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The rAmylase Project by Ellyn Daugherty

Western Blot to Identify α-Amylase

(Lab 6e)

(Cat. # BTNM-6E)



Developed in partnership with

The rAmylase Project by Ellyn Daugherty

Western Blot to Indentify α-Amylase (Lab 6e)

Teacher's Guide

The following laboratory activity is adapted from "Laboratory 6e: Western Blot to Identify 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.

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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).

- 4 nUView™ 10-well 10% Tris-Glycine Protein Gel, store at 4°C
- 1 bottle of 10X PAGE Electrophoresis Running Buffer (250ml)
- 8 tubes of 30µl 0.02mg/ml α-Amylase stock (from *Bacillus subtilis*), store at -20°C
- 8 tubes of 2X PAGE Sample Loading Dye Buffer (175µl), store at 4°C
- 8 tubes of 8µl PAGEmark™ Blue PLUS Protein Marker (sizing standards), store at -20°
- 4 Protein Binding Western Blot Membranes with padding
- 1 bottle of 20X Western Transfer Buffer (250ml)
- 1 bottle of 2X Blocking Buffer (2X NAP-Blocker) (250ml)
- 1 bottle of 10X MEM Wash Buffer (250ml)
- 1 tube of Primary α-Amylase Antibody conjugated to HRP (100µg)
- 1 bottle Western Blot (TMB) Colorimetric Assay Reagent (50ml)
- 8 Disposable Tubes (6ml)
- 50 Microcentrifuge Tubes (1.5ml)
- 24 Lid Locks

ADDITIONAL EQUIPMENT & MATERIALS REQUIRED

The following standard lab equipment should be available.

- P-1000 and P-200 Micropipets and tips
- Protein Electrophoresis/Western Blot Module Equipment (vertical gel boxes and power supplies)
- Heat block (preferred) or water bath set at 90°C
- Shaker/Rotator
- Washing Trays (large enough to fit the blot membrane, such as 12cm x 12cm)
- Methanol or isopropanol
- Beakers, flasks or other containers (4L, 2L, 1L and 500mL)
- Deionized Water
- Razor blade or scissors

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SPECIAL HANDLING INSTRUCTIONS

- Store the α-amylase, antibody and PAGEmark™ Protein Markers at -20°C.
- Store the protein gels, Blocking Buffer and HRP Substrate at 4°C
- All other reagents can be stored at room temperature.

*NOTE: This kit is sent with 4 "NG" nUView gels. These are considered universal gels and fit most 10X10cm gel box tanks. If requested at the time the order is placed, "NB" gels may be substituted for NG if the customer has Mini Protean gel tanks. If requested at the time the order is placed, "NN" gels may be substituted for NG if the customer has XCell SureLock gel tanks.

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 a large beaker (4L), pitcher, bucket or carboy "1X PAGE Electrophoresis Running Buffer," and add 250ml of the 10X PAGE Electrophoresis Running Buffer to the 4L container. Add 2250ml of deionized water to the buffer concentrate to make 2.5L of 1X PAGE Electrophoresis Running Buffer.
   NOTE: The amount of 1X Electrophoresis Running Buffer needed depends on the electrophoresis unit used.
   NOTE: Chill the buffer to 4°C to prevent overheating during electrophoresis. Place the chilled 1X PAGE Electrophoresis Running Buffer beaker on the communal lab table prior to use.

2. Label a 2L beaker "1X MEM Wash Buffer. Make 2L of 1X MEM Wash Buffer solution by adding 200ml of 10X MEM Wash Buffer and 1800ml of deionized water to the labeled beaker. Place the 1X MEM Wash Buffer beaker on the communal lab table.
   The MEM Wash Buffer is TBS (Tris buffered saline) supplemented with a mild detergent to aid in membrane washing.

3. The morning of the experiment, label a 500ml beaker "1X Blocking Buffer". Gently shake the s 2X Blocking Buffer (2X NAP-Blocker) bottle, add 200ml to the labeled beaker, and then add 200ml 1X MEM Wash Buffer. Into 8 labeled "1X Blocking Buffer" 50ml beakers, place 30ml of the 1X Blocking Buffer.

