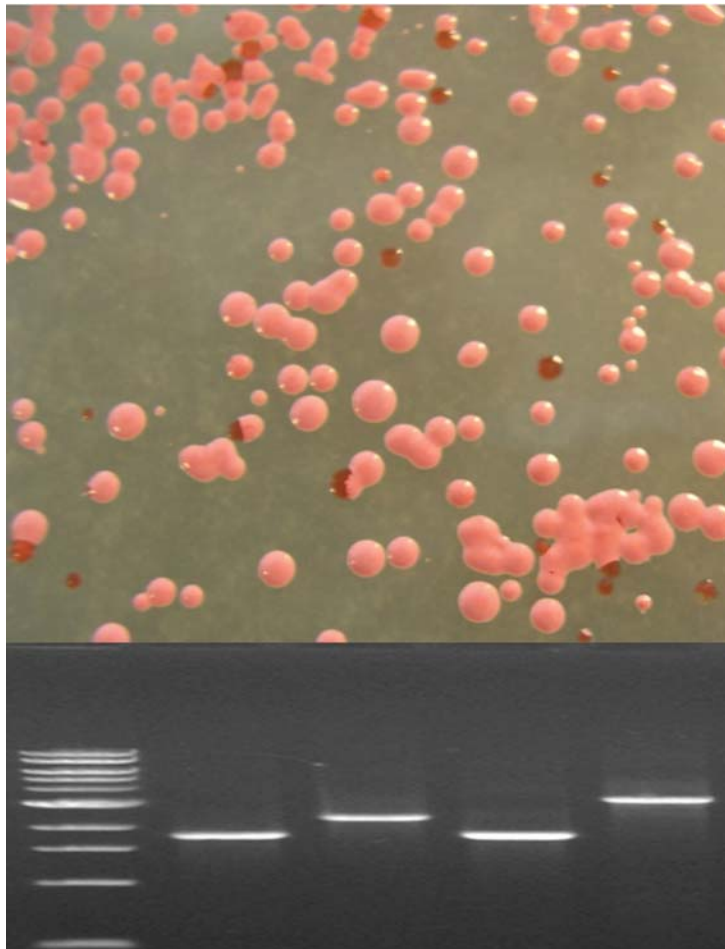


The Genotype–Phenotype Connection: Basic Molecular Genetics and Bioinformatics Skills

Teacher's Manual
and Student Guide



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*Photocopy the Student Guide as needed for use in your classroom

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Overview

Using the model microbe *Halobacterium* sp. NRC-1, students perform DNA extractions, PCR (polymerase chain reaction), and gel electrophoresis. Students also work with a stable mutant derivative, *Halobacterium* sp. KBT-1*.

Halobacterium is an archaeon and an extreme halophile (salt-loving organism). The microbe's brightly colored colonies and extreme lifestyle engage students and foster inquiry. As part of these laboratory exercises, students formulate questions and hypotheses to explain observed differences in colony phenotypes.

To detect genetic differences responsible for the different phenotypes, students use PCR to analyze DNA from either a defined mutant (KBT-1), an undefined mutant, a wild-type (NRC-1), or a sectored colony. To draw conclusions about any link between the molecular differences they detect and the phenotypes they observe, students pool their data. They then work through the questions in the Student Guide, demonstrating their comprehension of the concepts.

This kit is designed so that 25 individual students can each complete all parts of the lab, and we believe that the best learning occurs with this approach; however, the activities can be performed in small groups as well.

The Background Information section can be used by the teacher either to introduce or to review subtopics. Depending on your students' abilities and prior knowledge, you may want to omit sections of the background or, possibly, have advanced students research subtopics individually. The lab has been divided into four related sections that may be done on consecutive days, or several days apart.

*Named KBT because a Kansas biology teacher isolated it.

Objectives

Students will

- learn and practice sterile (aseptic) technique.
- study colony phenotypes.
- learn and practice pipetting techniques.
- extract DNA.
- perform polymerase chain reaction.
- perform agarose gel electrophoresis.
- analyze and draw conclusions from collected data.
- correlate genotype with phenotype.
- use basic bioinformatics tools.

Content Standards

To view the national and local standards met by this kit, visit www.carolina.com/correlations.

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Time Requirements

	Teacher prep before day of activity	Teacher prep on day of activity	Classroom time
Activity 1: <i>Halobacterium</i> exploration and cell lysis	Copy student guide for each student, as well as any needed sheets from appendices.	Set up student work area: 30 minutes.	~40 min
Activity 2: PCR	Program thermal cycler: ~10 minutes the first time you program it.	Aliquot Primer Mix as needed for student stations: ~5 minutes.	~30 min Thermal cycler running time: 1–2 hours for most machines.
Activity 3: Gel electrophoresis		Prepare buffer and pour gels (45–60 minutes). Aliquot DNA marker as needed for student stations.	Load gels: ~30 minutes. Run gels: 45 minutes to 1 hour. Stain gels: Process varies depending on stain used (30 minutes to overnight).
Activity 4: Data Comparison and Analysis	Obtain semilog graph paper (can be printed from many Web sites).	Photograph gels: 20 minutes.	~1 hour

Materials

The materials in this kit are supplied for use only with the exercises described in this kit. For the success of this lab, it is critical that your students conduct their exercises using safe and correct lab practices. Carolina Biological Supply Company disclaims all responsibility for other uses of these materials.

Included in the kit:

- 3 plates with colonies of *Halobacterium* sp. NRC-1 (wild type)*
- 2 plates with colonies of *Halobacterium* sp. KBT-1 (mutant)*
- 2 20-mL vials sterile water
- 25 PCR tubes containing Ready-To-Go™ PCR Beads, 0.2 mL capacity
- 1 vial Primer–Loading Dye Mix*
- Teacher's Manual with reproducible Student Guide
- in kits with GelGreen™:*
 - 1 30-μL vial 1-kb Ladder/DNA marker*
- in kits with CarolinaBLU™ or without electrophoresis materials:*
 - 4 30-μL vials 1-kb Ladder/DNA marker*

Kits with electrophoresis material also contain:

- 2 g agarose
- 150-mL 20× TBE buffer in a bottle
- 4 staining trays
- 4 pairs of disposable gloves

in kits with CarolinaBLU™:

- 1 250-mL bottle CarolinaBLU™ Final Stain
- 1 7-mL vial CarolinaBLU Gel and Buffer Stain

in kits with GelGreen™:

- 1 100-μL vial GelGreen
- 1 tube 1.5 mL 5 M NaCl

*If you requested that the perishable items ship separately, the kit contains an Order Form for prepaid delivery of the *Halobacterium* plates, Primer–Loading Dye Mix, and DNA marker. Follow the instructions on the form to place your request at least 2 weeks before your desired delivery date. Upon delivery, follow the storage instructions in this manual.

Needed, but not supplied:

- pipets (2- to 500-μL capacity) with sterile tips. Alternatively, if adjustable pipets are not available, fixed-volume pipetting devices for accurately pipetting 4, 10, 20, 100, and 500 μL may be used.
- resealable plastic bags or airtight plastic food containers
- permanent fine-tip markers
- stereomicroscope or hand lens (optional)
- microcentrifuge with adapters for holding 0.2-mL tubes[†]
- ice (in beakers or plastic cups per student)
- vortex (optional)
- thermal cycler
- power supplies
- gel electrophoresis chamber(s) (to accommodate enough gels for each student to load 1 lane plus extra lanes per gel for the DNA ladder and to reload misloaded samples)
- gel tray(s)
- gel comb(s)
- metric ruler
- a microwave, a heatable stir plate with stir bar, or a boiling waterbath
- UV transilluminator, fluorescent white light box, or blue light box
- UV safety goggles or blue light safety goggles or screen
- camera to capture gel images
- household bleach solution (10%) or autoclave
- gloves
- semilog graph paper
- lab coats or aprons (based on school policy)

[†]Adapters for 0.2-mL tubes can be made by removing the lids from 1.5-mL and 0.5-mL microcentrifuge tubes and nesting the smaller tubes in the larger ones in the centrifuge.

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Storage

Upon delivery, **store petri plates with *Halobacterium* colonies in the refrigerator in heavy-duty sealed plastic bags or sealed plastic containers (for up to 2 months)**. They must be tightly sealed because moisture loss leads to formation of salt crystals in the agar plates, interfering with observation of colonies.

Store the primer–loading dye mix and DNA marker in a freezer. Everything else can be stored at room temperature.

Store GelGreen™ in the dark.

Safety

Always ensure that each student understands and adheres to safe laboratory practices when performing any activity in the classroom or laboratory. Demonstrate the protocol for correctly using the instruments and materials necessary to complete the activities, and emphasize the importance of proper usage.

The American Type Culture Collection (ATCC) classifies *Halobacterium* sp. NRC-1 as a BioSafety Level 1 (BSL-1) organism. Materials classified at BSL-1 “are not known to cause disease in healthy adult humans.” *Halobacterium* sp. NRC-1 and KBT-1 do not survive outside their extreme environment, and few other organisms can survive the high salt of the media. Moreover, the organisms are destroyed by exposure to the low salt concentrations found in the human body and in tap water (and even in sea water). Even so, we always recommend treating microbes as though they might be pathogenic. We recommend use of personal protective equipment (gloves, goggles, and lab apron or coat) when working in a microbiology or chemistry laboratory setting. If some of the *Halobacterium* medium or culture does get past an apron and onto clothing, the characteristic odor can easily be washed out.

We suggest that you follow standard microbiology laboratory practice and autoclave labware (or soak it in a 1:9 solution [V:V] of household bleach and water) before disposal.

Background

The Student Guide includes the relevant background material.

To learn more about *Halobacterium* sp. NRC-1 and about molecular genetics in general, familiarize yourself with the HaloEd and MolGenT Web sites prior to these activities.

- HaloEd: <http://halo4.umbi.umd.edu/~haloed/> or access from a search engine by searching for “the HaloEd Project.” HaloEd was created to give educators, students, and the general public insight into Halophiles and their environment and to provide teaching tips.
- HaloWeb: <http://halo4.umbi.umd.edu> or access from a search engine by searching for “HaloWeb”. HaloWeb was created to facilitate viewing and understanding the genome of *Halobacterium* sp. NRC-1 and related organisms.

- MolGenT: <http://halo4.umbi.umd.edu/~haloed/MOLGENT/index.htm> (or access by searching for “MolGenT”). The Molecular Genetics Tutor at MolGenT is particularly helpful for students to strengthen their understanding and visualize the vital concepts required for this activity.

NOTES**Pre-labs**

The Pre-labs review concepts necessary for a more complete understanding and learning experience. To expand your students’ background knowledge, work through the pre-lab exercises either in class or as homework before each lab activity.

Teacher Preparation

1. Photocopy the Student Guide for each student.
2. For Activity 2:

Program the thermal cycler with the following times and temperatures:

- Step 1. 3 minutes at 94°C
- Step 2. 15 seconds at 94°C
- Step 3. 15 seconds at 60°C
- Step 4. 45 seconds at 72°C
- Step 5. Repeat steps 2–4 30 times.
- Step 6. 5 minutes at 72°C
- Step 7. Indefinitely held at 4°C (Note: Some machines do not have this capacity, in which case you must be present at the end of the cycle to transfer the samples to a refrigerator or freezer.)

Note: If you are using a machine that employs multiple blocks held at different temperatures (rather than the more common single-block machine that cycles between the different temperatures), use the program below:

- Step 1. 3 minutes at 94°C
- Step 2. 30 seconds at 94°C
- Step 3. 45 seconds at 60°C
- Step 4. 45 seconds at 72°C
- Step 5. Repeat steps 2–4 30 times.
- Step 6. 5 minutes at 72°C
- Step 7. Indefinitely held at 4°C

The PCR reaction takes approximately 1 to 2 hours to run. The amount of time varies with the machine.

3. For Activity 3:

Prepare 0.8% gel(s) and running buffer according to the volumes specified for your type of gel chamber.

The following directions and the materials in the kit are for making four gels for medium-sized gel chambers (gels approximately 9 × 9 cm).

Modify these instructions to suit the size of your gel chambers. The number of gels you require depends on the number of student samples and the number

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of wells on each gel. For each gel, reserve a lane for the 1-kb ladder (the molecular weight marker) and plan for an empty lane to allow students to reload if they make a mistake in loading the first time.

Preparation of 1× TBE buffer from the 20× TBE stock (provided)

Determine the number of gels and chambers required for your class.

- A. Determine the volume of buffer you need. Typically, you will need approximately 300–500 mL of 1× TBE buffer per gel chamber.
- B. To make 2.0 L of 1× TBE: combine 100 mL 20× TBE with 1900 mL of distilled or deionized water.

Preparation of agarose gels

Prepare the 0.8% agarose solution at least 40 minutes before running the gels; it takes about 15–30 minutes at room temperature for the gels to set. You may instead make gels a day ahead and store them covered with TBE buffer in the electrophoresis chambers until you are ready to use them. Review the *CarolinaBLU*[™] staining protocol in Appendix 7 to see if you want to add *CarolinaBLU* Gel and Buffer stain to your gels.

