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Using a Controlled Experiment to Identify Two Unknown Plasmids

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INTRODUCTION

Description

This activity can serve as an assessment following a unit on biotechnology. For a biotechnology unit including lecture and laboratory sessions on DNA structure, plasmids, restriction enzymes, gel electrophoresis, gel analysis following electrophoresis, and bacterial transformation, the student activity described below could serve as a "practicum" of sorts to assess what was learned in the unit. Alternatively, the protocol written in the instructor's notes could be given to the student as a traditional lab exercise.

Student Audience

This activity is suitable for a second-year high school biology course or Advanced Placement Biology.

Goals for the Activity

Students will

- demonstrate an understanding of recombinant DNA techniques (restriction enzyme digest, gel electrophoresis, staining gel);
- demonstrate skills needed to complete a gel electrophoresis and interpret a stained gel;
- solve a problem using the scientific method; and
- define "plasmid" and "restriction enzyme."

Recommended Placement in the Curriculum

Use the activity as a laboratory during a biotechnology unit or as an assessment at the completion of a biotechnology unit.

STUDENT HANDOUT
Using a Controlled Experiment to Identify Two Unknown Plasmids

Purpose

The purpose of this activity is to develop a reasonable experiment to identify unknowns in improperly labeled culture tubes—a common problem in the laboratory. Follow the scientific method as you develop your experiment. In addition, employ skills already developed in completing DNA digests, electrophoresis, and staining.

Scenario

You are a part-time laboratory assistant in a DNA research lab at the local university. One of the other laboratory assistants has left two culture tubes of the plasmids pAMP and pKAN unlabeled. These plasmids are to be used in an experiment to construct and analyze a recombinant DNA molecule. The plasmids are expensive and have been purchased with grant monies. Therefore, it is imperative that the contents of the tubes be properly identified. You have two restriction enzymes, *Bam*H I and *Hin*d III, to help you identify the plasmids. In addition, you have all the electrophoresis equipment in the lab available for your use.

Pre-Lab Questions

1. a) Define plasmid and give two examples used in this activity.
   b) Define restriction enzyme and give two examples used in this activity.

2. Predict what the stained gel would look like once you complete a controlled experiment using biotechnology techniques.

Safety, Handling, and Disposal

It is your responsibility to specifically follow your institution's standard operating procedures (SOPs) and all local, state, and national guidelines on safe handling and storage of all chemicals and equipment you may use in this activity. This includes determining and using the appropriate personal protective equipment (e.g., goggles, gloves, apron). If you are at any time unsure about an SOP or other regulation, check with your instructor.

When dealing with biological materials, take particular precautions as called for by the kit manufacturer or supplier.

The staining of the gel will be completed by the instructor at a designated sink area.

All tubes and tips used for storing or transferring DNA should be collected in a waste container and given to the instructor. Dispose of used reagents according to local ordinances.

Materials

<u>For the digest</u>

- 0.20 µg/µL pAMP
- 0.20 µg/µL pKAN
- unknown plasmid #1
- unknown plasmid #2
- *Bam*H I-*Hin*d III
- 2X restriction buffer

For the electrophoresis

- 0.1 mg/mL pAMP
- 0.1 mg/mL pKAN
- 0.1 mL unknown #1
- 0.1 mL unknown #2
- loading dye
- 0.8% agarose solution
- 1X tris-borate-EDTA (TBE) buffer
- 1 μg/mL ethidium bromide

Supplies and equipment

- 0.5- to 10-μL micropipetter and tips
- 1.5-mL tubes
- aluminum foil
- beaker for agarose
- beaker for waste/used tips
- weigh boat
- disposable gloves
- electrophoresis box
- masking tape
- permanent marker
- power supply
- test tube rack
- ultraviolet transilluminator
- 37°C water bath
- 60°C water bath for agarose

Procedure

Design a controlled experiment, given the scenario and materials above, to identify two unmarked tubes of plasmid. It is known that one tube is pKAN and the other is pAMP. You will be required to provide the steps used in your experiment. (See Question 1 below.)

