To close the yellow note, click once to select it and then click the box in the upper left corner.
To open the note, double click (Mac OS) or right click (Windows) on the note icon.

Characterization of the Bioluminescence (Lux$^+$) Gene in the Microorganism *E. coli*

Jennifer Gray, Madera Community College Center, Madera, CA

INTRODUCTION

Description

Students grow *E. coli* strain HB101, which contains the plasmid pUCD607 with the bioluminescence (Lux$^+$) gene. The plasmid containing the Lux$^+$ gene is isolated from the *E. coli*, then characterized by restriction analysis. The bioluminescence biochemical process and the nature of its phenotypic expression in *E. coli* HB101 is explained.

Student Audience

This activity is appropriate for high school AP biology majors, community-college biology majors, and four-year university majors in biology and molecular-biology-related courses.

Goals for the Activity

Students will

- understand the theory and methodology involved in the growth of microorganisms and the isolation and analysis of plasmid DNA,
- be able to identify DNA fragments using a standard,
- gain understanding of the biochemical process of bioluminescence, and
- gain understanding of phenotype and genotype as they relate to the expression of luciferase enzyme coded by the Lux$^+$ gene.

Recommended Placement in the Curriculum

This activity can be used as a module component in molecular biology and energy-releasing processes in biology, genetics, and biochemistry courses.

STUDENT HANDOUT
Characterization of the Bioluminescence (Lux$^+$) Gene in the Microorganism *E. coli*

Introduction

Bacterial bioluminescence (lux) exists in a wide variety of life forms including worms, mollusks, fish, diatoms, fungi, and bacteria. This lab utilizes bioluminescent bacteria that were genetically engineered from the cloned genes of the marine bacteria *Vibrio fischeri.* Bacterial bioluminescence is a useful phenotype, and it can be used as a marker for inactivation of cloning (no light), to estimate bacterial numbers, or to follow the spread of bacteria in plants as they move ahead of symptoms for early detection of crown gall disease. Several factors must be considered in the use of bioluminescence: the need to minimize background light, *E. coli* growth at temperatures below 33°C and 28°C, and in the exponential log phase (Shaw et al., 1986). Bioluminescence is catalyzed by an enzyme known as luciferase (Meighen, 1988). The bacterial enzyme is a heterodimer with a molecular weight of approximately 80,000 daltons and consists of α and β subunits with molecular weights of approximately 42,000 and 38,000 daltons, respectively. The active site is on the α subunit, although the β subunit is required for activity. Luciferase is a mixed-function oxidase that produces a blue-green light via the simultaneous oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain aldehyde (tetradecanal) by O_2.

$$FMNH_2 + O_2 + R\text{-}CHO \longrightarrow FMN + R\text{-}COOH + H_2O + light$$

The energy for light production is supplied by the oxidation of the aldehyde and $FMNH_2$. The actual mechanism of light emission is not clearly understood but is thought to result from the formation of a hydroperoxy flavin via the reaction of $FMNH_2$ and O_2. These molecules have been shown to emit light in the presence of aldehydes. (Meighen, 1988) Three additional enzymes are necessary to generate the aldehyde required in the reaction. The fatty acids for this fatty acid reductase enzyme complex are removed from the fatty acid biosynthesis pathway via the enzyme acyl-transferase. This enzyme reacts with acyl-ACP (acyl carrier protein) to release free fatty acids (R-COOH). The fatty acids are then reduced to an aldehyde by a two-enzyme system via the following reaction:

$$R\text{-}COOH + ATP + NADPH \longrightarrow R\text{-}CHO + AMP + PP + NADP^+$$

One enzyme, acyl-protein synthetase, activates the fatty acid via the cleavage of ATP to form R-CO-AMP. This serves as the substrate for the final enzyme, acyl-reductase, that catalyzes the NADPH-dependent reduction of the activated fatty acid to an aldehyde.

With the advent of recombinant DNA techniques, it has become possible to clone and determine the genetic organization of the bacterial bioluminescence genes from numerous species (see Meighen, 1988, 1991, and 1994 for examples). The α and β subunits of luciferase and the three enzymes required for aldehyde formation are encoded in a single operon (the lux operon) in all luminescent bacteria examined. The first two structural genes, lux C and lux D, code for the acyl-reductase and acyl-transferase, respectively. These are followed by lux A and lux B, which code for the α and β subunits of luciferase, and finally lux E, which codes for the acyl-protein synthetase. Two regulatory genes, lux I and lux R, have also been identified in *Vibrio fischeri* (which has recently been reclassified as *Photobacterium fischeri*). The lux I gene is on the same operon as the structural

genes, whereas lux R is transcribed in the opposite direction. Lux R codes for a transcriptional activator that binds the autoinducer synthesized by the lux I gene product (See Figure 1).

