WARD'S

Easy Transformation of *E. coli* using GFP Lab Activity Student Study Guide

BACKGROUND

DID YOU KNOW?

The phrase "transformation" comes from observations during a series of experiments studying pneumococcus by Fredrick Griffith in 1927. After observing a non-virulent strain of rough pnuemococcus change back to a smooth virulent strain, he attributed the change to a "transforming principle".

Introduction

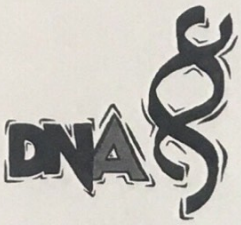
Transformation can be defined as the uptake and expression of foreign DNA by a living cell. In this laboratory, you will transform *Escherichia coli* (*E. coli*) cells with the Green Fluorescent Protein (GFP) gene from the pacific jellyfish - *Aquoriea victoria.* As its name implies, GFP is responsible for the jellyfish's natural ability to emit a green fluorescent wavelength of light. *E. coli* successfully transformed with GFP will also express this green fluorescence trait, easily observed when exposed to a hand-held UV light (blacklight). Using GFP, and other genes that visually "report" their expression, makes understanding the concept of transformation much easier for many students.

A Brief History of Transformation

The microbiologist Fredrick Griffith first described transformation in 1928. Griffith was studying two strains of pneumococcus bacteria and was interested in what makes some strains of bacteria harmful and other strains harmless. One strain called the S, or smooth strain, was lethal, causing pneumonia when injected into mice. The other strain, called R, or rough strain, did not cause any harmful effect when injected into mice. The smooth and rough strain designation refers to their growth pattern on plates. When Griffith heat-treated the S strain, by boiling, the "heat-killed" S strain pneumococcus lost its ability to cause any harm to the mice.

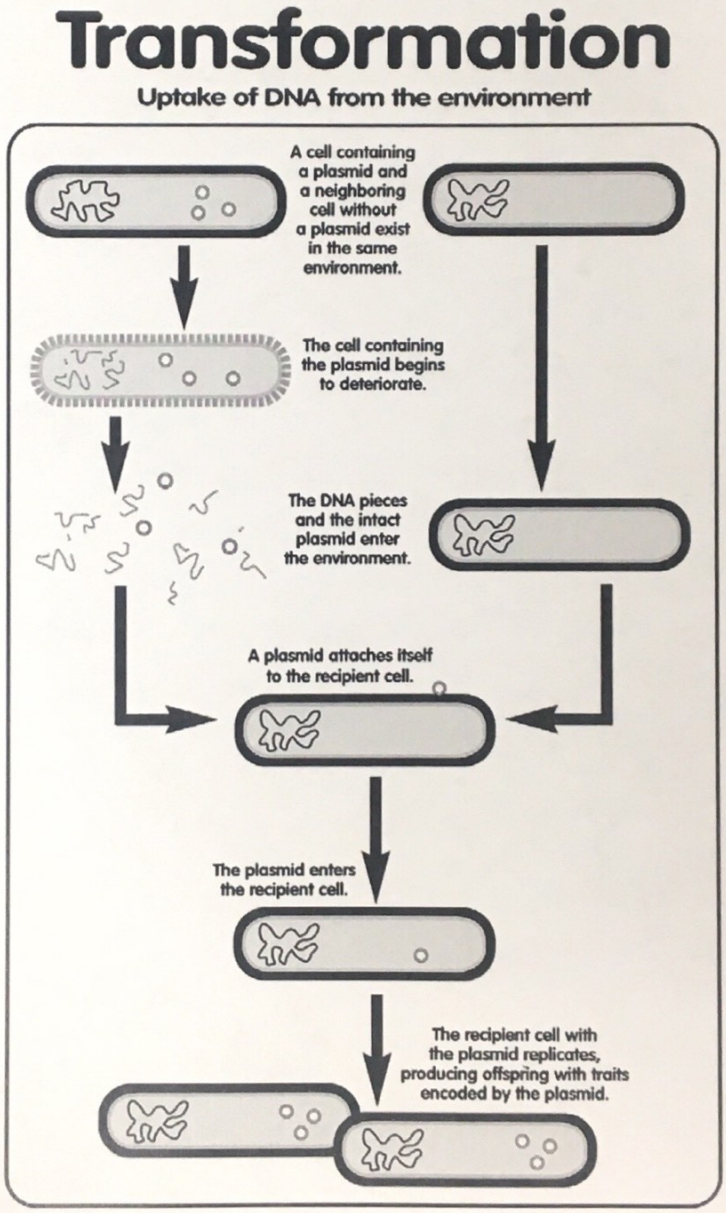
However, when Griffith combined the heat-killed S train with the live R strain, and injected this mixture into a mouse, the mouse developed pneumonia and died. This was a surprise, as both the R strain and heat-killed S strain by themselves were harmless to the mice. He repeated the experiment and found the same result. The bigger surprise came when Griffith cultured the pnuemococcus from the dead mice- he found they were killed by the live S strain! His conclusion- there must be some "transforming principle" in the heat-killed S strain that can restore the virulence in harmless R strain.

