

Dolan DNA Learning Center www.dnalc.org

Glowing Genes

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Background

History

A chromosome is a continuous DNA molecule that can be thousands or millions of base pairs long. The vast length of chromosomes posed a problem for scientists who were trying to isolate and study the stretches of DNA that make up genes. The discovery of restriction enzymes in 1962 gave scientist the "molecular scissors" they needed to isolate specific regions of DNA. By the end of the 1960s, DNA could be cut and pasted. But scientists needed a mechanism to copy it in order to maintain a large enough sample to work with. Because normal bacterial reproduction produces huge quantities of the desired DNA, *E. coli* was an ideal model organism. In 1971, scientists found an efficient way to introduce tiny loops of DNA called plasmids into *E. coli*. These loops of plasmid DNA can carry a package of foreign DNA into the host cell. Scientists can use plasmids containing a selected piece of DNA to transform, or change, the bacterium. Genetic engineering is a set of techniques used to manipulate DNA. In our experiment we will genetically engineer the bacteria cells by introducing two new genes.

Applications

In recombinant DNA technology, it is important to explore the possible applications within society. Diabetes is a genetic disease where the individual is not properly metabolizing glucose and may need to take injections of the hormone insulin. The pharmaceutical production of insulin uses similar procedures as the experiment we are conducting with our students. This technology is a safe alternative to the collection of insulin from the pancreas of cadavers and other organisms. While there are numerous branches of this research, for the purpose of our experiment, it is useful to focus on the relevance when treating and possibly curing genetic disease.

Genes and Proteins

A gene is a sequence of DNA that encodes the information to make a protein and to determine how much of a protein will be made. The genetic sequence for a protein is called a genotype. The observed result, or trait, is called the phenotype. Proteins have three major functions in cells: (1) they provide structure, (2) act as biological catalysts, called enzymes, and (3) serve as hormones. Structural proteins make human traits, or phenotypes. For example, keratin is the structural protein for hair. The chemical structure of keratin changes the shape of hair. Curly hair has a different chemical structure than straight hair.

The Green Fluorescent Protein

The Green Fluorescent Protein (GFP) gene is normally found in the Pacific jellyfish, *Aquaria victoria*. When the jellyfish are excited, GFP causes them to give off a fluorescent green light. This intensity of this protein is enhanced by UV light. By knowing the location of the gene and the sequence of chemicals in the jellyfish DNA, we are able to use appropriate restriction enzymes to cut out the GFP gene from the jellyfish genome. Once we have the gene isolated, we then glue it into a plasmid that contains a second gene. The second gene codes for a protein that enables the bacteria to break down the antibiotic ampicillin.



Host

Our host in this experiment is a type of bacteria called *Escherichia coli*. The specific strain that we will be using is called mm294 and is a safe organism with which to work. The bacteria used in this laboratory are unlikely to grow inside the human intestine if accidentally ingested. The strain was derived from *E. coli* that was originally isolated from the feces of a diphtheria patient at Stanford University in 1922.

E. coli is the most widely used organism in molecular biology for a number of reasons. They are prokaryotic organisms that provide a simple and well understood genetic environment. Their ability to store and express foreign DNA makes them a suitable host organism. The generation time is approximately thirty minutes and these small organisms are inexpensive and easy to grow. In addition, they can be stored as frozen stock.

Vector

A vector is a DNA molecule that is used as a vehicle to carry foreign DNA. We are using the simplest bacterial vectors, plasmids, which are small rings of DNA that exist independently of the main bacterial chromosome. They can be replicated and passed on to offspring. Plasmids can be used as tools to propagate large quantities of a given DNA sequence or as a means of getting a foreign gene expressed in a host cell.

Selection

The host cells are grown in media that contains the antibiotic ampicillin. Due to the selective media, only cells that contain a plasmid with resistance to the antibiotic can survive. By killing off cells that do not contain the plasmid, we are left with a pure population of cells that do contain the plasmid. Different antibiotics function in different ways. Ampicillin is a member of the penicillin family and interferes with cell wall formation. This antibiotic only kills cells that are actively dividing.