Questions

1. Provide a thorough protocol (step-by-step procedure) for your controlled experiment.

2. Explain how you would determine the proper identity of the unknowns from the stained gels.

3. a) Explain the presence of additional bands that might appear on the gel which were not predicted.
   b) Suggest a procedure to correct the problem of additional bands.

Suggested Reading

Freyer, G.A.; Micklos, D.A. *DNA Science: A First Course in Recombinant DNA Technology*; Cold Spring Harbor Laboratory: Burlington, NC, 1990.

Rennie, J. "Grading the Gene Tests," *Scientific American.* 1994, *270* (6), 88.

INSTRUCTOR NOTES
Using a Controlled Experiment to Identify Two Unknown Plasmids

Time Required
Two three-hour laboratory sessions should be allowed for this activity. During the first session, students will analyze the scenario and plan the protocol. During the second session, the activity will be completed.

Group Size
The suggested group size for this activity is two students. However, considering the limited supply of the numerous expensive materials and equipment needed you may prefer to have students work in groups of three or four. If this activity is used as an assessment tool, a group assessment would be acceptable.

Materials
The Bloom reference has an excellent appendix of recipes for reagents and solutions. Carolina Biological Supply Company is an excellent source of biotechnology supplies, including the plasmids and restriction enzymes.

The ethidium bromide staining method used in this lab is the most rapid, sensitive, and reproducible means of staining DNA. Since ethidium bromide is a mutagen, the staining procedure should be done by the instructor. The Bloom reference thoroughly describes the precautions and procedures for staining with ethidium bromide. The author recommends purchasing a ready-mixed stock solution from a supplier. This stock solution can be diluted to make a staining solution with concentration of 1 μg/mL ethidium bromide. Methylene blue can also be used to stain DNA. If methylene blue staining is preferred, the stated plasmid concentrations should be increased. The volumes stated in the lab remain the same in order to increase the concentrations of plasmids.

Suggested materials and equipment for each laboratory station
- 6 μL 0.20 μg/μL pAMP (for digest-known) (refrigerate all DNA)
- 6 μL 0.20 μg/μL pAMP (for digest-unknown)
- 6 μL 0.20 μg/μL pKAN (for digest-known)
- 6 μL 0.20 μg/μL pKAN (for digest-unknown)
- 5 μL 0.20 μg/μL pAMP (undigested)
- 5 μL 0.20 μg/μL pKAN (undigested)
- 8 μL *Bam*H I-*Hin*d III (Store enzymes in non-frost-free freezer.)
- 32 μL 2X restriction buffer (To make 1 mL, mix 200 μL 10X restriction buffer with 800 μL deionized water.)
- 6 μL loading dye (Purchase from supplier.)
- 1X TBE buffer (Add 2.25 L deionized water to 0.25 L 10X TBE electrophoresis buffer.)
- 0.8% agarose solution (Microwave 2.65 g agarose in 330 mL water to make more than enough agarose solution for the entire class; store agarose solution in 60°C water bath.)
- 0.5- to10-μL micropipetter and tips
- 1.5-mL tubes
- aluminum foil
- beaker for agarose
- beaker for waste/used tips
- weigh boat

- disposable gloves
- electrophoresis box
- masking tape
- permanent marker
- power supply
- test tube rack
- ultraviolet transilluminator
- 37°C water bath
- 60°C water bath for agarose

Materials used to disable stained gels and staining solution

- distilled water
- 0.05 M $KMnO_4$
- 0.25 N HCl
- 0.25 N NaOH

Safety, Handling, and Disposal

As the instructor, you are expected to provide students with access to SOPs, MSDSs, and other resources they need to safely work in the laboratory while meeting all regulatory requirements. Before doing this activity or activities from other sources, you should regularly review special handling issues with students, allow time for questions, and then assess student understanding of these issues.

