recipient of the Biotechnology Institute's National Biotechnology Teacher-Leader Award. She is the founder of the San Mateo Biotechnology Career Pathway (SMBCP). Started in 1993, SMBCP has instructed more than 7,000 high school and adult students. Annually, 30-40 SMBCP students complete internships with mentors at local biotechnology facilities.

About G-Biosciences: In addition to the Biotechnology by Ellyn Daugherty laboratory kit line and recognizing the significance and challenges of life sciences education, G-Biosciences has initiated the BioScience Excellence™ program. The program features hands-on teaching kits based on inquiry and curiosity that explore the fundamentals of life sciences and relate the techniques to the real world around us. The BioScience Excellence™ teaching tools will capture the imagination of young minds and deepen their understanding of various principles and techniques in biotechnology and improve their understanding of various social and ethical issues.

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Upon receipt, store the materials as directed in the package literature.

MATERIALS INCLUDED

This kit has enough materials and reagents for 8 lab groups (32 students in groups of 4).

- 8 tubes of 0.5mg/ml stock α-Amylase from *Bacillus subtilis* (2ml each)
- 8 tubes 1X PBS, pH 7.0 (4ml)
- 8 tubes of Unknown #1 (0.2mg/ml α-Amylase from *Bacillus subtilis*) (600µl each)
- 8 tubes of Unknown #2 (0.1mg/ml α-Amylase from *Bacillus subtilis*) (600µl each)
- 1 bottle Bradford Reagent [5X] (40ml)
- 50 Microcentrifuge Tubes (1.5ml)
- 8 Transfer Pipets
- 8 6ml Non Sterile Tubes for Mixing

ADDITIONAL EQUIPMENT & MATERIALS REQUIRED

- Spectrophotometer (Visible or UV/Vis)
- Spectrophotometer cuvettes (for the spectrophotometer used)
- Small Beakers (i.e. 50 ml beakers)
- 250ml Beaker
- P-1000 Micropipets and tips
- Deionized water

SPECIAL HANDLING INSTRUCTIONS

- Store all Amylase samples frozen at -20°C, until ready to use.
- Store the Bradford Reagent at 4°C, protected from light
- All other reagents can be stored at room temperature

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GENERAL SAFETY PRECAUTIONS

- The reagents and components supplied in the *The rAmylase Project*™ kits are considered non-toxic and are safe to handle (unless otherwise noted), however good laboratory procedures should be used at all times. This includes wearing lab coats, gloves and safety goggles.

- The teacher should 1) be familiar with safety practices and regulations in his/her school (district and state) and 2) know what needs to be treated as hazardous waste and how to properly dispose of non-hazardous chemicals or biological material.

- Students should know where all emergency equipment (safety shower, eyewash station, fire extinguisher, fire blanket, first aid kit etc.) is located and be versed in general lab safety.

- Remind students to read all instructions including Safety Data Sheets (SDSs) before starting the lab activities. A link for SDSs for chemicals in this kit is posted at www.gbiosciences.com

- At the end of the lab**,** all laboratory bench tops should be wiped down with a 10% bleach solution or disinfectant to ensure cleanliness.

- Remind students to wash their hands thoroughly with soap and water before leaving the laboratory.

TEACHER'S PRE EXPERIMENT SET UP

1. Label 8 small beakers, "1X Bradford Reagent." In a 250ml beaker, dilute the Bradford Reagent to 1X by adding 40ml of the 5X Bradford Concentrate to 160ml of deionized water. Mix thoroughly. Aliquot 25ml of 1X Bradford Reagent into the 8 labeled beakers.