- A. Again, assuming you are making four gels, add 2 g of agarose to a 500-mL flask.
- B. Add 250 mL 1× TBE buffer.
- C. Dissolve the agarose using one of the methods described below. Be aware that the time it takes to dissolve the agarose will vary greatly, depending upon the volume prepared and the equipment used.

Microwave: Heat the agarose and 1× TBE in a microwave until no particulate matter is seen either in the solution or stuck to the bottom after a gentle swirl of the hot flask (10–20 minutes). To prevent boiling over during heating, watch carefully and swirl the flask at the first sign of boiling and periodically thereafter.

Hot Plate: If possible, use a stirring hot plate (with a magnetic stir bar). Otherwise, swirl the solution frequently to prevent boiling over and scalding, and to help disperse the agarose. Heat until no particulate matter can be seen in suspension or stuck to the bottom of the flask.

Boiling Water Bath: Make sure the water in the water bath is at least level with the agarose solution in the flask. Heat until no particulate matter can be seen in solution or stuck to the bottom of the flask. This usually takes 5–10 minutes. Allow additional time for the water bath to come to boiling.

- D. On a level surface, pour an appropriate volume of the solution into each of the gel trays and add combs with the appropriate number of teeth.
 - E. Once the gels have set, place the gel tray into the electrophoresis chamber filled with TBE and gently pull the combs from the gels.
4. Staining of Gels

See Appendix 6 and 7 for staining protocols for *CarolinaBLU* and GelGreen.

Instructor Notes

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Activity 1

1. For efficient use of time, have some students observe and select *Halobacterium* colonies while others prepare their microcentrifuge tubes.
2. Students need only a small amount of *Halobacterium* to get good results. As long as the quantity they take is visible and is evenly dispersed in the water, they should get adequate results. If they take too much *Halobacterium*, their lane on the gel may appear smeared and overloaded.
3. Cell lysates should be frozen (–20°C) in a freezer (preferably a non–frost-free freezer, which maintains a constant temperature). If the lysate is left at room temperature too long, the enzymes (nucleases) in the lysate will digest the nucleic acids.
4. You may stop the lab for the day at the end of this activity or continue with the exercise.

Pre-lab for Activity 2

If you assign this as homework, you may want to lead a class discussion the following day to reinforce the concepts addressed in the online tutorial. The tutorial requires the QuickTime® application.

Activity 2

1. Before class, aliquot the vial of primer mix into several other microcentrifuge tubes to avoid bottlenecks in the classroom.
2. Have students write their initials on the hinge or lip of the tube lid as well as on the side of the tube. (Sometimes, writing rubs off the side.) The students are instructed to retain their cell lysate tube on ice in case you would like to do additional reactions using the same lysate. You may also store the lysates in the freezer for later use by the students or other classes. **Note:** Even if kept in the freezer, the nucleases will, over time, diminish the DNA’s quality such that it will not produce as much PCR product as it did originally.
3. See Appendix 1 for primer annealing locations.

Pre-lab for Activity 3

We recommend watching the suggested video as a class. If you assign it as homework, you may want to lead a class discussion the following day to reinforce what students watched in the video. Ask questions to make sure that your students understand

- the specifics of loading gel lanes with the pipets that your class will use.
- what happens if the bottom of the well is punctured.
- the importance of using a new pipet tip for loading each lane.

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Activity 3

1. Dilute the 20× TBE buffer to 1× and pour the gels, allowing them to solidify before class (15–30 minutes).
2. To avoid bottlenecks, aliquot the DNA marker into several microcentrifuge tubes before class.
3. Note that students are to load 5 μL of their PCR reaction and 5 μL of the DNA ladder if the gels are being stained with GelGreen or a stain with similar sensitivity (e.g., ethidium bromide), but they are to load 10 μL of their PCR reaction and 25 μL of the DNA ladder if the gels are being stained with CarolinaBLU™ or a similar stain.
4. If more than one gel is required per class period, distinguish the different gels to avoid confusion during analysis by loading the 1-kb ladder in different lanes on each gel and/or by removing a specific corner(s) of each gel once it has finished running. Make sure that students record the distinguishing characteristic of the gel on which their sample is loaded.
5. As a backup for the students' records, use a chart like the following to record students' gel lanes. Insert student name or initials and colony type in the appropriate box. Also indicate which lane contains the 1-kb ladder.

	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
Gel 1										
Gel 2										
Gel 3										
Gel 4										

6. Refer to the manufacturer's instructions for your gel chamber for the voltage to use and the approximate time to run the gel. For example, when using TBE buffer with a 0.8% gel in a standard Carolina gel chamber (item 213641 or 213654), run the gel(s) at 135 V for 45–60 minutes.
7. The gels must be stained right away. Appendices 6 and 7 provide instructions for using CarolinaBLU or GelGreen stain. Decide whether you or the students will stain the gels.

Activity 4

1. Appendix 4 includes a picture of an ideal gel. Should your students fail to get results, you may photocopy the picture to provide students with data to analyze. (When you copy the picture, you may wish to omit the caption identifying which species each amplified PCR product is from.)
2. Lead a class discussion asking students to point out the banding pattern in their lane and describe the colony they isolated. Collect the class data, using the chart in Appendix 5 or some other method, and make it available to the class for individual or group analysis.

Answers to Questions in the Student Guide

Activity 4

1. Calculate the approximate number of base pairs (bp) in your DNA fragment(s). See the student guide for the full question. Using the provided primers, the expected sizes of the bands generated from *Halobacterium* sp. NRC-1 (wild type) and *Halobacterium* sp. KBT-1 are 1792 bp and 2325 bp, respectively.

Student Analysis and Follow-up Questions

1. You observed the possible phenotypes of *Halobacterium* on the petri plates.

a. Describe each of the phenotypes observed.

The three main phenotypes are 1) pink and opaque, 2) red and translucent, and 3) sectored (mix of both within one colony).

b. What do you propose was the source or cause of the different phenotypes?

Pink colonies have gas vesicles; red colonies do not. The red colonies have mutations in the gas vesicle gene cluster region. The sectored phenotype results from a phenotype-altering mutation occurring in the gas vesicle gene cluster region of one or more of the daughter cells early in the growth of the colony. Subsequent daughter cells of the mutant pass on the red phenotype.

Note: From the Background reading, students should understand that the gas vesicle gene cluster of the red colonies may contain an insertion sequence that disrupted a gene region responsible for gas vesicle production. When gas vesicles are present, as they are in the wild-type (pink) colonies, light is bent as it moves from the denser cytoplasm, through the hollow vesicles, and then back into the cytoplasm. This refraction changes the colonies’ appearance (or phenotype) from the inherent red, translucent color (without gas vesicles) to opaque pink.

2. From your background reading, why do you think that *Halobacterium* can be lysed with the simple addition of water?

Like its environment, a *Halobacterium* cell’s interior is very salty (a minimum of 2.5 M NaCl). When the microbes are placed in water, the cell membrane becomes unstable. In addition, the water is highly hypotonic to the cell interior and floods into the cell.

3. You lysed your cells in water.

a. What cellular components would you expect to find in your lysate?

Nucleic acids (DNA and RNA), proteins, and lipids, among others.

b. Why was the cell lysate centrifuged?

The spinning crudely separates the insoluble cellular debris from the soluble cellular components such as the nucleic acids (DNA and RNA).

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- c. Why was 100 μL of supernatant transferred to another tube?

The supernatant contains the nucleic acids. Moving it to another tube helps ensure that the unwanted cellular components spun to the bottom of the tube are not added to the PCR reaction along with the DNA. These unwanted components include proteins such as nucleases (enzymes that break down DNA and RNA) as well as other components that may inhibit PCR.

4. List the necessary components in the PCR reactions and explain the purpose of each.

- PCR bead—contains Taq polymerase, the nucleotides necessary for synthesizing new DNA, and the salts and buffer needed for creating the appropriate conditions for Taq polymerase.
- DNA template—provides the starting material from which more DNA is made.
- Primer mix—provides both the forward and reverse primers needed to specifically target DNA replication to a specific sequence and to provide the starting point for the polymerase to begin DNA synthesis.

5. You mixed the PCR bead, primers, and cell lysate sample thoroughly before placing the reaction in the thermal cycler. Why was the mixing necessary?

Evenly mixing all of the needed reaction components ensures that the primers, template DNA, and polymerase are in close enough proximity for the reaction to proceed.

6. A polymerase chain reaction goes through several cycles of temperature changes. List the three steps of a cycle and describe what occurs during each one.

Denaturation—The DNA strands are heated so that they separate from one another.

Annealing—The primers anneal, or attach to their complementary region on the template DNA.

Extension—The polymerase extends the sequence out from the primer sequence, thus replicating the targeted sequence of the DNA in your sample.

7. Why did you place your nucleic acid extract and PCR reagents on ice until the PCR reaction was set up and placed in the thermal cycler?

Any remaining nucleases in the cell lysate might break down the DNA template. The cold temperature inhibits this. (Also, keeping the reaction on ice until placement in the thermal cycler prevents the polymerase from starting to synthesize DNA before the primer has annealed in the correct location.)

8. What are two purposes served by the loading dye?

- to weigh down the sample (your PCR reaction) so it will sink to the bottom of the well and not float up into the buffer when it is loaded on the gel.
- to provide a visual aid (the color) to make it easier to load the sample and to track how far the molecules on the gel have run. Being able to track how far the molecules on the gel have run helps you predict where fragments of a particular size are running on the gel so that you know when to turn off the power to the gel chamber.

9. You loaded the PCR samples into the wells of the gel electrophoresis chamber.
- Are the wells (and thus your DNA) nearer to the positive or negative electrode?
The wells are closer to the negative (black) electrode.
 - Is DNA positively or negatively charged? How does its charge impact how the gel chamber is set up?
Because DNA is negatively charged (due to the negatively charged phosphate group in each nucleotide), it must be loaded on the side of the gel nearest the negative electrode. Since like charges repel and opposite charges attract, the DNA will travel away from the negative electrode, through the gel, toward the positive electrode.
10. How many bands appeared in your lane? What are the bands made of?
Answers will vary. Most likely, the students will see one band, but occasionally they may see two (if the template DNA came from a sectored colony). Sometimes, they see no bands. Absence of bands may result from a deletion or major reorganization of the PCR priming sites, or from student error. The bands consist of the DNA that was amplified.
11. Look at the table with class results to determine if your band(s) ran a different distance into the gel than some of your classmate's bands or if you had a different number of bands than others. Think about the observations you originally made about the colony types. Can you find a correlation between the size and number of bands you and your classmates observed in each lane (the genotype) and the phenotypes of the colonies used to generate those bands? Using the class data, explain any correlation or lack of correlation you see.
- If the sample was from a pink colony from the wild-type *Halobacterium* sp. NRC-1 plate, they are most likely to have a single 1792-bp band.**
 - If the sample was from a red colony from the *Halobacterium* sp. KBT-1 plate, they are most likely to have a single 2325-bp band. This band confirms that the colony is KBT-1. Banding patterns different from 2325- or 1792-bp indicate a novel mutant.**
 - If the sample was from a red colony from the wild-type plate, a novel mutant may or may not have bands at 1792 or 2325 bp, depending on the nature of the mutation in the *gvp* gene cluster. A mutation might be an IS element, a deletion, an insertion, or a single- or multiple-nucleotide change in the *gvp* gene cluster.**
 - If the sample DNA was from a sectored colony (most likely to be found on a wild-type plate if at all), they might see one or two bands—a 1792-bp band (from the wild-type portion of the colony) and, if the mutation in the red portion of the colony is in a region amplified by the primer, a second band as well.**
12. Your gel band gives you information about the genotype of the colony you isolated. On the basis of your observations regarding the phenotype and genotype of your colony, what genotype (strain) of *Halobacterium* do you hypothesize you isolated (*Halobacterium* sp. NRC-1, KBT-1, or another)? Identify whether your colony was wild type (wt) or mutant (mut). What makes you conclude that?
- See the answer to question 11.**

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Optional Questions:

13. Write a paragraph clearly explaining how an IS element can cause a colony of *Halobacterium* sp. NRC-1 to appear red.

Students should cover the concepts included in the following paragraph:

An IS element can disrupt a *gvp* gene, causing one of the gas vesicle component proteins to be made incorrectly such that gas vesicles are unable to form, form aberrantly, or form in reduced numbers. The absence of the gas vesicles changes how light interacts with the colony, causing the colony to appear red and translucent rather than pink and opaque.

14. Occasionally, it is possible to find a red colony (mutant) that yields a band that appears to be the same size and sequence as the wild-type (*Halobacterium* sp. NRC-1) band. Explain how this could occur.

The detection of mutations by PCR amplification is limited by the primers (forward and reverse) used. If an insertion sequence or other mutation occurred outside the area amplified by the primers used in this lab, but within the *gvp* gene cluster, the amplified DNA fragment would have a wild-type sequence like NRC-1, even though the colony assayed was red.

15. The DNA sequence in the following figure shows the location of some of the genome region you just amplified using PCR. The region shown contains the gene sequence for *gvpA*1. (If you have trouble with these questions, review Pre-lab Activity 1.)

- a. Why are some letters capitalized?

In DNA notation, capitalization is used to indicate nucleotides that are coding regions, which will be translated into an amino acid sequence.

- b. Label the gene start codon for *gvpA* (usually ATG).

