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The rAmylase Project by Ellyn Daugherty

Restriction Digestion to Verify pAmylase Plasmid

(Lab 8b)

(Cat. # BTNM-8B)



Developed in partnership with

The rAmylase Project by Ellyn Daugherty

Restriction Digestion to Verify pAmylase Plasmid (Lab 8b)

Teacher's Guide

The following laboratory activity is adapted from "Laboratory 8b: Restriction Digestion to Verify pAmylase Plasmid" 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.

G BIOSCIENCES®

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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The rAmylase Project by Ellyn Daugherty

Restriction Digestion to Verify pAmylase Plasmid (Lab 8b)

Teacher's Guide

MATERIALS INCLUDED WITH THE KIT 4
ADDITIONAL EQUIPMENT & MATERIALS REQUIRED 4
SPECIAL HANDLING INSTRUCTIONS 4
GENERAL SAFETY PRECAUTIONS 5
TEACHER'S PRE-EXPERIMENT SET UP 5
PREPARATION OF AGAROSE GEL(S) 5
ALIQUOT AND DISTRIBUTION OF REAGENTS 6
TIME REQUIRED 6
NEXT GENERATION SCIENCE STANDARDS ADDRESSED 7
EXPECTED RESULTS 7
ANSWERS TO ADDITIONAL QUESTIONS 7
OBJECTIVES 8
BACKGROUND 8
MATERIALS FOR EACH GROUP 10
PROCEDURE 11
PART I: PREPARING DIGESTS 11
PART II: ANALYZING THE DIGESTS 12
DATA ANALYSIS & CONCLUSION 13
ADDITIONAL QUESTIONS 13

Restriction Digestion to Verify pAmylase Plasmid (Lab 8b)

Teacher's Guide

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 0.2µg/µl pAmylase2014 Plasmid (20µl each)
- 8 tubes of Restriction Digestion Buffer [10X] (15µl each)
- 8 tubes of *Hind*III Restriction Enzyme (8µl)
- 8 tubes of *EcoR*I Restriction Enzyme (8µl)
- 8 tubes of Sterile Deionized Water (dH_2O) (2ml)
- 1 bottle of TAE Buffer (50X) (40ml)
- 2 bottles of 0.8% agarose in 1X TAE (200 mL each)
- 2 vials LabSafe™ Nucleic Acid Stain (50µl)
- 8 tubes of DNA Loading Dye Buffer (6X) (50µl each)
- 8 tubes of DNAmark™ 1kbp Plus DNA Ladder (7µl each)
- 1.5ml Microcentrifuge Tubes (50)

ADDITIONAL EQUIPMENT & MATERIALS REQUIRED

The following standard lab equipment should be available.

- Beakers
- Micropipets and tips
- Heat block (preferred) or water bath set at 37°C
- DNA (Agarose) Electrophoresis Equipment (gel boxes and power supplies)
- Microwave
- UV Light box or transilluminator
- Trays (large enough to fit the gel, such as 12cm x 12cm)
- Deionized Water
- 8 Small Styrofoam cups packed with crushed ice

SPECIAL HANDLING INSTRUCTIONS

- Store plasmids, restriction enzymes and their buffer, DNA Loading Dye Buffer, the DNA Ladder and the Sterile Deionized Water at -20°C
- Store bottles of agarose with LabSafe™ Nucleic Acid Stain at 4°C.
- All other reagents can be stored at room temperature.
- Briefly centrifuge all small vials/tubes before opening to prevent waste of reagents.

Restriction Digestion to Verify pAmylase Plasmid (Lab 8b)

Teacher's Guide

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

Preparation of agarose gel(s)

- Agarose solution may be prepared and gels poured by the teacher before class or student groups may pour their own agarose gels from the agarose bottles teachers prepare. Agarose that has been liquefied maybe be stored in a shallow 60°C water bath until ready to use.
- Each group of 4 students will have 5 DNA samples plus a tube of standard markers. Thus a group needs 6 wells on a gel to run their samples. Ideally, each group should prepare and run their own 6-well gel. If equipment is limiting, several groups' samples may be run on a gel with more wells. Some gels allow for 10, 12, 20, and even 32 wells. For 8 groups, there needs to be 48 wells.
- For optimal results, the capacity of each well should be equal to or greater than 30µl. The agarose preparation below is for eight 40ml gels.