2. Distribute one of each of the following to each of the 8 lab groups:

    - 1 tube of 0.5mg/ml Amylase stock
    - 1 tube of Unknown Amylase #1
    - 1 tube of Unknown Amylase #2
    - 1 tube of 1X PBS, pH 7.0
    - 25ml of 1X Bradford Reagent
    - 5 Microcentrifuge Tube

TIME REQUIRED

- 30 minutes, pre-lab (preparation and sorting of reagents)
- Two 1-hour lab periods (to collect absorbance data, graph, and analyze the data)
- 30 minutes post lab analysis

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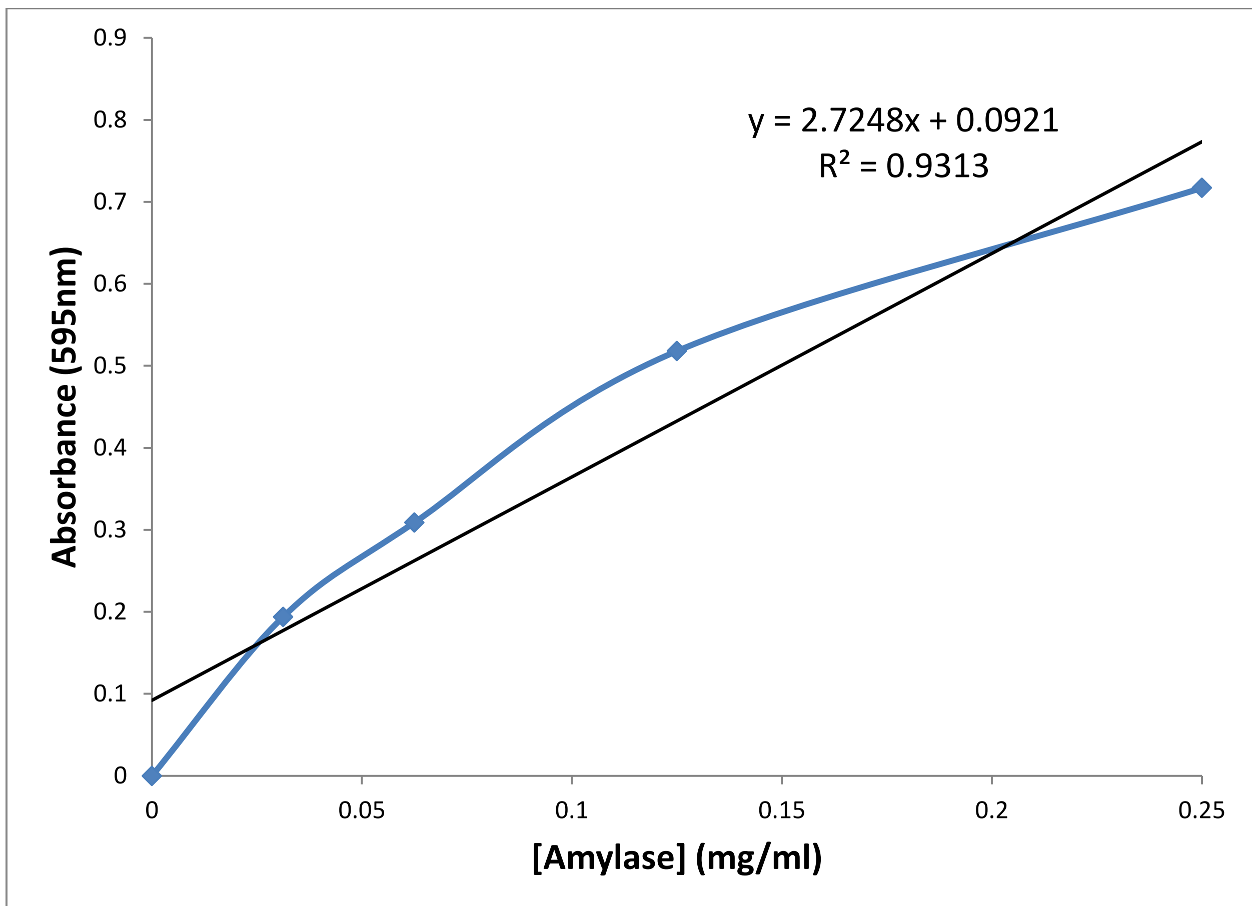
NEXT GENERATION SCIENCE STANDARDS ADDRESSED

