Name_____Period_____Assignment #_____

If you are allergic to penicillin you may not participate in this lab activity

INVESTIGATION 8: Transformation with Green Fluorescent Protein

How can we use genetic engineering techniques to manipulate heritable information?

See lecture questions 128, 130, 136-138

Annotate up to learning objectives and answer questions 1-16

Annotating Text
☐ **UNDERLINE** concepts you think might be useful for understanding or solving the problem
☐ **Box** information you think might be helpful for designing your investigation
☐ ← Write **notes** in the left margin
☐ → Write **questions and answers** in the right margin
Each paragraph (including each step of the procedures) must have something underlined or boxed, **AND** have something written in the margins (a question and/or note).

BACKGROUND

Are genetically modified foods safe? The science says yes, but the public (especially European public opinion) is fearful of the technology. There is ongoing public debate about whether it is safe to eat fruit and vegetables that are genetically modified to contain toxins that ward off pests. For instance, biotechnologists have succeeded in inserting a gene (*Bt*) from the bacterium *Bacillus thuringiensis* into the corn genome. When expressed, the *Bt* toxin kills caterpillars and controls earworms that damage corn, but is the corn safe for human consumption? Once again, the science says yes, but lay people remain fearful despite the fact that this technology reduces the amount of insecticides used!

Genetic information passed from parent to offspring via DNA provides for continuity of life. In order for information in DNA to direct cellular activities, it must be transcribed into RNA. Some of the RNAs are used immediately for ribosomes or to control other cellular processes. Other RNAs are translated into proteins that have important roles in determining metabolism and development, i.e., cellular activities and phenotypes (traits). When the DNA of a cell changes, the RNAs and proteins they produce often change, which in turn changes how that cell functions.

DNA inside a cell can change several ways. It can be mutated, either spontaneously or after the DNA replication machinery makes an error. Biotechnologists may cause an intentional mutation in a cell's own DNA as a way to change how that cell behaves.

One of the most powerful tools biotechnologists have is the ability to transfer DNA from one organism to another, and make it function there. With this tool, they can make cells produce novel protein products the cells did not make previously.

Examples of this powerful tool are all around us. Insulin that people take to control their blood sugar levels is often made from engineered bacteria. Some vaccines, as well as enzymes used for manufacturing denim jeans, are also made using engineered cells. In the near future, engineered bacteria and other cells being developed could help clean up spilled oil or chemicals, produce fuel for cars and trucks, and even store excess carbon dioxide to help slow global climate change. Can you think of other possible applications of genetic engineering?

Biotechnology and human manipulation of DNA raise several ethical, social, and medical issues, such as the safety of genetically modified foods. Once again, the science tells use genetically modified foods are safe and better for the environment than non-modified foods. Can you think of other issues to consider? This biotechnology depends on plasmids, small circles of DNA that were found first in bacteria. Plasmids allow molecular biologists to manipulate genetic information in a laboratory setting to understand more fully how DNA operates. Plasmids also let us move DNA from one bacterium to another easily.

In this experiment, you will transform a strain of competent *E. coli* which has no antibiotic resistance with supercoiled plasmid DNA which has a gene for antibiotic resistance. The plasmid produces the green fluorescent protein, because in addition to the antibiotic resistance gene, it contains the *gfp* gene.

Bacterial cells will be selected for the presence of plasmid by plating them on agar medium containing ampicillin. Only bacterial cells that take up the plasmid will survive selection on ampicillin agar plates and will produce green fluorescent colonies which will be visible under long wave U.V. light. The transformation efficiency will then be estimated.

Answer the following questions:

1) What causes mutations in bacteria and other organisms?

2) How could mutations affect plasmid function?

3) What is the function of plasmids in bacteria?

This investigation provides you with the opportunity to review, connect, and apply concepts that you have studied previously, including cell structure of bacteria; structure and function of cell membranes, enzymes, the relationship between DNA and RNA; transcription and translation; the operon model of the regulation of gene expression; evolution and natural selection; and interactions between organisms and their environment.

Basic Sterile Technique

When working with and culturing bacteria, it is important not to introduce contaminating bacteria or fungi into the experiment. Because these microorganisms are ubiquitous, i.e., you can find them everywhere — on fingertips, bench tops, lab tables, etc. — you must avoid these contaminating surfaces. When working with inoculation loops, bulb pipettes, micropipettes, and agar plates, do not touch the tips of them (or, in the case of agar, the surface itself) or place them directly onto contaminating surfaces. Be sure to wash your hands before beginning the procedure and after — and cover your sneezes. Do not eat, drink, apply cosmetics, or use personal electronic devices in the work area.

Working with E. coli

The host *E. coli* used in this investigation, plasmids, and the subsequent transformants created by their combination are *not* pathogenic (disease-causing) bacteria like the *E.coli* O157:H7 strain that has been in the news. However, handling *E. coli* requires appropriate microbiological and safety procedures. Your teacher will provide instructions, but these practices include, but are not limited to, the following:

- Decontaminating work surfaces once a day and after any spill of viable material with a 10% household bleach solution
- Decontaminating all contaminated liquid or solid wastes before disposal. This can be done in an autoclave (20 minutes at 121°C) or in a 10% bleach solution (soaked for 20 minutes).
- Washing your hands after handling organisms containing recombinant DNA and before leaving the lab
- Wearing protective eyewear and disposable gloves
  Not eating, drinking, applying cosmetics, or using personal electronic devices, such as iPods and cell phones, in the work area.