1. Prepare 1X TAE Electrophoresis Running Buffer: In a clean two-liter container, add the entire contents of the TAE buffer (50X) and add deionized water (1960ml) up to a total of two liters. Stir until thoroughly mixed.

2. Prepare agarose: Loosen the caps (so they vent steam and pressure when heating) on the 2 bottles of prepared agarose.

    *The agarose gets very hot, very quickly and can cause severe burns. Wear protective goggles when preparing agarose solution. Use heat-protective gloves when handling hot agarose solutions.*

3. One at a time, heat each solution in a microwave on 50% power for about 3 -4 minutes. Keeping the container away from your face, check to see that all the agarose has dissolved and no crystals of agarose are left unmelted. Heat for 1-minute intervals, if necessary, until agarose has dissolved.

Restriction Digestion to Verify pAmylase Plasmid (Lab 8b)

Teacher's Guide

4. Allow the agarose solution to cool to about 60°C. While it cools, orient the gel electrophoresis trays into position for casting (pouring) the gel(s). Some gel trays use tape on the ends, some use gates or buffer dams, and some are wedged into the gel box for pouring. Please refer to the manufacturer's instructions for how to set up and use your gel box.

5. To be done by the Instructor or by each lab group: Once the agarose has cooled to the point it can be held comfortably in one's hand (around 60°C), add 50µl of the LabSafe™ Nucleic Acid Stain to the warm agarose. Swirl gently to mix.

6. Quickly, pour the agarose with LabSafe™ Nucleic Acid Stain into one or more gel casting trays depending on your electrophoresis equipment (usually about 7mm thick). 6 wells are needed for each group. Using an appropriate size gel-well comb, place the comb in the hot agarose tray to create wells that will hold at least 30µl.

7. Let the gel(s) solidify, 10-20 minutes depending on the size of each gel. Once the gels have set, turn the casting tray (and the gel) into position so that the wells are on the negative side (black electrode) of the gel box. Fill the gel box and cover the gel with enough 1X TAE electrophoresis buffer to submerge the gel 1 cm. Remove the combs.
Note: *If needed, solidified gels can be left overnight, at room temperature, covered with buffer and with the combs still in the wells.*

Aliquot and Distribution of Reagents

 *Once the reagents have been thawed and/or resuspended they must be kept on ice. The reagents must remain on ice throughout the experiment. Centrifuge each tube before opening to collect all of each sample to the bottom of the tube.*

8. Pack 8 Styrofoam cups with crushed ice. These ice bathes will be used to keep each group's reagents cold.

9. Place each of the following tubes into each group's ice cup:

- pAmy2014
- 10X RE Buffer
- HindIII
- EcoRI
- dH2O
- 6X DNA Loading Dye
- DNA ladder

TIME REQUIRED

- 1 hour, pre-lab (preparation/aliquot of buffer, agarose, reagents)
- One 2- hour lab period (prepare digests, load, run and analyze gel)
- 30 minutes post lab analysis

Restriction Digestion to Verify pAmylase Plasmid (Lab 8b)

