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Producing a Strain of *E. coli* that Glows in the Dark

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INTRODUCTION

Description

In this exercise, students will create a luminescent population of bacteria by introducing into *Escherichia coli* (*E. coli*) a plasmid that contains the *lux* operon. This operon is found in the luminescent bacterium *Vibrio fischeri* and contains two genes that code for luciferase (the enzyme that catalyzes the light-emitting reaction) and several genes that code for enzymes that produce the luciferins (the substrates for the light-emitting reaction). The success of the transformation is readily apparent, since *E. coli* colonies that take up this plasmid glow in the dark. In another group, a control plasmid (pUC18) that does not contain the *lux* operon will be introduced into *E. coli*, and these cells will not glow in the dark. Both plasmids contain an ampicillin-resistant gene, and therefore both cell types will grow in the presence of the antibiotic. Each resistant colony growing on ampicillin-nutrient agar plates represents a single transformation event, and only those colonies that have taken up the plasmids will grow on the agar. The bioluminescent colonies represent bacteria that can successfully express a metabolic marker.

Student Audience

This exercise is suitable for middle and high school students who have discussed concepts related to DNA structure and function, the tools and methods of biotechnology, and present-day applications of those tools and methods.

Goals for the Activity

The main goals for this activity are to

- enable students to observe the experimental process called bacterial transformation,
- demonstrate the relationship between the genetic constitution of an organism and its physical attributes,
- enable students to observe the change in phenotype caused by the uptake and expression of a known plasmid sequence, and
- reinforce the need for sterile technique when working with bacteria.

Recommended Placement in the Curriculum

This exercise is best used in conjunction with a unit presenting information on the molecular basis of heredity, gene expression, the genetics of viruses and bacteria, and DNA technologies.

STUDENT HANDOUT

Producing a Strain of *E. coli* that Glows in the Dark

Purpose

The purpose of this exercise is to demonstrate phenotype changes in bacteria that have been transformed with an antibiotic-resistance gene and a metabolic marker and to introduce the concept of recombinant DNA and cloning vectors.

Discussion

Plasmids are small, circular DNA molecules that exist apart from the chromosomes in most bacterial species. Under normal circumstances, plasmids are not essential for survival of the host bacteria. However, many plasmids contain genes that enable bacteria to survive and prosper in specific environments. For example, some plasmids carry one or more genes that confer resistance to antibiotics. A bacterial cell containing such a plasmid can live and multiply in the presence of the drug. Indeed, antibiotic-resistant *Escherichia coli* (*E. coli*) isolated in many parts of the world contain plasmids that carry the genetic information for protein products that interfere with the action of many different antibiotics. In this laboratory, you will introduce a plasmid that contains an ampicillin-resistance gene into *E. coli*.

One plasmid that you will use is called pUC18. Plasmid pUC18 contains only 2,686 nucleotide pairs (molecular weight = 2×10^6). The small size of this plasmid makes it less susceptible to physical damage during handling. In addition, smaller plasmids generally replicate more efficiently in bacteria and produce larger numbers of plasmids per cell. As many as 500 copies of this plasmid may be present in a single *E. coli* cell. Plasmid pUC18 contains an ampicillin-resistance gene that enables *E. coli* to grow in the presence of the antibiotic. Bacteria lacking this plasmid, or bacteria that lose the plasmid, generally will not grow in the presence of ampicillin. The ampicillin-resistance gene of pUC18 codes for the enzyme beta-lactamase (penicillinase), which inactivates ampicillin and other penicillins.

In the laboratory, plasmids can be introduced into living bacterial cells by a process known as transformation. When bacteria are placed in a solution of calcium chloride (CaCl_2), they acquire the ability to take in plasmid DNA molecules. This procedure provides a means for preparing large amounts of specific plasmid DNA, since one transformed cell gives rise to clones that contain exact replicas of the parent plasmid DNA molecule. Following growth of the bacteria in the presence of the antibiotic, the plasmid DNA can be readily isolated from the bacterial culture.