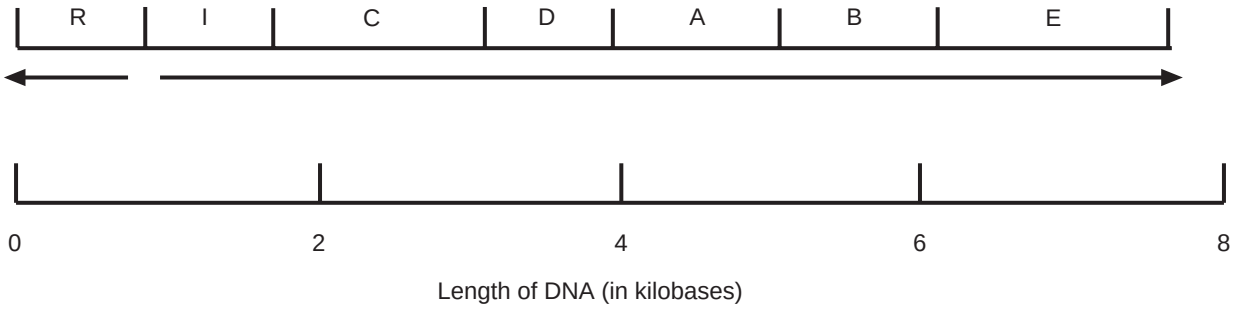

Figure 1: The lux operon of Vibro fischeri. Lux C codes for the acyl-reductase; lux D codes for the acyl-transferase; lux A and lux B code for the a and b subunits of luciferase, respectively; lux E codes for the acyl-protein synthetase; lux R and lux I code for regulatory proteins. Arrows under the operon indicate the direction of transcription.

Purpose

The purpose of this activity is to isolate and analyze the bioluminescence (Lux+) gene from *E. coli* strain HB101.

1. Grow HB101 *E. coli* (Lux+) bacterial strain.

2. Isolate and analyze the DNA plasmid pUCD607 containing the HB101 bioluminescence Lux+ gene.

Lab A. Plasmid Minipreparation of pUCD607

Plasmid DNA must be isolated from other molecules, such as lipids, proteins, and nucleic acids such as genomic DNA and RNA. The minipreparation is a simple and efficient procedure for isolating plasmid DNA. You should be familiar with the molecular and biochemical effects of each reagent used in the protocol.

- *Glucose-Tris-EDTA:* The tris buffers the cells at pH 7.9. EDTA binds divalent cations in the lipid bilayer, thus weakening the cell envelope.
- *SDS-sodium hydroxide:* This alkaline mixture lyses the bacterial cells. The detergent SDS dissolves the lipid components of the cell envelope as well as cellular proteins. The sodium hydroxide denatures the chromosomal and plasmid DNA into single strands. The intact circles of plasmid DNA remain intertwined.
- *Potassium acetate-acetic acid.* The acetic acid returns the pH to neutral, allowing DNA strands to denature. The large, disrupted chromosomal strands cannot rehybridize perfectly but instead collapse into a partially hybridized tangle. At the same time, the potassium acetate precipitates the SDS from the cell suspension, along with proteins and lipids with which it has associated. The renaturing chromosomal DNA is trapped in the SDS/lipid/protein precipitate. Only smaller plasmid DNA and RNA molecules escape the precipitate and remain in solution.
- *Isopropanol:* The alcohol rapidly precipitates nucleic acids but only slowly precipitates proteins. Thus, a quick precipitation preferentially brings down nucleic acids.
- *Ethanol:* A wash with ethanol removes some remaining salts and SDS from the preparation.
- Tris-EDTA: Tris buffers the DNA solution. EDTA protects the DNA from degradation by DNases (which catalyze hydrolysis reactions of DNA) by binding divalent cations that are necessary cofactors for DNase activity.

Materials

Culture and reagents

- *E. coli*/pUCD607 overnight culture
- glucose-Tris-EDTA (GTE)
- SDS-sodium hydroxide (SDS-NaOH)
- potassium acetate-acetic acid (KOAc)
- isopropanol
- 95–100% ethanol
- Tris-EDTA (TE)

Supplies and equipment

- 100- to 1,000-µL micropipet and tips
- 0.5- to 10-µL micropipet and tips
- 1.5-mL test tubes
- beaker of crushed ice
- beaker for waste and used tips
- 10% bleach or disinfectant solution
- clean paper towels
- hair dryer
- microfuge
- permanent marker
- test tube rack

Safety, Handling, and Disposal

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

Procedure

Prepare duplicate minipreps.

1. Shake the culture tube to resuspend *E. coli* cells.

2. Label two 1.5-mL test tubes with your initials. Use a micropipetter to transfer 1,000 µL *of E. coli* pUCD607 overnight suspension into each tube.