- HS-LS1: From Molecules to Organisms: Structures and Processes
- LS1.A: Structure and Function

For more information about Next Generation Science Standards, visit: http://www.nextgenscience.org/

EXPECTED RESULTS



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ANSWERS TO ADDITIONAL QUESTIONS

1. Without using the spectrophotometer, how can you estimate the concentration of amylase in the unknown samples?
   Answer: The concentration of amylase in the unknown samples can be estimated by doing the following: Prepare amylase-Bradford mixtures. The amount of blueness in the "unknowns" can be compared to the known solutions ("eye-balling"). These give a rough estimate of the concentrations.

2. On the best-fit standard curve, all the points are lined up, except the 0.5mg/mL sample. It is much lower than the rest of the line. Why might this be? Should the value be used in the standard curve?
   Answer: If the 0.5mg/mL reading is not in line with the others on the graph, the sample is probably so concentrated that it is hard to get an accurate absorbance reading. The reading is lower than it should be. If this happens, the reading should not be used in the standard curve because its changes the slope of the line.

3. A set of proteins is studied in the spectrophotometer. The linear regression ($y = mx + b$) gives a slope of $m = 0.93$ and the y-intercept (b) is zero. An unknown sample's absorbance is measured at 0.66 au. What is the approximate concentration of the unknown sample?
   Answer: $b = 0$, so $x = y/m$ and $x = 0.71$ mg/mL. The concentration of the unknown is approximately 0.71 mg/mL.

4. Amylase is a colorless molecule, thus, Bradford reagent was used in this experiment to make it visible in the Spec 20 D. Name at least one disadvantage to using the Bradford reagent on a suspected amylase sample.
   Answer: One disadvantage to using Bradford Reagent is that it denatures the protein in the sample, making it unusable. If there is a limited supply of the sample, the technician would not want to "waste" a sample in visible spectrophotometry.

5. Propose a method to detect colorless amylase in a sample and still be able to recover it, unaltered for future use.
   Answer: A UV spectrophotometer could be used to detect colorless amylase. The UV spectrophotometer does not alter the protein, and it is retrievable for future use.

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OBJECTIVES

What is the concentration of two unknown amylase solutions?

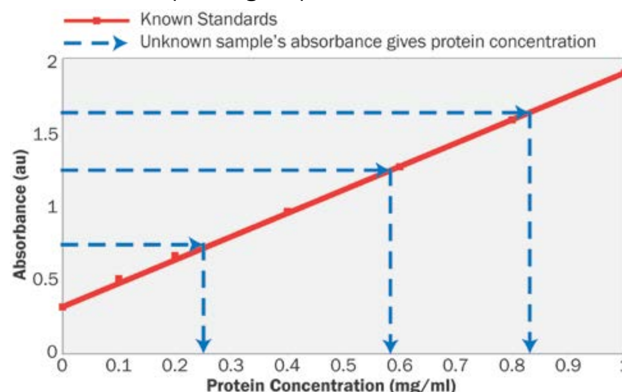
BACKGROUND

Often, the goal of a biotechnology company's product pipeline is to manufacture proteins of social or economic importance. The company must synthesize enough of the protein to market it at a profit.

The proteins are made in cells, usually ones that have been transformed with recombinant DNA (rDNA). The protein-making cells are grown in huge fermentation tanks. Often, the protein is released into the fermentation broth where the cells are grown, although sometimes the proteins are held within the cell. Either way, the protein has to be isolated from all the other thousands of proteins made by a typical cell.

Separating a protein of interest from other unwanted molecules is called purification. The biotech company wants purified proteins that are active, stable, and of a relatively high concentration. When proteins are purified from solutions (cytoplasm, fermentation broth, etc.), the product must be checked to see whether the protein is present and how much is present. At every step in the manufacture of a protein, the concentration must be determined. Protein concentration is usually measured in milligrams per milliliter (mg/ml) or micrograms per milliliter (µg/ml).