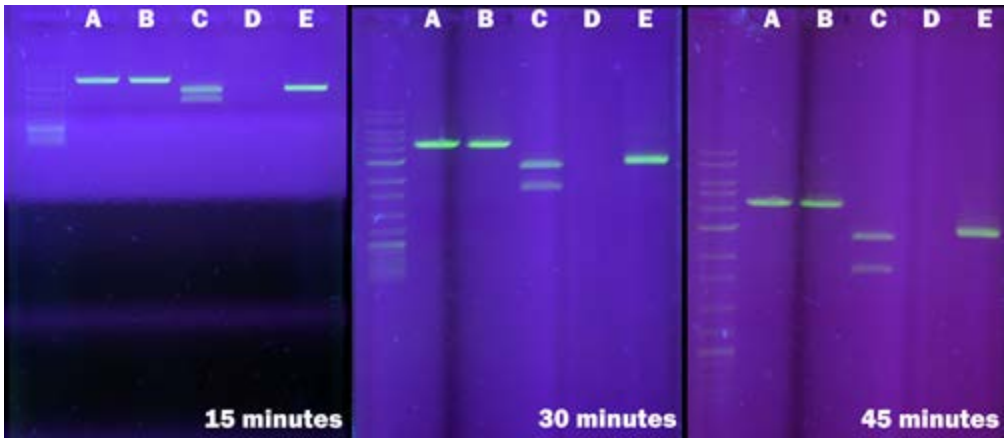
Teacher's Guide

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



The above expected result shows the gel image captured after 15, 30 and 45 minutes of electrophoresis at 115V.

ANSWERS TO ADDITIONAL QUESTIONS

1. The pAmy plasmid is provided in the stock tubes at 0.2µg/µl. What is the actual concentration of plasmid DNA (pDNA) in Tube A during the restriction digestion?
   Answer: The actual concentration of pAmy (pDNA) in tube A would be 0.2 µg/µl X 4 µl/20 µl total = 0.04 µg/µl.
2. What mass of pDNA is in Tube A?
   Answer: If there is 20µl of sample in the restriction digests, then there is 0.8µg of pAmy DNA in the tube (0.04 µg/µl X 20µl = 0.8µg).
3. A restriction map can be drawn showing the relative positions of the *EcoRI* restriction site to the *Hin*dIII site. To do this, determine the size of the fragments cut by each enzyme. Then, try to "fit" the pieces together like a jigsaw puzzle. In the past, restriction digestion mapping helped scientists determine the A, T, C, G sequence on a piece of DNA. Can you explain why?
   Answer: The map will be similar, but not exactly match the pAmylase2014. This is because the sizes of the fragments from the gel are only estimates. Since the restriction enzymes only recognize certain sequences, if a band is cut on the plasmid, we know the DNA sequence at that site.

Restriction Digestion to Verify pAmylase Plasmid (Lab 8b)

Student's Guide

OBJECTIVES

When a suspected sample of pAmylase is cut by restriction enzymes, do the resulting DNA fragments indicate that the sample has the characteristics of pAmylase?

BACKGROUND

Amylase is an enzyme that speeds the breakdown of starch to sugar. If genetic engineers see a need for large-scale production of the amylase enzyme to supply to several industrial customers, they can produce it in the lab.

One approach is to find naturally occurring bacteria (e.g., *Geobacillus stearothermophilus)* or fungi that make amylase. They could grow these "wild" bacteria or fungi and then extract the amylase protein from them. Often, though, the naturally occurring bacteria or fungi grow too slowly, or their production of amylase is too low for commercial use. Also, there is the possibility that a native amylase-producing bacterium could be dangerous or pathogenic.

Scientists already have, in culture, bacteria and fungi that they know how to grow safely in large quantities. These "model" organisms can be coaxed to produce proteins in large amounts. The bacterium grown by most genetic engineering companies is *E. coli.* One of the fungi is *Trichoderma.* If these model organisms can be given the foreign DNA that codes for amylase production, they might produce large amounts of amylase. They could be transformed into amylase producers. The amylase these genetically engineered cells make could then be isolated, purified, and sold for a profit.

To engineer cells to make amylase, one needs to construct a vector that can carry the amylase gene into the cells. To make a recombinant plasmid coding for amylase production, the amylase gene (just over 1660bp) is excised from a native source and pasted into an existing plasmid (pUC57). The new plasmid might be called pAmylase, pAmy for short, since it contains the amylase gene (see figure). Along with the amylase gene, pAmylase would also contain an ampicillin-resistance (Amp^R) gene since that was already present in the precursor plasmid vector, pUC57. If a bacteria cell, such as *E. coli,* receives this plasmid, it will gain two new phenotypes, amylase production, and ampicillin resistance.