3. Close the caps and place the tubes in a balanced configuration in the microfuge rotor. Spin for 1 minute to pellet cells.
   The cell pellet will appear as a small off-white smear on the bottom of the tube. Although the cell pellets are readily seen, the DNA pellets in step 14 are very difficult to observe. Get into the habit of aligning the tube with cap hinges facing up in the microfuge rotor. Then, the pellets should always be located at the tube bottom beneath the hinge.

4. Pour off the supernatant from both tubes into a waste beaker for later disinfection. *Be careful not to disturb cell pellets.* Invert the tubes and tap gently on the surface of a clean paper towel to drain thoroughly.

5. Add 100 µL GTE solution to each tube. Resuspend the pellets by pipetting solution in and out several times. Hold the tubes up to light to check that the suspension is homogeneous and that no visible clumps of cells remain.
   Accurate pipetting is essential to good plasmid yield. The volumes of reagents are precisely calibrated so that sodium hydroxide added in step 6 is neutralized by acetic acid in step 8.

6. Add 200 µL SDS/NaOH solution to each tube. Close the caps and mix the solutions by rapidly inverting the tubes five times.

7. Stand the tubes on ice for 5 minutes. The suspension will become relatively clear.

8. Add 150 µL *ice-cold KOAc* solution to each tube. Close the caps and mix solutions by rapidly inverting the tubes five times. A white precipitate will immediately appear.

9. Stand the tubes on ice for 5 minutes.

10. Place the tubes in a balanced configuration in a microfuge rotor, and spin for 5 minutes to pellet precipitate along the side of the tube.

11. Transfer 400 µL supernatant from each tube into two clean 1.5-mL tubes. *Avoid pipetting the precipitate.* Wipe off any precipitate clinging to the outside of the tip prior to expelling supernatant. Discard old tubes containing the precipitate.
    In step 11, the supernatant is saved and the precipitate is discarded. The situation is reversed in steps 14 and 17, where the precipitate is saved and the supernatant is discarded. Do step 12 quickly, and make sure that the microfuge will be immediately available for step 13.

12. Add 400 µL isopropanol to each tube of supernatant. Close the caps and mix vigorously by rapidly inverting the tubes five times. *Stand at room temperature for only 2 minutes.* (Isopropanol preferentially precipitates nucleic acids rapidly; proteins remaining in solution also begin to precipitate with time.)

13. Place tubes in a balanced configuration in the microfuge rotor, and spin for 5 minutes to pellet the nucleic acids. Align the tubes in the rotor so that the cap hinges point outward. The nucleic acid residue, visible or not, will collect under the hinge during centrifugation.

14. Pour off supernatant from both tubes. Be careful not to disturb the nucleic acid pellets. Invert tubes, and tap gently on surface of clean paper towel to drain thoroughly.
    The pellet may appear as a tiny smear or small particles on the bottom of each tube. Do not be concerned if the pellet is not visible; pellet size is not a predictor of plasmid yield. A large pellet is composed primarily of RNA and cellular debris carried over from the original precipitate. A smaller pellet often means a cleaner preparation. Nucleic acid pellets are not soluble in ethanol and will not resuspend during washing.

15. Add 200 µL 100% ethanol to each tube and close the caps. Flick the tubes several times to wash the pellets.

STOP POINT. STORE DNA IN ETHANOL AT 20°C UNTIL READY TO CONTINUE.

16. Place tubes in a balanced configuration in the microfuge rotor and spin for 2–3 minutes.

17. Pour off supernatant from both tubes. *Be careful not to disturb the nucleic acid pellets.* Invert the tubes and tap gently on the surface of a clean paper towel to drain thoroughly.

18. Dry the nucleic acid pellet by ONE of the following methods:
    - Direct a stream of warm air from a hair dryer into the open ends of the tubes for about 3 minutes. *Be careful not to blow the pellets out of the tubes.*
    - Close caps, and pulse tubes in microfuge to pool remaining ethanol. Carefully draw off drops of ethanol using a 1- to 10-mL micropipetter. Allow pellets to air dry at room temperature for 10 minutes.

19. At the end of the drying period, hold each tube up to light to check that no ethanol droplets remain. If ethanol is still evaporating, an alcohol odor can be detected. All ethanol must be evaporated before proceeding to step 20.

20. Add 15 µL TE to each tube. *Note: If you are using a 0.5- to 10-µL micropipetter, set to 7.5 µL and pipet twice.* Resuspend pellets by smashing with the pipet tip and pipetting in and out vigorously. Rinse down the side of the tube several times, concentrating on the area where the pellet should have formed during centrifugation (beneath cap hinge). Check that all DNA is dissolved and that no particles remain in the tip or on the side of the tube.