It would not be until 1944 and the elegant experiments of Oswald Avery, Colin MacLeod, and Maclyn McCarty, that Griffith's "transforming principle" would be identified. To the surprise of many scientists, Avery's lab identified the transforming principle as Deoxyribonucleic Acid - DNA. Many scientists at that time were expecting the molecule of heredity to be a protein - *not* DNA. In 1952, Alfred Hershey and Martha Chase provided the final proof that the molecule of heredity was indeed DNA in their now famous "blender experiment" using radiolabeled bacteriophage DNA (P^{32}) and protein (S^{35}).



DID YOU KNOW?

In 1944, Oswald Avery, Colin MacLeod, and Maclyn McCarty determined that the transforming principle described by Griffith was DNA.



William Studier

DID YOU KNOW?

BNL scientist William Studier developed a system that uses the bacterial virus T7's genetic "switch" for expressing genes in *E. coli*. This system, called the T7 promoter, has been genetically engineered into plasmid vectors for the expression of genes from many different organisms- including humans. This system played a key role in the development of the biotechnology industry.

In this laboratory, you will be using a modified version of Morton Mandel and Akiko Higa's protocol for transforming *E. coli*. This protocol was developed in 1970. While this technique provides more experimental control than Griffith's experiment, it should be noted that the exact mechanism of transformation by this protocol is not fully understood. In the Madel and Higa method, *E. coli* cells are washed in an ice-cold $CaCl_2$ solution to neutralize the normally negatively-charge phospholipids of the cell surface. It also neutralizes the negative charge of the DNA that you are attempting to transform the cells with. After an incubation period on ice, the cells are quickly heated in a 42° C water bath for a brief period. This step in the protocol is often referred to as the "heat shock" step. During this step, it is believed that a thermal gradient (temperature difference) helps to transport the foreign DNA into the cell. The cells are placed back on ice for a short period of time and then cultured on an agar plate.

The Host Cell - *Escherichia coli*

Escherichia coli (*E. coli*) is often referred to as the "workhorse" of the molecular biology world. *E. coli*'s cellular machinery is routinely used by molecular biologists to clone genes and express their products - proteins. They have earned "workhorse" status for several reasons:

- They reproduce quickly - you see results the next day.
- Laboratory strains (including the DH5 alpha strain used in this kit) are harmless.
- They are easy to grow.
- They are small, single celled prokaryotes.
- The Genome is sequenced.
- They have been used as a model organism for a long time.
- They are relatively easy to transform in the classroom setting.

The strain that is included with this kit is called DH5 alpha.

The Plasmid Vector - A Means of Gene Delivery

A plasmid is a small, circular, extra-chromosomal DNA molecule that can sometimes be found in bacteria. Plasmids were first discovered in bacteria that were living and growing outside of their normal environmental boundaries. For example, bacteria that would normally be killed by antibiotics, were now resistant to the antibiotics. Often, these resistant strains carried extra genetic material in the form of plasmids.