See the label on the following figure.

- c. Label the gene stop codon for *gvpA* (usually TAA, TGA, or TAG).

The stop codon for *gvpA*1 is TGA. See the label on the figure.

- d. How did you pick your start and stop codon, given that there were multiple codons like them in the sequence?

These were the first start codon and last stop codon of the amino acid coding region, indicated by the capital letters, so they were the most likely to be functional (i.e., actually used as a start and stop codon).

forward primer: agtctgtgggcgggtagct

5' atgaaaaactggctgagctcgcgggggaataaacacgattccgggtgtagtctgtgggcgggtagctagattgggtgaact 3'
 3' TACTTTTTGACCGACTCGAGCGCCCCCTTATTGTGCTAAGGCCACA**TCAGACACCCGCCACTCGA**TCTAACCCACTTGA 5'

forward primer annealing location

5' cactacttctctccagtcgatggcggttagagcactcccgactagtaggtgagggctttcttctgcttcacgactgtctaaga 3'
 3' **GTA**atgaagagaggtcagctaccgccatctcgtgagggctgatcatccactccgaaagaagcgaagtgtgacagattct 5'
end of gvpD1

IS Element (ISH2) inserts between these 2 nucleotides

5' agctttacactctccgtacttagaagtagcactcattacaggagacataacgactggtgaaaccatacacatc**ct**atgt 3'
 3' tcgaaatgtgagagcatgaatcttcatgctgagtaatgtcctctgtattgctgaccactttggtagtgttaggaataca 5'

Start codon for gvpA1

5' gatgcccagtatagttagagatgggttaatcccagatcacc**ATG**GCGCAACCAGATTCTTCAGGCTTGGCAGAAGTCC 3'
 3' ctacgggctcatatcaatctctaccaattagggcttagtggttaccgcttggctctaagaagtccgaaccgctctcagg 5'

5' TTGATCGTGTACTAGACAAAAGGTGTCGTTGTGGACGTGTGGGCTCGTGTGTCGCTTGTTCGGCATCGAAATCCTGACCGTC 3'
 3' aactagcacatgatctgtttccacagcaaacacctgcacaccgagcacacagcgaacagccgtagctttaggactggcag 5'

5' GAGGCGCGGGTCGTCGCCCTCGGTGGACACCTTCCTCCACTACGCAGAAGAAATCGCCAAGATCGAACAAGCCGAACT 3'
 3' ctccgcgcccagcagcggcgagccacctgtggaaggaggtgatgctcttcttttagcgggttctagcttgttcggcttga 5'

Stop codon for gvpA1

5' TACCGCCGGCGCCGAGGCGGCACCCGAGGCC**TGA**cgcacaggcctcccttcggcccggcgtaagggaggtgaatcgcttg 3'
 3' atggcgccgcggtccgcccgtgggctccggactgctgtcggaggggaagccgggcccgcattccctccacttagcgaac 5'

start of gvpC1

5' caaaccatactattaacacccttctcgggtacacactaatccc**ATG**AGTGTACAGACAAACGCGACGAGATGAGTACTG 3'
 3' gtttggtatgataattgtgggaagagccatgtgtgattagggtagctcacagtgtctgtttgctgctctactcatgac 5'

A portion of the wild-type *Halobacterium* sp. NRC-1 gvpA1 gene region sequence amplified using the primers in this kit.

16. Translate the capitalized sequence for *gvpA1* (from question 15) into an amino acid sequence. Use the sequence editor function on HaloWeb to help you do this.

Be aware that some students may remember from the pre-lab exercise that they can get the amino acid sequence directly from the HaloWeb site without having to use the sequence editor.

Go to HaloWeb and click the “search” tab. Search for *gvpA1*, get the sequence, and copy and paste it into the sequence editor window—the top strand sequence (all in capital letters) for *gvpA1* from the start to stop only. Click “translate it,” and the sequence editor gives the following amino acid sequence:

**M A Q P D S S G L A E V L D R V L D K G V V V D V W A R V S L V G I E I L T V E A
 R V V A A S V D T F L H Y A E E I A K I E Q A E L T A G A E A A P E A**

M = methionine, A = Alanine, Q = Glutamine, etc.

17. When the *gvpA1* gene region was amplified from KBT-1 and sequenced, the following sequence was obtained. Note how the KBT-1 sequence differs from the NRC-1 sequence used for question 15. How might such a difference affect the phenotype?

forward primer: agtctgtgggCGGGTgagct

5' atgaaaaactggctgagctcgcgggggaataacacgattccgggtgtagtctgtgggCGGGTgagctagattgggtgaact 3'
 3' TACTTTTTGACCGACTCGAGCGCCCCCTTATTGTGCTAAGGCCACA**TCAGACACCCGCCCACTCGA**TCTAACCCACTTGA 5'

forward primer annealing location

5' cactacttctctccagtcgatggcggtagagcactcccgactagtaggtgaggtcttcttcgcttcacgactgtctaaga 3'
 3' GTAatgaagagaggtcagctaccgccatctcgtgagggctgatcactccgaaagaagcgaagtgtgacagattct 5'

end of *gvpD1*

5' agctttacactctccgacttagaagtaacgactcattacaggagacataacgactggtgaaac**ccatacacatcc****cattcg** 3'
 3' tcgaaatgtgagaggtcagctaccgccatctcgtgagggctgatcactccgaaagaagcgaagtgtgacagattct**gtaagC** 5'

5' **tcttttagttaagaaatcgcgtagacagcggtaggatctcttcgctgtgcaagacgCGGCTgagatctcttttgataatatt** 3'
 3' **agaaatcaattcttttagcgactgtcgccatcctagagaagcgacacgcttctcgCGCACTctagagaaaactattataa** 5'

3' **ttaacctaaatcgaaataagacgataatcttacCGTgcaccCGTgcacgtaatttctaagagCGTctaagactatggctc** 5'
 5' **aattggatttagctttattctgctattagaatggcagctgggCCacgtgcataaagattctcgcagattctgataccgag** 3'

3' **gaacccaaatgggCGTctccatccgaactgaactcgttgatgaactcgattcactcgtcogatgagtggtcagatctcggc** 5'
 5' **cttggttttaccCGcagaggtaggcttgacttgagcaactacttgagctaagtgagcagctactcacaagcttagagcct** 3'

3' **gcaagccgctccgagatcgttgaagccatcctcagcagatattttcagaacgatgaagacccaaatcaaacagacgCGgaga** 5'
 5' **cgttcggCGagggctctagcaactcCGgtaggagtgtcgtataaaaagtcttgcactctcgtggtttagtttgtctgCGctct** 3'

3' **gctgattatccgcaacagaaaacgctctaactcgttagagaagttcgtgcaccgagtgcaCGaacttcttagagcgtcatt** 5'
 5' **cgactaataggCGttgtcttttgcgagattgagcatctctcaagcagctggctcagctgcttgaagaatctcgcagtaa** 3'

3' **caaaaccagcacttagctaaattaatocggattggcttgggCGaatagaaaatctctctcactgttcagccctacgc** 5'
 5' **gttttggtgctggaatcgatttaattatagcctaaccagaaccCGcttactctttagaagagaagtgacaagctcgggatgCG** 3'

3' **catgtggCGgtttcgtcttaactaaagacgaatgCCatacacatcc**ttatgtgatgcccagatagattagagatgggt 5'
 5' **gtacaccgCCaaagcagagaattgattctcgtctacCGgtatgtgtagg**aatacactacgggctcatatcaatctctacca 3'

Start codon for *gvpA1*

5' taatcccagatcacc**ATG**CGCAACCAGATTCTTCAGGCTTGGCAGAAGTCCTTGATCGTGTACTAGACAAAGGTGTCTG 3'
 3' attagggcttagtggttaccCGgttggcttaagaagtcgaaccgctctcaggaaactagcacatgatctggtttccacagc 5'

5' TTGTGGACGTGTGGGCTCGTGTGTCGCTTGTTCGGCATCGAAATCCTGACCGTCGAGGCGCGGGTTCGTCGCCGCTCGGTG 3'
 3' aacacctgcacaccgagcacacagcgaacagccgtagctttaggactggcagctccgCGccagcagcggcgagccac 5'

5' GACACCTTCTTCCACTACGCAGAAGAAATCGCCAAGATCGAACAAGCCGAACCTTACCGCCGGCGCCGAGGCGGCACCCGA 3'
 3' ctgtggaaggaggtgatgctctctttagcgggtcttagcttggcttggcttgaatggCGccgCGgctccgCGctgggct 5'

Stop codon for *gvpA1*

5' GGCC**TGA**cgcacaggcctcccttcggccCGgctaagggaggtgaatcgcttgcaaacatactattaacacccttctcg 3'
 3' ccggactgCGgtcCGgaggaagcCGgccgcatccctccacttagcgaacgcttggatgataattgtgggaagagc 5'

start of *gvpC1*

5' ggtacacactaatcccATGAGTGTACAGACAAACGCGACGAGATGAGTACTG 3'
 3' ccatgtgtgattagggactcacagtgctgcttggcgtgctctactcatgac 5'

Halobacterium sp. KBT-1 sequence of *gvpA1* gene region. The region of boldface text indicates an insertion sequence (IS) in the promoter region of *gvpA*. The nucleotides between which the sequence inserted are shaded gray. The underlined sequence on each side of the insertion is the direct repeat. The direct repeat results from the insertion element's copying the 12 nucleotides located just before the insertion site and repeating them at the end of the IS as it inserts.

Most importantly, this sequence contains an insertion sequence that disrupted the genome just before the *gvpA1* gene. The disruption likely caused a reading frame shift, which led to this species’ inability to form gas vesicles.

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18. On the HaloWeb genomes page (<http://halo4.umbi.umd.edu/>), click on *Halobacterium* NRC-1, and then on the Resources link. Click on the Insertion Sequence elements (IS elements). On the basis of the size difference between the sequences listed for questions 15 and 17, which IS element do you predict may have inserted? Note that the length of the IS elements as shown in the chart does not include either direct repeat.

ISH2. This is determined by first finding the difference in length between the sequences (which, not counting the added 12-bp direct repeat, should be 533 bp). Students then subtract from 533 the 12 bp from the second direct repeat, for a count of 521 bp. On the Web site, scroll down the “length” column to find an IS element that matches that length. ISH2A, ISH2B, ISH2C, and ISH2D are all acceptable answers. Note: Some students may note that the ISH2 elements in the chart have direct repeats that are 10 bp rather than the 12 bp they see in their sequence. For a given IS element, direct repeats are usually a characteristic length, but some variation occurs.

Extension Activities

- Use bioinformatics tools to design your own primers for amplifying the gas vesicle protein genes. Do this in theory, or have a commercial lab produce your primers so you can try them out.

To design a primer to amplify a sequence, do the following:

- Look at the top strand only and select about 20 bases before (5′) and after (3′) the region of interest and highlight them. By convention, the top strand is depicted 5′ to 3′ from left to right. In addition, if the gene you are working with is on the top strand, the top strand is referred to as the coding, or sense strand, and the bottom strand is referred to as the noncoding or antisense strand.
- If the gene is located on the top strand, name the primer 5′ of the sequence you want to amplify the *forward* primer (i.e., *gvpA1* forward). Because DNA replication proceeds 5′ to 3′, the sequence of the primer 5′ to the gene will have a sequence identical to a sequence in the coding strand and will be the reverse complement of a sequence in the noncoding strand.
- To design the reverse primer (e.g., *gvpA1*Reverse), paste the 3′ sequences you highlighted on the coding strand (the upper strand) into the sequence editor and click on “reverse complement” and then “Do it.” Remember that the sequence editor can be found on the left sidebar on the NRC-1 HaloWeb page (<http://halo4.umbi.umd.edu/cgi-bin/haloweb/nrc1.pl>).
- The output sequence in the lower window will be the reverse complementary sequence to the 3′ end sequence that you entered. This

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is the sequence you use to order the reverse primer. It is standard when placing primer orders to write the sequence 5' to 3'

- f. In the laboratory, the following guidelines are followed to design an effective primer:
 - The primer has a relatively low GC content (<60%) because the GC bond is harder to break than the AT bond.
 - The primer does not have a run of bases of the same kind, especially Gs or Cs (e.g., GGGG).
 - The primer has no predicted hairpin turns (usually the ordering Web site can provide information regarding this), especially not at the 3' end.
 - Most importantly, the primer sequence is unique—found nowhere else in the genome. Check for this by BLASTing the desired primer sequence using the HaloWeb page (the BLAST tool can be found on the left sidebar; see “BLAST NRC-1 genome”). The primer sequence should have homology only to your targeted region. If it is not unique to the region, then choose another set of bases further 5' or 3' from the region of interest.
2. Experiment with how gas vesicles from *Halobacterium* sp. NRC-1 respond to pressure. First, obtain a liquid culture of *Halobacterium* sp. NRC-1. Wearing safety goggles and lab aprons, fill two microcentrifuge tubes to the brim with a stationary phase (pink) liquid culture of *Halobacterium* sp. NRC-1. Quickly snap the cap shut on one of them and observe. What happens to the color of the culture in the closed tube? What do you think happened to the gas vesicles? You can look at the cells under the phase microscope and see the difference in their appearance. Also, you can let the two tubes sit for a few weeks on the bench and then see what happens.
3. *Halobacterium* offers many engaging topics for research. For instance, what is the connection between this archaeon and the search for life on other planets? How is *Halobacterium* metabolism different from that of other organisms? Research these or other aspects of *Halobacterium* that may have sparked your interest.

