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The rAmylase Project by Ellyn Daugherty

Direct ELISA of Bacterial α-Amylase

(Lab 6e)

(Cat. # BTNM-6D)

Developed in partnership with

The rAmylase Project by Ellyn Daugherty

Direct ELISA of Bacterial α-Amylase (Lab 6e)

Teacher's Guide

The following laboratory activity is adapted from "Laboratory 6e: Direct ELISA of Bacterial Alpha-Amylase" 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 WITH THE KIT

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

- 8 tubes of 0.08mg/ml Amylase stock solution (400µl)
- 8 tubes of 1X PBS (4ml)
- 8 tubes of 0.002mg/ml Amylase ELISA Unknown 1 (400µl)
- 8 tubes of 0.00005mg/ml Amylase ELISA Unknown 2 (400µl)
- 1 bottle of 10X ELISA Washing Buffer (60ml)
- 1 bottle of 2X Blocking Buffer (2X NAP-Blocker) (60ml)
- 1 tube of Primary α-Amylase Antibody conjugated to HRP
- 1 Tube (50ml)
- 1 bottle ELISA Colorimetric Assay Substrate (25ml)
- 8 tubes of ELISA Stop Solution (1ml)
- 24 ELISA Plate Assay Strips
- 1 ELISA Plate Assay Strip Holder
- 50 Microcentrifuge tubes (1.5ml)
- 24 tubes (6ml)

ADDITIONAL EQUIPMENT & MATERIALS REQUIRED

The following standard lab equipment should be available.

- Clear Tape
- Shaker/Rotator
- Spectrophotometer (i.e. Spectronic 20D+) (optional)
- Spectrophotometer cuvettes (i.e. 13 X 100mm test tubes for a Spectronic 20D+) (optional)
- Graduated cylinders
- Beakers (1L, eight 250ml, and 50ml)
- P-1000 Micropipets and tips (3 sets 96 tip trays/group)
- P-100 Micropipets and tips
- Deionized water
- Microplate Reader (optional)

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SPECIAL HANDLING INSTRUCTIONS

- Store ELISA Washing Buffer, Blocking Buffer (2X NAP-Blocker) & ELISA Substrate at 4°C.
- Store ELISA standard samples, ELISA Unknown samples and the antibody at -20°C.
- All other reagents can be stored at room temperature.
- ELISA Substrate is light sensitive and should be kept dark (i.e. foil-wrapped) until ready to add to the ELISA plates.
- Briefly centrifuge all small vials/tubes before opening to prevent waste of reagents.

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. Make a 1X ELISA Washing Buffer solution by adding the bottle of 10X ELISA Washing Buffer (60ml) to a 1-liter container. Bring up to final volume of 600ml with deionized water. Label eight beakers with "1X ELISA Wash Buffer." Add 60ml of 1X ELISA Wash Buffer to each of the 8 beakers.
   The ELISA Washing Buffer is 1XPBS (Phosphate buffered saline) supplemented with a mild detergent (Tween-20) to aid in membrane washing.

2. The morning of the experiment, gently shake the supplied Blocking Buffer (2X NAP-Blocker) bottle to mix and then combine with 60ml 1X ELISA Washing Buffer. Label eight 6ml tubes with "1X Blocking Buffer." Add 6ml of 1X Blocking Buffer (NAP-Blocker) to each 6ml tubes.

3. Briefly centrifuge the antibody tube to collect the antibody in the bottom of the tube. Add 500µl 1X ELISA Washing Buffer solution to the antibody and gently pipette up and down to completely mix. Transfer the all the (500µl) antibody to a 50ml tube and add 25ml 1X NAP-Blocker (Step 2). Gently mix. Label eight 6ml tubes "Diluted antibody" and add 3ml of the diluted antibody to each 6ml tube.

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4. Label eight 6ml tubes "ELISA Substrate" and add 3ml of the ELISA Substrate, a colorimetric assay substrate, into each of the eight 6ml tubes. Keep the ELISA substrate in the dark (in a drawer, refrigerator, or wrapped in foil) until ready to use.