Plasmids usually provide the microbe host with some selective advantage, such as the antibiotic resistance just described. Once a plasmid enters the cell, the cellular machinery is used to replicate the plasmid, sometimes as many as 800 copies per cell. These are called high-copy-number plasmids. A plasmid is relatively small, typically only a few thousand base pairs in length. This is small compared with the

DNA length of *E. coli*'s complete genome, about 4.6 million base pairs. Plasmids can also be exchanged between two microbial cells, including two microbes of different species. Some scientists believe, because that of their ability to replicate inside of a host cell, plasmids may even be an ancestral form of viruses.

Today, scientists use the unique attributes of plasmids, especially their ability to enter cells, replicate, and carry selected genes such as antibiotic resistance genes, to their advantage. In bacterial transformation experiments, plasmids are used as a vector. A vector, in this sense, is a means of delivering your gene of interest, the jellyfish gene GFP. The plasmid in this laboratory has been genetically engineered to carry the GFP gene. If your plasmid vector enters an *E. coli* cell, the cell will express the green fluorescent protein trait and fluoresce when exposed to UV light.

Expressing Foreign Proteins in *E. coli* - A Brookhaven National Laboratory Breakthrough

An important breakthrough in biotechnology came from the basic research of Brookhaven National Laboratory scientist William Studier. Through his study of gene regulation in the bacteria virus T7, Studier was able to develop a strong and controllable system for expressing proteins of interest in *E. coli.* The T7 expression system, developed and patented at Brookhaven National Laboratory in the 1980's, is used worldwide by academia and industry to produce large amounts of specific proteins within bacterial cells.

The plasmid used in this laboratory contains elements of the T7 expression system. One critical element of the T7 expression system consists of a DNA sequence from the T7 virus, called a gene promoter sequence. This DNA sequence is used to drive, or "promote", the expression of a gene of interest. In this laboratory, our gene of interest is the Green Fluorescent Protein (GFP). In *E. coli* cells, T7 is a very strong promoter of gene expression. Thus, successfully transformed *E. coli* will fluoresce a bright green color when exposed to UV light.

Today, biotechnology and pharmaceutical companies use the T7 expression system to produce recombinant proteins critical for the treatment of several human disorders. In addition to recombinant human insulin, other therapeutic proteins produced under the T7 expression system include recombinant clotting-factor proteins used to treat hemophilia, and recombinant human growth hormone (HGH) used to treat pituitary dwarfism.

DID YOU KNOW?

The Hershey-Chase experiment was performed just down the road from Brookhaven National Laboratory at Cold Spring Harbor Laboratory.

Your Protein of Interest- The Green Fluorescent Protein

In this lab you will transform *E. coli* with plasmid DNA that carries a gene coding for the green fluorescent protein (GFP) trait of the Pacific jellyfish- *Aquoriea victoria.* As its name suggests, GFP is a protein that will fluoresce when exposed to blue light with a 488nm wavelength. In the jellyfish, a second protein called Aequorin provides the bioluminescence (blue light) required for GFP fluorescence. Therefore, it is important to think of bioluminescence and fluorescence as two related, yet independent capabilities. Surprisingly, scientists do not fully understand the biological role of bioluminescence and fluorescence in the jellyfish.

DID YOU KNOW?

High-copy-number plasmids, such as the plasmid used in this kit, can replicate after entering the *E. coli* cell. It is believed that each transformed cell may host 800 copies of the plasmid per *E. coli* cell.

A Means of Selection

Relatively speaking, bacterial transformation is a rare event when using the Mandel and Higa protocol. Most of the cells will not be transformed. To compensate for the low probability of a cell being transformed, the plasmid DNA also carries a gene conferring resistance to the antibiotic ampicillin. In addition to expressing GFP, cells that are transformed can grow in the presence of ampicillin. Therefore, you will culture your *E. coli* on luria agar plates that also contain ampicillin. Cells that are transformed will be able to grow and produce green, ampicillin-resistant colonies. The cells that fail to uptake the plasmid DNA will not be able to grow in the presence of the antibiotic. Furthermore, these cells will die when they attempt to undergo cell division. By culturing your experiment in the presence of ampicillin, you select for the growth of only the cells that have been transformed. It is often helpful to think about selection in this way- every colony on the agar with ampicillin plate represents one cell that you successfully transformed. Each colony equals one successful act of transformation.