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4. Remove 500µl 1X Blocking Buffer from the remaining stock of 1X Blocking Buffer and transfer to the vial of anti α-amylase antibody-HRP. Gently pipette up and down to mix and then transfer the entire content of the vial back to the 160ml 1X Blocking Buffer Stock. When need in the procedure, supply each group with 20ml diluted anti-α-amylase antibody-HRP directly onto their blot membrane.

5. Label a 4L container, "1X Western Transfer Buffer." Make 1X Western Transfer Buffer by adding 200ml 20X Western Transfer Buffer plus 800ml of methanol (or isopropanol) to the 4L container. Add 3 liters of deionized water to make a final volume of 4 liters. Mix thoroughly. Into 8 labeled "1X Western Transfer Buffer" 50ml beakers, place 20ml of the 1X Blocking Buffer.

6. Supply each group with 10ml methanol to hydrate the protein binding membrane. The membrane is PVDF and is hydrophobic.

7. Label the eight 6ml tubes "TMB Substrate" and place 5ml Western Blot (TMB) Colorimetric Assay Reagent into each tube.

8. Distribute the following items to each lab group:

   - 6 Microcentrifuge Tubes (1.5ml)
   - 1 tube of 0.02µg/µl α-Amylase (30µl)
   - 1 tube of 2X Sample Loading Dye Buffer (175µl)
   - 1 nUView™ 10-well 10% Tris-Glycine Protein Gel (Shared with two groups)
   - 1 tube of PAGEmark™ Protein Marker (sizing standards)
   - Vertical Gel Box/Electrophoresis Apparatus with Western Blot Module and pads (shared with another lab group)
   - 300V Power Supply (shared with another lab group)
   - 1X Western Transfer Buffer (20ml)
   - A Large Tray (big enough to assemble the blot cassette)
   - 10ml Methanol (Shared with two groups)
   - 1 Protein Binding Western Blot Membrane with padding (Shared with two groups)
   - 1 beaker of 1X Blocking Buffer (NAP-Blocker) (30ml)
   - 1 tube of Western Blot (TMB) Colorimetric Assay Reagent (5ml)

TIME REQUIRED

- 1 hour, pre-lab (preparation of samples)
- 3- hours (to load and run gel and set up and run the transfer)
- 2- hour (visualization of blot and post lab analysis)

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



ANSWERS TO ADDITIONAL QUESTIONS

1. After developing the blot, no bands are visible anywhere on the membrane. List several reasons why few or no bands may be visible on the blot?
   Answer: Answers will vary but the most likely reasons for no bands on the membrane are poor transfer from the gel to the membrane, poor antigen recognition by the antibody, or low antigen concentration.

2. Describe why having just the right concentration of antibody in the blot is important. What might the blot look like if too much antibody is added?
   Answer: If too much antibody is added, it is likely that there would be random binding of antibody all over the membrane, and the background color of the membrane might be too blue to see low concentrations of antigen.

3. This Western blot takes several days to complete (if you work all the way through). Suggest a step in the protocol where a change may still give the expected results but might cut down the overall time to completion. Explain what effect the change may have on the blot bands.
   Answer: The answers may vary, but one strategy is that the transfer may be sped up a tiny bit by increasing the voltage/current and shortening the time slightly (maybe 1 hour at 65 mAmp).

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OBJECTIVES

What concentrations of α-amylase can be identified on a Western blot?

BACKGROUND

Studying proteins on a denaturing SDS/polyacrylamide gel can reveal the approximate molecular weight and number of polypeptides in a protein. Once the banding pattern for a protein is known, it can be used to identify the protein in future samples.

However, since there are so many proteins with similar molecular weights, a method to identify a specific protein on a gel is valuable. The Western blot technique allows for the recognition and visualization of a particular protein on a PAGE gel. In a sample of hundreds of proteins, as in cell extracts or in broth cultures, a Western blot can recognize bands of a particular protein in a mixture.

Western blotting utilizes antibody-antigen specificity. A PAGE gel with samples containing a protein of interest is run. At the end of the gel run, a positively charged membrane (i.e., nitrocellulose) is laid over the gel and the gel/membrane sandwich is placed in a transfer box. When current is applied, proteins on the gel move to the membrane (blot). Antibodies can be used to recognize the blotted proteins.