21. Pool DNA/TE solutions into one tube.

STOP POINT. FREEZE THE DNA/TE SOLUTION AT -20°C UNTIL READY TO CONTINUE. THAW BEFORE USING.

22. Clean up.

Results and Conclusions

1. Consider the three major classes of biologically important molecules: proteins, lipids, and nucleic acids. Which steps of the miniprep procedure act on proteins? on lipids? on nucleic acids?

2. What aspect of plasmid DNA structure allows it to denature efficiently in step 8?

3. What other kinds of molecules, in addition to plasmid DNA, would you expect to be present in the final miniprep sample? How could you find out?

Lab B. Restriction Analysis of pUCD607

The purpose of this laboratory is to analyze isolated plasmid pUCD607. The protocol uses the restriction enzyme *Eco*R I to digest plasmid pUCD607. DNA that is cut with restriction enzymes will leave a specific electrophoresis pattern. The plasmid used in this laboratory was genetically constructed at University of California, Davis, in the Plant Pathology department of Dr. Clarence I. Kado, Joe J. Shaw, and Peter M. Rogowsky.

Materials

Reagents

- miniprep DNA/TE
- 0.1 µg/µL pUCD607
- *Eco*R I
- 5X restriction buffer/RNase
- distilled water
- loading dye
- 0.8% agarose
- 1X Tris-Borate-EDTA (TBE) buffer
- 1 µg/µL ethidium bromide (or 0.025% methylene blue)

Supplies and equipment

- 0.5- to 10-µL micropipetter and tips
- 1.5-mL test tubes
- aluminum foil
- beaker for agarose
- beaker for wasted and used tips
- 10% bleach solution
- camera and film
- electrophoresis box
- masking tape
- microfuge
- Parafilm® or waxed paper
- permanent marker
- plastic wrap
- power supply
- disposable rubber gloves
- goggles with UV filter
- test tube rack
- ultraviolet transilluminator or other UV source
- light box
- 37°C water bath
- zipper-type plastic bags
- dark construction paper

Safety, Handling, and Disposal

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

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

Visualization of DNA fragments uses ethidium bromide, which is a mutagen and suspected carcinogen. Although it is used in very dilute concentrations, handle as directed by the instructor. Disposal of ethidium bromide: RETURN TO BIOHAZARD LIQUIDS CONTAINER, according to local ordinances.

I. Set up restriction digest

1. Use a permanent marker to label four 1.5-mL test tubes, in which restriction reactions will be performed:
   - Mini– = miniprep, no enzymes
   - Mini+ = miniprep + *Eco*R I
   - pUCD607+ = pUCD607 + *Eco*R I
   - pUCD607– = pUCD607, no enzymes

2. Use Table 1 as a checklist while adding reagents to each reaction. Read down each column, adding the same reagent to all appropriate tubes. *Use a fresh tip for each reagent.* Refer to the detailed directions that follow.

Table 1: Reagent Checklist Matrix

Tube	Miniprep DNA	pUCD607	Buffer/ RNase	*Eco*R I	Distilled H_2O
Mini–	5 µL	—	2 µL	—	3 µL
Mini+	5 µL	—	2 µL	2 µL	1 µL
pUCD607+	—	5 µL	2 µL	2 µL	1 µL
pUCD607–	—	5 µL	2 µL	—	3 µL

3. Collect the reagents and place them in a test tube rack on a lab bench.

4. Add 5 µL miniprep DNA to the tubes labeled Mini– and Mini+.

5. Use a *fresh tip* to add 5 µL pAMP to the tubes labeled pUCD607+ and pUCD607–.

6. Use a *fresh tip* to add 2 µL restriction buffer/RNase to a clean spot on each reaction tube.

7. Use a *fresh tip* to add 2 µL *Eco*R I to tubes labeled Mini+ and pUCD607+.

8. Use a *fresh tip* to add proper volumes of distilled water to each tube.

9. Close the tops of the tubes. Pool and mix reagents by pulsing the tubes in a microfuge or by sharply tapping the tube bottoms on a lab bench.

10. Place the reaction tubes in a 37°C water bath and incubate them for 30 minutes only. *Do not overincubate. During longer incubation, DNases in the miniprep may degrade plasmid DNA.*

STOP POINT. FOLLOWING INCUBATION, FREEZE THE REACTION AT -20°C UNTIL READY TO CONTINUE. THAW REACTIONS BEFORE CONTINUING TO SECTION III, STEP 1.

II. Cast 0.8% agarose

1. Seal the ends of the gel-casting tray with tape and insert a well-forming comb. Place the gel-casting tray out of the way on a lab bench so that the agarose poured in the next step can set undisturbed.
   The gel is cast directly in the box in some electrophoresis apparatuses.