When dealing with biological materials, take particular precautions as called for by the kit manufacturer or supplier.

Always use disposable gloves and goggles when working with ethidium bromide. Use the following procedure to decontaminate stained gels and solution:

1. Add water to reduce the concentration to less than 0.5 mg/mL.
2. Add 1 volume of 0.05 M $KMnO_4$ and mix carefully.
3. Add 1 volume of 0.25 N HCl and mix carefully.
4. Let the solution stand at room temperature for several hours.
5. Add 1 volume of 0.25 N NaOH and mix carefully.
6. Discard the decontaminated solution down a sink drain. Drain the decontaminated gel and discard in the trash.

$KMnO_4$ is an irritant. Wear gloves and goggles. Solutions should be handled in a fume hood.

All materials used to store and/or transfer DNA should be soaked in a bleach solution for several hours before discarding. Lab benches should be wiped clean with a bleach solution. Dispose of used reagents according to local ordinances.

Points to Cover in the Pre-Lab Discussion

Pre-lab questions provided in the Student Handout give students a starting place. This activity should only be done following a unit on biotechnology where restriction digests and electrophoresis have been performed. Skill in these areas will be required. In a pre-lab discussion, procedures for digestion and electrophoresis should be briefly reviewed. References should be available for students to conduct research on the two plasmids and the effect of the two enzymes on them.

Procedural Tips and Suggestions

Organize the materials on the lab benches prior to the activity. Prepare tubes of known and unknown plasmids prior to the activity, making sure all tubes are labeled correctly. To save time, the agarose solution could be prepared and incubated in proper amounts for the gel chambers. The buffers and loading dye should also be prepared for each group in labeled tubes.

Sample Results

1. The "digested pAMP" control should show two distinct bands of 784 base pairs and 3,755 base pairs.

2. The "digested pKAN" control should show two distinct bands of 1,861 base pairs and 2,332 base pairs.

3. One "digested unknown" should be a restriction "fingerprint" of pAMP and the other should be a restriction "fingerprint" of pKAN.

4. An ideal digest for pAMP and pKAN can be found on page 118 of the Bloom reference.

Plausible Answers to Pre-Lab Questions

1. a) Define plasmid and give two examples used in this activity.
   A plasmid is a small ring of DNA that carries accessory genes separate from those of a bacterial chromosome (Campbell). Two plasmids used in this activity are pAMP and pKAN.

   b) Define restriction enzyme and give two examples used in this activity.
   *A restriction enzyme is a degradative enzyme that recognizes and cuts up DNA that is foreign to a cell (Campbell). Two examples used in this activity are Bam*H I *and Hin*d III.

2. Predict what the stained gel would look like once you complete a controlled experiment using biochemistry techniques.
   The digested pAMP *would show two distinct bands of 784 base pairs and 3,755 base pairs. The digested* pKAN *would show two distinct bands of 1,861 base pairs and 2,332 base pairs. The "unknown #1 digest" would match one of the digested plasmids, and the "unknown #2 digest" would match the other.*

Plausible Answers to Questions

1. Provide a thorough protocol (step-by-step procedure) for your controlled experiment.
   A sample procedure is provided on the following pages. An option for this activity could be to provide students with this sample procedure and have them follow it.

2. Explain how you would determine the proper identity of the unknowns from the stained gels.
   Look for a "restriction fingerprint" for each unknown when compared to the digests of the pAMP and pKAN. Identify the unknowns accordingly.

3. a) Explain the presence of additional bands that might appear on the gel which were not predicted.
   The presence of any additional bands indicates some degree of incomplete digest.

   b) Suggest a procedure to correct the problem of additional bands.
   Add another 1 μL Bam*H I*-Hin*d III solution and incubate 20 minutes longer.*

Sample Procedure

I. Cutting the DNA: setting up restriction digests

1. Obtain reaction tubes with the following labels: “pAMP control,” “ pKAN control,” “ unknown plasmid #1,” and “ unknown plasmid #2.”