Since the protein molecules are submicroscopic, we must measure the proteins using indirect methods. To determine the concentration of a solution, one produces a standard curve that plots the absorbance of solutions at *known* concentrations. First, the technician prepares solutions of known concentrations and reads their absorbance at a given wavelength. The technician produces a "best-fit" straight line, representing how the concentration affects the absorbance by the molecule being tested. The absorbance of an *unknown* solution is then determined. From the "best-fit" straight-line standard curve, the concentration of the unknown solution is determined based on where the absorbance value intersects the standard curve (see Figure).



Standard Curve of Protein Concentration versus Absorbance.

The absorbance of the sample is directly proportional to the number of molecules present (concentration).

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MATERIALS FOR EACH GROUP

Supply each group with the following components. Some components will be shared by the whole class and should be kept on a communal table.

- 1 tube of Amylase Stock Solution (0.5mg/ml)
- 1 tube of Amylase Unknown #1 (600µl)
- 1 tube of Amylase Unknown #2 (600µl)
- 1 tube of 1X PBS Buffer, pH 7.0 (4ml)
- 1 beaker of 1X Bradford Reagent (25ml)
- 5 Microcentrifuge Tubes (1.5ml)

ADDITIONAL MATERIALS FOR EACH GROUP

The following standard lab equipment should be available for each group.

- P-1000 Micropipets and tips
- Spectrophotometer (Visible or UV/Vis)
- Spectrophotometer cuvettes (for the spectrophotometer used)
- Paper towels

PROCEDURE

I. Prepare the Standard (Known) Samples

Prepare a serial dilution of the *known* samples starting with the0.5mg/ml of amylase stock solution.

1. Label five microcentrifuge tubes No. 1 through 5.

2. Add 500µl of 0.5mg/ml of amylase stock solution to Tube No. 1.

3. Add 500µl of 1XPBS Buffer to each of the test tubes No. 2 through 5.

4. Take 500µl of the 0.5mg/ml of amylase stock solution and add it to tube No. 2. Gently pipette it up and down to mix. What is the concentration of protein in this tube?

5. Take 500µl of the solution in test tube No. 2 and add it to test tube No. 3. Gently pipette it up and down to mix. What is the concentration of protein in this tube?

6. Continue making these dilutions with the remaining tubes. After preparing test tube No. 5, discard the extra 500µl so that all tubes No. 1 through 5 contain the same volume of solution (500µl). Record the concentration of tubes #1-5 in your notebook. What type of serial dilution have you performed (i.e., 1:1, 1:2, 1:3, 1:4, or 1:5)?

7. These 5 tubes contain the "known" standard samples of known concentration that are used to create the standard curve used in calculating the unknown samples' concentrations.

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II. Prepare the Samples for Spectrophotometry

- Make sure you are familiar with how to use the spectrophotometer in your lab. To take absorbance reading of all the samples, you must work quickly when using the spectrophotometer.
- For use in the visible spectrophotometer the samples (as well as buffer blank and the unknown samples) must be transferred to appropriate tubes for mixing with the colored protein indicator (Bradford Reagent). If the spectrophotometry cuvettes are an appropriate size, they can be used for the mixing, and then for spectrophotometry. If not, then each sample and Bradford Reagent is mixed in a separate test tube and then transferred to a cuvette for analysis.

1. Transfer all of the standard samples to labeled "mixing tubes". Label these tubes with their actual concentration in mg/ml (0.5, 0.25, 0.125, 0.0625, 0.03125, respectively.).

2. Learn how to use the model of spectrophotometer in your lab. Warm up and calibrate your spectrophotometer (using the blank), absorbance data will be taken at a wavelength of 595nm. Set the spectrophotometer to a wavelength of 595 nm.

