

Transformation of the bacterium *E. coli* using a gene for Green Fluorescent Protein

Background

In molecular biology, transformation refers to a form of genetic exchange in which the genetic material carried by an individual cell is altered by incorporation of foreign (exogenous) DNA. This foreign DNA may be derived from unrelated species and even other kingdoms, such as bacteria, fungi, plants or animals, which would otherwise be inaccessible to an organism.

Bacteria and yeast have been transformed with human genes to produce proteins that are useful in treating human diseases and disorders e.g. the production of insulin. Some bacteria have been modified such that they are able to digest oil from accidental spills. Bacteria are single celled organisms that can easily pass information between one another and thus changes in genetic make-up are rapidly passed on to subsequent generations.

Transformation is usually more difficult with multicellular organisms such as plants, in which it is necessary to either alter many cells with the new piece of DNA or to alter just a single cell and then induce it to produce a whole, new plant.

Genetic transformation of plants and other organisms does occur naturally. Bacteria and viruses can move DNA (or RNA) into an organism and cause profound changes. Examples are *Agrobacterium tumefaciens* (for plants) and HIV (for Humans).

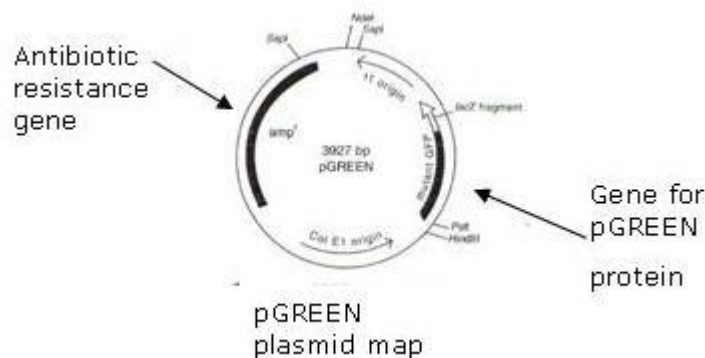
The bacterium you will be transforming, *E.coli*, lives in the human gut and is a relatively simple and well understood organism. Its genetic material consists mostly of one large circle of DNA 3-5 million base pairs in length, with small loops of DNA called plasmids, usually ranging from 5,000-10,000 base pairs in length, present in the cytoplasm. It is these plasmids that bacteria can transfer back and forth, allowing them to share genes among one another and thus to naturally adapt to new environments.

The ability of bacteria to maintain these plasmids and replicate them during normal cell multiplication is the basis of cell transformation. The plasmids are used as “gene taxis” in transformation events to bring DNA of interest into the cell where it can integrate into the genome (or remain as a plasmid within a bacterium) and be translated into proteins not normally found in that organism.

In this lab, Green Fluorescent Protein (GFP) from the bioluminescent jellyfish *Aequorea victoria* has been incorporated into a plasmid along with a gene for

resistance to the antibiotic ampicillin. The GFP is actually located in discrete spots around the bell margin of the jellyfish and will fluoresce under certain conditions. When inserted into a plasmid and used for the transformation procedure, the transformed bacteria will express their newly acquired jellyfish gene and produce the fluorescent protein, which causes them to glow green under ultraviolet light. The mutant form of GFP used in pGREEN makes the bacteria a yellow-green color even in white light.

This plasmid contains an ampicillin-resistance gene in addition to the GFP gene. Ampicillin is an antibiotic and works by preventing *E. coli* from constructing cell walls, thereby killing the bacteria. When the ampicillin-resistance gene is present it directs the production of an enzyme that blocks the action of the chemical and the bacteria are able to survive. Bacteria without the plasmid and hence the resistance gene are unable to grow on a plate containing ampicillin in the medium and only the transformants will survive.



GFP is also used in research as a reporter molecule. It can be linked to the protein that you are interested in studying, and this protein can then be followed through changes in expression of the linked GFP.

Objectives

1. Understand recombinant techniques and the transformation procedure using the heat shock method.
2. Understand how we can screen for a gene of interest and the importance of marker or reporter genes in molecular biology experiments.
3. Investigate how DNA can be transferred to another organism and the change in phenotype (physical characteristics) that may result from such a transfer.
4. Learn the importance of the sterile techniques that are used to handle bacteria and the decontamination necessary when the experiment is complete.
5. Learn how to calculate transformation efficiency.