Before we transform *E. coli* cells with pAmylase2014 DNA, we want to confirm that the DNA we are using has the characteristics of pAmylase2014. This can be done by restriction digestion. The pAmylase2014 plasmid has a size of approximately 4295 bp. It contains a single restriction site for the *Hind*III restriction enzyme. It also contains a single restriction sites for the *Eco*RI restriction enzyme. The sites are at specific positions on the circular plasmid.The products of the restriction digestion by these enzymes can be seen on an agarose gel.

Restriction Digestion to Verify pAmylase Plasmid (Lab 8b)

Student's Guide

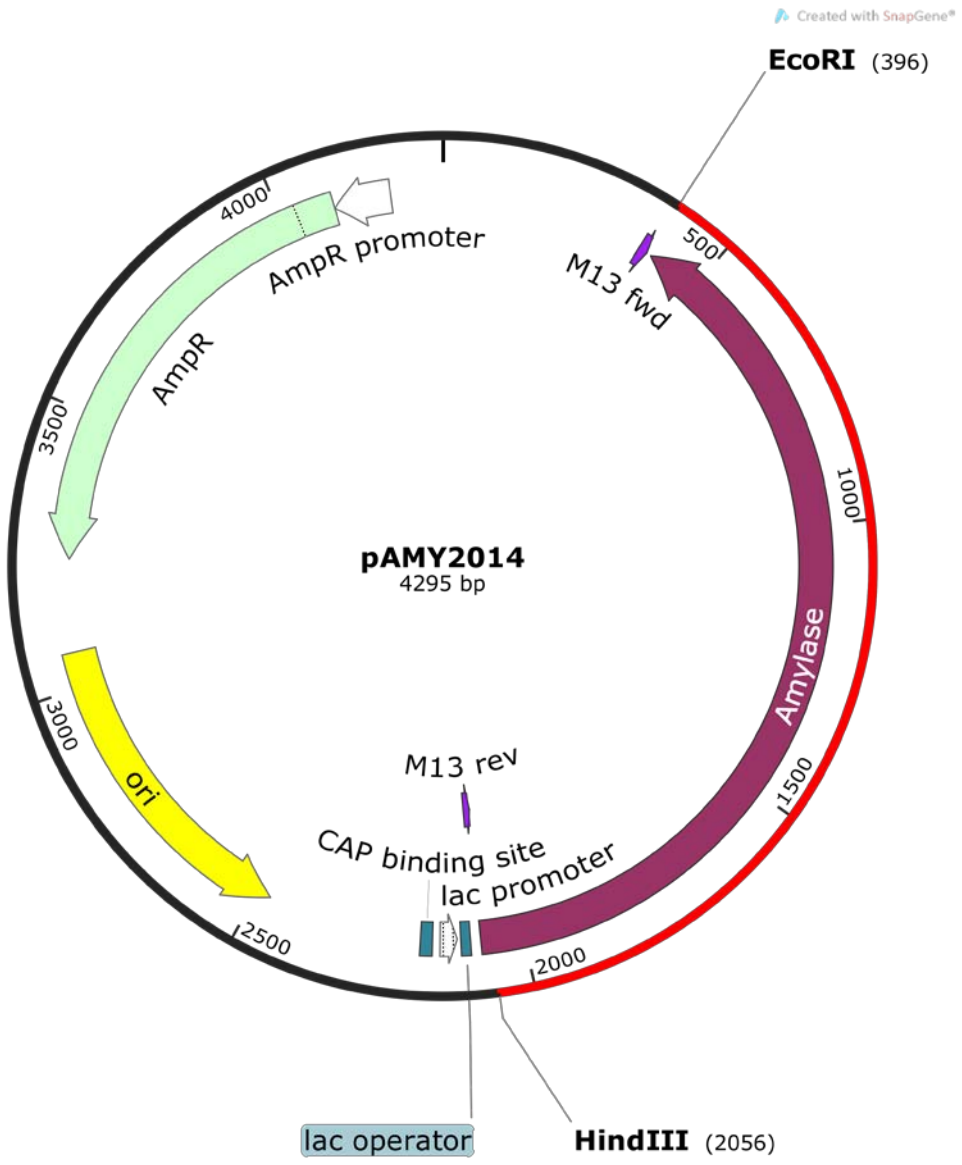


Figure: pAmylase2014 Plasmid. pAmylase contains the amylase gene. In cells, the amylase gene is transcribed and amylase is produced. Starch clearing occurs on Luria Bertani (LB) starch agar plates around colonies that are transformed with pAmylase. An Amp^R gene is also part of pAmylase. The Amp" gene allows a second way to detect that the plasmid got into cells, since only *E. coli* cells transformed with the Amp^{1}^ gene will grow on ampicillin agar.