3. In your notebook, create a data table to record the absorbance data for all the amylase samples of known concentration (amylase protein standards). Create another data table to record the absorbance of the unknown amylase samples.

4. Place 500μl of unknown sample #1 in a mixing tube. Label this tube "U1."

5. Place 500μl of unknown sample #1 and unknown #2 in a mixing tube. Label this tube "U2."

6. Place 500μl of the 1X PBS Buffer in a mixing tub. Label this tube "B", for blank.

7. *Do not go onto step 7 until the spectrophotometer you will be using is free and ready for you to use. Once you add Bradford Reagent to the samples the absorbance of each must be read in 3 minutes.*

8. Using a transfer pipet and without touching the inside of each tube, add 2.5ml of 1X Bradford Reagent to the blank, each of the standard known samples, and the two unknown samples. Be careful to not touch the solutions in the tubes, spill or accidentally contaminate one sample with another. Gently mix thoroughly without letting the mixture bubble. Within 3 minutes use the spectrophotometer to observe (take absorbance readings) of all the samples.

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III. Creating a Protein Concentration Standard Curve

1. With the spectrophotometer set at 595 nm, use the Blank/Bradford mixture to set the absorbance to 0 au.

2. Quickly, read the absorbance of each standard/Bradford sample and each unknown/Bradford sample at 595nm. Record this absorbance data (in au) in the appropriate data table.

   As you collect data, look to see if the absorbance of each standard "makes sense." If a sample has twice the concentration of molecules as a second sample, it would be expected to absorb twice as much light as the second sample.

3. After collecting the absorbance data, use the absorbance of the known standard samples to make a "standard curve" graph. Plot the concentration (mg/ml) on the x-axis and absorbance (au) on the y-axis. Add the absorbance data points for the known standards. The points should "line up" since the absorbance is correlated to the number of molecules present to absorb the light passing through a sample. However, there is an optimal range of concentrations a spectrophotometer can detect. If a sample's concentration is "too high" the spectrophotometer will report it erroneously lower than it actual is. This data will skew the standard curve line. Pick the best 3-4 data points to use for the best-fit standard curve line and ignore samples that are obviously skewing the line.

4. Make a "best fit" straight line through the data points of the known standard samples. The best-fit straight-line estimates what a sample of amylase molecules will absorb within a range of concentrations.
   NOTE: *If you are creating the graph using Microsoft® Excel®, pull down "Chart" on the "Menu," select "Add Trendline," and select "Linear Regression." It will add a "best-fit" straight line for you. Double-click on the "Best-fit Trendline." Select "Options." Click on "axis through 0" and "show equation of the line." This will determine the "equation of the line" to be used later.*

IV. Determining Protein Concentration Using a Standard Curve

1. Using this best-fit standard curve, estimate the concentration of your "unknowns." Show (draw in) where the absorbance value for each "unknown" intersects the following: the y-axis, the "best-fit" straight line, and the x-axis. See the Background Section of this lab sheet for an example of how the best-fit graph might look.
   Record these estimates of each "unknown's" concentration in the data table of unknown amylase samples.

2. Your eye can do a fair job of estimating where the best-fit standard curve should be drawn and estimating the concentration of the unknown samples but in industry, the concentration of a sample is calculated mathematically, using the best fit straight line and the linear regression equation (equation of the line), as shown below. This method allows technicians to determine the concentrations of many "unknowns" quickly.

$$y = mx + c$$

y = the absorbance of the unknown sample
m = the slope of the best-fit straight line
x = the concentration of the unknown sample
c = the y-intercept

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NOTE: *The equation of the best-fit standard curve line was determined and placed on the graph above in step 4. For your line, what is the slope of the line and what is the y-intercept?*
You might expect the slope to be close to 1 since the rise of your line should be close to the run of the line if the absorbance increases proportionally to the concentration.
You would expect the y-intercept to be close to 0 since a concentration of 0mg/ml should give a 0 au absorbance reading.