Materials

For each lab group

- Gloves
- Safety goggles if desired
- Waterproof marker
- Microtube rack
- 6 disposable pipettes
- 6 disposable inoculating loops
- Foam cup with crushed ice
- 2 LB plates
- 2 LB + Amp plates

Groups Will Share:

- Squirt bottle containing 10% bleach
- Microtube filled with 50 Mm CaCl_2 (keep on ice)
- Microtube filled with Luria broth

Common materials

- 20 μl micropipette for instructor use
- Water bath (with floating tube rack)
- Streak plates of *E.coli*
- pGREEN plasmid (0.005 $\mu\text{g}/\mu\text{l}$)
- Crushed ice
- Distilled water
- 37°C incubator
- Parafilm
- Large container with 10% bleach

Precautions

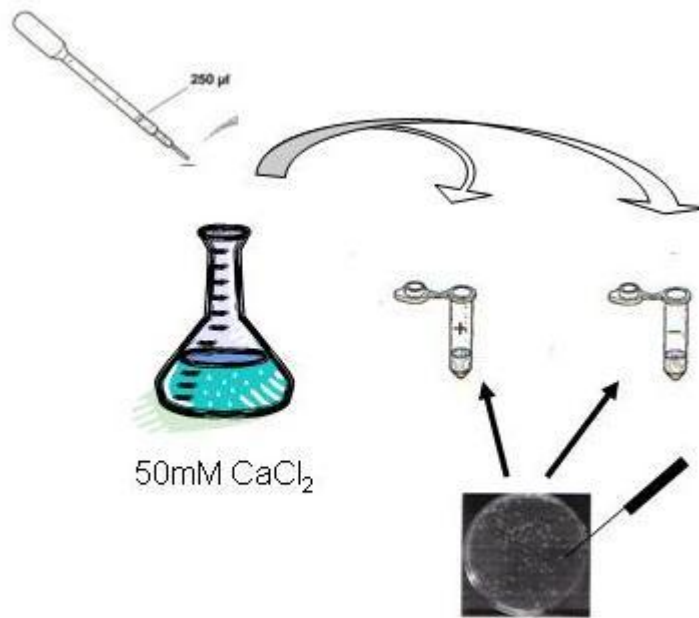
Review the safety instructions with your teacher to ensure that you know how to handle the cultures and equipment safely. At the completion of the lab dispose of all materials by soaking in 10% bleach solution and then draining and placing in the trash. Clean the lab benches with the bleach solution and remember to **wash your hands before leaving the lab.**

Procedure

Day 1:

1. Make sure that you have all of your group's materials and that you know which other group you will be sharing with and where the bleach containers are located for clean-up at the end
2. Put on gloves and safety goggles (if you have them).
3. Label two sterile microtubes: one "+" and the other "-". Write your lab group's name or initials on each tube.

- Using a disposable pipette, add 250 μl of 50mM CaCl_2 solution to each tube (“+” and “-”) and immediately place them both on ice.