Plasmids are very useful tools for the molecular biologist because they serve as gene-carrier molecules. A basic procedure of recombinant DNA technology consists of joining a gene of interest to plasmid DNA to form a hybrid, or recombinant, molecule that is able to replicate in bacteria. In order to prepare a recombinant molecule, the plasmid and gene of interest are cut at precise molecules and spliced together. After the hybrid plasmid molecule has been prepared, it is introduced into *E. coli* cells by transformation. The hybrid plasmid replicates in the dividing bacterial cells to produce an enormous number of copies of the original gene. At the end of the growth period, the hybrid molecules are purified from the bacteria, and the original gene of interest is recovered. This method has enabled scientists to obtain large quantities of more than 1,000 specific genes, including the genes for human interferon, insulin, and growth hormone.

DNA technology has triggered advances in almost all fields of biology by enabling biologists to tackle specific questions with finer tools. By far the most ambitious research project made possible is the Human Genome Project, a multibillion-dollar effort to determine the nucleotide sequence of the entire human genome. The entire human genome will be characterized by cloning in specialized vectors and by analyzing the clones to determine where their inserted DNA molecules reside on the 23 chromosomes of the human genome. Such an analysis, aided by research on smaller genomes, produces a physical map of all the genes in the human genome. This map will be pivotal in research concerning the diagnosis of human genetic disorders and other diseases, the application of gene therapy, and the development of vaccines and other pharmaceutical products.

DNA technology also offers applications to the study of forensic science, problems relating to the environment, and the ever-growing need for better, more productive agriculture. DNA testing has refined the process of forensic analysis because not as much tissue is required and guilty individuals can be identified with a higher degree of certainty. With recent advances in restriction fragment length polymorphism (RFLP) analysis, polymerase chain reaction (PCR), and variable number of tandem repeats (VNTRs), the probability that two people will have exactly the same DNA fingerprints is very small. (For legal cases the probability is between one chance in 100,000 and one in a billion, depending on the number of genomic sites compared.)

Environmental research has seen an increase in the applications of genetics engineering. Microorganisms are being used to extract heavy metals from mining waste; clean up the environment; provide another source for metals such as lead, copper, and nickel; recycle wastes; and detoxify toxic chemicals. Sewage treatment facilities rely on the ability of microbes to degrade many organic compounds into nontoxic forms. Chlorinated hydrocarbons are a real problem, and biotechnologists are attempting to engineer microorganisms that can be incorporated into the treatment process to degrade these compounds. Eventually the microorganisms may be incorporated into the manufacturing of these toxic chemicals, thus preventing their release as waste.

Agricultural applications of DNA technology include research in the areas of animal husbandry and manipulating plant genes. Products of recombinant DNA methods are already being used to treat farm animals. These include new or redesigned vaccines, antibodies, and growth hormones. Bovine growth hormone (BGH), made by *E. coli*, increases milk production by approximately 10% in milk cows and improves weight gain in beef cattle. Transgenic organisms (organisms that contain genes from other species) have been developed for potential agricultural and medical uses (e.g., organ transplants, production of human hormones, etc.). Genetic engineers working with plant genes commonly use DNA vectors to move genes from one organism to another. Several companies have developed strains of wheat, cotton, and soybeans carrying a bacterial gene that makes the plants resistant to herbicides used by farmers to control weeds. Tomatoes containing genes that retard spoilage have been approved by the Food and Drug Administration (FDA). A number of crop plants have been engineered to be resistant to infectious pathogens and pest insects. Increasing the ability of bacteria to “fix” nitrogen or transferring the ability to fix nitrogen directly to plants would reduce or eliminate the need for expensive and environmentally detrimental fertilizers.

DNA technologies have many uses in fields as diverse as medicine, agriculture, forensic science, ecology, and industry. The early successes in research point to a revolution just around the corner. Using these technologies wisely will become a challenge of the next century. (Excerpts taken from Campbell, 1996, Ogden and Adams, 1989, Micklos and Bloom, 1989, and Anderson, 1995.)

