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The rAmylase Project by Ellyn Daugherty

Using a Miniprep Kit for Plasmid Isolation

(Lab 8g)

(Cat. # BTNM-8G)



Developed in partnership with

The rAmylase Project by Ellyn Daugherty

Using a Miniprep Kit for Plasmid Isolation (Lab 8g)

Teacher's Guide

The following laboratory activity is adapted from "Laboratory 8g: Using a Miniprep Kit for Plasmid Isolation" from *Biotechnology: Laboratory Manual* 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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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)

- 1 vial Ampicillin (25mg)
- 1 vial Sterile Water (2ml)
- 1 tube "E. coli transformed with pAmy2014" Agar Stab
- 1 tube sterile LB Broth (2ml)
- 2 bottles sterile LB Broth (2 x 30ml)
- Sterile 200-250ml culture flask
- 8 Transfer pipets (Large)
- 8 Sterile Capped Culture Tubes
- 50 Microcentrifuge Tubes (1.5ml)
- 1 bottle Cell Suspension Solution (10ml)
- 1 vial LongLife™ RNase (50µl)
- 8 tubes of "Lysis Buffer" (600µl)
- 8 tubes of "Neutralization Buffer" (800µl)
- 8 tubes DNA Wash (2ml)
- 16 GET™ Plasmid Columns (Mini)
- 8 tubes of "TE Buffer" (100µl)
- 8 Nucleic dotMETRIC™ Assay strips
- 8 Nucleic DotMETRIC™ Dye (0.6ml)
- 8 dotMETRIC™ Standard

ADDITIONAL EQUIPMENT & MATERIALS REQUIRED

- Heat block or Water bath or beaker and thermometer
- High-speed Microcentrifuge (15,000xg or higher) for 1.5-2ml microcentrifuge tubes
- Micropipets (P1000, P100 or P200 and P10 or P20) and sterile tips
- Disinfectant towels
- Disposal container with 10% bleach
- Shaking Incubator for overnight cultures

SPECIAL HANDLING INSTRUCTIONS

- Store "E. coli transformed with pAmy2014" agar stab at 4°C. This is stable for ~1 month if stored correctly.
- Store AMP (Ampicillin) and LongLife™ RNase frozen until ready to use.
- All other components can be stored at room temperature.
- Briefly centrifuge all small vials before opening to prevent waste of reagents.

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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 before opening to prevent waste of reagents.
- Preheat a heat block or water bath to 37°C prior to starting the preparation of the bacterial broth cultures.
- Preheat a shaking incubator to 37°C prior to starting the preparation of the bacterial broth cultures.
- Wipe down the lab tabletop with disinfectant towels before starting broth cultures.
- Make sure to use the correct concentration of ethanol at the specified step in the protocol.
- Place all bacteria-contaminated pipets and tips into a 10% bleach solution. Allow contaminated items to soak for 30 minutes before disposing.

Preparation of LB Broth

1. Transfer the entire contents of both LB Broth bottles to a sterile 200-250ml culture flask.

2. Using a sterile micropipette tip, rehydrate the ampicillin by transferring 250µl sterile water to the vial of ampicillin. Dissolve the ampicillin by inverting the tube several times.

3. Using a sterile micropipette tip, transfer 60µl ampicillin to the LB broth and swirl to mix.

Prepare Bacterial Stocks

1. Using a sterile micropipette, transfer 0.8ml LB broth from the 2ml LB Broth vial to the bacterial agar stab and incubate at 37°C for 30 minutes.

2. Vigorously shake or vortex for 1-2 minutes, then transfer, with a sterile micropipette, 0.6ml LB broth from the agar stab to the 200-250ml culture flask containing the LB Broth with ampicillin.

3. Incubate the culture flask containing the LB Broth with ampicillin and cells at 37°C with shaking (250 rpm) overnight.

4. The following day, aliquot 7ml of the overnight culture into each of the 8 tubes labeled, "*E. coli/*pAmy".

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Additional Preparation and Distribution of Reagents

1. Add the entire contents of the LongLife™ RNase to the bottle of Cell Suspension Solution. Invert gently to mix. Label 8 microcentrifuge tubes "CSS" for Cell Suspension Solution and add 1ml of the store Cell Suspension Solution to the microcentrifuge tubes. Store Cell Suspension Solution at 4°C until ready to use.