5. Distribute the following materials to each lab group.

    - 1 tube of 0.08mg/ml Amylase stock solution (400µl)
    - 1 tube of 1X PBS (5ml)
    - 1 tube of Amylase Unknown 1 (500µl)
    - 1 tube of Amylase Unknown 2 (500µl)
    - 1 beaker of 1X ELISA Washing Buffer, 100ml
    - 1 tube 1X Blocking Buffer (NAP-Blocker) (10ml)
    - 1 tube of diluted Primary α-Amylase Antibody/HRP (4ml)
    - 1 tube ELISA Colorimetric Assay Substrate (5ml)
    - 1 tube of ELISA Stop Solution (1ml)
    - 3 ELISA Plate Assay Strips
    - Six 1.5ml Microcentrifuge tubes

TIME REQUIRED

- 1hour pre-lab to prepare and dispense reagents and buffers.
- Two 1 hour lab periods to complete the ELISA procedure
- 1 hour lab period to analyze and discuss the ELISA results

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/

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EXPECTED RESULTS

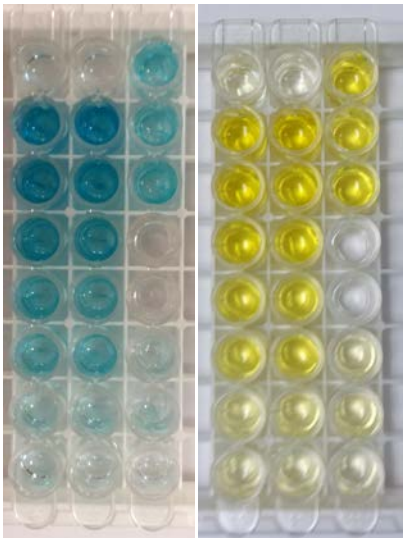

Figure: The expected results above show the ELISA plate before and after addition on the stop solution.

ANSWERS TO ADDITIONAL QUESTIONS

1. Why it is important to wash the wells after every step?
   Answer: Washing removes any proteins that have not bound to the micro-wells and any antibodies that have not bound to their targets, thus preventing unbound proteins (either antigen or antibodies) from giving false positive result
2. After developing an ELISA, all wells are colored. Give reasons that all wells, including the negative control would be colored.
   Answers will vary but might include cross-contamination, inadequate blocking, inadequate washing, or timing in stopping the color development.
3. After developing an ELISA, none of the wells are colored. Give reasons that all wells, including the negative control might not be colored.
   Answers will vary but might include inadequate antibody, pH may be off and antibody or protein binding is affected, and/or the ELISA substrate is compromised.
4. Explain what might happen if the pH of the 1X PBS was at pH 5.5 instead of pH 7.4.
   Answer: The antibody-antigen recognition, as well as the binding of the antigen to the plates is pH dependent. If the pH is not correct, then the charges on these molecules may change and prevent binding.
5. Describe why enzymes are used in immunoassays.
   Answer: Enzymes are used as they are able to rapidly catalyze substrates to produce either a chemiluminescence or colorimetric product, allowing the quantity of antibody, and therefore antigen, to be calculated.

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OBJECTIVES

What is the concentration of bacterial α-amylase in 2 unknown samples as determined by a direct ELISA?

BACKGROUND

How can a technician know that a particular protein of interest is present in a solution, and how can the concentration of that protein be determined? A technique that is commonly used in research and manufacturing to recognize the presence and concentration of protein in a solution is called an ELISA.

ELISA stands for enzyme-linked immunosorbent assay. In an ELISA, an antibody is used to recognize (immuno-, from immunological) and bind (-sorbent) a particular molecule (called an antigen), let's say amylase. Both the antibody and antigen are colorless, so an enzyme that can cause a colored reaction is attached onto the antibody (enzyme-linked). In this way, when an antibody with its enzyme attaches to an antigen, a reagent can be added and converted by the enzyme to a measurable color.

ELISAs are used to show the presence and concentration of specific proteins during protein production and purification. Two types of ELISAs, direct and indirect, are commonly used in research and manufacturing. The difference between them is that a direct ELISA uses a single antibody to recognize an antigen and an indirect ELISA uses two antibodies.

In this direct ELISA, a single, specific antibody (anti-α-amylase) binds with a specific antigen molecule (α-amylase). Conjugated onto the antibody is the enzyme horseradish peroxidase (HRP), which causes a colorimetric reagent, tetramethylbenzidine (TMB), to change colors from clear to blue. To stop the reaction, acid is added to denature the HRP enzyme. The TMB turns to a stable yellow color in acid. When the conjugated antibody is bound to the antigen and the reagent changes colors, a technician can measure the color change and infer the presence and concentration of the antigen (amylase) by comparing it to known standards samples that have also been through the ELISA protocol.