Materials

Per class

- control plasmid (pUC18)
- plasmid *lux*
- 20 petri dishes containing ampicillin-nutrient agar
- nutrient broth
- 25 inoculating loops
- 19 large, sterile transfer pipets
- 18 sterile tubes
- calcium chloride (CaCl₂) solution
- *E. coli* (DH5)
- water bath maintained at 37°C
- ice bath
- light-free room
- sterile micropipets
- 10% bleach solution, soapy water, or disinfectant (such as Lysol®)

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. Dispose of used reagents according to local ordinances.

When dealing with biological materials, take particular precautions as called for by the kit manufacturer or supplier. A commensal organism of *Homo sapiens*, *E. coli* is a normal part of the bacterial fauna of the human gut. It is not considered pathogenic and is rarely associated with any illness in healthy individuals. Adherence to simple guidelines for handling and disposal makes work with *E. coli* a nonthreatening experience for instructor and students.

Wear gloves when handling bacteria. When pipetting a suspension culture, keep your nose and mouth away from the tip end to avoid inhaling any aerosol that might be created.

Avoid overincubating plates. Because a large number of cells are inoculated, *E. coli* is generally the only organism that will appear on plates incubated for 12–24 hours at 37°C. However, with longer incubation, contaminating bacteria and slower-growing fungi may arise. If students will not be able to observe plates following initial incubation, refrigerate plates to retard growth of contaminants.

Always re flame the inoculating loop or cell spreader one final time before setting it down on the lab bench. Wipe down lab benches with 10% bleach solution, soapy water, or disinfectant (such as Lysol) at the end of the activity. Wash your hands before leaving the laboratory.

Collect bacterial cultures, as well as tubes, pipets, and transfer loops that have come into contact with the cultures. Disinfect these materials as soon as possible after use. Contaminants, often smelly and sometimes potentially pathogenic, can be cultured over a period of several days at room temperature. Disinfect bacteria-contaminated materials in one of two ways:

- Treat with 10% bleach solution (5,000 ppm available chlorine). Immerse contaminated pipets, transfer loops, and open tubes directly in a sink or tub containing bleach solution. Plates should be placed in a sink or tub and flooded with bleach solution. Let materials stand in bleach solution for 15 minutes or more. Then drain excess bleach solution, seal the materials in a plastic bag, and dispose of them as regular garbage.
- Autoclave these items at 121°C for 15 minutes. Tape three or four culture plates together and tightly close tube caps before disposal. Collect contaminated materials in an autoclavable bag and seal the bag before autoclaving. Dispose of the materials as regular garbage.

Procedure

In this exercise, plasmid *lux* and a control plasmid (pUC18) will be introduced into *E. coli* by transformation. There are four basic steps to the procedure:

- A. Treat bacterial cells with CaCl_2 solution in order to enhance the uptake of plasmid DNA. Such CaCl_2 -treated cells are said to be “competent.” (This step should be performed by the instructor before or during the laboratory session.)
- B. Incubate the competent cells with plasmid DNA.
- C. Select those cells that have taken up the plasmid DNA by growth on an ampicillin-containing medium.
- D. Examine the cultures in the dark.

A. Preparation of competent cells

(These steps should be performed by the instructor.)

1. Place a vial of CaCl_2 solution and the tube of *E. coli* in the ice bath.
2. Using a sterile pipet, transfer about 0.5 mL CaCl_2 solution to the tube containing the bacteria.
3. Using the same pipet, transfer the contents of this tube back into the vial that contains most of the CaCl_2 solution.
4. Tap the vial with the tip of your index finger to mix the solution.
5. Incubate the cells for about 10 minutes on ice. The cells are then called competent because they can take up DNA from the medium. If desired, the cells can be stored in the CaCl_2 solution for 12–24 hours.