2. Store Neutralization Buffer tubes at 4°C until ready to distribute to student groups.

3. In addition to one each of an overnight culture tube of *E. coli/*pAmy2014 broth culture and CSS, distribute following to each of the 8 lab groups:

   - A tube of "Lysis Buffer"
   - A tube of "Neutralization Buffer".
   - A tube of DNA Wash
   - A tube of "TE Buffer"
   - 2 GET™ Plasmid Columns (Mini)
   - 1 Transfer pipets (Large)
   - 1 Nucleic dotMETRIC™ Assay strips,
   - 1 tube Nucleic DotMETRIC™ Dye
   - 1 dotMETRIC™ Standard
   - 2 Microcentrifuge tubes (1.5ml)

TIME REQUIRED

- 2 hours (teacher preparation of cultures and aliquot of buffers and wash solutions)
- 2-hour lab period (students mini-prep)
- 1-hour lab period for miniprep plasmid analysis (G-Biosciences Nucleic dotMETRIC™ Assay), longer if conducting restriction digestion/gel run and/or UV spectrophotometry
- 1 hour 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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ANSWERS TO ADDITIONAL QUESTIONS

1. In biotechnology, "time is money." If one procedure works as well as another and costs less, that is the procedure that will be used. Are there any steps in this miniprep protocol that might be modified to give similar results in a shorter time? Suggest a modification and explain how you might test it.
   Answer: The answers will vary but suggestions might include using higher concentrations of one buffer or another, or using more cells in each miniprep sample, or trying to decrease incubation or spin times.
2. A concentration of 0.005 µg/µL is usually required for a transformation. Did your miniprep yield enough (30µL) of a sufficient concentration (0.005µg/µL) of plasmid for another transformation?
   Answers will depend on the success of the miniprep.
3. Is there any evidence of RNA contamination of the pAmylase2014 mini-prep sample? How do you know? Why is RNA contamination a problem and what can be done about it?
   Answer: The answers will vary but RNA contamination is common in minipreps. RNA contamination appears as a smear of sample around 500bp on an agarose gel. Also, RNA contamination can be detected by using UV spectrophotometry. Additional RNase treatment should rid a sample of RNA.

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OBJECTIVES

How well is pAmylase2014 extracted from transformed *E. coli* cells using a commercial plasmid DNA isolation kit?

BACKGROUND

Plasmid DNA that is suitable for recombinant DNA work and PCR can be recovered from transformed cells using a minipreparation (miniprep) procedure. The goal of a miniprep is to isolate 10 to 25µg of plasmid in a volume of 25 to 50µL of buffer or sterile, deionized water. The plasmid DNA should be fairly pure with spectrophotometer absorbance values that give Abs_{260}/Abs_{280} of about 1.8.

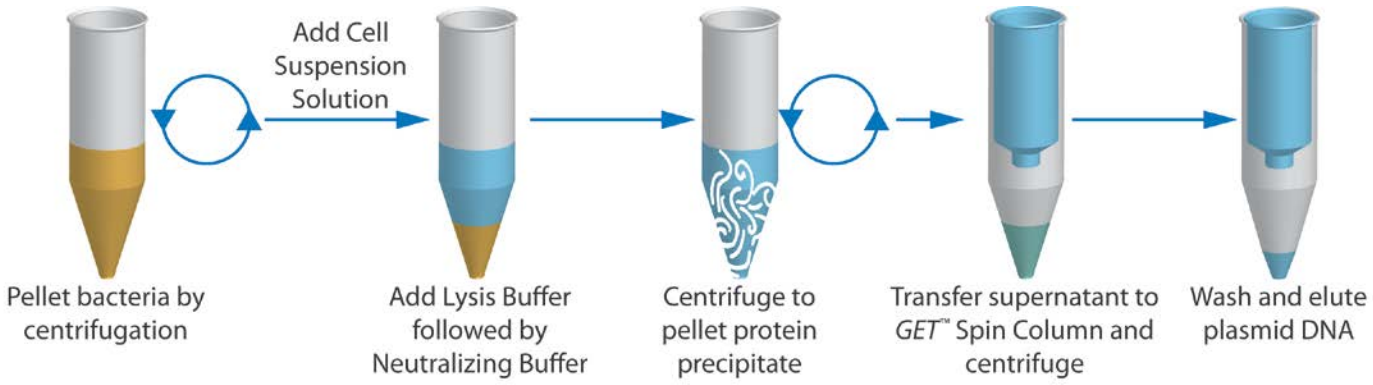


Figure: A General Miniprep Scheme.

Technicians can produce all the reagents needed for a miniprep, such as in the alkaline lysis miniprep, but to decrease the amount of time and increase the yield, companies have developed commercial miniprep kits. A kit makes a miniprep faster, easier, and fairly economic. The kits vary slightly from one manufacturer to another, but the basic steps are the same.