2. Carefully pour enough agarose solution into the casting tray to fill it to a depth of about 5 mm. The gel should cover only about one-third the height of comb teeth. Use a pipet tip to move large bubbles or solid debris to the sides or end of the tray while the gel is still liquid.

3. The gel will become cloudy as it solidifies (about 10 minutes). Be careful not to move or jar the casting tray while the agarose is solidifying. Touch a corner of the agarose away from the comb to test whether the gel has solidified.

4. When the agarose has set, unseal the ends of the casting tray. Place the tray on the platform of the gel box so that the comb is at the negative (black) electrode.

5. Fill the box with TBE buffer to a level that just covers the entire surface of the gel.
   Too much buffer will channel the current over the top rather than through the gel, increasing the time required to separate the DNA. TBE buffer can be used several times; do not discard. If using buffer remaining in the electrophoresis box from a previous experiment, rock the chamber back and forth to remix ions that have accumulated at either end. Buffer solution helps to lubricate the comb. Some gel boxes are designed such that the comb must be removed prior to inserting the casting tray into the box. In this case, flood the casting tray and gel surface with running buffer before removing the comb. Removal from a dry gel can cause tearing of the wells.

6. Gently remove the comb, taking care not to tear the wells.

7. Make certain that the sample wells left by the comb are completely submerged. If “dimples” appear around the wells, slowly add buffer until they disappear.

STOP POINT. COVER THE ELECTROPHORESIS TANK AND SAVE THE GEL UNTIL READY TO CONTINUE. THE GEL WILL REMAIN IN GOOD CONDITION FOR AT LEAST SEVERAL DAYS IF IT IS COMPLETELY SUBMERGED IN BUFFER.

III. Load gel and electrophorese

1. Add loading dye to each reaction. Do ONE of the following:
   - Add 1 mL loading dye to each reaction tube. Close the tube tops and mix by tapping the tube bottoms on a lab bench, pipetting in and out, or pulsing in a microfuge. Make sure the tubes are placed in a balanced configuration in the rotor.
   - Place four individual droplets of loading dye (1 mL each) on a small square of Parafilm or wax paper. Withdraw the contents from the reaction tube and mix with a loading dye droplet by pipetting in and out. Immediately load the dye mixture according to step 2. Repeat successively, using a clean tip for each reaction.

2. Use a micropipetter to load the entire contents of each reaction tube into a separate well in the gel, as described below. Use a *fresh tip* for each reaction.
   A piece of dark construction paper beneath the gel box will make the wells more visible.
   a. Steady the pipet over the well using two hands.
   b. If there is air in the end of the tip, carefully depress the plunger to push the sample to the end of the tip. (If an air bubble forms a "cap" over the well, the DNA/loading dye will flow into the buffer around the edges of the well.)
   c. Dip the pipet tip through the surface of the buffer, center it over the well, and gently depress the pipet plunger to slowly expel the sample. The sucrose in the loading dye increases the density of the sample, causing it to sink to the bottom of the well. *Be careful not to punch the tip of the pipet through the bottom of the gel.*

3. Close the top of the electrophoresis box, and connect the electrical leads to a power supply, anode to anode (red-red) and cathode to cathode (black-black). Make sure both electrodes are connected to the same channel of the power supply.

4. Turn the power supply on, and set it to 100–150 volts. The ammeter should register approximately 50–100 milliamperes. If current is not detected, check the connections and try again.
   *Alternatively, set the power supply on a lower voltage and run the gel for several hours. When running two gels off the same power supply, the current is double that for a single gel at the same voltage. The Eco*R I *digest yields two bands. The small fragment runs directly behind the purplish band of bromophenol blue (equivalent to approximately 300 bp), followed by the large lux-containing fragment.*

5. Electrophorese for 20–40 minutes. Good separation will have occurred when the bromophenol blue band has moved 4–6 cm from the wells. If time allows, electrophorese until the bromophenol blue band nears the end of the gel. Stop the electrophoresis before the bromophenol blue band runs off the end of the gel.

6. Turn off the power supply, disconnect the leads from the inputs, and remove the top of the electrophoresis box.

7. Carefully remove the casting tray from the electrophoresis box, and slide the gel into a disposable weigh boat or other shallow tray labeled with your name.

STOP POINT. COVER THE ELECTROPHORESIS TANK AND SAVE THE GEL UNTIL READY TO CONTINUE. THE GEL CAN BE STORED IN A ZIPPER-TYPE PLASTIC BAG AND REFRIGERATED OVERNIGHT FOR VIEWING/PHOTOGRAPHING THE NEXT DAY. HOWEVER, OVER LONGER PERIODS OF TIME, THE DNA WILL DIFFUSE THROUGH THE GEL, AND THE BANDS WILL BECOME INDISTINCT OR DISAPPEAR ENTIRELY.