References and Resources

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NOTES

Web Sites

At the time of this printing, the Web sites given below were active.

- <http://halo4.umbi.umd.edu/>
- <http://halo4.umbi.umd.edu/~haloed/>
- <http://halo4.umbi.umd.edu/~haloed/MOLGENT/index.htm>
- <http://www.ncbi.nlm.nih.gov/>
- <http://www.nobelprize.org/>

Related Products

Following is a list of related items available from Carolina Biological Supply Company. For more information, please refer to the most recent **Carolina™ Science** catalog, call toll free 800-334-5551, or visit our Web site at **www.carolina.com**.

154771	Basic Microbiology Skills Kit, Part 1
154772	Basic Microbiology Skills Kit, Part 2
154776	Antibiotics in Action Kit
154773	Extremely Easy DNA Extraction Kit
154770	Introduction to Life in an Extreme Environment Kit
821449	<i>Halobacterium</i> Broth, 5-mL tubes, Pack of 10
154777	Culture Packet of Brine Salt Inclusions
154800	<i>Halobacterium</i> sp. NRC-1, Tube
154801	<i>Halobacterium</i> sp. NRC-1, Plate

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The Genotype–Phenotype Connection: Basic Molecular Genetics and Bioinformatics Skills

Background

Nucleic Acids

Deoxyribonucleic Acid (DNA) is a polymer of nucleotide subunits. Each nucleotide contains a 5-carbon sugar (deoxyribose), a negatively charged phosphate group, and a nitrogenous base. Because of the way the nucleotides that form the DNA molecule are linked together, one end of the DNA molecule ends in a phosphate and the other in a hydroxyl group. The end with the phosphate group is always referred to as the 5' end, and the end with the hydroxyl group, as the 3' end. The 3' and 5' designations are derived from how the carbons in an organic molecule are described; they are described as either 1', 2', 3', etc., depending upon their position in the molecule. The hydroxyl group at the 3' end of a DNA molecule is attached to the 3' carbon on the deoxyribose sugar. The phosphate group at the 5' end is attached to the 5' carbon on the deoxyribose sugar. This 5' and 3' notation is important when studying the replication of DNA and designing primers for molecular biology experiments. New nucleotides are always added to the end with the hydroxyl group. Thus, a single-stranded piece of DNA (such as a primer) can be extended only from the 3' end (5' to 3').

When the nucleotides are joined together to form a polymer strand of DNA, the sugar and phosphate groups link in an alternating pattern to form a sugar–phosphate backbone. DNA is a double-stranded structure in which the two strands of DNA are joined together in an antiparallel arrangement, meaning that one strand runs in the 5' to 3' direction and the other runs in the 3' to 5' direction. The nitrogenous bases are complementary to each other, with adenine (A) pairing with thymine (T), and guanine (G) pairing with cytosine (C).

For the sake of simplicity, the DNA double helix is often depicted in a two-dimensional form (like a drawing of a ladder), but the molecule is in fact helical (like a twisted ladder) with 10.5 base pairs per turn as described by Francis Crick, James D. Watson, and Maurice Wilkins who shared a Nobel prize in 1962 for the discovery of the DNA double helix (Watson and Crick, 1953). Their discovery was made possible by X-ray diffraction images created by Rosalind E. Franklin.

During the process of transcription, DNA is used as a template to create strands of messenger ribonucleic acid (mRNA). Like DNA, RNA is a polymer composed of four types of nitrogenous bases (in RNA, uracil is used in place of thymine). Unlike DNA, RNA is a single strand, and the bases are attached to ribose rather than to deoxyribose. Messenger RNA codes for the amino acids that form proteins. In this process of translation, carried out by the ribosomes, the mRNA nucleotides are read in groups of three. Each of these triplet codons designates a specific amino acid to be added to the growing polypeptide chain. Deciphering this process secured Robert W. Holley, H. Gobind Khorana, and Marshall W. Nirenberg a Nobel Prize in 1968 (Khorana, 1965).

Genotype and Phenotype

The central dogma of molecular genetics is that information is transferred from DNA to mRNA to protein. The genotype of an organism is the nucleotide sequence of its DNA. The nucleotide sequence dictates,

through transcription and translation, which proteins are produced. The proteins carry out many of the specified functions of the cell and thus determine the organism's phenotype.

This laboratory exercise uses the microorganism *Halobacterium* and focuses on how the cell's genotype, with respect to the gas vesicle protein (*gvp*) gene cluster, dictates whether or not the cells produce intracellular gas-filled vesicles. Depending on the DNA sequence of the *gvp* genes, a cell colony generally has one of three possible phenotypes:

- pink and opaque (wild-type colonies of *Halobacterium* sp. NRC-1)
- red and translucent (mutant derivatives of *Halobacterium* sp. NRC-1, such as KBT-1)
- sectored (sectors of pink and red cells within one colony).

The pink color results from the presence of gas vesicles, and the red color results from their absence.

Colony Phenotype

Each cell deposited on the surface of an agar plate may give rise to a colony by exponentially dividing into “identical” daughter cells (clones) via binary fission. Colony morphology, the visual appearance of colonies, has long been used by microbiologists to determine the nature of organisms that they observe. Details they look for include

- the perimeter of the colony. (Is it smooth, wavy, rough, or uneven?)
- the surface of the colony. (Is it glossy, matte, smooth, or rough?)
- the height of the colony. (Is it raised or flat?)
- the color of the colony.
- opacity or transparency of the colony.

Haloarchaea

Haloarchaea are thought to be ancient life forms. They are classed in the Archaea, one of the three domains of life (the other two are the Eukaryotes and the Bacteria). You may sometimes see the Archaea referred to by their original name, the Archaeobacteria, but the Archaea are no longer considered “bacteria” at all (though both domains consist of prokaryotes). Many archaeons are extremophiles, organisms that live in unusual environments (e.g., extremes of heat or cold or pressure or—in the case of haloarchaeons—salinity).

Halobacterium is a halophile (“halo” for salt, “phile” for lover). In this lab activity, you will work with *Halobacterium* sp. NRC-1 (wild type) and *Halobacterium* sp. KBT-1 (a mutant derivative). As in the naming of other organisms, “sp” refers to “species.” These microbes require extremely high salt concentrations in order to maintain the stability of their membrane. They grow in media with NaCl concentrations up to 5.2 M (saturation). Their ability to grow in environments with high salt concentrations and their mechanisms for handling other harsh environmental factors (e.g., toxic metals and high levels of radiation) allow these organisms to persist in surprising places. They can sometimes even be isolated from table and rock salt. However, the unique adaptations required to survive these extreme conditions make them incapable of surviving in what humans consider “normal” environments. *Halobacterium* sp. NRC-1 lyses (bursts open) when salinity levels around it are lowered to less than about 2 M NaCl. For comparison, sea water has a total salinity of 0.6 M, while human blood contains only 0.14 M NaCl.

Halobacterium sp. NRC-1 has been studied for decades and was among the first microbes to have its genome sequenced (Ng et al., 2000). The DasSarma laboratory led the sequencing effort and created the

HaloWeb genome Web site, <http://halo4.umbi.umd.edu/>. At HaloWeb, anyone can explore the whole genome of *Halobacterium*—from gene locations, to gene sequences, to protein sequences, and more. The site also includes access to other, more recently derived haloarchaeal genomes (SL DasSarma et al., 2010.)

Gas Vesicles

When *Halobacterium* sp. NRC-1 cultures are observed on an agar plate, most colonies appear pink (pink is the “wild-type” phenotype). However, red or sectored colonies may also be found. A sectored colony looks like a red or pink pie with one or more slivers of the other color in it. The red color is the inherent color of the cells. However, the wild-type cells produce a type of organelle called a gas vesicle (GV), which refracts light, changing the red translucent appearance of the colonies or liquid cultures to an opaque pink. Refraction is the bending of light as it moves through materials of different density, such as the cytoplasm and the hollow gas vesicles of the cell.

Gas vesicles allow the cells to float to the surface of their briny habitats (like the Great Salt Lake in Utah). Access to oxygen or light often allows the organisms to produce energy most efficiently. These extraordinary microbes obtain energy through a variety of means. Their most efficient use of energy is aerobic respiration, but they are also capable of phototrophy, a process of obtaining energy directly from sunlight. In addition, they can obtain energy anaerobically.

Gas vesicles occur in a few groups of bacteria and archaea. A GV consists of a membrane made solely of protein formed into a pouch and filled with ambient gas. In *Halobacterium*, the 13 genes that code for gas vesicle proteins (GVPs) are organized into two divergent operons (operons are sets of genes that are expressed together; divergent means transcription from the operons goes in opposite, or divergent, directions) (DasSarma, et al., 1994; DasSarma and Arora, 1997).

Transposable Genetic Elements

In 1983, at age 81, Barbara McClintock received a Nobel Prize for her work in discovering transposable elements (McClintock, 1961; Fedoroff, 1984). Transposable elements are segments of DNA that may relocate in a genome, often showing little, if any, specificity as to where in the genome it integrates. Some transposable elements actually make a copy of themselves that inserts into a different part of the genome, while the original transposable element stays in place.

You have transposable genetic elements in your genome. More than 10% (by mass) of your human genome can be attributed to transposable elements. These relocating segments of DNA include the *Alu* gene family of mobile elements (Cook et al., 2011). In prokaryotes, there is a specific class of transposable elements known as insertion sequence (IS) elements.

When an IS element inserts into a vital region of the genome, the effect of this mutation may be observed phenotypically. For example, in the case of *Halobacterium* sp. NRC-1, disruption of critical areas in any of the genes coding for gas vesicle proteins can cause a change in color of the organism (approximately 1% of NRC-1 colonies spontaneously show evidence of this kind of genotypic–phenotypic change).

Research into the apparent ease with which *Halobacterium* changes from a pink to a red phenotype led to the discovery of transposable insertion sequences in *Halobacterium*. It was found that the IS elements commonly inserted at certain “hot spots,” such as in the gas vesicle gene region. These insertion sequences were named Insertion Sequences in Haloarchaea (ISH) (DasSarma et al. 1987, Jones et al. 1989, DasSarma 1989).

Polymerase Chain Reaction

Polymerase chain reaction (PCR) is the primary way scientists amplify (make copies of) genetic sequences to get a quantity sufficient for various types of analysis (such as visualization on a gel). Forensics, medical diagnostics, and environmental testing laboratories use PCR for DNA fingerprinting and species identification. The method uses primers and the enzyme DNA polymerase along with a series of temperature changes to amplify DNA. The Taq polymerase used in PCR is heat stable.

The primers used in PCR are short segments of DNA (around 20 bp) that are designed to amplify a specific genetic sequence. Two primers are used: a forward primer and a reverse primer. The forward primer is composed of sequence from the 5' end of the coding strand of the sequence to be amplified, and the reverse primer is the reverse complement of the 3' end of the coding strand. **Note:** *By convention the top strand is depicted 5' to 3' from left to right. If the gene you are working with is on the top strand, the top strand is referred to as the coding strand. If the gene is on the bottom strand, the bottom strand is the coding strand.* Using the primers as a starting point, the DNA polymerase synthesizes DNA 5' to 3'. After multiple rounds of amplification, the polymerase chain reactions result in amplification only of the DNA that is between the forward and reverse primers.

PCR involves a series of temperature changes applied to denature double-stranded DNA bonds, to anneal primers, and then to replicate DNA. This series of temperature change steps is repeated multiple times.

Details of the steps of PCR can be described as follows (these steps are also demonstrated in MolGenT at <http://halo4.umbi.umd.edu/~haloed/MOLGENT/d03.htm>):

1. **The denaturation step:** The double-stranded DNA used as the template is denatured by heating to a high temperature, usually 94°C. Separating the two strands allows the primers to bind.
2. **The annealing step:** The temperature is reduced to allow the primers to anneal to their complementary sequences on the denatured single strands of DNA.
3. **The extension step:** The temperature is brought up to 72°C, and DNA polymerase extends new strands of DNA, using each primer as a starting point and the single strands of DNA as the templates. The series of steps is repeated, and the number of new DNA strands doubles after each round. Going through 30 rounds can produce, from one double-stranded molecule, over a billion copies of the desired DNA fragment ($2^{30} = 1,073,741,824$).

The optimal annealing temperature for a given set of primers depends on the length of the primers and on the GC content (a larger proportion of guanine–cytosine bonds typically requires a higher annealing temperature).

