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The rAmylase Project by Ellyn Daugherty

Confirming the Amylase Gene Using PCR

(Lab 13h)

(Cat. # BTNM-13H)



Developed in partnership with

The rAmylase Project by Ellyn Daugherty

Confirming the Amylase Gene Using PCR (Lab 13h)

Teacher's Guide

The following laboratory activity is adapted from "Laboratory 13h: Confirming the Amylase Gene Using PCR" 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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The rAmylase Project by Ellyn Daugherty

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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 0.08µg/µl pAmylase2014 (7µl)
- 8 tubes of 0.1µg/µl *Geobacillus stearothermophilus* genomic DNA D1 (5µl)
- 8 tubes of 0.01µg/µl *Geobacillus stearothermophilus* genomic DNA D2 (5µl)
- 8 tubes of 3' Amylase PCR Primer (Reverse primer, "P_R") (250pmoles)
- 8 tubes of 5' Amylase PCR Primer (Forward primer, "P_F") (250pmoles)
- 8 tubes of Taq Polymerase 2X Master Mix (MM) (110µl)
- 8 tubes of Sterile Water (Nuclease free) (2ml)
- 8 tubes of 6X DNA Loading Dye Buffer (50µl each)
- 8 tubes of DNAmark™ 1kbp Plus DNA Ladder (7µl each)
- 36 PCR tubes (200µl)
- 1 bottle of 50X TAE Buffer (40ml)
- 2 bottles of 0.8% agarose in 1X TAE (200mL each)
- 2 vials LabSafe™ Nucleic Acid Stain (50µl)

ADDITIONAL EQUIPMENT REQUIRED

The following standard lab equipment should be available.

- Beakers/Bottles (2L, 500ml)
- Micropipets and tips
- Heat block (preferred), 37°C or water bath set at 60°C
- DNA (Agarose) Electrophoresis Equipment (gel boxes and power supplies)
- UV Light box or transilluminator
- Trays (large enough to fit the gel, such as 12cm x 12cm)
- Deionized Water
- Thermocycler (PCR Machine)
- 8 Small Styrofoam cups packed with crushed ice

SPECIAL HANDLING INSTRUCTIONS

- Store 5' and 3' primers, Taq Polymerase Master Mix, DNAmark™ 1kbp Plus DNA Ladder and both plasmid and genomic DNA at -20°C
- Store the LabSafe™ Nucleic Acid Stain at 4°C.
- 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

- Briefly centrifuge all small vials/tubes of defrosted reagents before opening (to prevent waste of reagents).

 *All components used in the polymerase chain reaction should be kept on ice. The students' labeled reagents and sample tubes can be placed in a small amount of crushed ice at their workspace. A small paper or Styrofoam cup works well to hold crushed ice and tubes.*

Program Thermocycler

 *NOTE: This kit is designed so that each group of 4 students prepares 4 PCR reactions. Ideally each student sets up their own PCR reaction for a total of 32 samples in the class. If your thermocycler is unable to accommodate 32 samples, then run 16 samples in the thermocycler and then the 2nd 16 samples can be kept chilled (on ice) until the 1st run is done. Once the thermocycler run is complete, the samples should be kept in the refrigerator until gel loading dye is be added to the tubes prior to the electrophoresis.*

1. Follow the manufacturer's instructions for your thermocycler. Determine if there is enough room in the thermocycler heat block for the number of samples to be run. If 2 runs are necessary, plan timing accordingly.
2. Use the menu on the thermocycler display to run the following program:

- 1 Cycle of 95°C for 2 minutes (Initial Activation)
- 33 Cycles:
    - 95°C for 30 seconds (Denaturation of template strands)
    - 60°C for 45 seconds (Annealing of primer to template)
    - 72°C for 45 seconds (Extension of synthesis strands)
- 1 Cycle of 72°C for 5 minutes (Final extension)
- 1 Cycle of 4°C infinitely. (Storage)

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Preparation of agarose gel

- 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 4 PCR samples plus a tube of standard markers. Thus a group needs 5 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.
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.