Getting Started

DNA provides the instructions necessary for the survival, growth, and reproduction of an organism. When genetic information changes, either through natural processes or genetic engineering, the results may be observable in the organism. These changes may be advantageous for the long-term survival and evolution of a species, but it also may be disadvantageous to the individuals who possess the new genetic information.

In bacteria, genetic variation does not happen by mutation alone. It also can be introduced through the lateral (horizontal) transfer of genetic material between cells. Some bacteria undergo conjugation, which is direct cell-to-cell transfer. Other bacteria acquire DNA by transduction (viral transmission of genetic information). The third route is transformation, which is uptake of “naked” DNA from the environment outside the cell. (You may have previously studied transformation in a different context. In an experiment conducted in 1928, Frederick Grifith, seeking a vaccine against a virulent strain of pneumonia, suggested that bacteria are capable of transferring genetic information through transformation. Little did Grifith know that his work would provide a foundation for genetic engineering and recombinant DNA technology in the 21st century.) Answer the following questions:

4) To transform an organism to express new genetic information, do you need to insert the new gene into every cell in a multicellular organism or just one?

5) Explain your answer to the previous question.

6) Which organism is best suited for total genetic transformation — one composed of many cells or one composed of a single cell?

7) Explain your answer to the previous question.

8) Can a genetically transformed organism pass its new traits on to its offspring?

9) To get this information, which would be a better candidate for your investigation — an organism in which each new generation develops and reproduces quickly or one that does this more slowly?

10) Based on how you answered the first two sets of questions, what organism would be a good choice for investigating genetic transformation — a bacterium, earthworm, fish, or mouse?

If your answer to the last question is "bacterium," you are on the right track. Genetic transformation of bacteria most often occurs when bacteria take up plasmids from their environment. Plasmids are not part of the main DNA of a bacterium. They are small, circular pieces of DNA that usually contain genes for one or more traits that may be beneficial to survival. Many plasmids contain genes that code for resistance to antibiotics like ampicillin and tetracycline. Antibiotic-resistant bacteria are responsible for a number of human health concerns, such as methicillin-resistant *Staphylococcus aureas* (MRSA) infections. Other plasmids code for an enzyme, toxin, or other protein that gives bacteria with that plasmid some survival advantage. In nature, bacteria may swap these beneficial plasmids from time to time. This process increases the variation between bacteria — variation that natural selection can act on. In the laboratory, scientists use plasmids to insert "genes of interest" into an organism to change the organism's phenotype, thus "transforming" the recipient cell. Using restriction enzymes, genes can be cut out of human, animal, or plant DNA and,

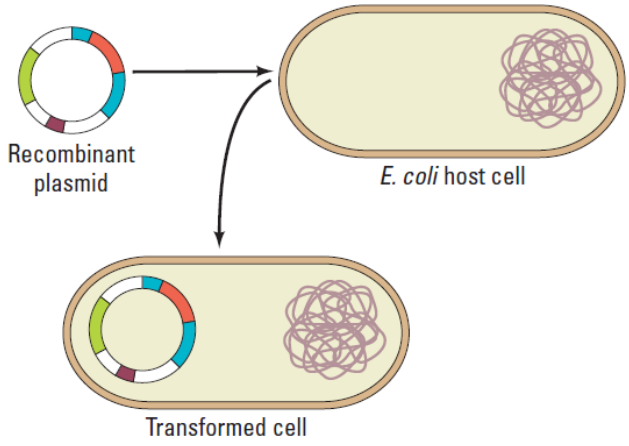
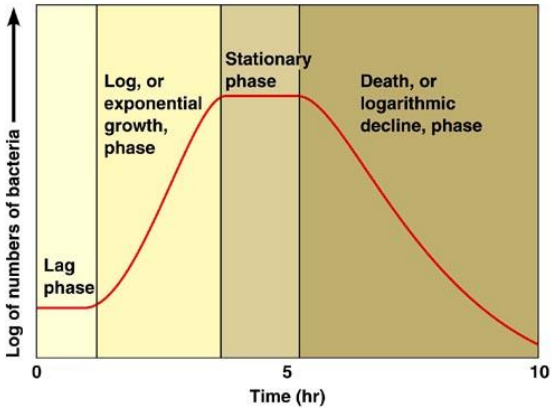
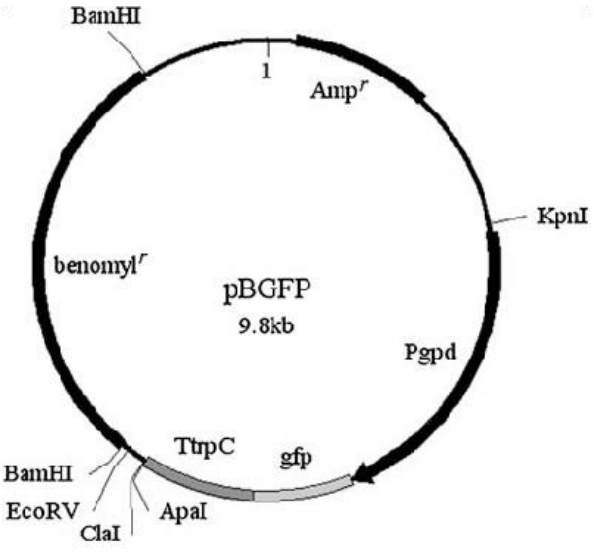