In this kit, an overnight broth culture is grown. The plasmids of interest are found within the cells so the cells are pelleted out of the broth in a centrifugation step. Next, the cells are resuspended in a buffer (Cell Suspension Solution [CSS]) to maintain pH and bind ions that could interfere with further procedures. *RNase is included to degrade the RNA when the cells are lysed.*

Next the cells are lysed (exploded) using a lysis buffer containing sodium hydroxide and SDS (sodium dodecyl sulfate). The sodium hydroxide denatures the plasmid and chromosomal DNA into single strands. SDS, an ionic (charged) detergent dissolves the phospholipids in the membrane causing lysis and release of the bacteria contents, including the DNA, into the solution.

The lysed cell mixture contains a large amount of protein contamination. A potassium acetate solution is added to precipitate contaminant proteins which leaves fairly clean plasmid DNA in the supernatant. In addition, the potassium acetate neutralizes the solution allowing the renaturation of the DNA. The large chromosomal DNA is captured in the precipitate, whereas the small plasmid DNA remains in solution.

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Next, the DNA supernatant is put on a column that has resin beads the binds the plasmid sample. Alcohol washes of the column remove remaining contaminants. Finally, the plasmid is removed (eluted) from the column using TE Buffer and collected in a clean tube.

A technician who conducts minipreps regularly should compare the time involved and the yield of plasmid isolated using various miniprep kits and protocols. In this lab, a commercially available miniprep kit is used to isolate pAmylase plasmid from transformed *E. coli* cells grown in LB/amp broth. The plasmid yield is measured and evaluated against the expected yield of about 30µg./30µl miniprep elution. Concentration and purity of the eluted plasmid may be analyzed using UV spectrophotometry. The pAmylase2014 plasmid may also be analyzed using a restriction digestion (Restriction Digestion to Verify the pAmylase Plasmid" Lab 8b Kit (Cat. # BTNM-8B).

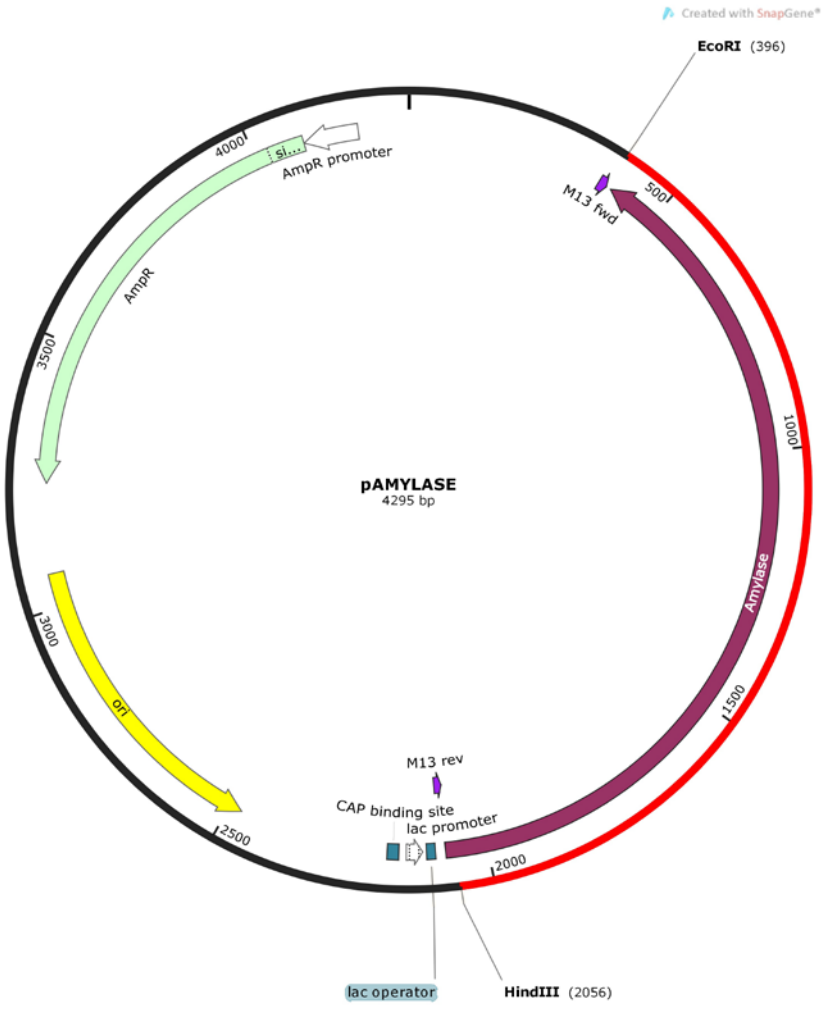


Figure: pAmylase2014 Plasmid. pAmylase contains the amylase gene. In cells, the amylase gene is transcribed and amylase is produced. Starch clearing occurs on Luria Bertani (LB) starch agar plates around colonies that are transformed with pAmylase. An Amp^R gene is also part of pAmylase. The Amp" gene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