B. Uptake of DNA by competent cells

1. Label one small tube “C DNA” (for control DNA) and one tube “L DNA” (for plasmid *lux*).
2. Place the two tubes in an ice bath.
3. Using a sterile micropipet, add 10 μL control plasmid to the tube labeled “C DNA” and 10 μL plasmid *lux* to the tube labeled “L DNA.”

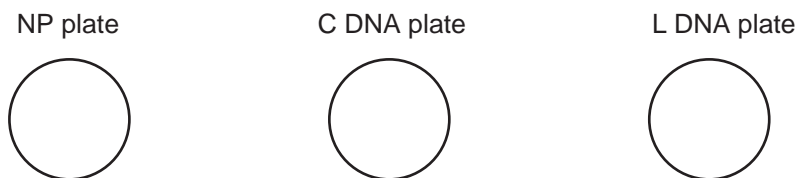
4. Gently tap the tube of competent cells with the tip of your index finger to ensure that the cells are in suspension. Then, using a sterile transfer pipet, add 6 drops of the competent cells (approximately 120 μL) to each of the two tubes. Tap each of these tubes with the tip of your index finger to mix these solutions, and store both tubes on ice for 10–15 minutes. The competent cells, which are suspended in the CaCl_2 solution, will now begin to take up the plasmid DNA. During this time, one member of the class should obtain two additional tubes. Add 6 drops of competent cells to each tube and label the tubes “NP” (no plasmid).
5. Transfer the tubes to a water bath preheated to 37°C , and allow them to sit in the bath for 5 minutes.
6. Use a sterile pipet to add about 0.7 mL nutrient broth to each tube and incubate the tubes at 37°C for 30–45 minutes. This incubation period allows the bacteria time to recover from the CaCl_2 treatment and to begin to express the ampicillin-resistant gene on the plasmid.

C. Selection of cells that have taken up the plasmid by growth on an ampicillin-containing medium

1. Obtain four ampicillin-nutrient agar plates from your instructor. Label one plate “C DNA,” another plate “L DNA,” and the remaining plates “NP.”
2. Using a sterile pipet, remove 0.25 mL mixed bacterial suspension from the “C DNA” tube, remove the lid from the “C DNA” plate, and dispense the bacteria onto the agar. Use an inoculating loop to spread the bacteria evenly onto the agar surface.
3. Transfer 0.25 mL bacterial suspension from the “L DNA” tube to the “L DNA” plate and spread these cells onto the agar surface as described in the previous step.
4. Cells from the two tubes that did not contain plasmids (NP) should be plated onto two plates (NP) as described in steps 2–3.
5. Replace the lids on the plates, and leave the plates at room temperature until the liquid has been absorbed (about 10–15 minutes).
6. Invert the plates and incubate them in the light-free room at room temperature.

Data Analysis and Study Questions

1. Predict the growth on the following plates by marking “+” for growth and “–” for no growth in the circles below.



2. Colonies should appear in about 2–3 days at room temperature. Plates must be viewed at that time, since bioluminescence decreases with time after colony formation. Allow at least 3 minutes for the eyes to adjust to the dark in a light-free room. View your plates and the plates of your classmates in the dark and then in the light. Record your results in the following table. Were the results as expected? Explain possible reasons for variations from expected results.

Plate Designation	# Colonies	Bioluminescent Colonies
NP (no plasmid)		
Control plasmid (pUC18)		
Plasmid <i>lux</i>		

3. Why do the cells transformed with pUC18 and plasmid *lux* grow in the presence of ampicillin?
4. Name one enzyme that is produced by cells transformed with plasmid *lux* that is not produced by the cells transformed by pUC18.
5. Remembering that plasmid size will affect the efficiency of transformation, which plate would be expected to show the fewest colonies?

Suggested Readings

Access Excellence®. About Biotech. www.accessexcellence.org//AB/index.html (accessed April 4, 2000).

National Human Genome Research Institute Glossary of Genetic Terms. www.nhgri.nih.gov/DIR/VIP/Glossary/pub_glossary.cgi (accessed April 4, 2000).

The University of Arizona. The Biology Project. www.biology.arizona.edu/ (accessed April 4, 2000).