Electrophoresis

Electrophoresis is a process by which fragments of DNA can be separated by size. During electrophoresis, DNA is drawn through an agarose gel matrix by an electric current. Agarose is a highly purified seaweed extract that forms a gel when heated in liquid and cooled. The agarose is dissolved in buffer and heated. When the solution is poured into the gel tray, it solidifies, forming a matrix through which the DNA is run. DNA has an overall negative charge due to the negatively charged phosphate groups in its backbone. When placed into the wells at the end of the gel nearer the negative electrode, the DNA moves through the gel toward the positive electrode. DNA fragments of different size move through the gel at different rates. A gel is generally run until the fastest (shortest) fragments almost reach the positive electrode. Fragments of the same size form a discrete band since they pass through the gel matrix at the same rate. The largest fragments form bands closest to the loading wells, and the smallest fragments form bands

farthest away. A DNA ladder, a set of DNA markers containing fragments of known size, is loaded into one lane of a gel. By comparing the locations of the amplified PCR fragments with those of the marker bands, one may determine the approximate sizes of the amplified fragments.

The gel is made with and immersed in a salt buffer to provide the ions necessary to carry electric current through the gel. In this exercise, we will use TBE buffer (Tris–borate EDTA). To make the gel-loading process go more smoothly in the classroom, loading dye is added directly to the reaction mix prior to PCR. Sugar in the loading dye makes the DNA sample denser than the buffer so that it sinks to the bottom of the well. The loading dye used in this lab also contains a dye called cresol red (which, unlike most dyes, does not inhibit the action of Taq polymerase). On a 0.8% gel, cresol red runs through the gel at approximately the same rate as a 1.5-kB DNA fragment. By watching the progress of the dye through the gel, one knows when to turn off the power supply.

Bioinformatics

Bioinformatics is a science involving the computer storage and analysis of molecular biology data. Bioinformatics came about after the amount of data generated by experiments worldwide became so overwhelming that biologists needed help analyzing, organizing, and archiving it. For example, the *Halobacterium* sp. NRC-1 genome is about 2.5 million base pairs in size, and codes for about 2500 proteins. Imagine trying to organize and analyze the data that generated this entire sequence without the aid of computers and programmers.

This lab will use bioinformatics to evaluate information regarding the gas vesicle gene region. Bioinformatics tools such as those found on HaloWeb (<http://halo4.umbi.umd.edu/>) will help as you explore the genotype of the colorful colonies of *Halobacterium* sp. NRC-1 and its mutants.

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The Genotype–Phenotype Connection: Basic Molecular Genetics and Bioinformatics Skills

Introduction

Following these instructions, you will use the model microbe *Halobacterium* to perform four related activities: 1) *Halobacterium* Exploration and Cell Lysis (including identification of colony phenotype), 2) Polymerase Chain Reactions (PCR), 3) Gel Electrophoresis, and 4) Data Comparison and Analysis. In the analysis, you and your classmates will compare data. Your careful observations of the *Halobacterium* colony phenotypes at the beginning of the lab will be critical in deciphering your results later.

Activity 1: *Halobacterium* Exploration and Cell Lysis

Pre-lab

HaloWeb is a haloarchaeal-specific bioinformatics resource tool that has been publicly available for over a decade. It can be used to explore the genomes of several Haloarchaea, including *Halobacterium* sp NRC-1.

Use HaloWeb to explore *Halobacterium* gas vesicle genes.

1. Go to the HaloWeb entry page (<http://halo4.umbi.umd.edu> or access from a search engine, by searching for “HaloWeb”).
2. Click on “*Halobacterium* sp. NRC-1” to go to the individual genome web page.
3. Click on the “search” tab to search the genome.
4. The “both” button is already preselected for you.
5. Type in “gvpA1” into the annotation search box, in order to search for the *gvpA1* gene related to gas vesicle assembly.
6. Click on the “search” button on the end of the page or hit return.
7. Select “gvpA1,” one of the gas vesicle protein genes that is integral in the formation of gas vesicles. You may choose *gvpA1* for either replicon (plasmid).
8. Now you are on the gene web page. Here you can explore all aspects related to the gene and the regions around it. Try out some of the options listed on the page:
 - “Get data for this gene” gives the DNA sequence of *gvpA1* as well as the amino acid sequence.
 - Either “BLAST” option could be chosen if you had a DNA/RNA or protein sequence you wanted to compare to the *gvpA1* nucleotide or an amino acid sequence to examine for similarities or differences.
9. You can expand or shrink the region you want to see on the map. For example, use the drop-down menu “Number of genes in this neighborhood” and select 23 genes for the region you want to see. Scroll down to see all 23 genes in the *gvpA1* gene region. Just below the chart of surrounding genes, scroll down to look at the gene map. Notice that there are other *gvp* genes surrounding *gvpA1*. Notice the location of *gvpD1*. Also note that there are insertion elements (ISH8-1, ISH3-2, etc.) just downstream of *gvpA1*. You may wish to select fewer than 23 genes or more than 23 genes in the area around *gvpA1* to get a different view.

10. To see the nucleotide and consequent amino acid sequences for the gene sequence you have chosen in the neighborhood of *gvpA1*, click on the “submit” button at the bottom of the screen. **Note:** your computer must be able to open a pop-up for this function, so be sure to check if pop-ups are enabled if at first you do not get a sequence.
11. Look at the DNA sequence that has come up in the pop-up window. You will notice that some of the bases are capitalized and some are not. Bases that are transcribed into RNA are capitalized; untranscribed areas, known as intergenic regions, are shown in lowercase.

Note: You may stop here. However, if time permits and you want to learn more about how to use HaloWeb, proceed with steps 12–15.

12. Go back to the gene Web page and select “show top strand only” at the bottom of the screen, then hit “submit” as before. Now you will get a single-stranded version of the gene region.
13. You can also see if there are other, similar regions in the genome. Select and copy the whole or partial sequence and then click on the “BLAST NRC-1 Genome” link on the main page.

The BLAST function on the HaloWeb site is carried out at the NCBI (National Center for Biotechnology Information). A BLAST allows you to compare a DNA sequence with those already in the genome databases. Potentially homologous (similar) regions between the sequence you submit and any sequences in the database are identified, and scores are assigned to indicate the likelihood that the sequences are truly homologous.

14. Paste your sequence into the “Enter Query Sequence” box and then click the “BLAST” button near the bottom of the page. Your sequence is the query sequence (referenced below).
15. Click on “View report” to see the NCBI results. (The report may come up on its own.)
 - “Graphic summary” shows you how similar the sequence you copied is to other sequences within *Halobacterium* sp. NRC-1.
 - The “Descriptions” chart includes a query coverage that tells what percentage of your query sequence is found elsewhere in NRC-1.
 - “Alignments” gives you a base-pair alignment of your query to the subject (*Halobacterium* sp. NRC-1). Vertical lines between the two sequences indicate exact matches. Spaces between the vertical lines mean there was not an exact match. Dashes tell you that there was a misalignment and that space was added to get the sequences to align from that point forward.

Materials

- 2 sterile microcentrifuge tubes, 1.5-mL capacity
- fine-tip permanent marker
- 3 petri plates with *Halobacterium* NCR-1 culture(s), shared among the class
- 2 petri plates with *Halobacterium* KBT-1 culture(s), shared among the class
- 1 micropipet for measuring 100 μ L and 500 μ L (per student or student group) with sterile tips
- sterile distilled water
- vortexer (not essential)
- microcentrifuge
- dissecting scope or hand lens (not essential)
- gloves

Laboratory Procedure

1. Set up a clean, sterile work area as your teacher instructs.
2. Get your two microcentrifuge tubes. With a permanent marker, label one tube “#1” and the other “#2 extract.” Set the tube labeled “#2 extract” aside for later use.
3. Add 500 μL of sterile distilled water to your microcentrifuge tube labeled “#1.”
4. Observe a petri plate of *Halobacterium* colonies by holding it up to a light. To make more detailed observations, use a hand lens or a stereomicroscope.
5. Describe at least two different-looking colonies. Include color and colony morphology (e.g., Do the colonies have smooth or irregular borders? Are the colonies raised or flat? Are they matte or glossy? Are they opaque or translucent?)

Colony description (color, etc.)	Colony morphology observations

6. While observing the various *Halobacterium* colonies, select one colony from which to extract DNA. Use a permanent marker on the bottom outside of the petri dish to circle your colony and mark it with your initials. Your colony information will be compared with that of your classmates. The compilation of all the data will allow you to form a hypothesis regarding the genotype of your colony.
7. Observe your colony with a hand lens or stereomicroscope, if available. Use varying amounts of light on the scope or hold the plate up to the light for thorough examination. It is critical that you carefully observe and accurately record the features of your colony:

Describe in detail the appearance of your colony.	Diagram your colony, noting color.

8. Using a micropipet with a sterile tip (or a disposable sterile plastic micropipet), pick up your colony from the surface of the agar using a swiping motion (be careful not to stab the agar.) Depending on the colony's size, you may not need to take all of it. As long as you have a visible amount of material on your pipet tip, you have enough. Make sure not to touch your pipet tip to any other colony or surface.
9. Put the pipet tip (containing the colony) into your tube labeled #1. Pipet up and down at least 10 times in the distilled water to remove your colony from the tip. Continue this action until you no longer see your colony on the tip or colony fragments in the water. Make the solution homogeneous.
10. Securely close the cap of your microcentrifuge tube.
11. Allow your microcentrifuge tube to sit undisturbed on the lab bench for 5 minutes. This will ensure that the cells from your colony are all lysed. You now have "cell lysate" in your tube. If you let the cell lysate stand too long, the DNA may be degraded by cellular enzymes called DNases.
12. Vortex (or tip the tube side to side) until mixed.
13. Spin your tube of cell lysate in a microcentrifuge at maximum speed for 1 minute to pellet the insoluble cellular fragments (a pellet may not be visible.) The water-soluble components of the cell, including the DNA, will be in the supernatant (liquid above the pellet).
14. Transfer 100 μ L of the supernatant into your unused tube labeled "#2 extract." (To avoid disturbing the material at the bottom, pipet carefully from the very top when collecting the supernatant.)
15. Follow your teacher's instructions for storage of the #2 extract tube and disposal of tube #1.
16. Answer questions 1–3 in Analysis and Follow-up Questions.

Activity 2: PCR

Pre-lab

Go to the MolGenT (Molecular Genetics Tutor) Web site to watch the unit on PCR:
<http://halo4.umbi.umd.edu/~haloed/MOLGENT/index.htm>

Note: This activity requires that you have QuickTime installed on your computer in order to view the units.

1. Go to Part D: Recombinant DNA and Society.
2. Click on the PCR Amplification tutorial link (on the left menu) and view the animation. This narrated animation demonstrates how PCR works.

Materials

your microcentrifuge tube from Activity 1, containing nucleic acid extract (labeled “#2 extract”)
1 0.2-mL PCR tube containing a PCR Ready-To-Go Bead™
fine-tip permanent marker
micropipet (4- to 20- μ L capacity) with sterile tips
ice (in beaker or plastic cup)
1 vial Primer Mix, shared among several students
access to thermal cycler

Procedure

1. Set up a clean work area as your teacher instructs.
2. Collect your individual supplies. The white bead in the PCR microcentrifuge tube contains the reagents necessary for synthesizing the new DNA, including a DNA polymerase called Taq polymerase, dNTP's, and buffer components.
3. Keep your microcentrifuge tube of cell lysate from Activity 1 on ice. It should remain on ice while you are working.
4. Label your PCR tube (containing the bead) with your initials as your teacher instructs. You will be adding reagents to this tube from here on. Work with this tube on ice as much as possible until you place it in the thermal cycler.
5. Obtain Primer–Loading Dye Mix from your teacher. It contains a forward primer, a reverse primer, and a purple-pink loading dye. Pipet 20 μ L of the Primer Mix into your PCR tube.
6. Allow the tube with the PCR bead and primer to sit on ice for 1–2 minutes.
7. With a new sterile tip (or new microcapillary pipet), add 4 μ L lysate from the microcentrifuge tube (#2 extract) from Activity 1 to the liquid in the bottom of the PCR tube and gently pipet up and down several times to mix the contents. Keep this tube on ice until you place your tube into the thermal cycler.
8. Follow your teacher's instructions for placement of your PCR tube into the thermal cycler.
9. Answer questions 4–7 in the Analysis and Follow-up Questions.

Activity 3: Gel Electrophoresis

Pre-lab

Watch the Carolina Video (5 minutes: 46 seconds) explaining how to load a gel. Go to www.carolina.com. Under Teacher Resources, select How-to & Techniques, and find the video titled “Loading a Gel for Electrophoresis.”

Determine which type of pipet you will be using (micropipet, fixed-volume pipet, microcapillary, etc.).

Materials

per student

- your PCR tube from Activity 2 (it has already undergone PCR)
- ice (in beaker or plastic cup)
- micropipet (for pipetting 10 μL) with sterile tips, or 1 disposable microcapillary pipet
- stain
- staining tray
- gloves

to share

- metric ruler
- gel electrophoresis chamber
- 1 \times TBE running buffer
- agarose gel poured and ready
- 1 vial 1 kb DNA ladder
- white light or blue light

Procedure

1. Obtain your individual materials. Keep your PCR tube on ice when possible during the following steps.
2. Your teacher or another student should pipet 5.0 μL (if you are using GelGreen™ or a stain of similar sensitivity) or 25 μL (if you are using CarolinaBLU™ or a stain of similar sensitivity) of the DNA ladder into the appropriate lane.
3. Following your teacher’s instructions, carefully pipet your PCR sample into your assigned gel well. Load 10 μL if you are staining with CarolinaBLU. Load 5 μL if you are using GelGreen.
4. Because each student is evaluating a different colony, it is important to know which lane in the gel belongs to you. Record the gel and the lane number in which your sample is loaded.