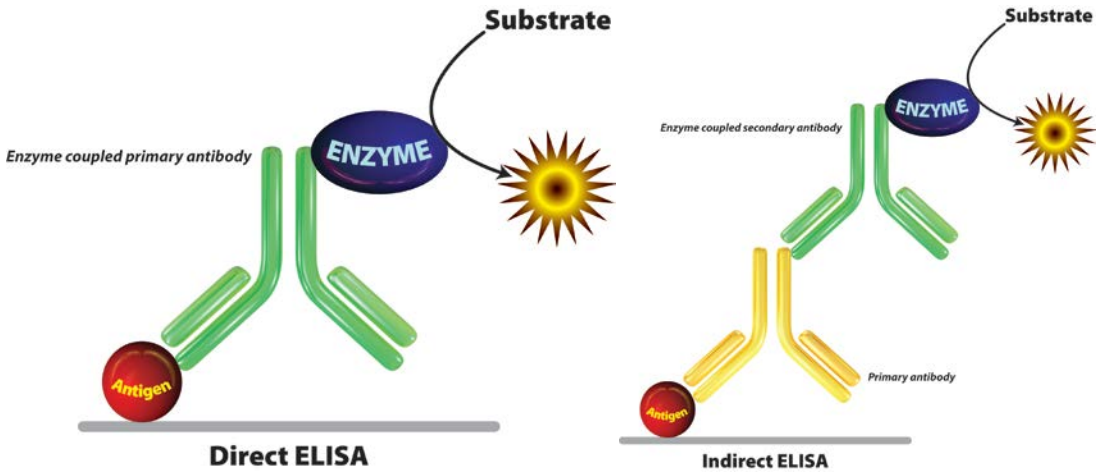

Figure: **Direct ELISA**: Anitgen binds directly to well surface, enzyme-tagged antibody binds to antigen, enzyme on antibody produces color when substrate applied. **Indirect ELISA**: Anitgen binds directly to well surface, primary antibody binds antigen, enzyme-tagged secondary antibody binds to antigen, enzyme on secondary antibody produces color when substrate applied.

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MATERIALS FOR EACH GROUP

Each lab group needs:

- 1 tube of 0.08mg/ml Amylase stock solution (400µl)
- 1 tube of 1X PBS (5ml)
- 1 tube of Amylase Unknown 1 (500µl)
- 1 tube of Amylase Unknown 2 (500µl)
- 1 beaker of 1X ELISA Washing Buffer, 100ml
- 1 tube 1X Blocking Buffer (NAP-Blocker) (10ml)
- 1 tube of diluted Primary α-Amylase Antibody/HRP (4ml)
- 1 tube ELISA Colorimetric Assay Substrate (5ml)
- 1 tube of ELISA Stop Solution (1ml)
- 3 ELISA Plate Assay Strips
- Six 1.5ml Microcentrifuge tubes

Components shared by the whole class and kept on a communal table, include:

- P-1000 Micropipette and tips (3 sets 96 tip trays/group)
- P-100 Micropipette and tips
- Clear Tape
- Shaker/Rotator
- Spectrophotometer (i.e. Spectronic 20D+) (optional)
- Spectrophotometer cuvettes (i.e. 13 X 100mm test tubes for a Spectronic 20D+) (optional)
- Microplate Reader (optional)

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PROCEDURE

1. Prepare the "known" ELISA Standards by making 1:4 serial dilutions from the Amylase Stock. [0.08mg/ml].

    a. Label 6 microcentrifuge tubes with initials and the labels: 1:4, 1:16, 1:64, 1:254, 1:1,024, and 1:4,096.

    b. As indicated in the table below, prepare the dilutions of the 0.08mg/ml amylase stock solution by adding the amount of 1X PBS buffer and then the volume of the specified amylase solution listed to each tube. Pipette up and down to mix each sample completely before using it for the next dilution.

Known Standard Sample Dilution	1X PBS Buffer	Amylase Solution
1:4	300µl	100µl Amylase Stock
1:16	300µl	100µl 1:4 Dilution
1:64	300µl	100µl 1:16 Dilution
1:256	300µl	100µl 1:64 Dilution
1:1,024	300µl	100µl 1:256 Dilution
1:4,096	300µl	100µl 1:1,024 Dilution

    What is the concentration (mg/ml) of amylase in each of these known standard solution tubes?

    What is the mass (mg) of amylase in each of these known standard solutions?

2. *Antigen Binding*: Use two strips of clear tape to tape 3 ELISA plate strips together. Align the tabs and wells to resemble a 3 x 8 plate grid. Each group will need one 3 x 8 plate grid. Place Assay Strips on a clean surface and label 1-3.

3. Bind the Amylase Standards (knowns) and unknown samples (antigens) to the ELISA strips by adding 125µl of each known standard sample or each Unknown sample to the specified wells as shown in the table below.
   NOTE: There are 2 assay replications of each known sample and 3 replications of each unknown sample.
   When adding any reagent to the well, ensure you use a clean pipette tip and the pipette tip is at the bottom of the well. This prevents artifacts occurring due to residual reagents remaining on well walls.