OBJECTIVES

- Understand the process of transformation and its purpose
- Perform transformation of E. coli using GFP plasmid DNA
- Calculate transformation efficiency

MATERIALS

DID YOU KNOW?

The term "aseptic technique" refers to the sterile transfer of a culture from one medium to another without contamination of the culture, either medium, or the surroundings. The proper use of aseptic technique is critical in microbiology.

MATERIALS NEEDED PER GROUP

1 disposable innoculating loop
1 *E. coli* starter plate
2 Bacti-spreaders
1 beaker of ice/ice water
2 Luria + ampicillin agar plates
2 microcentrifuge tubes 51mM $CaCl_2$ (300 µl)
1 microcentrifuge tube pGFP plasmid DNA (10 µl)
1 marker

SHARED MATERIALS

10 µl micropipette
100 µl micropipette

PROCEDURE

1. Obtain a cup of fresh crushed ice (or ice cubes with water filling in the spaces).

2. Obtain a 1.5 mL microcentrifuge tube containing 300 µl $CaCl_2$ and label it "+ pGFP DNA." This is your experimental tube for transforming the bacteria with the jellyfish gene GFP.

3. Obtain a 1.5 mL microcentrifuge tube containing 300 µl $CaCl_2$ and label it "– Control." This tube will not receive any plasmid DNA. It is your negative control.

4. Place both tubes in your beaker of ice.

5. Obtain an *E. coli* starter plate. Using an inoculating loop, transfer about ¼ loop-full of *E. coli* from the plate to each tube. Each time, be careful not to damage the agar plate as you collect the *E. coli* that grows on its surface. Each time, twirl the loop rapidly between your thumb and index finger to make sure the *E. coli* cells come off the loop and into the $CaCl_2$.

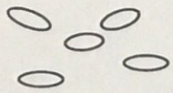
6. The $CaCl_2$ in each tube should be cloudy with *E. coli* cells after twirling the inoculating loop. That's good. Use your micropipette, set on 100 µl, to mix the cells with the $CaCl_2$ completely. Do this by going up-down to the first thumb stop on the micropipette 4-5 times. Your mixture should be a cloudy white color. If still clear, add more *E. coli* cells. You may use the same tip during the mixing. Try not to make bubbles (no need to go crazy).

7. Place both tubes, each contain the $CaCl_2$ / *E. coli* mix, back on ice.

8. With a fresh tip, transfer 10 µl of pGFP plasmid DNA to the tube labeled "+ pGFP DNA" only. Mix this tube by vigorously tapping the tube until you create what looks like a little "tornado". This is called finger vortexing. Alternatively, you can give a quick pulse on an eletric vortex. Tap the tube on the desk to make sure the $CaCl_2$ / *E. coli* / plasmid DNA mix settles back to the bottom of the tube and is not stuck in the cap.

DO NOT add plasmid DNA to your negative control!

9. Place both tubes on ice and incubate on ice for 15 minutes.



DID YOU KNOW?

A single rod-shaped *E. coli* cell is approximately .7 microns wide and 1.8 microns long.

10. While the tubes are on ice, obtain two Luria + ampicillin plates from your teacher. Label the bottom of one plate with your initials, the date, and "+ pGFP DNA". Label the bottom of the second plate with your initials, the date, and "- control". Flip both plates over.

The tubes can stay on ice until you are ready for the next step.

11. After 15 minutes perform a "heat shock". Instantly remove both tubes from ice and incubate them in your hands for 3 minutes. Shake your hands for 2-3 seconds while the tubes are incubating. After 3 minutes of "hand-incubating", place them back on ice for 1 minute.

12. Transfer 100 µl of your "- control" tube to the negative control plate. Use a bacti-spreader to spread the liquid across the entire plate.

13. Using a fresh tip, transfer 100 µl of your "+ pGFP DNA" tube to the "+ pGFP DNA" plate. Use a fresh bacti-spreader to spread the liquid across the entire plate.