8. Stain and view the gel using ONE of the methods described in Sections IVA and IVB.
   Staining may be performed by an instructor in a controlled area when students are not present.

IVA. Stain gel with ethidium bromide and view

Caution: Review safe handling of ethidium bromide. Wear rubber gloves when staining, viewing, and photographing the gel and during cleanup. Confine all staining to a restricted sink area.

l. Flood the gel with ethidium bromide solution (1 μg/mL), and allow it to stain for 5–10 minutes. *Staining time increases markedly for thicker gels. The ethidium bromide solution may be reused to stain 15 or more gels. When staining time increases markedly, disable the ethidium bromide solution.*

2. Following staining, use a funnel to decant as much ethidium bromide solution as possible from the staining tray back into the storage container.

3. Rinse the gel and tray under running tap water.

4. If desired, the gel can be destained in tap water or distilled water for 5 minutes or more to help remove background ethidium bromide from the gel.

STOP POINT. STAINING INTENSIFIES DRAMATICALLY IF RINSED GELS SIT OVERNIGHT AT ROOM TEMPERATURE. STACK STAINING TRAYS, AND COVER THE GEL WITH PLASTIC WRAP TO PREVENT DESICCATION.

5. View under an ultraviolet transilluminator or other UV source.
Caution: Ultraviolet light can damage eyes. Never look at unshielded UV light source with the naked eye. View only through a filter or safety glasses that absorb harmful wavelengths.

6. Clean up and wash your hands before leaving the lab.

IVB. Stain gel with methylene blue and view

1. Wear rubber gloves during staining and cleanup.

2. Flood the gel with 0.025% methylene blue, and allow it to stain for 20–30 minutes.

3. Following staining, use a funnel to decant as much methylene blue solution as possible from the staining tray back into the storage container.

4. Rinse the gel in running tap water. Let the gel soak for several minutes in several changes of fresh water. DNA bands will become increasingly distinct as the gel destains.
Destaining time is decreased by rinsing the gel in warm water with agitation.

STOP POINT. FOR BEST RESULTS, CONTINUE TO DESTAIN OVERNIGHT IN A *SMALL VOLUME* OF WATER. (THE GEL MAY DESTAIN TOO MUCH IF LEFT OVERNIGHT IN A LARGE VOLUME OF WATER.) COVER THE STAINING TRAY TO RETARD EVAPORATION.

5. View the gel over a light box; cover the surface of the light box with plastic wrap to prevent staining. By convention, DNA gels are “read” from left to right, with the sample wells oriented at the top. The area extending from the well down the edge is termed a “lane.” Thus, reading down a lane identifies fragments generated by a particular restriction reaction. Scanning across lanes identifies fragments that have comigrated the same distance down the gel and are thus of like size.

Questions

1. Why is water added to the tubes in Part I?

2. What is the function of a compromise restriction buffer?

3. What are the two functions of loading dye?

4. How does ethidium bromide stain DNA? How does this relate to the need to minimize exposure to humans?

5. Troubleshooting electrophoresis: What will occur
   a. if the gel box is filled with water instead of TBE buffer?
   b. if water is used to prepare the gel instead of TBE buffer?
   c. if the electrodes are reversed?

6. Troubleshooting gels: What effect will be observed in the stained bands of DNA in an agarose gel
   a. if the casting tray is moved or jarred while the agarose is solidifying in Part II, step 3?
   b. if the gel is run at very high voltage?
   c. if a large air bubble or clump is allowed to set in the agarose?
   d. if too much DNA is loaded in a lane?

7. Linear DNA fragments migrate at rates inversely proportional to the $\log_{10}$ of their molecular weights. For simplicity's sake, base-pair length is substituted for molecular weight.

   a. The matrix in Figure 2 gives the base-pair size of λ DNA fragments generated by a *Hin*d III digest:

*Hin*d III		*Eco*R I		
Dis.	Act. bp	Dis.	Cal. bp	Act. bp
	27,491[a]			
	23,130[a]			
	9,416			
	6,557			
	4,361			
	2,322			
	2,027			
	564[b]			
	125[c]			

Figure 2: Base-Pair Matrix. [a]Pair appears as a single band on the gel. [b]Band may not be visible in methylene-blue stained gel. [c]Band runs off the end of the gel when the bromophenol blue is approximately 2 cm from the end of the gel. When present on the gel, the band is not detected by methylene blue and is usually difficult to detect with ethidium bromide staining.