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

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

   - 1 tube of 0.08µg/µl pAmylase2014 (7µl)
   - 1 tube of 0.1µg/µl *Geobacillus stearothermophilus* genomic DNA D1 (5µl)
   - 1 tube of 0.01µg/µl *Geobacillus stearothermophilus* genomic DNA D2 (5µl)
   - 1 tube of 3' Amylase PCR Primer (Reverse primer, "P_R") (250pmoles)
   - 1 tube of 5' Amylase PCRPrimer (Forward primer, "P_F") (250pmoles)
   - 1 tube of Taq Polymerase 2X Master Mix (MM) (110µl)
   - 1 tube of Sterile Water (Nuclease-free) (2ml)
   - 1 tube of 6X DNA Loading Dye Buffer (50µl each)
   - 1 tube of DNAmark™ 1kbp Plus DNA Ladder (7µl)
   - Four PCR tubes (200µl)

Also have available or distribute the following to each group:

- Trays (large enough to fit the gel, such as 12cm x 12cm)
- 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

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

- 1X TAE Buffer (Gel Electrophoresis Buffer)
- UV Light box or transilluminator
- Deionized Water

TIME REQUIRED

- 1 hour pre-lab (preparation and aliquot of buffer, agarose and samples)
- 1 hour lab period (student preparation of PCR samples, load, and start PCR run); more time needed if doing 2 runs
- 2-hour lab period (load, run, and photograph gel)
- 30 minutes post lab analysis

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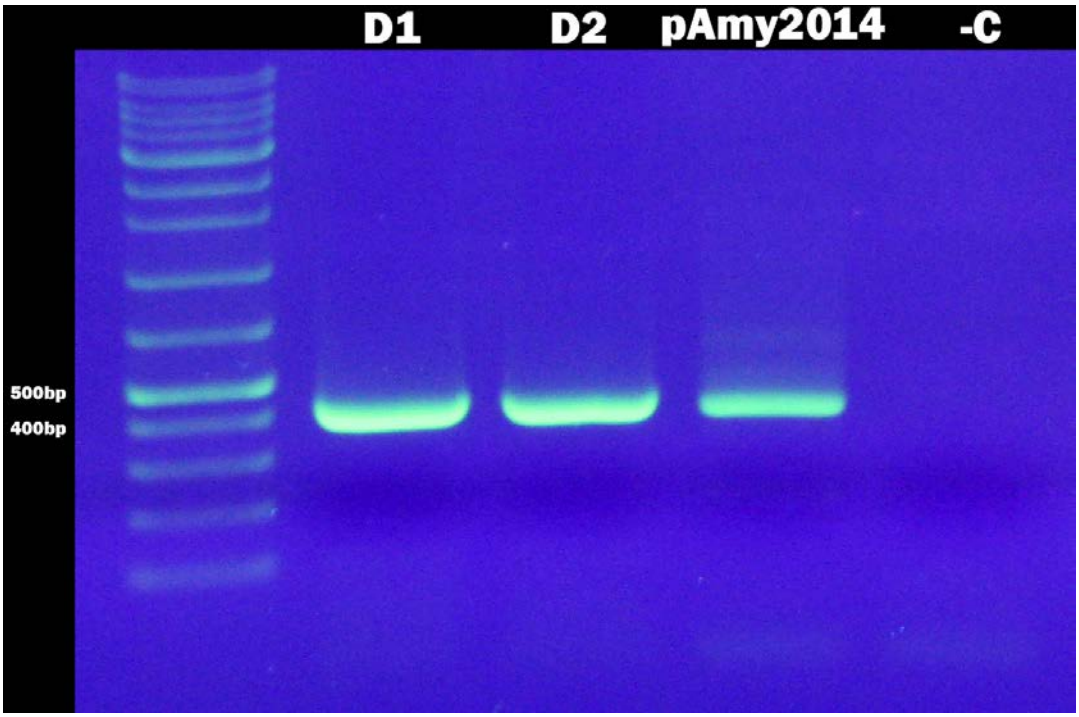
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NEXT GENERATION SCIENCE STANDARDS ADDRESSED