Use y = mx + c to calculate the concentrations of the unknown samples, and add these data to the unknown samples data table. Using y = mx + c is easy since the line equation gives you "m" and "b" and you have determined "y" for each unknown.

Which calculation do you have most confidence in, the one determined by drawing on the graph, or the on determined using the equation? Explain why.

1. Collect the concentration determinations for each unknown (y = mx + c) from the other groups in the class (multiple replications). Put these values into a new data table. Calculate the average concentration of each sample for all the replications of the experiment. These averages are the best guess of the true concentrations of the "unknowns."
2. How good are your data? Are they accurate, reliable? How well does your data "match" the multiple replications of the experiment? Compare your values to the averages, the mean, and the range of values.

3. In the lab during R&D, if data are within 10% of the value expected, they are often considered "close enough." Determine the amount of deviation your measurements have (in %) from the average values. To do this, use the following equations.

$$\frac{\text{(your sample's value - the class' average for the sample)}}{\text{the class' average for the sample}} \text{ X } 100 = \underline{\qquad\qquad} \text{ \% deviation for unknown No 1 from the class average}$$

$$\frac{\text{(your sample's value - the class' average for the sample)}}{\text{the class' average for the sample}} \text{ X } 100 = \underline{\qquad\qquad} \text{ \% deviation for unknown No 2 from the class average}$$

Is your determination of each "unknown's" concentration "close enough"?

4. Commonly, sample data are analyzed to see how much they deviate from average data. Scientists usually consider data within 1 deviation above or below the average to be valid. Determine the standard deviation (SD) of each group of amylase samples (multiple replications) using the following formula.

$$\text{Standard Deviation} = \sqrt{\sum \frac{(\text{Average} - \text{sample reading})^2}{\#\text{ of samples}}}$$

If you are using Microsoft® Excel® for your data table, it is relatively easy to calculate the SD for a group of numbers. Highlight an empty cell, and enter the following equation: = STDEV (highlight the numbers you want to analyze) Press "Enter." Add and subtract the SD to your class average. Do your own samples' data fall within the SD?

The range of acceptable concentration determinations is the average, + and - the SD. For example, if the average for a group of data is 0.48mg/ml, and the SD is 0.07, then the range of acceptable data is 0.55 mg/ml down to 0.41mg/ml. If a sample does not fit in this range, then the data either do not support the hypothesis or they are erroneous.

Should your individual determinations of the "unknown's" concentration be accepted as valid? The smaller the SD for the collection of samples, the more reproducibility there is in the measurements. With a small SD, you can have confidence that the group of measurements reflects the concentrations of the samples.

DATA ANALYSIS & CONCLUSION

In a written concluding statement (1-3 paragraphs), discuss the results of the experiment, including your best estimate of the concentration of each unknown amylase solution. How much confidence do you have in the estimations? Explain. Discuss possible errors in data collection and analysis that could lead to erroneous or misleading results. What variables are hard to control in this experimental design? What could you do to have more confidence in your concentration estimations? Of what value is determining the concentration of a protein solution? Where in industry might it be used? Give several examples.

ADDITIONAL QUESTIONS

1. Without using the spectrophotometer, how can you estimate the concentration of amylase in the unknown samples?
2. On the best-fit standard curve, all the points are lined up, except the 0.5mg/mL sample. It is much lower than the rest of the line. Why might this be? Should the value be used in the standard curve?
3. A set of proteins is studied in the spectrophotometer. The linear regression ($y = mx + b$) gives a slope of $m = 0.93$ and the y-intercept (b) is zero. An unknown sample's absorbance is measured at 0.66 au. What is the approximate concentration of the unknown sample?
4. Amylase is a colorless molecule, thus, Bradford reagent was used in this experiment to make it visible in the Spec 20 D. Name at least one disadvantage to using the Bradford reagent on a suspected amylase sample.
5. Propose a method to detect amylase in a colorless sample and still be able to recover it, unaltered, for future use.

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