2. Use the matrix below to add reagents to each tube. Change the tip on the micropipetter for each reagent.

Table 1

Tube	pAMP Control	pKAN control	unk #1	unk #2	2X buffer	*Bam*H I-*Hin*d III
digested pAMP	5.5 µL	–	–	–	7.5 µL	2 µL
digested pKAN	–	5.5 µL	–	–	7.5 µL	2 µL
digested unk #1	–	–	5.5 µL	–	7.5 µL	2 µL
digested unk #2	–	–	–	5.5 µL	7.5 µL	2 µL

3. After adding all reagents, close the top of the tube and sharply tap the bottom of the tube on the lab bench.

4. Place the four reaction tubes in a 37°C water bath for 30 minutes or longer.

II. Pouring the gel

1. Tape the ends of the gel bed with masking tape. Make sure you have a good seal.

2. Insert comb in notches near the end of the bed. Place the bed on a flat, level surface. It should not be moved until the gel has solidified (about 15–20 minutes).

3. Pour agarose into the bed to a depth of 5 mm. The gel should cover only about one-third of the height of the comb.

4. After the agarose solidifies, unseal the ends of the bed. Place the agarose gel bed on the platform in the gel box so that the comb is at the negative (black) electrode.

5. Fill the box with electrophoresis (TBE) buffer so that the gel is barely covered with it.

6. Gently remove the comb.

7. Once the comb has been removed, check to see that the buffer surface is smooth without “dimples.”

III. Loading the gel and electrophoresis

1. Use a permanent marker to label four clean 1.5-mL tubes: “digested pAMP,” “digested pKAN,” “digested UNK #1,” and “digested UNK #2.”

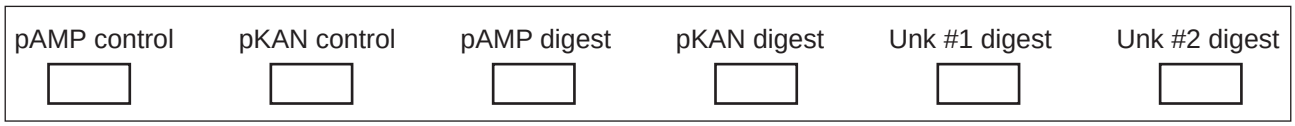
2. Remove the four tubes from the 37°C water bath. (They should have been incubating for at least 30 minutes.)

3. Transfer 5 μL of each digested plasmid into its appropriate tube (labeled in step 1 above).

4. Obtain 1.5-mL tubes containing pAMP and pKAN plasmids that have not been digested. Use a permanent marker to label these tubes "uncut pAMP" and "uncut pKAN."

5. Add 1 μL loading dye to all six tubes. Close the tops of the tubes and tap the bottom of each tube on the lab bench to mix.

6. Load the entire contents of each tube into a separate well in the gel as shown. Use a clean tip for each sample.



7. Electrophorese at 75 volts until the bromophenol blue bands have moved 30–40 mm from the wells.

8. Turn off the power supply, remove the casting tray from the electrophoresis box, and transfer the gel to a disposable weigh boat for staining.

IV. Staining the gel (to be completed by instructor)

1. Flood the gel with ethidium bromide solution and allow it to stain for 5–10 minutes.

2. View the gel under an ultraviolet transilluminator.

Caution: Wear disposable gloves when staining and viewing gels. Confine all staining to a restricted sink area.

V. Cleanup

Decontaminate the gel and any staining solution that is not to be reused. Wash your hands before leaving the laboratory.

Extensions and Variations

Depending on time and level of instruction, students could continue with these plasmids to construct a recombinant plasmid that contains both ampicillin- and kanamycin-resistant genes.

References

Bloom, M.V.; Freyer, G.A.; Micklos, D.A. *Laboratory DNA Science: An Introduction to Recombinant DNA Techniques and Methods of Genome Analysis;* Benjamin/Cummings: Menlo Park, CA, 1996.

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