Gel (if more than one gel is run during your class period):	I pipetted my sample into Lane #:

5. Run your gel according to your instructor's directions. Before staining, measure the distance from the front edge of the well to the front edge of the dye front and record here.

_____ cm

6. Follow your teacher’s instructions for staining your gel. Staining makes the DNA visible.
7. Answer questions 8–10 in the Analysis and Follow-up Questions.

Activity 4: Data Comparison and Analysis

Materials (per student unless otherwise noted)

- photos of various gels (or gels themselves)
- semilog graph paper
- metric ruler

Procedure

1. Calculate the approximate size in base pairs (bp) of your DNA fragment(s).
 - a. You will interpolate the molecular weights of your PCR products by using a standard curve created using the 1-kb ladder. For this exercise, use either a photo of the gel or a representation created by placing a piece of clear acetate over the gel and tracing each band. The sizes of the DNA bands in the ladder are given in the figure to the side.
 - b. Using your own gel, a photograph, or a representation of the gel traced on acetate, carefully measure the distance (in centimeters) each DNA band in the DNA ladder traveled from the well. Record these distances in the appropriate space of the “Distance traveled” row of the following table.
 - c. Also measure the distance traveled by the band(s) in the lane loaded with your sample. Measure from the front edge of the well to the front edge of each band. Enter the distances into the table under “PCR product.”



Bands in DNA marker.

Note: You may have only one band in your lane.

DNA band	0.5 kb	1.0 kb	1.5 kb	2.0 kb	3.0 kb	4.0 kb	5.0 kb	6.0 kb	8.0 kb	10.0 kb	PCR product	PCR product	PCR product
Distance traveled													
R_f													
	Base-pair sizes:												

- d. Calculate the R_f for each fragment. To do this, divide the distance traveled by a given band by the distance traveled by the dye front (measured in step 5 of Activity 3). R_f is used when calculating molecular weight.
- e. Set up semilog graph paper with R_f as the x-axis (arithmetic axis) and base-pair length as the y-axis (logarithmic axis). Then, plot the R_f versus the base-pair length for each of the DNA bands in the marker.
- f. Connect the data points with a line of best fit. This best-fit line is the standard curve.
- g. Locate on the x-axis the R_f for one of the DNA bands in the lane loaded with your sample. Using a ruler, draw a vertical line from this point to its intersection with the standard curve.
- h. Extend a horizontal line from this point on the standard curve to the y-axis. This gives the base-pair size of this band. Record the size in the data table above.
- i. Repeat steps g and h for each band in the lane loaded with your sample and record the size(s).

2. Share with your classmates the observations you recorded about the morphology of your colony (while it was still on the agar plate). While your classmates are looking at the gel (or photo), indicate the location of your lane and describe the size of the band(s) in that lane.
3. Record the class data in a chart so that it can be easily observed. Your teacher may provide you with a chart to use.
4. Answer questions 11 and 12 in the Analysis and Follow-up Questions. Your teacher may also assign some or all of the Optional questions, 13–18.

- b. Is DNA positively or negatively charged? How does its charge impact how the gel chamber is set up?
10. How many bands appeared in your lane? What are the bands made of?
11. Look at the table with class results to determine if your band(s) ran a different distance into the gel than some of your classmate's bands or if you had a different number of bands than others. Think about the observations you originally made about the colony types. Can you find a correlation between the size and number of bands you and your classmates observed in each lane (the genotype) and the phenotypes of the colonies used to generate those bands? Using the class data, explain any correlation or lack of correlation you see.
12. Your gel band gives you information about the genotype of the colony you isolated. On the basis of your observations regarding the phenotype and genotype of your colony, what genotype (strain) of *Halobacterium* do you hypothesize you isolated (*Halobacterium* sp. NRC-1, KBT-1, or another)? Identify whether your colony was wild type (wt) or mutant (mut). What makes you conclude that?

15. The DNA sequence in the following figure shows the location of some of the genome region you just amplified using PCR. The region shown contains the gene sequence for *gvpA1*. (If you have trouble with these questions, review Pre-lab Activity 1.)
- Why are some letters capitalized?
 - Label the gene start codon for *gvpA* (usually ATG).
 - Label the gene stop codon for *gvpA* (usually TAA, TGA, or TAG).
 - How did you pick your start and stop codon, given that there were multiple codons like them in the sequence?

forward primer: agtctgtgggcggtgagct

```

5' atgaaaaactggctgagctcgcgggggaataaacacgattccggtgtagtctgtgggcggtgagctagattgggtgaact 3'
3' TACTTTTTGACCGACTCGAGCGCCCCCTTATTGTGCTAAGGCCACATCAGACACCCGCCACTCGATCTAACCCACTGA 5'
    
```

forward primer annealing location

```

5' cattaacttctctccagtcgatggcggtagagcactcccgactagtaggtgaggctttcttcgcttcacgactgtctaaga 3'
3' GTAatgaagagaggtcagctaccgccatctcgtgagggtgatcatccactccgaaagaagcgaagtgctgacagattct 5'
    
```

end of *gvpD1*

IS Element (ISH2) inserts between these 2 nucleotides

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5' agctttacactctccgtacttagaagtagcactcattacaggagacataacgactggtgaaaccatacacatccttatgt 3'
3' tcgaaatgtgagaggcatgaatcttcctgctgagtaatgtcctctgtattgctgaccactttggatgtgtaggaataca 5'
    
```

```

5' gatgcccagatagttagagatgggttaatcccagatcaccaATGGCGCAACCAGATTCTTCAGGCTTGGCAGAAGTCC 3'
3' ctacgggctcatatcaatctctacccaattagggctagtggttaccgcgttggctctaagaagtccgaaccgtcttcagg 5'
    
```

```

5' TTGATCGTGTACTAGACAAAGGTGTCGTTGTGGACGTGTGGGCTCGTGTGTCGCTTGTGGCATCGAAATCCTGACCGTC 3'
3' aactagcacatgatctgtttccacagcaacacctgcacaccgagcacacagcgaacagccgtagcttttaggactggcag 5'
    
```

```

5' GAGGCGCGGGTCGTCGCCGCTCGGTGGACACCTTCCTCCACTACGCAGAAGAAATCGCCAAGATCGAACAAGCCGAAC 3'
3' ctccgcgcccagcagcggcggagccacctgtggaaggaggtgatgcgtcttcttttagcgggttctagcttgttcggcttga 5'
    
```

```

5' TACCGCCGGCGCCGAGGCGGCACCCGAGGCCTGAcgcacaggcctcccttcggcccggcgtaagggaggtgaatcgcttg 3'
3' atggcggccgcggctccgcccgtgggctccggactgctgtccggaggaagccgggcccgcattccctccacttagcgaac 5'
    
```

start of *gvpC1*

```

5' caaaccatactattaacacccttctcgggtacacactaatcccATGAGTGTACAGACAAACGCGACGAGATGAGTACTG 3'
3' gtttggtatgataattgtgggaagagccatgtgtgattagggtagctcacagtgtctgtttgcgctgcttactcatgac 5'
    