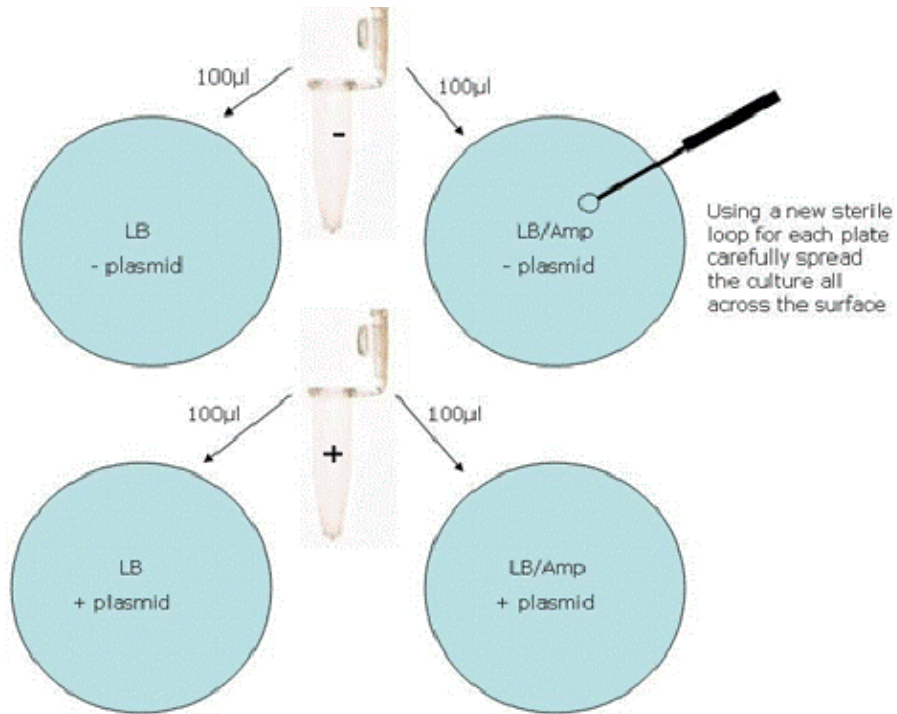
- Use a sterile plastic loop to transfer one or two 3 mm bacterial colonies or an equivalent amount of smaller colonies from the streak plate to the “+” tube. Do not pick up an agar as it may inhibit the transformation process.
- Immerse the loop tip into the calcium chloride solution in the “+” tube and vigorously spin the loop to dislodge the cell mass and disperse the entire mass into the calcium chloride solution. **Note: No visible clumps of cells should remain. This step is critical to obtaining good results.**
- Place the loop into the bacterial waste container to kill the bacteria that remain on it.
- Close the tube lid and put the tube on ice.
- Follow steps 5 to 8 and use a new sterile loop to transfer a mass of cells to the “-” tube. Both tubes should now be on ice.
- Using a sterile inoculating loop pick up one loopful (10 μl) of pGREEN and add directly into the CaCl_2 in your “+” tube. Your teacher may do this for you. Following the addition of the plasmid, close the tube lid and tap gently with your finger to mix. Try not to splash the suspension up the sides of the tube. Tap the base of the tube gently on the desktop to make all the liquid moves to the bottom of the tube.
- Return the “+” tube to the ice. **DO NOT** add the plasmid to your “-” tube. Incubate both tubes on ice for 15 minutes.

12. While the cells are incubating, your teacher will pass a UV lamp over the pGREEN DNA solution. Note your observations on the student activity sheet and complete questions 1-3.
13. Following incubation, "heat shock" the cells. It is essential that the cells receive a sharp and distinct shock.
 - a. Carry the ice container with the tubes to the 42 °C water bath.
 - b. Remove both tubes from ice and immediately hold them in the water for 90 seconds. Three-fourths of the tube should be under water.
 - c. After heat shock, immediately return the tubes to ice. Let them remain on ice for at least one minute.

Stop Point: The tubes can be refrigerated overnight and removed just prior to the beginning of the next lab. If you will not finish the lab today, give the tubes to your teacher for overnight storage. **Clean up:** Place used loops etc in the bacterial waste container. Spray workspace with bleach solution and wipe down. Wash hands before leaving lab.

14. Use a disposable pipette to add 250 µl of Luria broth to both the "+" and "-" tubes. Make sure that you add to the "-" tube first so as to avoid cross-contamination of the plasmid. Discard this pipette into the bacterial waste.
15. Close lids, and gently tap tubes to mix. Place in a rack and incubate for 10 minutes at room temperature.
16. Label the **BOTTOM** of your media plates while the tubes are incubating. Make sure to also include your lab group name and the date.
17. Label 1 LB plate and 1 LB/Amp plate "+**PLASMID**" and label 1 LB plate and 1 LB/Amp plate "-**PLASMID**"
18. Use a disposable pipette to add 100 µl of cell suspension from the "- tube" to the "**LB - PLASMID**" plate, and another 100 µl to the "**LB/Amp - PLASMID**" plate. Discard the pipette and "- tube" into the bacterial waste container.
19. **Using a new sterile loop for each plate** spread the suspensions evenly around the surface of the agar by quickly skating the flat surface of a new sterile loop back and forth across the plate surface. Turn the plate a quarter turn and go back and forth several more times.

20. Use a new sterile pipette to transfer 100µl of cell suspension from the "+" tube to the appropriate plate and spread as above.