   b. Using your gel, carefully measure the distance (in mm) the *Hin*d III fragments migrated from the origin. Measure from the front edge of the well to the front edge of each band. Enter the distances into the matrix. Alternatively, measure distances on an overhead-projected image of the methylene-blue-stained gel.

c. Label each pair of *Hin*d III fragments with its kilobase pair (kbp) size. For example, 27,491 bp equals 27.5 kbp.

d. Set up a sheet of semilog graph paper with the distance migrated as the *x* (arithmetic) axis and log of base-pair length as the *y* (logarithmic) axis. Then, plot distance migrated versus base-pair length for each *Hin*d III fragment.

e. Draw a best-fit line for the data.

f. Locate on the *x* axis the distance migrated by the first *Eco*R I fragment. Using a ruler, draw a vertical line from this point to its intersection with the best-fit data line.

g. Now extend a horizontal line from this point to the *y* axis. This gives the base-pair size of this *Eco*R I fragment.

h. Repeat steps f and g for each *Eco*R I fragment. Enter results in the matrix in the calculated base-pair (Cal. bp) columns for each digest.

i. Enter the actual base-pair size of each *Eco*R I fragment (as provided by your instructor) into Act. bp column of the matrix.

j. For which fragment sizes was your graph most accurate? For which fragment sizes was it least accurate? What does this tell you about the resolving ability of agarose gel electrophoresis?

Suggested Reading

Campbell, N.A. *Biology*, 3rd ed.; Benjamin/Cummings: Redwood City, CA, 1993.

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

Winfrey, M.R.; Rott, M.A.; Wortman, A.T. *Unraveling DNA: Molecular Biology for the Laboratory;* Prentice-Hall: Upper Saddle River, NJ, 1997.

INSTRUCTOR NOTES
Characterization of the Bioluminescence (Lux$^+$) Gene in the Microorganism *E. coli*

Time Required
Two (3-hour) laboratory sessions
- Day 1: Lab A. Plasmid Minipreparation of pUCD607
- Day 2: Lab B. Restriction Analysis of pUCD607

Group Size
A maximum group size of 20 students working in groups of two works well.

Materials
Materials and reagents needed are listed at the beginning of each lab.

Materials used to disable stained gels and staining solution
- 0.5 M $KMnO_4$
- distilled water
- 2.5 N HCl
- 2.5 N NaOH

Safety, Handling, and Disposal
As the instructor, you are expected to provide students with access to SOPs, MSDSs, and other resources they need to safely work in the laboratory while meeting all regulatory requirements. Before doing this activity or activities from other sources, you should regularly review special handling issues with students, allow time for questions, and then assess student understanding of these issues. When dealing with biological materials, take particular precautions as called for by the kit manufacturer or supplier.

Lab A and B
- Aliquoting reagents: It is safest to prepare separate aliquots of the enzyme, DNA, and buffer in 1.5-mL test tubes. Each aliquot should contain slightly more than will be required for each lab. Following the experiment, the tubes are discarded, and new aliquots are made. Although this practice may appear wasteful, it prevents cross-contamination that invariably occurs if aliquots are reused or shared.

Lab B
- Handling of ethidium bromide: The protocols limit the use of ethidium bromide to a single procedure that can be performed by an instructor in a controlled area. Handled responsibly, the dilute solution used for gel staining poses minimal risk. Ethidium bromide is a mutagen and a suspected carcinogen. The following are precautions for handling and decontaminating ethidium bromide:

    1. Always wear gloves when working with ethidium bromide solutions or stained gels.

    2. Limit ethidium bromide use to a restricted sink area.

3. Following gel staining, use a funnel to decant as much as possible of the solution into a storage container for reuse or decontamination and disposal.

4. Disable stained gels and used staining solution according to accepted laboratory procedure. One method is given below.
   a. If necessary, add sufficient water to reduce the concentration to less than 0.5 mg/mL.
   b. Add 1 volume of 0.5 M $KMnO_4$ and mix carefully.
      Caution: $KMnO_4$ is an irritant and is explosive when in contact with an oxidizable substance. Solutions containing $KMnO_4$ should be handled in a chemical hood.
   c. Add 1 volume of 2.5 N HCl and mix carefully.
   d. Let stand at room temperature for several hours.
   e. Add 1 volume of 2.5 N NaOH and mix carefully
   f. Discard the disabled solution down a sink drain. Drain the disabled gels and discard them in regular trash.

Dispose of used reagents and the gels and solutions containing ethidium bromide according to local ordinances.

The greatest risk is to inhale ethidium bromide powder when mixing a stock solution. Purchasing a ready-mixed stock solution is recommended. The stock solution is diluted to make a staining solution with the required final concentration.