Antibodies (i.e., anti-α amylase) can be purchased that recognize the unique structure of a specific antigen molecule (i.e., α-amylase) and bind to it. Using antibodies that have attached colored markers, enzymes, or other reporter molecules, a technician can visualize an antigen of interest on the blot.

For this blot, a PAGE gel is run with samples of amylase at different concentrations. The gel is blotted onto a nitrocellulose membrane using a Western-blot transfer unit. The membrane is treated with an anti-alpha amylase antibody that binds only to alpha-amylase. The antibody has an enzyme [horseradish peroxidase (HRP)] attached to it. When tetramethylbenzidine (TMB) is washed over the blot, HRP changes the TMB from clear to blue, and blue bands are visible on the membrane where amylase is found.

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MATERIALS FOR EACH GROUP

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

- 6 Microcentrifuge Tubes (1.5ml)
- 1 tube of 0.02µg/µl α-Amylase (30µl)
- 1 tube of 2X Sample Loading Dye Buffer (175µl)
- 1 nUView™ 10-well 10% Tris-Glycine Protein Gel (Shared with another group)
- 1 tube of PAGEmark™ Protein Marker (sizing standards)
- Vertical Gel Box/Electrophoresis Apparatus with Western Blot Module and pads (shared with another lab group)
- 300V Power Supply for use with gel box (shared with another lab group)
- 1X Western Transfer Buffer (20ml)
- A Large Tray (big enough to assemble the blot cassette)
- 10ml methanol (Shared with two groups)
- 1 Protein Binding Western Blot Membrane with padding (Shared with another group)
- 1 beaker of 1X Blocking Buffer (NAP-Blocker) (30ml)
- 1 tube of Western Blot (TMB) Colorimetric Assay Reagent (5ml)

Some components will be shared by the whole class and should be kept on a communal table.

Caution: Never use latex gloves when using a heat block or water bath. A 90° heat block or water bath can cause severe burns. Use lid locks to keep tubes closed. Lid locks have handles to make for safer handling. If using a hot water bath, place tubes in a floating rack so the students do not come in contact with the near boiling water.

- Heat block (preferred) or water bath set at 90°C
- 1X PAGE Electrophoresis Running Buffer (shared with whole class), 250-300ml per gel box
- Large weigh boats or trays (about 10x10cm), for transferring and staining gels and blots and for setting up blot unit
- 1X MEM Wash Buffer
- Deionized water
- Lid Locks

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ADDITIONAL MATERIALS FOR EACH GROUP

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

- P-100 and P-20 Micropipets and tips
- Microcentrifuge
- Gloves and goggles
- Paper towels
- Glassware (250ml and 50ml beakers, glass rods)
- Forceps

PROCEDURE

Prepare Samples and Buffers

1. Label 4 microcentrifuge tubes with the amylase concentrations listed in the table below. Add 25µl of 2X PAGE Sample Loading Dye Buffer to each tube.

2. Starting with the 0.02µg/µl amylase stock solution, prepare a serial dilution of the α-amylase adding 25µl of the amylase sample specified in the table. Pipette samples up and down to mix thoroughly before transferring a volume to the next dilution. Store prepared samples at 4°C if not using during the same lab period.

Final α-amylase concentration (µg/µl)	Amount and concentration of α-Amylase to add (µl)	2X PAGE Sample Loading Dye Buffer (µl)
0.01	25µl of 0.02µg/µl α-amylase	25
0.005	25µl of 0.01µg/µl α-amylase	25
0.0025	25µl of 0.005µg/µl α-amylase	25
Negative Control	25µl of 1X MEM Wash Buffer	25

3. Label a beaker "1X MEM Wash Buffer". Place about 240ml of 1X MEM Wash Buffer in the beaker.

Protein Electrophoresis

The protein electrophoresis and Western transfer will be performed together with another group.

1. Set up a vertical electrophoresis gel box as directed in the following steps. Use a nuView 10% PAGE gel. In your notebook, record the gel concentration, the lot number, and the expiration date.

2. Cut open the packet and drain the preservative from the cassette.

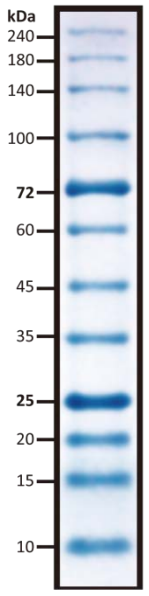
3. Notice how the wells of the gel are numbered and there is a line at the bottom of each well. Using a permanent marker to shade the well numbers makes the wells easier to see during loading.