- 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



ANSWERS TO ADDITIONAL QUESTIONS

1. When the gel of the PCR products is run, a 388-bp band of lighter intensity is seen in the negative control sample. List several reasons why this unexpected result could occur.
   Answer: No PCR product or DNA of any kind (except primer) should be in the negative control sample. DNA in the negative control sample is evidence of contamination, either in one of the reagents (water or master mix, primers) or during micropipeting.
2. When the gel of the PCR products is run, the only bands seen anywhere on the gel are in the 100-bp sizing standard sample. Which reactant or ingredient missing in the PCR reaction could yield this result if accidentally left out of the PCR reaction tubes?
   Answer: If there is no DNA or even primer, seen on the gel, then the most likely missing ingredient is primer.
3. When the gel of the PCR products is run, a bright smear of DNA bands is up high on the gel near the well. Explain how this unexpected result could occur.
   Answer: Long strands of genomic DNA might stay in the wells or load into the gel but not move far. If a bright smear of DNA bands is up high on the gel, the concentration of template DNA is probably too high.

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OBJECTIVES

Using the PCR protocol developed for amylase gene amplification in pAmylase2014, can a 388bp sequence of the amylase gene be recognized in extracted *Geobacillus stearothermophilus* DNA?

BACKGROUND

The pAmylase2014 (pAmy) plasmid is a recombinant plasmid containing the amylase gene. It is used to transform *E. coli* cells into amylase producers. It is believed that pAmy was constructed by inserting the amylase gene from the bacterium *Geobacillus stearothermophilus* into an existing plasmid (pUC57).

After a transformation of *E. coli* cells with pAmylase2014, the plasmid DNA can be extracted out of the transformed cells using a miniprep protocol (Cat. # BTNM-8G). Confirmation of the plasmid as pAmy may be done through restriction digestion (Cat. # BTNM-8B). In addition, the presence of the amylase gene on pAmy (or in the source DNA from the original bacterium) may be confirmed through PCR.

To conduct a PCR reaction to recognize the amylase gene, primers are designed to recognize a specific, unique region (in this case, a 388bp section) on the target DNA. Using a thermal cycling protocol and a master mix of *Taq* polymerase, dNTPs, and buffer, the target amylase DNA region may be amplified. The amplified DNA is visible on an agarose gel as a band at 388bp.

The amylase gene PCR protocol used in this activity was developed using a known sample of pAmylase2014. The primers were designed to recognize a 388bp section of the plasmid's amylase gene. Theoretically, the amylase PCR protocol developed for pAmy should also recognize the same sequence in the original amylase gene in DNA extracted from the original source bacterium, *Geobacillus stearothermophilus* (also called *Bacillus stearothermophilus).* In this activity, genomic DNA will be tested by PCR for the presence of the amylase gene.



Figure: pAmylase2014 Plasmid. pAmylase2014 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.

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MATERIALS FOR EACH GROUP

Supply each group with the following components.

- 1 tube of 0.08µg/µl pAmylase2014 (7µl)
- 1 tube of 0.1µg/µl *Geobacillus stearothermophilus* genomic DNA D1 (5µl)
- 1 tube of 0.01µg/µl *Geobacillus stearothermophilus* genomic DNA D2 (5µl)
- 1 tube of 3' Amylase PCR Primer (Reverse primer, "P_R") (250pmoles)
- 1 tube of 5' Amylase PCR Primer (Forward primer, "P_F") (250pmoles)
- 1 tube of Taq Polymerase 2X Master Mix (MM) (110µl)
- 1 tube of Sterile Water (Nuclease free) (2ml)
- 1 tube of 6X DNA Loading Dye Buffer (50µl each)
- 1 tube of DNAmark™ 1kbp Plus DNA Ladder (7µl)

Also distribute the following to each group:

- Four PCR tubes (200µl)
- Trays (large enough to fit the gel, such as 12cm x 12cm)
- 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

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

- 1X TAE Buffer (gel electrophoresis Buffer)
- UV Light box or transilluminator
- Deionized Water

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PROCEDURE

- Use sterile technique throughout the procedure. Use a laminar flow hood, if available, for work with bacteria.
- Keep cells on ice unless otherwise directed.
- Dispose of all biohazards appropriately.

I. PCR Reactions

1. Transfer 25µl Sterile Water (Nuclease free) to the 5' Amylase Primer tube. Resuspend the primer by gently pipetting up and down.

2. Transfer 25µl Sterile Water (Nuclease free) to the 3' Amylase Primer tube. Resuspend the primer by gently pipetting up and down.