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MATERIALS FOR EACH GROUP

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

- 1 Culture Tube with *E. Coli* transformed with pAmy2014 Overnight Culture
- 2 Microcentrifuge Tubes (1.5ml)
- 1 transfer pipet (3ml)
- 1 tube of Cell Suspension Solution containing RNase (CSS) (1ml)
- 1 tube of Lysis Buffer (600µl)
- 1 tube of Neutralization Buffer (800µl)
- 1 tube of DNA Wash (2ml)
- 2 GET™ Plasmid Columns (Mini)
- 1 tube of TE Buffer (TE) (100µl)
- 1 Nucleic dotMETRIC™ Assay strips,
- 1 tube Nucleic DotMETRIC™ Dye
- 1 dotMETRIC™ Standard

PROCEDURE

The protocol below is written for a lab group of 4 students (working as 2 pairs) to perform two minipreps of a single overnight culture sample. After harvesting cells, the student group uses alkaline lysis and commercial DNA spin columns to recover the pAmylase2014 from the transformed cells in the broth culture. By the end of the miniprep protocol, a group's goal is to recover approximately 20µg of pAmylase2014 DNA from each miniprep.

I. Collect Bacteria

1. Label two 1.5ml microcentrifuge tubes with initials, one for each student pair.

2. Finger-flick the tube of overnight culture to resuspend the *E. coli* transformed with pAmylase2014 cells containing before taking a sample. Using a transfer pipet, transfer 1.5ml of the overnight culture into each labeled tube. Place the transfer pipette on a paper towel when not being used.

3. Close the cap, and place the tubes in a **balanced** configuration in a tabletop centrifuge. Make sure all tubes have the lid hinge facing the center of the centrifuge. Centrifuge at high speed (greater than 7,000xg) for 3 minutes to pellet the bacteria cells.

4. Pour off the supernatant (broth) from the tubes and into a waste beaker with 10% bleach solution. **.** Invert the tubes, and gently tap on the surface of a small piece of clean paper towel to thoroughly drain the supernatant. **Be careful not to disturb the cell pellets.**

5. Using the transfer pipet, add another 1.5ml volume of the *E. coli* transformed with pAmylase2014 cells to the centrifuge tubes with the pellet of cells from step 4. Spin again for 3 minutes to pellet more cells. Dispose of the transfer pipet in the disinfectant waste.

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6. Pour off the supernatant (broth) from the tubes and into a waste beaker with 10% bleach solution. **Be careful not to disturb the cell pellets.** Invert the tubes, and gently tap on the surface of a small piece of clean paper towel to thoroughly drain the supernatant. Dispose of this paper towel in the bleach as well.

II. Bacterial Lysis & Protein Precipitation

The plasmid DNA extraction kit use optimized buffers and solutions to perform alkaline lysis to explode the cells and rid the sample of protein contamination.

7. Add 250µl Cell Suspension Solution with RNase (CSS) to the bacterial cell pellets and mix the contents by flicking the tubes or pipetting several times. Hold the tubes up to the light to check that the suspension is homogeneous and that no visible cell clumps remain.

8. Add 250µl Lysis Buffer and mix the contents by gently inverting the tubes 4-5 times, until the lysate is clear. Do not shake. Vigorous shaking will cause the bacterial genomic DNA to shear (break) and this may contaminate the plasmid DNA.

9. Allow the tubes to incubate for 3 minutes only to allow for complete RNA digestion.

10. Add 350µl of chilled Neutralization Buffer (NB) and mix the contents by inverting the tubes 8-10 times. The Neutralization Buffer is also a precipitation buffer. A thick white precipitate of denature protein will become visible.

11. Place the microcentrifuge tubes in **a balanced** configuration in a centrifuge and spin at 15,000xg (maximum speed) for 15 minutes to pellet the precipitate along the side of tube.