4. Study the gel box to understand how it is put together and which side is the front. Put the gel(s) in the box with the high side facing out, so that the labeled side faces the front of the gel box. When running a gel, a tight seal must be

formed between the gel cassette and the gasket of the running frame to prevent buffer and current leakage from the inner buffer chamber (where the gel wells are exposed to the buffer).

5. Pour sufficient 1X Electrophoresis Running Buffer into the inner buffer chamber of the gel running apparatus to completely cover the sample wells. Fill the outer tank with running buffer to ensure the bottom of the gel cassette is covered (3-4 cm). Make sure that buffer is being held in the inner chamber and that the chamber is not leaking. Make adjustments as necessary to prevent leakage. Gently rinse the wells of the gel thoroughly with running buffer (2 times the well volume) to remove air bubbles and to displace any storage buffer.

6. The PAGEmark™ Protein Markers are ready-to-use and do not require boiling. 5µl will be loaded into a well on the gel. The PAGEmark™ Protein Marker consists of a mix of twelve Prestained proteins of molecular weight 240, 180, 140, 100, 72, 60, 45, 35, 25, 20, 15 and 10kDa. The molecular weight markers are used to determine molecular weights of the amylase protein.



4-20% Tris-Glycine

7. A few minutes before loading samples into the gel well in step 8 of the Protein Electrophoresis, seal the tubes with the supplied lid-locks and boil the protein samples for 3 minutes in a 90°C heat block. After boiling, centrifuge the samples for 20 seconds to bring down the condensation from sides of tubes.

8. Load the protein marker and samples on the gel as per the table below.

Well #	Volume of Sample with 2X Sample Prep Loading Dye to be loaded in a Well
1	Group 1: 5µl of PAGEmark™ Protein Marker
2	Group 1: 25µl of 0.01µg/µl α-amylase
3	Group 1: 25µl of 0.005µg/µl α-amylase
4	Group 1: 25µl of 0.0025µg/µl α-amylase
5	Group 1: 25µl of Negative Control
6	Group 2: 5µl of PAGEmark™ Protein Marker
7	Group 2: 25µl of 0.01µg/µl α-amylase
8	Group 2: 25µl of 0.005µg/µl α-amylase
9	Group 2: 25µl of 0.0025µg/µl α-amylase
10	Group 2: 25µl of Negative Control

9. Run the gel at 250 volts (no more than 80mA) for about 30 minutes* until the blue dye front is 0.5-1cm from the bottom of the gel.
   * The nUView™ Tris-Glycine Protein gels are designed to be run at 200-250 volts but no more than 80mA. Having the current above 80mA could melt the gel. At 80mA the gel run is over in about 30 minutes. The gels run that fast only when one gel is run on a power supply at a time. If 2 or more gels/gel boxes are run on the same power supply then the gel run time will be significantly longer.

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10. Set up and label a washing tray (dish, tray or large weigh boat) with 20ml of 1X Western Transfer Buffer.

11. When the electrophoresis run is completed, remove the gel cassette(s) from the tank according to the manufacturer's instructions. Rinse the cassette in water. To open the cassette, pry apart from one of the cassette edges. Pull the top plate of the cassette away from the bottom plate. The two halves will snap apart completely, exposing the gel.

12. Transfer the gel into a dish containing 20ml of 1X Western Transfer Buffer. Invert the plate with the gel on it over the buffer and touch the gel to the buffer. The gel will slip into the 1X Western Transfer Buffer. Loosen the gel at the bottom with transfer buffer, if necessary. Do not let the gel sit in the transfer buffer for more than 10 minutes.