Restriction Digestion to Verify pAmylase Plasmid (Lab 8b)

Student's Guide

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.

- 1 tube with pAmylase2014 (pAmy)
- 1 tube of 10X Restriction Digestion Buffer (10X RE Buffer)
- 1 tube of *Hind*III Restriction Enzyme
- 1 tube of *Eco*RI Restriction Enzyme
- 1 tube of Sterile Deionized Water (200µl)
- 1 tube of DNA Loading Dye Buffer (6X) (50µl each)
- 1 tube of DNAmark™ 1kbp Plus DNA Ladder (7µl each)
- 5 Microcentrifuge tubes (1.5ml)
- Trays (large enough to fit the gel, such as 12cm x 12cm)

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

- Agarose gel electrophoresis apparatus and power supply
- P-20 and/or P-10 micropipettes and tips
- P-200 and/or P-100 micropipettes and tips
- Heat block (preferred) or water bath set at 37°C
- UV Light box or transilluminator
- Deionized Water

Restriction Digestion to Verify pAmylase Plasmid (Lab 8b)

Student's Guide

PROCEDURE

- **Keep reagents on ice.** The reagents and enzymes are temperature sensitive.
- **Use sterile technique.** DNA is easily destroyed by contaminant enzymes. Do not touch micropipet tips with your fingers. Contaminants might also inhibit restriction enzyme performance.
- **Pipet slowly and carefully.** You are using tiny volumes of reagents. These are easily measured incorrectly. Small pipetting errors can have a significant impact on the results.

Part I: Preparing Digests

1. Label five 1.5ml microcentrifuge tubes, A through E. The restriction digests will take place in these tubes. Keep the tubes on ice.

2. Add reagents to each reaction tube as shown in table below. Pipet each reagent directly into the solution that is already in the tube. Make sure to watch the end of the pipet tip to ensure that all of each reagent is added. Change tips for each delivery. Buffer goes into the tube before the enzyme. **Always add enzyme last.**

Tubes	Sterile H_2O (µl)	10X Restriction Buffer (µl)	pAmylase (µl)	Enzyme (µl)	Total Reaction Volume (µl)
A	12	2	4	2 *Hind*III	20
B	12	2	4	2 *Eco*RI	20
C	10	2	4	2 *Hind*III + 2 *Eco*RI	20
D	14	2	-	2 *Hind*III + 2 *Eco*RI	20
E	14	2	4	-	20

3. Tightly close the caps on Tubes A through E. Give each tube a 1 to 2 second pulse in the microfuge to mix and pool reactants. (Be sure the tubes are in a balanced arrangement.)

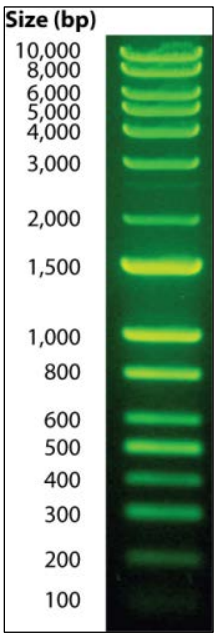
4. Incubate the restriction digests at 37°C for 15-30 minutes.