- Viewing stained cells: Transillumination gives superior viewing of gels stained with either ethidium bromide or methylene blue. A mid-wavelength (260–360 nm) ultraviolet (UV) lamp emits in the optimum range for illuminating stained gels. Avoid short-wavelength lamps, whose radiation is most dangerous.
  Caution: Ultraviolet light can damage the retina of the eye. Never look at an unshielded UV light source with the naked eye. View only through filter or safety glasses that absorb harmful wavelengths.

Points to Cover in the Pre-Lab Discussion

Provide a safety discussion for Labs A and B.

Procedural Tips and Suggestions

Storing and handling restriction enzymes

The enzymes are most stable at cold temperatures, losing activity if warmed for any length of time. Following the guidelines below will increase the success of the labs.

a. Store the enzymes in a NON-frost-free freezer with a constant temperature between -10 and -20°C. If a frost-free freezer must be used, store the enzymes in their Styrofoam® shipping container within the freezer to help maintain constant temperature.
b. Split large shipments of enzyme into several smaller aliquots of 50–100 µL in 1.5-mL test tubes. Label the test tubes with type, concentration, and date received.
c. Remove the enzymes from the freezer directly down into crushed or cracked ice in an insulated ice bucket or cooler. Keep the enzymes on ice at all times during preparation, and return them to the freezer immediately after use.
d. Keep aliquots of enzymes, buffer, and DNA in a cooler filled with ice while in use.
e. Although it is good technique to set up restriction digests on ice, little loss of enzyme activity occurs during brief setup time when reactions are set up in a test tube rack at room temperature.

Storing DNA and buffer

Repeated freezing and thawing damages DNA. Restriction buffer is best kept frozen and is not affected by freezing and thawing. Tris-Borate-EDTA (TBE) electrophoresis buffer can be reused several times. Collect and store in a large carboy. If different gels will be run over several days, store the buffer in an electrophoresis box with the cover in place. Prior to reusing, rock the buffer back and forth to reequilibrate ions that differentially accumulate at either end during electrophoresis. Groups of restriction enzymes operate under different conditions of salt and pH. To simplify procedures, use a "compromise" restriction buffer—a universal buffer that is a compromise between the conditions preferred by various enzymes.

All buffers are used at a final concentration of lX. Rely on standard $C_1V_1 = C_2V_2$ formula to determine how much buffer to add to obtain a 1X solution:

volume of buffer	concentration of buffer	=	total volume of reaction	1X buffer
(5 µL)	(2X)	=	10 µL	(1X)
(1 µL)	(10X)	=	10 µL	(1X)

For convenience, use 2X restriction buffer whenever possible—it can save a pipetting step to add water to bring a reaction up to 10 µL total volume. It is also easier and more accurate to pipet 5 µL than 1 µL.

Diluting DNA

DNA for short-term use can be diluted with distilled or deionized water. However, dilute with Tris-EDTA (TE) buffer for long-term storage. The EDTA in the buffer binds divalent cations, such as Mg^{2+}, that are necessary cofactors for DNA-degrading nucleases. Always dilute DNA to the concentration specified by the protocol.

1. Determine the total volume of DNA required by multiplying the number of experiments by the total volume of DNA per experiment, including overage.

2. Use this total volume required in the volume formula, along with the desired final DNA concentration and the concentration of the stock DNA. Solve for V_1, the volume of stock DNA needed in the dilution.

3. Add water or TE to make the total volume of final solution.

Aliquoting reagents

It is safest to prepare separate aliquots of enzyme, DNA, and buffer in 1.5-mL test tubes for each experiment. Each aliquot should contain slightly more than will be required for the lab. Following the experiment, the tubes are discarded, and new aliquots are made for the next experiment. This prevents cross-contamination and provides consistent results.

For aliquots of restriction enzymes, add 1 µL extra when 1–3 µL are called for and 2 µL extra when 4–6 µL are actually needed. The overage aids in visualizing the reagent in the tube and allows for

small pipetting errors. Do not make small aliquots of enzymes more than 1–2 days in advance. Also, make sure that the tubes are fully submerged in ice while awaiting use.

Aliquots of DNA and buffer should be approximately 20% larger than volume actually needed. This allows for overpipetting and other mishaps. Considering that DNA is generally the most expensive reagent, you may prefer to aliquot the exact amount and add other reagents directly to the DNA tube.

Colored 1.5-mL test tubes are very handy for color-coding each reagent aliquot.

Working with *E. coli* HB101 to optimize lux expression:

1. Measure at log phase.

2. Grow at 28–30°C.

3. Use LB amp agar plate.

4. Minimize background light when viewing.

Viewing stained cells

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A fluorescent light box for viewing slides and negatives provides ideal illumination for methylene-blue-stained gels. An overhead projector may also be used. Cover the surface of the light box or projector with plastic wrap to keep liquid off the apparatus.

References

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