Western Blotting Procedure

 *CAUTION: Do not handle the membrane unless wearing gloves. With gloved handles hold the membrane at the edges.*

1. Follow the manufacturer's instructions for assembling of the Western blot apparatus. A general guideline is outlined below. It is important that the blot sandwich apparatus, filter paper, gel, blot membrane, and pads stay submerged in 1X Western Transfer buffer until the blot apparatus is assembled.

    a. In a suitably large tray, soak 2 fiber pads and the 2 sheets of padding from the Protein Binding Blot Membrane in 20ml of 1X Western Transfer buffer.

    b. In a second tray, place the Protein Binding Blot Membrane (a PVDF membrane) and add 10ml methanol. The PVDF membrane is hydrophobic and has to be treated with methanol to allow for optimal protein binding.

    c. Open the plastic blot holder and place it in the plastic tray with the negative electrode (black side) down. Make sure the blot holder will be submerged in 1X Western Transfer buffer.

    d. Place one fiber pad on the cassette, followed by one sheet of padding. Ensure no air bubbles are trapped. Use a glass rod to roll out air bubbles.

    e. While wearing gloves, quickly pick up the gel (at the bottom) and lower it onto the filter paper. Make sure it is wet with 1X Western Transfer buffer and that no air bubbles are trapped. Use a glass rod to roll out air bubbles.

    f. Next using the forceps, lower the wet Protein Binding Blot Membrane onto the gel. Ensure no air bubbles are trapped. Use a glass rod to roll out air bubbles.

    g. Lay the second piece of padding, then the fiber pad onto the stack and close and snapping the cassette shut. The positive electrode (red) end closes last.

2. Transfer the cassette to the Western blot apparatus, fill the apparatus with transfer buffer, close the apparatus, and start the protein transfer. Run the transfer for the amount of time and current given by your teacher/ instructor, typically 65mA for 1.5-2 hours.

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 *NOTE: The orientation of the "sandwich" is essential. The wrong orientation will result in the proteins moving away from the membrane and being permanently lost. The gel should be nearest to the cathode (the negative terminal) and the Protein Binding Membrane should be nearest to the anode (positive terminal).*

3. On completion of transfer, open the cassette (thinking about where the protein bands should be on the blot membrane) and carefully remove the Protein Binding Blot Membrane with forceps and place in a washing tray.

4. Cut the membrane between lanes 5 and 6, using the markers as a reference. Transfer one half to a different washing tray and proceed as your original group. Make sure the side that has the proteins bound to it is facing up.

Protein Detection

1. Add 20ml 1X MEM Wash Buffer and gently rotate the tray for 5 minutes.

2. Discard the 1X MEM Wash Buffer and add the 30ml 1X Blocking Buffer (NAP-Blocker) to block the antibody from binding to non-specific, non-amylase sites. Incubate with gentle shaking, on a shaker/rotator, for 30-60 minutes at room temperature.
   NOTE: The membrane covered with 1X Blocking Buffer can be left overnight at 4°C.

3. Discard 1X Blocking Buffer (NAP-Blocker) and (have the teacher) add 20ml of α-amylase antibody-HRP solution to the Protein Binding Blot Membrane and incubate for 30-60 minutes at room temperature with gentle shaking.

4. Discard the antibody solution and wash 3 times, 10 minutes each, with 20ml of 1X MEM Wash Buffer.

5. Discard 1X MEM Washing Buffer and add 5ml of HRP Substrate (TMB solution) to the Protein Binding Blot Membrane. Allow the substrate to react at room temperature with gentle shaking until suitable color intensity is observed (approx 1-5 minutes).

6. Pour off the substrate and add deionized water to stop the color reaction when several protein bands of varying concentration are visible and distinct. Record and describe the results. Take a photograph of the developed blot and print copies for all lab partners.

7. Pour off the substrate and add deionized water to stop the color reaction when several protein bands of varying concentration are visible and distinct. Record and describe the results. Take a photograph of the developed blot and print copies for all lab partners.

DATA ANALYSIS & CONCLUSION

Evaluate your ability to perform a Western blot that distinguishes between different concentrations of amylase in solution. Were the different concentrations of amylase appropriately blue? What concentrations of amylase were visible on the blot? What was the lowest concentration to be detected? Look at other blots. How does your blot result compare to the results of others? Of what value is Western blotting in protein research and manufacturing?

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ADDITIONAL QUESTIONS

1. After developing the blot, no bands are visible anywhere on the membrane. List several reasons why few or no bands may be visible on the blot?

2. Describe why having just the right concentration of antibody in the blot is important. What might the blot look like if too much antibody is added?

3. This Western blot takes several days to complete (if you work all the way through). Suggest a step in the protocol where a change may still give the expected results but might cut down the overall time to completion. Explain what effect the change may have on the blot bands.

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