5. Store the digests at 4°C until they are used in the electrophoresis.

Restriction Digestion to Verify pAmylase Plasmid (Lab 8b)

Student's Guide

Part II: Analyzing the Digests



6. Line up Tubes A through E in a rack.
7. Add 4µl of DNA loading dye to each tube A-E. Change tips each time.
8. Give the tubes a 1 to 2 second pulse in the microfuge to mix and pool reactants.
9. The agarose gels should have been prepared prior to this step. Each student takes turn in loading a sample into a well. A reference lane will also be loaded with a 1kb DNA ladder. The DNA ladder is composed of pieces of DNA of known size (in base pairs). They are used to determine (mark) the size of the pieces of DNA in the unknown samples (Tubes A-E). See the image for DNA bands visible in the standard markers).
10. Load all 24µl of each sample tube into a well of your gel. Load Tube A into Well 1, B into Well 2, etc. Change tips each time.
11. Load 5µl of the 1kb DNA Ladder to your sixth well.
12. Run the gel at 115 V for approximately 15-45 minutes. To check the separation of the fragments, turn off the power and carefully transfer the gel to a UV Light box. If separation is not adequate, return the gel to the gel box and continue to run.
    The agarose gel has a dye within it that binds the DNA strands and is visible under ultra violet light. These stains are safer alternatives to ethidium bromide staining. Wear UV Safety Specs when working around the UV Light Box.
13. While you wait, draw a diagram in your notebook showing the sample that was loaded into each lane, and the concentration and voltage of the gel run.
14. Photograph your gel for a permanent record. Glue the photo in the data section of your notebook. Above the photograph, give the gel a title that distinguishes it from other gel data.
15. On the gel photograph, place a label at the well of what was loaded into each lane. Also, identify and label the sizes of each standard marker in the 1kb ladder.
16. Below each lane, report the size of the DNA fragment pieces by estimating the size of the unknowns as compared to the known DNA standard pieces in the ladder:
    - You can roughly estimate the lengths of the unknown bands by "eyeballing" the position of the unknown bands versus the known standard bands.
    - Make a better estimate (more quantitative) by plotting the data on semilog graph paper with the standard fragment sizes along the y-axis and the distance they travel from the well along the x-axis. Draw a best-fit straight line through these data to produce a standard curve. To estimate the sizes of the unknowns, look at the intersection of the distance the unknowns traveled.

Restriction Digestion to Verify pAmylase Plasmid (Lab 8b)

Student's Guide

- You can make an even better estimate by letting Microsoft® Excel® create the standard curve for you. Open a Microsoft® Excel® spreadsheet. Make a two-column data table with the standard fragment sizes in the first column and the distance the fragments traveled in the second column. Using the Chart menu, plot the data on an XY scatter line graph. Give the graph a title. Label the axis. Double click on the x-axis to view the "Format Axis." Choose "Scale" and "logarithmic scale." Set the minimum on "100." This plots the x-axis logarithmically to straighten the line. Next, click on the line. Choose "Add a Trendline" from the Chart menu. It will give a linear trendline. Click on the trendline. Select "Type" and "Logarithmic." Choose "Options," "Display equation on the chart," and "Display R-squared value on the chart." This gives a best-fit, straight line, standard curve, plus the equation of the line on the graph. To calculate a size for an unknown fragment, measure the distance traveled on the gel "y," and solve for In(x). When you get In(x), use ƒx to determine "exp (x)" for the In (x) value, and it will give the size of the unknown fragment.

DATA ANALYSIS & CONCLUSION

Are the observed bands in the gel what was expected after restriction digestion? Do the digestion fragments indicate that the plasmid sample studied here has the characteristics of the pAmylase2014 plasmid? Give evidence for your statement. Do all groups in the class have similar data? Why or why not? Discuss the possible errors that could lead to varying results from one team to another. If the digestion confirms that the sample is pAmylase2014, why is that information valuable?

ADDITIONAL QUESTIONS

1. The pAmy plasmid is provided in the stock tubes at 0.2µg/µl. What is the actual concentration of plasmid DNA (pDNA) in Tube A during the restriction digestion?

2. What mass of pAmylase2014 is in Tube A?

3. A restriction map can be drawn showing the relative positions of the *EcoRI* restriction site to the *Hin*dIII site. To do this, determine the size of the fragments cut by each enzyme. Then, try to "fit" the pieces together like a jigsaw puzzle. In the past, restriction digestion mapping helped scientists determine the A, T, C, G sequence on a piece of DNA. Can you explain why?

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