14. Let the cells penetrate into the agar for 3 minutes.

15. Incubate upside down at room temperature for two-three days or overnight at 37 °C in an incubator.

16. Observe the plates under a UV light. Count and record the number of fluorescent colonies on each plate. Record your results in the Analysis section.

Cleanup and Disposal

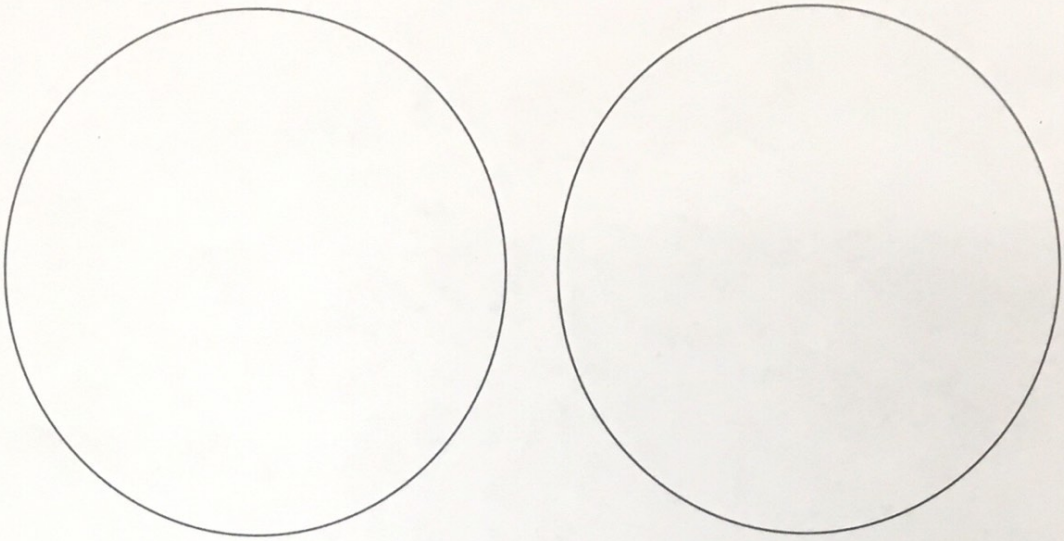
All materials containing bacterial growth and all materials that have come in contact with bacterial growth must be decontaminated prior to disposal. All contaminated materials should be placed in the provided biohazard bag and autoclaved at 121 °C , 15 psi, for 15 minutes. If an autoclave is not available, soak all contaminated materials in bleach solution, allowing them to stand for 45 to 60 minutes prior to disposal.

DID YOU KNOW?

One of ten national laboratories overseen and primarily funded by the Office of Science of the U.S. Department of Energy (DOE), Brookhaven National Laboratory conducts research in the physical, biomedical, and environmental sciences, as well as in energy technologies and national security.

Name:_____
Group: _____
Date: _____

ANALYSIS



- Control

+ pGFP

Transformation efficiency is the number of resistant colonies per microgram of plasmid. Using the directions below, calculate transformation efficiency.

Total mass of plasmid used
(total mass = volume x concentration .01 µg/ µl) _____

Total volume of suspension _____

Fraction of cell suspension put on plate
(µl on plate/total volume) _____

Total mass of plasmid in fraction
(mass of plasmid x fraction on plate) _____

Number of colonies per µg of plasmid
(# of colonies counted/mass of plasmid put on plate) _____

Name:_____
Group: _____
Date: _____

ASSESSMENT

1. Define bacterial transformation.

2. Why is *E. coli* such a convenient host cell for transformation in biotechnology?

3. Define a vector. What type of vector is used in this protocol?

4. In this laboratory, how did you select for culturing only transformed cells? What happened to the cells you failed to transform?

5. Define the term "reporter gene". What reporter gene was used in this laboratory?

6. What is a microbial colony? What does one colony represent on your experimental (+ pGFP) Luria ampicillin agar plate?

7. To make the *E. coli* "competent" at being transformed, the cells are re-suspended in $CaCl_2$ solution. What is the purpose of the $CaCl_2$?

8. Why would scientists transform *E. coli* with plasmids?