Transformation

In order to begin the bacterial transformation, we must first create bacterial cells that are competent to take up the plasmids. We start by growing the bacteria overnight on a media containing Luria broth. By using sterile technique, we transfer the *E. coli* cells and suspend them in a calcium chloride solution. After placing the competent cells and the plasmids in the same tube, we put the tube at 0°C. While the exact mechanism by which the cells take up the plasmid DNA is unknown, one hypothesis is that the plasmids pass through zones of adhesion. These regions are located where the outer and inner cell membranes are fused to pores in the cell wall and are only present in growing cells. By placing the tubes on ice, the distribution of charged phosphates in the cell membranes is stabilized. The role of the positively charged calcium ions is to shield the negatively charged phosphate ions of the outside on the bacterial cell. This increases the probability of the plasmids moving through the adhesion zones. The transfer of the tubes to 42°C in the heat shock creates a thermal gradient between the inside and outside of the cell that physically helps to move the DNA through the adhesion zone. After placing the tubes on ice the bacteria are transferred to a media containing Luria broth and ampicillin. The cultures are then placed at 37°C overnight.



Description of Activity

This one-hour laboratory provides children in grades 6-8 with the opportunity to perform a genetic engineering experiment. Students transfer a gene for green fluorescent protein (GFP) normally found in the Pacific jellyfish *Aequorea victoria*, into bacteria. GFP glows bright green when exposed to UV light, which in effect produces glowing bacteria! This lesson can be used as a springboard into a discussion of the ethical implications of genetic engineering in regards to treatment and/or prevention of human genetic disease as well as techniques currently used to produce human proteins such as insulin.

Learning Outcomes

Students will:

- learn about the role of a host, vector, and mode of selection in transformation.
- understand concepts such as gene splicing and heat shock.
- discuss the numerous applications of genetic engineering.
- recognize the significance of gene therapy in treating and preventing genetic disease.

Assumptions of Prior Knowledge

Students should be familiar with the structure and function of DNA, and understand the relationship between DNA, genes, and proteins.

Misconceptions

Many students assume that all bacteria are virulent. It is important to emphasize that the bacterial strain in this experiment is harmless.

Students may also believe that it is possible to genetically change a multicellular organism by simply injecting or ingesting DNA.

Lesson

Become familiar with the DNA Interactive (DNAi) Internet site (<u>www.dnai.org</u>) and how to navigate through it. Take time to explore the chapter titled **Manipulation** for background information on recombinant DNA technology and transformation.

Materials and Equipment

- Racks or cups to hold tubes (one per group)
- Permanent markers (one per group)
- Tubes of sterile glass beads (one per group)
- 15ml snap cap tubes (one per group)
- 1.5ml tubes (one per group)
- Large micropipettors (100-1000µl)* (one per group)
- Small micropipettor (0.1-10µl)
- Large micropipettor tips*
- Small micropipettor tips*
- Empty beakers or cups for waste (one per group)
- Beaker or cups of ice (one per group)
- Masking tape (one per group)
- Agar Petri plates with freshly grown mm294 (one per group)
- Bunsen burners (one per group)
- Metal inoculating loops or sterile plastic inoculating lops (one per group)
- Agar Petri plates made with Luria Broth and ampicillin (one per group)
- 1 water bath
- 1 hand-held UV light

*If micropipetters are unavailable, plastic droppers may be used instead.

Reagents

- 50mM calcium chloride
- Aliquots of 10µl GFP plasmid in 1.5 ml tubes (one per group)
- Aliquots of 260µl mm294 resuspended in calcium chloride on ice (one per group)

Recipes

Ampicillin 10 mg/ml

- Add 1g of ampicillin sodium salt (m.w. = 371.40) to 100ml of dH₂O in a clean 250ml flask.
- Stir to dissolve.
- Pass solution through a Nalgene filter for sterilization.
- Store at 4°C (up to 3 months)



Glowing Genes

1M Calcium Chloride (CaCl₂)

- Dissolve 11.1g of anhydrous CaCl₂ (m.w. = 146.99) into 80ml of deionized or distilled water.
- Add deionized or distilled water to make a total volume of 100ml of solution.
- Store at room temperature indefinitely.