```

A portion of the wild-type *Halobacterium* sp. NRC-1 *gvpA1* gene region sequence amplified using the primers in this kit.

16. Translate the capitalized sequence for *gvpA1* (from question 15) into an amino acid sequence. Use the sequence editor function on HaloWeb to help you do this.

17. When the *gvpA1* gene region was amplified from KBT-1 and sequenced, the following sequence was obtained. Note how the KBT-1 sequence differs from the NRC-1 sequence used for question 15. How might such a difference affect the phenotype?

forward primer: agtctgtgggcggtgagct

5' atgaaaaactggctgagctcgcgggggaataaacagattccgggtgtagtctgtgggcggtgagctagattgggtgaact 3'
 3' TACTTTTTGACCGACTCGAGCGCCCCCTTATTGTGCTAAGGCCACA**TCAGACACCCGCCACTCGA**TCTAACCCACTGA 5'

forward primer annealing location

5' cattaacttctctccagtcgatggcggtagagcactcccgactagtaggtgaggtttcttccgcttcacgactgtctaaga 3'
 3' GTAatgaagagaggtcagctaccgccatctcgtgagggctgatcatccactccgaaagaagcgaagtgtgacagattct
end of gvpD1

5' agctttacactctccgtacttagaagtagcactcattacaggagacataacgactggtgaaaccatacacatcc**cattcg** 3'
 3' tcgaaatgtgagaggtcatgaatcttcctgctgagtaatgtcctctgtattgctgaccactttgggtatgtgtagg**gtaagC** 5'

5' **tcttttagttaagaaatcgcgtagacagcggttaggatctcttcgctgtgcaagacgcggtgagatctcttttgataatatt** 3'
 3' **agaaatcaattcttttagcgcactgtcgccatcctagagaagcgacacgcttctgcgccgactctagagaaaactattataa** 5'

3' **ttaacctaaatcgaaataagacgataatcttacgctgcaccggtgcacgtaatttctaagagcgttctaagactatggctc** 5'
 5' **aattggatttagcttattctgctattagaatggcagctgggccagctgcataaagattctcgcagattctgataccgag** 3'

3' **gaaccaaaaatggcgctctccatccgaactgaactcggtgagtaactcgattcactcgtcgaatgagtggttcagatctcggga** 5'
 5' **cttggttttaccgcagaggttaggcttgacttgagcaactacttgagctaagtgcagcagctactcacaagcttagagcct** 3'

3' **gcaagccgctccgagatcggtgaagccatcctcagcagataatttccagaacgatgaagaccaaatacaacagacgcgaga** 5'
 5' **cgttcggcgaggtcttagcaacttcggtaggagtgctcgtataaaagtcttgctacttctgggttagttagtctcgcgctct** 3'

3' **gctgattatccgcaacagaaaacgctcctaactcgtagagaagttcgtgcaccgagtgcaacgaacttcttagagcgtcatt** 5'
 5' **cgactaataggcgttgctcttttgcgagattgagcactcttcaagcagctggctcagctgcttgaagaatctcgcagtaa** 3'

3' **caaaaccagcacttagctaaattaatatcggattggcttggggcgaatagaaaatcttctcttccactgttcagccctacgc** 5'
 5' **gttttgggtgctggaatcgaatataatagcctaaccagaaccgcttatactttagaagagaagtgacaagtcgggatgcg** 3'

3' **catgtggcggtttcgtcttaactaaagacgaatgcccatacacatccc**ttatgtgatgcccgagtatagtttagagatgggt 5'
 5' **gtacaccgccaagcgagaattgatttctgcttacgggtatgtgtagg**aatatacactacgggctcatatcaatctetacca 3'

Start codon for gvpA1

5' taatcccagatcacc**ATG**GCGCAACCAGATTCTTCAGGCTTGGCAGAAGTCCTTGATCGTGTACTAGACAAAGGTGTCG 3'
 3' attagggctagtggttaccgcttgggtctaagaagtccgaaccgctcttcaggaactagcacaatgatctgtttccacagc 5'

5' TTGTGGACGTGTGGGCTCGTGTGTCGCTTGTGGCATCGAAATCCTGACCGTCGAGGCGCGGGTCGTCGCCGCCCTCGGTG 3'
 3' aacactgcacaccgagcacaacagcgaacagccgtagctttaggaactggcagctccgcgccagcagcggcgagccac 5'

5' GACACCTTCCTCCACTACGCAGAAGAAATCGCCAAGATCGAACAAGCCGAACTTACCGCCGGCGCCGAGGCGGCACCCGA 3'
 3' ctgtggaaggaggtgatgCGTcttcttttagcggttctagcttgttcggcttgaatggcggccgCGGctccgCGtgggct 5'

Stop codon for *gvpA1*

5' GGCCTGAcgcacaggcctcccttcggccccggcgtaagggaggtgaatcgcttgcaaaccatactattaacacccttctcg 3'
 3' ccggactgCGTgtccggaggggaagccgggCGcattccctccacttagcgaacgtttggtatgataattgtgggaagagc 5'

start of *gvpC1*

5' ggtacacactaatccc**ATGAGTGT**CACAGACAAACGCGACGAGATGAGTACTG 3'
 3' ccatgtgtgattagggTactcacagTgtctgtttgCGctgctctactcatgac 5'

Halobacterium sp. KBT-1 sequence of *gvpA1* gene region. The region of boldface text indicates an insertion sequence (IS). The nucleotides between which the sequence inserted are shaded gray. The underlined sequence on each side of the insertion is the direct repeat. The direct repeat results from the insertion element's copying the 12 nucleotides located just before the insertion site and repeating them at the end of the IS as it inserts.

18. On the HaloWeb genomes page (<http://halo4.umbi.umd.edu/>), click on *Halobacterium* NRC-1, and then on the Resources link. Click on the Insertion Sequence elements (IS elements). On the basis of the size difference between the sequences listed for questions 15 and 17, which IS element do you predict may have inserted? Note that the length of the IS elements as shown in the chart does not include either direct repeat.

Appendix 1: Primer locations in the *gvpA1* gene region

forward primer: 5' agtctgtgggcggggtgagct 3'

5' atgaaaaactggctgagctcgcgggggaataaacacgattccgggtgtagtctgtgggcggggtgagctagattgggtgaact 3'
 3' TACTTTTTGACCGACTCGAGCGCCCCCTTATTGTGCTAAGGCCACATCAGACACCCGCCCACTCGATCTAACCCACTTGA 5'

forward primer annealing location

5' cattaacttctctccagtcgatggcggttagagcactcccgactagtaggtgaggttttcttcgcttcacgactgtctaaga 3'
 3' GTAatgaagagaggtcagctaccgccatctcgtgagggctgatcatccactccgaaagaagcgaagtgtgacagattct 5'

end of *gvpD1*

IS Element (ISH2) inserts between these 2 nucleotides

5' agctttacactctccgtacttagaagtagcactcattacaggagacataacgactggtgaaaccatacacatccttatgt 3'
 3' tcgaaatgtgagagcatgaatcttcatgctgagtaatgtcctctgtattgctgaccactttggatgtgtaggaataca 5'

gvpA1

5' gatgcccagtatagttagagatgggttaatcccagatcaccaATGGCGCAACCAGATTCTTCAGGCTTGGCAGAAGTCC 3'
 3' ctacgggctcatatcaatctctaccaattagggcttagtggttaccgcgttggctctaagaagtccgaaccgtcttcagg 5'

5' TTGATCGTGTACTAGACAAAAGTGTGCTTGTGGACGTGTGGGCTCGTGTGTCGCTTGTTCGGCATCGAAATCCTGACCGTC 3'
 3' aactagcacatgatctgtttccacagcaacacctgcacaccgagcacacagcgaacagccgtagctttaggactggcag 5'

5' GAGGCGCGGGTTCGTCGCCCTCGGTGGACACCTTCCTCCACTACGCAGAAAGAAATCGCCAAGATCGAACAAAGCCGAAC 3'
 3' ctccgcgcccagcagcggcgagccacctgtggaaggaggtgatgctcttcttttagcggttctagcttgttcggcttga 5'

5' TACCGCCGGCGCCGAGGCGGCACCCGAGGCCTGACgcacaggcctcccttcgcccggcgtaagggaggtgaatcgcttg 3'
 3' atggcgccgcggtctccgctggtctccgactgctgtccggaggaagccgggcccgcattccctccacttagcgaac 5'

gvpC1

5' caaaccataactattaacacccttctcgggtacacactaatcccATGAGTGTACAGACAAACGCGACGAGATGAGTACTG 3'
 3' gtttggtatgataattgtgggaagagcccatgtgtgattagggtagctcacagtgtctgtttgctgctctactcatgac 5'

5' CCCGCGATAAGTTCGCAGAAATCACAGCAGGAGTTCGAATCATACTGACGAGTTCGAGCCGATATCACGGCAAAGCAA 3'
 3' gggcgctattcaagcgtcttagtgctcctcaagcttagtatgagactgctcaaacgctcggctatagtgccgtttcgtt 5'

5' GACGATGTCAGCGACCTTGTTCGATGCGATCACCGACTTCCAGGCGGAGATGACCAACACGACGGATGCATTTACACATA 3'
 3' ctgctacagtgcgtggaacagctacgctagtggtgaaggtccgcctctactggttgtgctgcctacgtaaaagtgtgat 5'

5' TGGTGACGAGTTCGCCGCTGAGGTTGACCACCTCCGTGCCGATATTGACGCCAGCGGGACGTGATCCGTGAGATGCAGG 3'
 3' accactgctcaagcggcgactccaactggtggaggcacggctataactgcgggtcgccctgcactaggcactctacgtcc 5'

5' ATGCGTTCGAGGCATATGCTGACATCTTCGCTACAGATATCGCAGACAAACAAGATATCGGCAATCTTCTGGCTGCGATT 3'
 3' tacgcaagctccgtatacagctgtagaagcgatgtctatagcgtctgtttgttctatagccggttagaagaccgacgctaa 5'

5' GAGGCGCTCCGAACAGAGATGAACTCAACCCACGGGGCATTCGAAGCATATGCGGACGACTTCGCAGCCGATGTCGCTGC 3'
 3' ctccgcgaggcttgtctctacttgagttgggtgccccgtaagcttcgtatacgcctgctgaagcgtcggctacagcgacg 5'

5' GCTCCGTGATATATCTGATCTGGTTGACGCAATCGACGACTTCCAAGAGGAATTCATCGCCGTGCAGGACGCATTTGACA 3'
 3' cgaggcactatatagactagaccaacgctcggttagctgctgaaggttctccttaagtagcggcacgtcctgcgtaaaactgt 5'

5' ACTACGCTGGTGAATTCGATGCGGAGATCGACCAGCTCCACGCTGCCATCGCTGACCAGCACGACAGCTTCGACGCTACC 3'
 3' tgatgcgaccactgaagctacgcctctagctggctcagaggtgcgacggtagcagctggctgctgctcgaagctgcgatgg 5'

5' GCGGACGCCCTTCGCAGAGTACCGAGATGAGTTCTATCGCATAGAGGTGGAAGCACTGCTTGAGGCGATCAACGACTTCCA 3'
 3' cgcctgcggaagcgtctcatggctctactcaagatagcgtatctccaccttcgtgacgaactccgctagttgctgaaggt 5'

5' GCAGGACATCGGTGACTTCCGAGCGGAGTTTCAAACGACTGAGGACGCGTTTCGTTGCCTTCGCCCGTGACTTCTAgatgg 3'
 3' cgtcctgtagccactgaaggctcgctcaaactttgctgactcctgcaagcaacggaagcgggactgaagataccgg 5'

5' ACGAGATCACGGCCGAGGAAGGCGCCGCCGAAGCGGAAGCCGAACCCGTCGAGGCTGACGCGGACGTGAAGCGGAAGCA 3'
 3' tgctctagtgcggctccttccgcgggcgttcgccttcggcttgggcagctccgactgagcctgcagcttcgccttcgt 5'

5' GAAGTCTCTCCAGACGAAGCTGGCGGAGAATCCGCCGGTACCGAGGAAGAAGAGACAGAGCCGGCCGAGGTGGAAACAGC 3'
 3' cttcagagaggtctgcttcgaccgctccttagggcgccatggctccttctctctgtctcggccggctccacctttgtcg 5'

5' GGCTCCAGAAGTAGAGGGGAGTCTTGCAGACACGGCAGACGAAGCGGAAGATACGGAAGCAGAGGAGGAGACAGAGGAAG 3'
 3' ccgaggtcttcatctcccctcaggacgctgtgcccgtctgcttcgccttctatgccttcgtctcctcctctgtctcctc 5'

5' AGGCACCGGAAGACATGGTGCAGTGCCGGGTGTGCGGCGAATACTATCAGGCCATCACGGAGCCCCATCTCCAGACCCAT 3'
 3' Tccgtggccttctgtaccacgtcacggcccacacgccgcttatgatagtcggtagtgctcggggtagaggtctgggta 5'

gvpN1

5' GATATGACGATTCAGGAGTACCGCGACGAGTACGGTGTAGGATGTCCCCCTTCGGCCGGATGATAAAACATGAcgaacgag 3'
 3' ctatactgctaagtctcatggcgctgctcatgccactcctacagggggaagccggcctactatthttgtactgcttctc 5'

reverse primer annealing location

5' tcccgtaaagcaaggtacgagggctgcagatccgctcctcacgcgcgacaagaaacaggggcgatcacagagccgtga 3'
 3' agggcatttgcttccatgctcccagcgtctagggcagggagtgcgccgctgttctttgtcccgcgtagtgtctcggcact 5'

reverse primer: 5' acggctctgtgatcgcccct 3'

5' tgataaggagatcgagcgtctcgagagggcagaacgacgctcgtggccaggagtcgtctaccacgtcgacgaggg...
 3' actattcctctagctcgagagctctccgtcttgctgagcaccggctcctcagcagatgggtgcagctgctccc...

Appendix 2: Glossary

Amino Acid (AA). Monomer unit of proteins. Amino acids have the same basic structure, with the exception of an “R group” that varies. The R group gives each amino acid a different chemical property.

Amplify. In PCR, the process of making more copies of a specific segment of DNA.

Annealing. Attachment by base pairing of one strand of nucleotides to its complementary strand (such as a DNA primer to its complementary DNA template).

Antiparallel. A term used to describe DNA strands that run in opposite directions in a double-stranded molecule.

Archaea. A domain of prokaryotic life distinct from the domain Bacteria. Archaea includes many organisms from extreme environments, including halophiles, methanogens, and thermophiles.

Bacteria. The domain of “true” bacteria; prokaryotic, single-celled microbes, many with a cell wall made of peptidoglycan.

Bioinformatics. The use of computers and related tools to store, manage, and analyze biological data. The use of a computer in this way is especially common in molecular biology, genetics, and genomics.

Clones. Organisms that are genetically identical.

Codon. A group of three nucleotides in a DNA or RNA polynucleotide chain that (depending upon their sequence) serves as either a start or stop signal for the translation of a protein or as a code for a specific amino acid to be added to the growing polypeptide during translation.

Colony. A visible clonal population of microbial cells on an agar or other surface, resulting from many generations of cell division of a founder (mother) cell.

Complementary sequences. In DNA, nucleotide sequences that can be joined by base pairing to form double-stranded DNA.

Denature. To separate double-stranded DNA into two single strands, usually by heating or exposure to alkaline conditions.

Deoxyribonucleic acid (DNA). A chemical substance consisting of two polynucleotide chains forming a double helix with Watson–Crick pairing of the nucleotide bases. DNA functions as the hereditary material of the cell. It contains genetic information in the form of triplet codons.

DNA polymerase. An enzyme that functions in the replication and repair of DNA.

Domain. One of the three broadest categories of life forms in current biological taxonomy: Eukaryotes, Bacteria, and Archaea.

Double helix. The structure of DNA, in which two chains are wrapped around each other like the sides of a twisted ladder.

DNA ladder. Also called a DNA marker. A sample of DNA containing multiple DNA fragments, most of which increase in size in regular increments. For example, a ladder may have fragments of 500 bp, 1000 bp, 1500 bp, etc. When the fragments are separated using gel electrophoresis, they create a banding pattern that resembles a ladder and can be used for measuring the size and quantity of other DNA fragments on the gel.

Electrophoresis. The process by which molecules are separated by size or charge by being drawn through a matrix by an electric current (e.g., DNA fragments are often separated by size by drawing them through a matrix formed by agarose).

Eukaryotes. The domain also referred to as “Eukarya.” These cells have nucleic acid surrounded by a nuclear membrane, and membrane-bound organelles. The eukaryotic organisms are often, but not always multicellular.

Forward primer. A short sequence of DNA designed to amplify a specific genome region. The forward primer is composed of a sequence from the 5' end of the coding strand of the sequence that a person wants to amplify. Both a forward and a reverse primer are needed amplify the template DNA.

Gas vesicles (GVs). Organelles found in certain microorganisms, including *Halobacterium* sp. NRC-1. They consist of a rigid protein structure, filled with ambient gas. These vesicles give buoyancy to *Halobacterium*, allowing it to float in its hypersaline aquatic environment and thus gain access to more oxygen and sunlight.

Gel. For the purposes of this lab, the word refers to the agarose gel used in gel electrophoresis.

Genome. All of the genetic information contained in one cell.

Genotype. The genetic makeup of an organism, either in total or in terms of one or a few sets of genes.

***Halobacterium* sp. NRC-1.** An extremely halophilic archaeon found in extremely salty environments all over the world. It is used as a model organism for research and teaching.

Halophile. An organism that requires a salt concentration greater than that of seawater for optimal growth. Those that require nearly saturated salinity are called “extreme” halophiles.