III. Spin Column DNA Purification

12. Heat the tube of TE buffer for 5-10 minutes in a 55°C heat block so it is ready to use at step 15.

13. Ensure the GET™ Plasmid Mini column is in a collection tube, then transfer the clear supernatant (~850µl) from the microcentrifuge tube (Step 11) and apply to the column. The clear supernatant contains the plasmid DNA. Make sure to not transfer any of the white protein precipitate to the spin column.

14. Place the spin columns with their collection tubes in **a balanced** configuration in a centrifuge and spin for 60 seconds, at maximum speed and discard the flow through.

15. Wash the column by adding 450µl DNA Wash and centrifuge as in step 14. Discard the flow through. Repeat this step again.

16. Perform a final spin of the column for 60 seconds to remove any residual DNA Wash from the sides of the column. Discard this flow through wash as well.

17. Place the column over a clean 1.5ml microcentrifuge tube. Elute (remove) the plasmid DNA from the column by adding 30µl of the pre-warmed TE Buffer directly to the column membrane. Incubate for 2 minutes then centrifuge in **a balanced** configuration at maximum speed for 60 seconds.

18. Label the microcentrifuge tube of eluted pAmylase2014 DNA. If time allows, continue to the analysis of the miniprep product. If necessary, store the miniprep plasmid product at -20°C until ready to analyze or use.

IV. Analysis of Miniprep Product Yield and Purity

19. The G-Biosciences Nucleic dotMETRIC™ Assay is a quick way to determine the presence and approximate amount of plasmid DNA in the miniprep product.
    - Dot two 1µL samples of each eluted plasmid sample (1cm apart) on the dotMETRIC™ Assay strips. You should have 4 dots per group
    - Add 500µL of Nucleic DotMETRIC™ Dye (cover all samples) and leave on for 1 minute.
    - Using forceps to hold the strip, wash (dunk) the strip in water to remove excess dye.
    - Compare the color density of the nucleic acid spots to the dotMETRIC™ Standard.

20. Analysis of the miniprep product is traditionally done using UV spectrophotometry.

    - Use a UV spectrophotometer and a 50µl adaptor or a 50µl cuvette. Calibrate the UV spec to 260nm.
      NOTE: *If a 50µl adaptor is not available then samples from different groups can be pooled to a reach a volume that can be used in the cuvettes available.*
    - Add 5µl miniprep DNA and 45µl water to the cuvette and mix by pipetting. Do not forget to multiply your final concentration by 10 to allow for the dilution factor.
    - To calculate the concentration of DNA in a sample, use a simple ratio. It is known that 50µg/ml of pure, double-stranded DNA absorbs approximately 1 au of light at 260 nm. One can determine the concentration of an unknown DNA sample using the following equation:

$$\frac{50\mu g/ml}{1\ au\ at\ 260nm} = \frac{X\mu g/ml}{the\ absorbance\ of\ sample\ at\ 260nm}$$

An added advantage of using a UV Spectrophotometer is that the absorbance at 280nm can also be measured. The 280nm absorbance value estimates the amount of protein contamination in the DNA sample. Suitable purity levels for plasmid DNA preps are calculated as a ratio of A_{260} : A_{280}. For DNA a ratio of ~1.8 is deemed "pure" DNA.

21. An alternative comparison method is to perform a restriction digest and agarose gel electrophoresis analysis. The purified plasmids may be used and analyzed with the G-Biosciences kit, "Restriction Digestion to Verify the pAmylase Plasmid" (Cat. # BTNM-8B).

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DATA ANALYSIS & CONCLUSION

What is the mass (µg) and concentration of (µg/µl) of plasmid extracted in the mini-prep your group conducted? How close did the sample come to the expected yield? Knowing that a restriction digestion of the plasmid requires at least 1µg of plasmid, did your mini-prep produce enough plasmid to run a restriction digestion and an electrophoresis? Explain. What is the yield of your mini-prep in comparison to other groups in the class? Describe steps in the procedure that could easily cause technician error and result in lower yield.

ADDITIONAL QUESTIONS

1. In biotechnology, "time is money." If one procedure works as well as another and costs less that is the procedure that will be used. Are there any steps in this miniprep protocol that might be modified to give similar results in a shorter time?
2. A concentration of 0.005µg/µL is usually required for a transformation. Did your miniprep yield enough (30µl) of a sufficient concentration (0.005µg/µL) of plasmid for another transformation?
3. Is there any evidence of RNA contamination of the pAmylase2014 mini-prep sample? How do you know? Why is RNA contamination a problem and what can be done about it?

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