50 mM Calcium Chloride

- Mix 50ml of 1M Calcium Chloride into 950ml dH2O.
- Pass solution through a Nalgene filter for sterilization.
- Test solution with a bacterial transformation.

Luria-Bertani (LB) Broth (1L)

- 10g Tryptone
- 5g-yeast extract
- 10g NaCl
- 875µl 4N NaOH (4g NaOH /100ml of dH₂O)
- Mix ingredients into 1L of dH₂O.
- Pour into glass bottles (100ml each)
- Autoclave for 25 minutes.
- For LB + Amp, add 1ml of Ampicillin (10mg/ml) to 50 ml of LB solution after it has been autoclaved and cooled. Store at 4°C.

Luria-Bertani Agar

- 10g Tryptone
- 5g yeast extract
- 10g NaCl
- 875µl 4N NaOH (4g NaOH /100ml of dH₂O)
- 15g Bacto-agar
- Mix ingredients into 1L of dH₂O
- Autoclave for 25 minutes
- Let solution cool to 55°C and pour into sterile petri plates.

Luria-Bertani Agar with ampicillin

- After autoclaving, cool solution to 55°C
- Add 10ml of Ampicillin (solution: 10mg/ml)
- Mix and pour into plates.
- When completely solidified store all agar plates in refrigerator at 4°C.

Purchasing information

- Ampicillin Sodium Salt Sigma-Aldrich Biological
- Miscellaneous chemical reagents Fisher Scientific

- Glass Beads- Fisher Scientific
- Microcentrifuge tubes (1.5ml)-USA Scientific
- Petri Dishes- Fisher Scientific
- Transfer pipettes-VWR International
- PGFP (pGreen) plasmid Carolina Biological Supply Company
- mm294 bacteria Carolina Biological Supply Company
- Hand-held UV light Home Depot

Before Class

- Streak fresh mm294 (starter plates) on Petri dishes containing Luria Broth. Place upside down in an incubator at 37°C overnight.
- Aliquot 12µL of plasmid into 1.5mL tubes (1 per group).
- Set water bath to 42°C.
- Use sterile technique to carefully transfer several colonies of mm294 from a fresh starter plate into 2ml calcium chloride solution and resuspend bacteria with a large micropipetter.
- Add 6 more ml of calcium chloride, and resuspend again.
- Make 260ul aliquots of bacteria into 15mL snap-cap tubes (1 per group).

During Class

- Recount the historical breakthroughs of Frederick Griffith and Oswald Avery, and their work on the "transforming principle". For more information, see <u>www.DNAi.org</u> > Timeline > Oswald Avery.
- It is important that students understand the cascade of events when a gene is inserted into a living cell. The host cell will "read" the additional gene like a recipe to make a new protein, and that protein will in turn confer a new trait. Use physical examples such as melanin (pigment), keratin (hair and nails) or collagen (skin elasticity and connective tissue) to demonstrate this connection. Classes can also discuss the affect of environment on the production of certain proteins, such as melanin production and U.V. light.
- Discuss the process of genetic engineering and give a basic overview of the things needed to successfully transform bacteria cells, including a host (mm294), vector (pGFP, or plasmid containing the GFP gene) and means of selection. Make sure to point out that mm294 are a strain of *E.coli* bacteria that are harmless.
- Explore the difference between the genetic engineering of unicellular and multicellular organisms. Point out that because bacteria are



Glowing Genes

prokaryotic, the transformation process is much less complex.

- Discuss that the pGFP vector not only carries the GFP gene, but also carries the AMP gene, for ampicillin resistance. Make sure students understand
- The importance this second gene plays in the selection of bacterial growth, and discuss that the bacteria will be grown on selective AMP Petri plates for that reason.
- Explain and demonstrate each set of steps in the procedure.
- Explain that the cells are suspended in calcium chloride to neutralize the bacterial cell membrane and plasmid DNA.
- Describe the heat shock process, and the important role it plays by creating a thermal gradient across the membrane. This gradient sweeps small plasmids into the cell through openings, or zones of adhesion, in the membrane.
- Ask students to predict what the results will look like. Remind them that on a selective plate, only transformed cells will survive and reproduce.
- Discuss the applications of this technology, such as GMO's (Genetically Modified Organisms) and gene therapy. One great success story for gene therapy is the development of new treatments for the disease called SCIDS (Severe Combined Immunodeficiency Syndrome).