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Well #	Assay Strip 1	Assay Strip 2	Assay Strip 3
1	Blank (PBS)	Blank (PBS)	Unknown 1
2	Amylase Stock	Amylase Stock	Unknown 1
3	1:4	1:4	Unknown 1
4	1:16	1:16	-
5	1:64	1:64	-
6	1:256	1:256	Unknown 2
7	1:1,024	1:1,024	Unknown 2
8	1:4,096	1:4,096	Unknown 2

What is the mass (mg) of amylase in each of these known standard solution assay wells?

4. Incubate the ELISA plate at room temperature for 15 minutes, gently rocking the plate ensuring no splattering.

5. After incubation, discard liquid from wells by inverting plate and gently tapping out the liquid over a sink or tray. Remove excess antigen by repeatedly tapping the plate on a stack of paper towels.

6. *Blocking Stage*: Add 250µl 1X Blocking Buffer (NAP-Blocker) into each well and incubate with shaking for 30-60 minutes at room temperature.

If necessary, this is a convenient stopping point. Wrap the ELISA plate in plastic film, such as saran wrap or cling film, and store in a fridge. Do not store longer than a week.

7. After incubation, discard Blocking Buffer (NAP-Blocker) as in Step 5 and wash each well by rinsing with 250µl of ELISA Washing Buffer. Rock the plate gently for at least 1 minute for each wash. Repeat wash four more times.

8. *ELISA Antibody*: Add 125µl of the Antibody solution to each well and incubate with shaking for 15-30 minutes at room temperature. Ensure you change the pipette tip each time and the pipette touches the bottom of the well on addition of antibody.

If necessary, this is a convenient stopping point. Wrap the ELISA plate in plastic film, such as saran wrap or cling film, and store in a fridge. Do not store longer than overnight.

9. After incubation, discard antibody solution and wash each well with 250µl of ELISA Washing Buffer as before. Repeat wash four times.

10. Before adding ELISA Colorimetric Assay Substrate, make sure the Stop Solution used in step 12 is ready to use. Add 125µl ELISA Colorimetric Assay Substrate to each well and shake for 5-15 minutes at room temperature. Monitor the plate to observe a blue color change.

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11. As blue color develops, examine the wells and note the intensity of the blue color of the unknown sample wells compared to the known ELISA Standards. The reaction should be stopped in step 12 before the samples turn "too" blue.

12. Before the negative control samples turn blue and when there is a varying amount of blue in the known samples, most concentrated turning the most blue but little or no blue in the lowest concentration standards, quickly add 30µl ELISA Stop Solution (0.5M HCl) to each well. Swirl the contents of the well before changing tips and adding acid to the next well. The acid turns the blue TMB to a yellow color and denatures the HRP so that no further reaction occurs. The amount of yellow color, compared with the negative control, is an indication of the relative amount of protein that the ELISA antibody recognizes.

13. Record the degree *of* yellow color for all the replications of each test (0 = no yellow/clear; 5 = lemon yellow).

Estimate the concentration of each Unknown sample based on color of the ELISA known standards.

What is the mass (mg) of amylase in each of the unknown solution samples?

14. Photograph the plate from the top and from the side for another record of the yellow change in the wells.

15. If a plate reader is available, transfer the strips to the supplied strip holder and read the plate at 450-475nm and record the data. If no plate reader is available, several samples (from different lab groups) of the same concentration may be pooled and read in a UV spectrophotometer at 450-475nm.

16. Create a best-fit linear graph that shows the relationship between the known concentrations of amylase (in µg/mL) in the standards and their absorbance.

17. Use the best-fit linear graph to estimate the concentration of Ul and U2. If the graph was produced in Microsoft® Excel®, determine the equation of the line (y=mx+b) using "Add Trendline" (on the Chart menu). Use the equation of the line to calculate the concentration of each unknown.

DATA ANALYSIS & CONCLUSION

What is your estimate of the concentrations of amylase in Unknown Samples 1 and 2?

In a concluding statement, evaluate your ability to perform an ELISA that distinguishes between different concentrations of the protein amylase. Were the different concentrations of the known samples appropriately yellow, or in the case of the negative control, not yellow? Explain the coloration in the multiple replications of each known standard sample. Are the ELISA results "good enough" to estimate the concentration of the two unknown samples? Describe technical errors that could lead to fallacious results. How could you minimize these errors? Describe the importance of using the ELISA techniques in protein manufacturing, research and medical applications.

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ADDITIONAL QUESTIONS

1. Why it is important to wash the wells after every step?
2. After developing an ELISA, all wells are colored. Give reasons that all wells, including the negative control would be colored.
3. After developing an ELISA, none of the wells are colored. Give reasons that all wells, including the negative control might not be colored.
4. Explain what might happen if the pH of the 1X PBS was at pH 5.5 instead of pH 7.4.
5. Describe why enzymes are used in immunoassays.

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