Insertion sequences (IS elements). (also see transposable element). DNA sequences that insert into the genome; also known as “jumping genes.” When IS elements insert into a new location of the genome, they cause mutations that can bring about phenotypic change.

Insertion Sequences in Haloarchaea (ISH). Insertion sequences in Haloarchaea, (first identified in *Halobacterium*) that commonly insert at certain “hot spots,” such as in the gas vesicle gene region.

Loading dye. Pigments and sugars (e.g., sucrose, ficoll, glycerol) mixed and added to a DNA sample before it is run on a gel. The pigment makes it easier to see the sample during loading and to judge the location of the DNA fragments on the gel. The sugar adds density to the sample so that it sinks to the bottom of the well.

Lysate. The solution/mixture of cell components that results after cells are broken open (lysed).

Lyse. To cause a cell to break open such that its contents are released and the cell dies (the breaking is referred to as “lysing”).

mRNA. Messenger RNA is a polynucleotide chain of ribonucleotides transcribed from DNA. Through the process of translation by ribosomes, it is used to make proteins.

Mutation. A change in the genetic code. A mutation can result from the addition or removal of one or more nucleotides into the DNA sequence. These types of mutations are called insertion or deletion mutations, respectively. Mutations can also occur if one or more base pairs in the DNA sequence are changed to a different base pair or pairs.

Nitrogenous base. One of three components that makes up a nucleotide. A DNA nucleotide can contain any of four different nitrogenous bases: adenine (A), thymine (T), guanine (G), or cytosine (C). Adenine pairs with thymine, and guanine pairs with cytosine. (In RNA, uracil is found in place of thymine.)

Nucleotide. Any one of the building blocks of DNA (deoxyribonucleotides) or RNA (ribonucleotides). A nucleotide is composed of a phosphate group, a sugar, and a nitrogen base.

Operon. A group of linked genes or segments of DNA that are transcribed together and often regulated together.

Phenotype. Observable characteristic or trait of an organism, e.g., brown, blue, or green eyes in people, or colony color or opacity in microorganisms.

Phototrophy. An energy-generating pathway similar to, but distinct from photosynthesis that uses sunlight and the protein bacteriorhodopsin.

Polymer. A chain of monomer units that link together, usually through dehydration synthesis, to make a longer strand. Proteins, carbohydrates, DNA, and RNA are all polymers.

Polynucleotide. A chain of nucleotides forming a strand of DNA or RNA.

Polymerase Chain Reaction (PCR). A process for amplifying a specific segment of DNA from a few copies of that DNA. PCR is a powerful tool which simplifies and, in many cases, makes possible, the manipulation and analysis of specific sequences of DNA.

Precipitate. To cause a dissolved material to come out of a solution or suspension, often so that it can be collected at the bottom of a vessel.

Primer. In DNA replication, a short RNA or RNA/DNA fragment that serves to prime DNA synthesis. DNA polymerase attaches the first nucleotide in DNA synthesis to the primer. In PCR, an artificially made DNA fragment (oligonucleotide) is used as a primer for DNA synthesis. See also “forward primer” and “reverse primer.”

Protein. A biomolecule made of chains of amino acids. Peptide bonds link the amino acids in the chain. Proteins can function as enzymes, structural molecules, and signal molecules and can have other functions as well.

Reverse primer. The reverse complement of the 3' end of the coding strand to be amplified by PCR. See also “forward primer.”

Ribonucleic Acid (RNA). A chemical substance found in all cells, which is synthesized by using parts of the DNA sequence in the cell as a template. RNA is synthesized in a process called transcription. One type of RNA, mRNA, is used as a template in translation; other types of RNA serve other functions.

Sectoried colony. A colony with different phenotypes, pink or red, segregated into discrete sections of the colony.

Transcription. The process of DNA being used as a template to form a new RNA molecule with a sequence complementary to the original DNA template. The process is carried out by RNA polymerase.

Translation. The process in which messenger RNA (mRNA) is used to code for the synthesis of an amino acid chain. This process is carried out by the ribosome, which reads the mRNA nucleotides in groups of three. Each group of three nucleotides is called a codon and designates which amino acid is to be added to the growing polypeptide chain. Different codons code for different amino acids.

Transposable element. Segment of DNA that can either relocate to other areas of a genome, or copy and insert the copy into other areas of a genome.

Appendix 3: Troubleshooting

A student had a band that is larger or smaller than KBT-1 or NRC-1.

For examples of this, see lanes 7, 9, and 10 in the following gel photo. Also see the answer to student question 11. Overloading a lane may also alter the apparent mobility of DNA bands in the sample.

The bands are so faint, they are difficult to see.

This could be caused by students' having very little DNA extracted and thus very little amplified. It may also be a result of a student's not getting enough of the sample into the well of the gel.

The bands are very bright, and there is a great deal of smearing visible.

This is likely caused by loading too much DNA in the lane. Either too much template DNA was added to the PCR reaction, or too much DNA was loaded into the lane. Students may end up with too much template DNA if they used too much *Halobacterium* for their DNA isolation.

Some of my students do not have any band(s) in their gel lane.

All of the following are possible explanations for this occurrence:

- Students made a pipetting error when they were setting up their PCR reaction.
- When the students removed the supernatant during the DNA purification, they removed part of the "pellet" at the bottom of the tube. The pellet contains nucleases that can degrade the DNA. It may also contain other cellular components that inhibit the PCR reaction. Nuclease activity is more of a problem if the sample is not chilled sufficiently. Chilling slows the activity of the enzyme.
- There was a loading error.

Other possible problems are illustrated in the gel photo shown here:

- Lanes 2 and 3 show bands amplified from NCR-1 and KBT-1 respectively.
- Lanes 4, 6, and 8 appear empty, suggesting that either the student did not successfully load the lane or that their PCR reaction did not work.
- Lane 11 is empty, but the two bands in the adjacent lane 10 suggest the possibility that lane 10 is double-loaded and one student loaded the wrong lane. Two bands may also be seen if the student who loaded lane 10 sampled a sectorized colony or if multiple genetic rearrangements occurred.
- In lane 12, there is a faint band above the main band. There are two main potential causes: 1) a student picked and amplified DNA from a sectorized colony in which cells with a red phenotype are not numerous, or 2) the PCR reaction was contaminated. (Also, see lane 11 explanation, above).
- Lanes 5, 7, 9, 10, and, to a certain extent, lane 12 all have too much DNA loaded in them. Overloading causes the smearing and the background bands seen in these lanes.

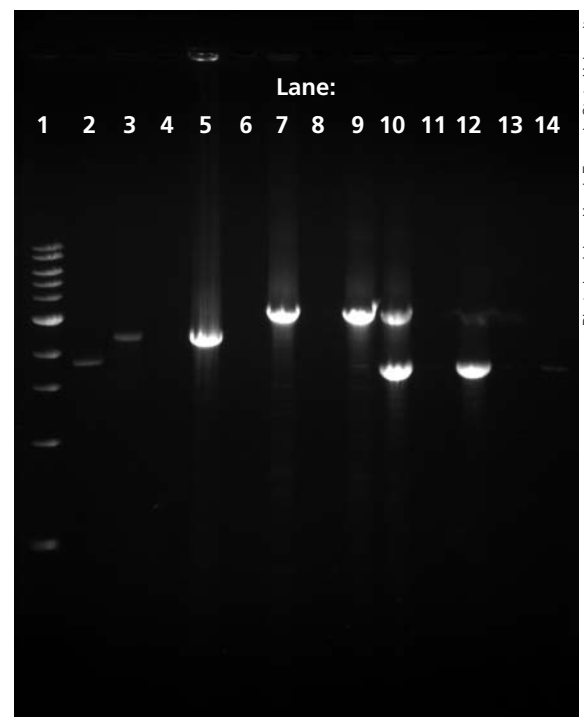
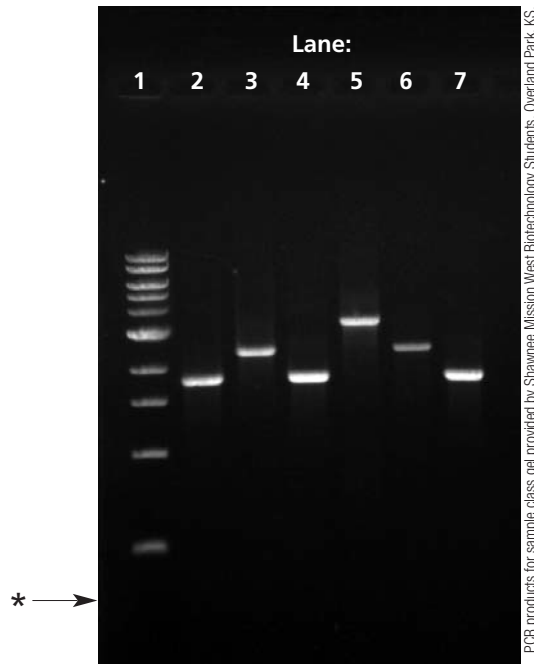


Photo by Jolene Hund, Emporia State University.

Some possible student mistakes that may occur in the classroom.

Appendix 4: Sample Class Gel Image



A sample class gel image with DNA marker and PCR products from *Halobacterium* sp. NRC-1, KBT-1, and unknown mutant.

Lane 1: 1 kb DNA ladder (DNA marker)

Lane 2: *Halobacterium* sp. NRC-1 PCR product

Lane 3: *Halobacterium* sp. KBT-1 PCR product

Lane 4: *Halobacterium* sp. NRC-1 PCR product

Lane 5: an unknown red mutant PCR product containing an insertion sequence larger than KBT-1 yet still found within the region amplified by the primers in this kit.

Lane 6: *Halobacterium* sp. KBT-1 PCR product

Lane 7: *Halobacterium* sp. NRC-1 PCR product

*If you use this image for Question 1 in the Data and Comparison section, assume that the dye front is here.

Appendix 6: Staining with GelGreen™*

Note: View and photograph gels stained with GelGreen between 1 and 2 hours after staining. If left in solution longer, the bands begin to diffuse and are not as sharp. If bands are faint, diffusion can make them more difficult or even impossible to see.

Storage and Handling:

Store the 10,000× aqueous solution in the dark at room temperature. Also store the 3× working solutions in the dark at room temperature, and they will remain good for at least a year. If precipitation of the dye occurs, heat the solution in a 45–55°C water bath for 2 minutes and/or vortex.

Safety Information:

Studies by the manufacturer and independent labs have shown GelGreen to be nonmutagenic, noncytotoxic, and unable to cross the cell membrane barrier; however, as is prudent when using any chemical in the classroom, we suggest using gloves and any other required personal protective equipment when handling this stain. Ensure that the appropriate orange filter or orange safety goggles are kept between the viewer's eyes and the blue light when viewing the gel.

Staining Protocols:

1. To make the needed volume of 3× staining solution, dilute the GelGreen as follows:

Mix:	15 μL	GelGreen 10,000× stock solution
	5 mL	1 M NaCl
	45 mL	distilled water

2. Place the gel in a suitable polypropylene container or staining tray and add just enough of the 3× staining solution to cover the gel.
3. Soak the gel for 30 minutes, with constant, gentle agitation if possible. If constant, gentle agitation is impossible, agitate every 5–10 minutes by hand.

Viewing and Photographing the Gel

1. Because GelGreen has a UV absorption peak between 250 nm and 300 nm and a stronger adsorption peak around 500 nm, it can be visualized with either a 254-nm UV transilluminator or a "blue light" transilluminator.
2. Photograph the gel using any suitable imaging equipment. Use a long path green filter such as a SYBR** filter or GelStar** filter for photography. *Note: Some "blue light" transilluminators incorporate filters appropriate for photography.* The stain's peak emission occurs between approximately 500 and 550 nm.

*GelGreen and its uses are covered by pending US and international patents.

**SYBR is a registered trademark of Molecular Probes Inc., and GelStar is a registered trademark of FMC.

Appendix 7: Staining with CarolinaBLU™

Pre-staining: Adding CarolinaBLU to the Gel and Buffer

CarolinaBLU can be used to stain the DNA while the gel is running. The stain will not be as intense as the final stain, and final staining will still be required to visualize all of the bands. However, staining while the gel is running increases the sensitivity of the stain and allows students to visualize at least some of the DNA bands while the gel is running.

To stain the gel while it is running, add CarolinaBLU gel and buffer stain in the amounts indicated below. The amount of stain to use depends upon the voltage used for electrophoresis. Note: Do not use more stain than recommended. This leads to precipitation of the DNA in the well, and can create artifactual DNA bands in the agarose gel.

Gels containing CarolinaBLU may be prepared 1 day ahead of the lab. However, gels stored longer tend to fade and lose their ability to stain DNA bands during electrophoresis. Note: If you add stain to the gel you must also add stain to the buffer.

Add the stain to the agarose gel in the amounts indicated in the table below.

Voltage	Agarose Volume	Stain Volume
<50 volts	30 mL	40 µL
	50 mL	70 µL (1 drop)
	400 mL	533 µL (8 drops)

>50 volts	50 mL	70 µL (1 drop)
	400 mL	640 µL (9 drops)

Add the stain to the buffer in the amount indicated in the table below.

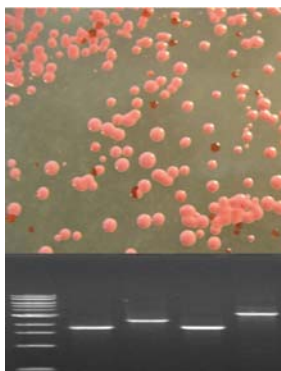
Voltage	Buffer Volume	Stain volume
<50 volts	500 mL	500 µL (7 drops)
	2.6 L	2.6 mL (37 drops)

>50 volts	500 mL	960 µL (14 drops)
	2.6 L	5 mL (71 drops)

After you add the stain to the agarose, swirl to mix and then pour the gel immediately. Store the gels covered with a small amount of buffer containing CarolinaBLU (leave the masking tape or dam in place to contain the buffer). Keep them in a plastic bag or a plastic container with a lid. Another option is to store them covered with buffer (with CarolinaBLU) in the gel box with the lid on.

Post-staining

1. Cover the electrophoresed gel with the CarolinaBLU™ Final Stain and let sit for 20–30 minutes. Agitate gently (optional).
2. After staining, pour the stain back into the bottle for future use. (The stain can be used 6–8 times.)
3. Cover the gel with deionized or distilled water to destain. Chloride ions in tap water will cause the staining to fade.
4. Change the water 3 or 4 times over the course of 30–40 minutes. Agitate the gel occasionally.
5. Bands that are not immediately present will become more apparent with time and will reach their maximum visibility if the gel is left to destain 4–5 hours to overnight in just enough water to cover the gel. Gels left overnight in a large volume of water may destain too much.



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