3. Label four 0.2mL (200µl) PCR tubes with "D1," "D2," "+C," and "-C," respectively and initials of your lab group.

4. Using a micropipette with a fresh aerosol-plug tip each time, add PCR reagents in the order listed on the table below, adding one reagent directly into the other.

Reagent	Tube "DI" (µl)	Tube "D2" (µl)	Tube "pAmy2014" (µl)	Tube "-C" (µl)
Sterile Water (Nuclease free)	11	11	10	15
2X Master Mix	25	25	25	25
DNA sample (from DI, D2, or +C)	4	4	5	0
Forward primer, 10µM	5	5	5	5
Reverse primer, 10µM	5	5	5	5

5. Pool each 50µl sample with a 2-second centrifuge spin.

6. Place labeled tubes in a programmed thermocycler to run the following Amylase Gene PCR program.

    a. 1 Cycle of 95°C for 2 minutes (Initial Activation)

    b. 33 Cycles:

        i. 95°C for 30 seconds (Denaturation)

        ii. 60°C for 45 seconds (Annealing)

        iii. 72°C for 45 seconds (Extension of strands)

    c. 1 Cycle 72°C for 5 minutes (Final extension)

    d. 1 Cycle of 4°C forever. (Storage)

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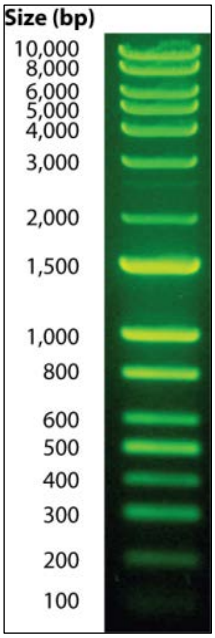
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7. If a loading grid diagram is available, record the position of each sample on a loading grid (see example below).

PCR: _____ PERIOD: _____ DATE: _____

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

8. After the thermal cycling reaction is complete, PCR samples can be refrigerated at 4°C or prepared for electrophoresis.



II: Electrophoresis and Analysis of PCR Products

1. To prepare the samples for electrophoresis, add 8µl of 6X DNA Loading Dye Buffer to each tube. Change tips each time.
2. Give the tubes a 1 to 2 second pulse in the microfuge to mix and pool reactants..
3. Load samples (25µl of each) in the first four wells of a 2% agarose gel in 1X TAE Buffer.
4. Load 5µl of DNAmark™ 1kbp Plus DNA Ladder sizing standards in Lane 5. Change micropipette tips with each load.
5. Run the gel at 115 V for approximately 1 hour until the front loading dye goes at least halfway on the gel. 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.
6. Once the blue dye front has migrated ¾ the length of the gel, turn off the power and carefully transfer the gel to a UV Light box. Turn on the UV light and observe the DNA bands on the gel.
   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.

   CAUTION: Wear UV Safety Specs when working around the UV Light Box.

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9. 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.

10. 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 DNAmark™ 1kbp Plus DNA Ladder sizing standards.

11. 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.

    - 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 fx to determine "exp (x)" for the In (x) value, and it will give the size of the unknown fragment.

12. Identify the PCR product bands on the gel. Also, identify any excess primer bands on the gel.

DATA ANALYSIS/CONCLUSION

Discuss how well the PCR protocol amplified a 388-bp band from the positive control (pAmylase sample). Is there any PCR product in the unknown samples (diluted *Geobacillus stearothermophilus* gDNA samples)? If yes, what is the size of this PCR product(s)? Did either of the dilutions amplify better than the other? What can be concluded from the bands in the unknown sample lanes?

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ADDITIONAL QUESTIONS

1. When the gel of the PCR products is run, a 388-bp band of lighter intensity is seen in the negative control sample. List several reasons why this unexpected result could occur.

2. When the gel of the PCR products is run, the only bands seen anywhere on the gel are in the 100-bp sizing standard sample. Which reactant or ingredient missing in the PCR reaction could yield this result if accidentally left out of the PCR reaction tubes?

3. When the gel of the PCR products is run, a bright smear of DNA bands is up high on the gel near the well. Explain how this unexpected result could occur.

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