21. Rest the plates on the bench for 10 minutes to allow the cell suspension to absorb into the media.
22. Wrap Parafilm around your four plates to seal the lids. Place the plates upside down in an incubator or at room temperature. The results will be ready to observe in 24 hours if incubated at 37°C or in 48-72 hours at room temperature.
23. After tips and tubes have sat in bleach solution for at least 15 minutes, pour liquid in bacterial waste container down drain. Collect tips and tubes in a plastic bag and discard in the normal garbage. Spray down workspace with bleach solution. Wash hands before leaving lab.

Day 2

1. Retrieve your plates from incubator or other storage area and check for growth. Complete the remainder of the activity sheet you began on day 1 of the lab.

2. Open the Petri plates and immerse in the large container of bleach solution your instructor has provided. Spray down workspace with bleach solution. Wash hands before leaving lab.

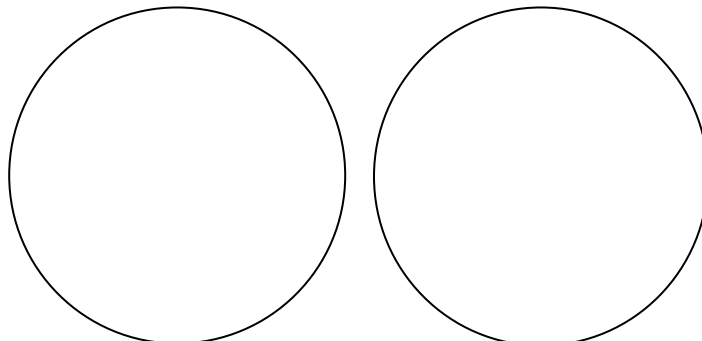
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Student Activity

1. What color was the pGREEN plasmid DNA when we exposed it to the UV light?
2. Will all of the plates have bacteria growing on them?
3. Explain your answer to question 2.

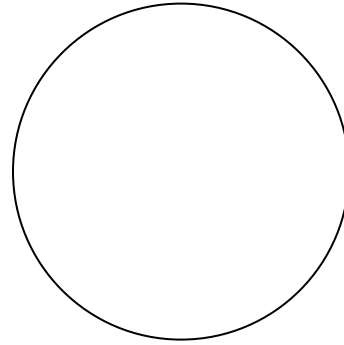
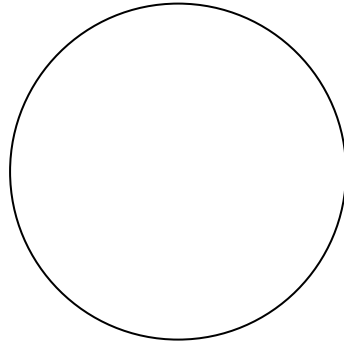
3. Now observe the results on the Petri dishes without removing the lids. Count how many colonies are present on each plate. If there are too many to count record "lawn". Record your results on the diagrams below.

1. LB -plasmid



2. LB +plasmid

3. LB/Amp
-plasmid



4. LB/Amp
+plasmid

5. Were your results different from what you expected? Explain what you think might have happened.

6. Which plates are the control plates? Why do we need the control plates?

7. Only one plate has transformants. This is the experimental plate. Which one is it? Why do transformants only grow on this plate?

8. Shine the UV light on the plate with the transformants. What color are the bacteria? Compare this answer to what you observed when the light was shined onto the pGREEN plasmid. Is there a difference? Why?
9. Were all of the bacteria that we started with transformed? Compare the growth on plates 2 and 4 and then explain your answer.
10. Transformation efficiency is defined as the number of transformed colonies per microgram (μg) of plasmid DNA. In order to determine the efficiency of the transformation we need to know the mass of plasmid that was spread on the plate and relate this to the number of transformed colonies that were observed on the experimental plate.

The formula for determining the transformation efficiency is:

Total number of colonies growing on the LB/amp per μg of plasmid DNA used for the transformation

a) To determine the initial amount or mass of plasmid DNA:

Total amount of DNA (μg) = concentration ($\mu\text{g}/\mu\text{l}$) X volume (μl)

*Remember, you used 10 μl of plasmid DNA at a concentration of 0.005 $\mu\text{g}/\mu\text{l}$.

b) To determine fraction of DNA solution spread:

Volume of solution spread on plate divided by total amount in tube = fraction spread

c) To determine the mass of DNA spread on the experimental plate:

Total mass of DNA [μg] X fraction spread = Mass of DNA spread

d) Number of colonies per μg of plasmid DNA:

Number of colonies observed divided by mass of DNA spread = Transformation efficiency