Analysis and Discussion

- Following the completion of the lesson, the teacher will collect the Petri plates and stack them upside down in an incubator at 37°C overnight. If an incubator is unavailable, the plates can be stacked upside down at room temperature for 2-4 days.
- When growth is visible, the class should examine the results and review the experiment. Bacterial colonies should glow when exposed to black light, and are assumed to be ampicillin resistant if they are able to survive and reproduce on a selective plate. Any abnormal results can be discussed to determine possible causes of error.
- After several days, the plates might have small white colonies surrounding the larger colonies of transformed bacteria. These are called satellite colonies, and are able to grow as a result of the ampicillin in the Petri plate expiring over time. They are un-transformed bacteria that were able to temporarily "hibernate" in a hostile environment.
- Ask students if this technique could be used to produce human proteins. It is not uncommon for students to have relatives or friends that have various forms of diabetes. Diabetics that use insulin to

control their disease are taking insulin produced though recombinant DNA technology inside bacteria!

Further Explorations

A wonderful way to enhance the knowledge acquired in this lab is to have students choose a genetic disease to research, and propose the role gene therapy could play in treatment or prevention. For information on specific genetic diseases, go to <u>www.ygyh.org</u>. Students should design a unique way of illustrating what they have learned. The class can work collaboratively and present the material to one another.

Ask students to research the possible applications of genetic engineering and to find out about existing regulations. Students will share their opinions on the subject and will be asked to support their point of view with information gathered from reliable resources. This can be an open discussion or an organized class debate.

Discuss the genetic engineering of multicellular organisms, such as mice. How can a new gene be introduced into a multicellular organism? Why might a scientist want to introduce a new gene into an organism like a mouse? Research how monoclonal antibodies can be produced in mice and used to treat human diseases such as rheumatoid arthritis.

Resources

Books:

DNA Science. Micklos, David A., Freyer, Greg A. Cold Spring Harbor Press, New York, 2003.

Internet Sites:

http://www.dnai.org/index.htm

A Dolan DNA Learning Center Internet site This site describes the past, present and future of DNA science.

http://www.ygyh.org/

A Dolan DNA Learning Center Internet site This site is a multimedia guide to genetic disease.



Glowing Genes

Correlations

New York State

NYS Standard 4: Science The Living Environment

- Living things are both similar to and different from each other and nonliving things.
- Organisms inherit genetic information in a variety of ways that result in continuity of structure and function between parents and offspring.
- Individual organisms and species change over time.
- Human decisions and activities have a profound impact on the physical and living environment.

National Science Standards

Content Standard C: Life Sciences Reproduction and Heredity

- Hereditary information is contained in genes, located in the chromosomes of each cell. Each gene carries a single unit of information. An inherited trait of an individual can be determined by one or by many genes, and a single gene can influence more than one trait. A human cell contains many thousands of different genes.
- The characteristics of an organism can be described in terms of a combination of traits. Some traits are inherited and others result from interactions with the environment.

AAAS Benchmarks

Chapter 5: The Living Environment Standard B: Heredity

- Some new gene combinations make little difference, some can produce organisms with new and perhaps enhanced capabilities, and some can be deleterious.
- Genes are segments of DNA molecules. Inserting, deleting, or substituting DNA segments can alter genes. An altered gene may be passed on to every living cell that develops from it. The resulting features may help, harm, or have little or no effect on the offspring's success in its environment.

Standard D: Interdependence of Life

- Most microorganisms do not cause disease, and many are beneficial.
- Changes in an organism's habitat are sometimes beneficial to it and sometimes harmful.