References

Anderson, J. "Producing a Strain of *E. coli* that Glows in the Dark," *Modern Biology*. 1995.

Campbell, N.A. *Biology*; Benjamin/Cummings: Menlo Park, CA, 1996.

Micklos, D.A.; Bloom, M.J. *DNA Science: A First Laboratory Course in Recombinant DNA Technology*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.

Ogden, R.; Adams, D.A. "Recombinant DNA Technology: Basic Techniques," *Carolina Tips*. 1989, 52, 13–16.

Ogden, R.; Adams, D.A. "Recombinant DNA Technology: Applications," *Carolina Tips*. 1989, 52, 17–20.

INSTRUCTOR NOTES

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Time Required

The activity may be conducted using the following time table:

Day	Time Needed	Activity
10 days before lab	75 min	Prepare LB agar plates.
1– 2 days before lab	15 min	Streak starter plates.
1 day before lab	20 min	Brief students on lab.
lab day	30 min	Set up student work areas.
lab day	45 min	Perform colony transformation experiment.
2– 3 days after lab	30 min	Discuss results in class.

Group Size

The activity may be conducted in groups of two or three students.

Materials

If the author's instructions call for use of a commercial kit and specific materials provided in that kit, we strongly recommend that you use the recommended materials to attain the desired results. When using a commercial kit, read and follow the instructions provided by the kit manufacturer. The author's procedure provided in this activity is not necessarily intended to duplicate or reproduce the manufacturer's instructions. Rather, the procedure has been provided by the author as a summary of the general steps to follow.

This lab kit can be purchased from Modern Biology, Inc., 111 North 500 West; West Lafayette, Indiana 47906. 1-800-733-6544, FAX 1-765-743-7612.

Per class

- 110 μ L control plasmid (pUC18)
- 110 μ L plasmid *lux*
- 20 Petri dishes containing ampicillin-nutrient agar
- 10 mL nutrient broth
- 25 inoculating loops
- 19 large sterile transfer pipets
- 18 sterile tubes
- 0.6 mL CaCl_2 solution
- 0.5 mL *E. coli* (DH5)
- water bath maintained at 37°C
- ice bath
- light-free room
- sterile micropipets
- (optional) 37°C air incubator
- 10% bleach solution, soapy water, or disinfectant (such as Lysol®)

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. A commensal organism of *Homo sapiens*, *Escherichia coli* (*E. coli*) is a normal part of the bacterial fauna of the human gut. It is not considered pathogenic and is rarely associated with any illness in healthy individuals. Adherence to simple guidelines for handling and disposal makes work with *E. coli* a nonthreatening experience for instructor and students.

Wear gloves when handling bacteria. When pipetting a suspension culture, keep your nose and mouth away from the tip end to avoid inhaling any aerosol that might be created.

Avoid overincubating plates. Because a large number of cells are inoculated, *E. coli* is generally the only organism that will appear on plates incubated for 12–24 hours at 37°C. However, with longer incubation, contaminating bacteria and slower-growing fungi may arise. If students will not be able to observe plates following initial incubation, refrigerate plates to retard growth of contaminants.

Always reflare the inoculating loop or cell spreader one final time before setting it down on the lab bench. Wipe down lab benches with 10% bleach solution, soapy water, or disinfectant (such as Lysol) at the end of the activity. Wash your hands before leaving the laboratory.

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- Autoclave these items at 121°C for 15 minutes. Tape three or four culture plates together and tightly close tube caps before disposal. Collect contaminated materials in an autoclavable bag and seal the bag before autoclaving. Dispose of the materials as regular garbage.

Dispose of used reagents according to local ordinances.

Points to Cover in the Pre-Lab Discussion

Discuss the concept of bacterial transformation:

- production of competent cells;
- incubation of cells and uptake of plasmid DNA;
- selection of cells using an ampicillin-containing medium;
- examination of cultures; and
- contamination prevention through use of sterile techniques.