Chapter 10-Historical Perspectives Standard I: Discovering Germs

 In medicine, as in other fields of science, discoveries are sometimes made unexpectedly, even by accident. But knowledge and creative insight are usually required to recognize the meaning of the unexpected.





Making Glowing Bacteria

The Green Fluorescent Protein (GFP) gene is normally found in the Pacific jellyfish. When the jellyfish are excited, GFP causes them to give off fluorescent green light. In this experiment you will add the jellyfish gene to *E.coli* bacteria cells. Adding this new gene to *E.coli* gives them the ability to glow fluorescent green when exposed to ultra-violet light. When the *E.coli* are transformed they get the jellyfish gene, and express the jellyfish trait! This technique is called genetic engineering, it is used to move genes from one organism into another.



- 1. Use a permanent marker to label one tube "+" and another "-"
- 2. Use a large (100-1000 μ L) micropipettor to add **250 \muL** of calcium chloride solution to each tube.
- 3. Place the tubes in a beaker of ice.
- 4. Follow these steps to transfer *E.coli* cells from a petri plate to **both** tubes:

a. Hold a metal loop in a flame to sterilize it.

b. Gently scrape up several colonies with the sterile loop. Be careful not to dig at or take up any agar.

c. Carefully place the loop into the calcium chloride solution and shake off the cells. You should be able to see the small lump of cells sitting on the bottom of the tube.

d. Using the large micropipettor, mix the cells by pressing the button **gently** up and down.

e. Return the tubes to ice after mixing.



- 5. Use a small (0.5-10 μ L) micropipettor to add **10** μ L of **GFP plasmid** directly into the "+" tube.
- 6. Return the "+" tube to ice and let it sit on ice until the next step.
- 7. **Heat Shock** the cells:

a. Carry the beaker of ice, with your tubes in it, to a water bath. Remove both tubes from the ice and **immediately** place them in a **42°C** (115°F) water bath for **90** seconds.

b. Immediately return the tubes to ice for 1 minute.

- 8. Place both tubes in the rack on your desk to rest at room temperature.
- 9. Label 2 petri plates as follows:

- GFP and + GFP

don't forget to write your initials on each plate!

10. Use a large micropipettor to transfer the bacteria cells onto the plates:

a. Transfer **100** μ L of the bacteria in the "-" tube onto the - **GFP** plate.



THE HEAT SHOCK.

b. Transfer 100 μ L of the bacteria in the "+" tube onto the + GFP plate.

11. Spread the cells over the surface of the agar:

a. Clamshell the lid and carefully pour the glass beads onto each plate.

b. Use a **gentle** swirling motion to move the glass beads around the whole surface of the plate. This spreads the cells evenly over the agar surface.

12. Store the plates upside down, in a dark place, at room temperature.



Making **Glowing** Bacteria

The **Green Fluorescent Protein** (**GFP**) gene is normally found in certain Pacific jellyfish. When these jellyfish are excited, **GFP** causes them to give off **fluorescent green light.** In this experiment you will add the jellyfish gene to *E.coli* bacteria cells. Adding the new gene to *E.coli* gives them the ability to glow **fluorescent green** when exposed to **ultra-violet light**. When the *E.coli* are transformed they get the jellyfish gene, and express the jellyfish trait! This technique, used to move genes from one organism into another, is called **genetic engineering**.



Combine 10 uL of plasmid containing the GFP gene with the *E.coli* cells.

Heat Shock the cells:

a. Remove the tube from the beaker of ice and immediately place it in a 42°C water bath for 90 seconds.

b. Immediately return the tube to ice for 1 minute, then place it in a rack at room temperature.

Transfer 3 drops of the newly transformed bacteria cells onto the agar surface of a petri plate. Be sure to clam shell the lid of the petri plate.

Spread the cells over the surface of a petri plate:

a. Clam shell the lid of the petri plate and carefully pour glass beads onto the plate.

b. Use a gentle swirling motion to move the glass beads around the entire surface of the petri plate. This spreads the bacteria cells evenly on the agar surface where they will grow.



Store plates upside down at room temperature.



The heat shock.