Stress the importance of proper disposal of used equipment and materials to prevent the spread of bacteria. Review the proper use of a micropipet. Note that an inoculating loop can be used to dispense 10 μL (10 μL = one loop full).

Procedural Tips and Suggestions

A room with lights out and shades drawn does not provide enough darkness to observe luminescence. The room must be in total darkness to the point of not seeing your hand in front of your face.

Luminescence is apparent at 18–24 hours after transformation when plates are incubated at 37°C or at 2–3 days at room temperature. After these times, the amount of light emitted decreases.

Luminescence can be maintained if the cells that carry the *lux* plasmid are subcultured every 3–4 days on agar-ampicillin plates.

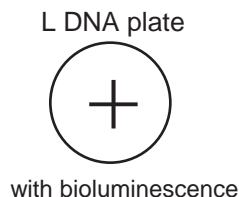
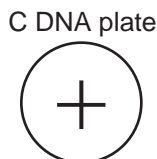
Instead of using the microliter pipets, the inoculating loops can be used to dispense 10 μL (10 μL = one loop full).

During the uptake of DNA by competent cells, have an activity to occupy your students. The 30–45 minutes it takes for the cells to incubate can be a good time to discuss the process of bacterial transformation.

Preparation of competent cells by the instructor can save time in the lab session.

Plausible Answers to Questions

1. Predict the growth on the following plates by marking “+” for growth and “–” for no growth in the circles below.



2. Colonies should appear in about 2–3 days at room temperature. Plates must be viewed at that time, since bioluminescence decreases with time after colony formation. Allow at least 3 minutes for the eyes to adjust to the dark in a light-free room. View your plates and the plates of your classmates in the dark and then in the light. Record your results in the following table. Were the results as expected? Explain possible reasons for variations from expected results.

Plate Designation	# Colonies	Bioluminescent Colonies
NP (no plasmid)	None	No
Control plasmid (pUC18)	20 to 200	No
Plasmid <i>lux</i>	20 to 200	Yes

If bioluminescent colonies are growing, then students can just answer “yes.” If no bioluminescent colonies appear to be growing on the plates, then the students should answer “no” and provide possible explanations for the results. The following might be an acceptable explanation: There were no bioluminescent colonies growing on the L DNA plate. The bacterial cells might not have been competent to uptake the plasmid. There could have been contamination due to not using sterile technique. The plasmid might not have been of the proper concentration.

3. Why do the cells transformed with pUC18 and plasmid *lux* grow in the presence of ampicillin? *Both plasmids grow because they contain an ampicillin-resistance gene that codes for a product (beta-lactamase) that breaks down ampicillin.*
4. Name one enzyme that is produced by cells transformed with plasmid *lux* that is not produced by the cells transformed by pUC18.
Luciferase.
5. Remembering that plasmid size will affect the efficiency of transformation, which plate would be expected to show the fewest colonies?
*The plasmid *lux* plate would be expected to show the fewest colonies because that plasmid is larger than plasmid pUC18 and would be more difficult for the cells to absorb. The larger plasmid *lux* is also more susceptible to physical damage and probably replicates less efficiently.*

Extensions

This lab could be performed using a variety of different plasmids that are on the market today. Many biological supply companies sell a host of recombinant DNA plasmids that would be suitable to demonstrate bacterial transformation.

Extensions of this lab could include the following:

- Analyze the effects of growth temperature and time on the glowing process. This analysis can be carried out by replating the transformed cells onto fresh nutrient-ampicillin agar plates.
- Isolate plasmid *lux* from transformed cells using plasmid DNA isolation techniques. The plasmids can then be digested with various restriction endonucleases to identify the plasmids that were cloned.

References

- Anderson, J. "Producing a Strain of *E. coli* that Glows in the Dark," *Modern Biology*. 1995.
- Campbell, N.A. *Biology*; Benjamin/Cummings: Menlo Park, CA, 1996.
- Micklos, D.A.; Bloom, M.J. *DNA Science: A First Laboratory Course in Recombinant DNA Technology*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.
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