Figure 1. Transformation of Bacteria

using plasmids as vectors (carriers of genetic information), inserted into bacteria. If transformation is successful, the recipient bacteria will express the newly acquired genetic information in its phenotype (Figure 1).



For transformation to occur, bacterial cells must be in a particular physiological state, referred to as competency, in which the bacterial cell wall is made permeable to macromolecules such as DNA. Competency can occur naturally in certain species of *Haemophilus* and *Bacillus* when the levels of nutrients and oxygen are low. Bacteria are most competent during the early log phase of their growth curve. Competent *Haemophilus* cells express a membrane-associated transport complex that binds and transfers certain DNA molecules from the medium into the cell where they are then integrated into the bacterial chromosome and expressed. In nature, the source of the external DNA is from other cells that have died and their cell walls lysed to release their DNA into the surrounding medium.



Much current research in molecular biology involves the transformation of E. coli, an organism that does not naturally enter a state of competency. *E. coli* can artificially be made competent when treated with chloride salts of the metal cations calcium, magnesium and rubidium. In addition, abrupt transition between heat and cold can induce competency. It is believed that metal ions and temperature changes affect the structure and permeability of the cell wall and membrane, allowing DNA molecules to pass through. Due to their unstable cell walls, competent *E. coli* cells are fragile and therefore must be treated carefully.

To ferry foreign genes into bacteria, plasmids are usually used. Plasmids are self-replicating extrachromosomal, double-stranded circular DNA molecules found in many strains of bacteria. Many plasmids contain genes that provide resistance to various antibiotics, including tetracycline, kanamycin, and ampicillin (amp). Ampicillin is a derivative of penicillin that inhibits bacterial growth by interfering with the synthesis of bacterial cell walls. The product of the ampicillin resistance gene is the enzyme β-lactamase. This enzyme is secreted by transformed cells into the surrounding medium, where it destroys ampicillin. Due to this extracellular secretion, cells that are not transformed are able to undergo limited growth in the zones surrounding transformed, antibiotic-resistant cells. Colonies consisting of these untransformed cells are called “satellites:, since they only appear aroung larger colonies of transformed cells. Larger plating volumes and longer

incubation times increase the number of satellite colonies.

Plasmids naturally exist as supercoiled molecules. The two strands of DNA in the supercoiled molecule wind around each other to produce a condensed, entangled structure when compared to relaxed (non-supercoiled) DNA (Figure 2). Competent *E. coli* cells are sensitive to the conformation of the DNA they will accept. Supercoiled DNA gives the highest transformation efficiencies.

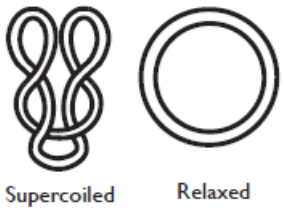


Figure 2: Supercoiled and circular forms of plasmid DNAs

In this experiment, the goal is to express fluorescent proteins (GFP) in transformed bacterial cells (Figure 3). To begin this process, there must be a means of "turning on "the cloned GFP gene in the recombinant plasmid. In order to have an "off/on" switch for controlling expression, the gene is placed under the control of a DNA sequence known as a promotor.

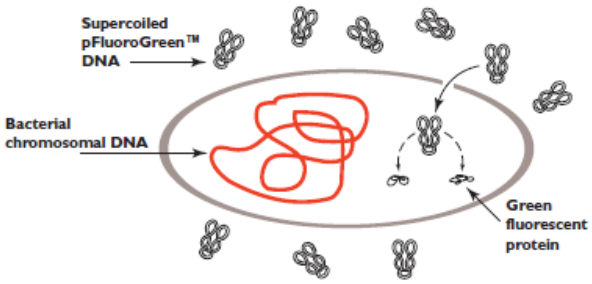


Figure 3:
Overview of transformation and protein expression.

A promoter is a sequence of DNA that typically occurs just in front (upstream) of the DNA coding sequence (the sequence that specifies the amino acid sequence for a protein). The chromosome of the host bacterial strain used in this experiment has been genetically engineered to contain the gene for RNA polymerase, which is under control of the *lac* promoter, and can be turned on (induced) by the presence of a small molecule called (iPTG (isopropyl-beta-D-thiogalcopyranoside). IPTG binds to and inactivates an inhibitor protein known as the *lac* repressor.

Quick Reference Abbreviations

GFP	Green fluorescent protein
pGFP	Plasmid for GFP expression
gfp	Gene for green fluorescent protein

The sequence of events required to turn on expression of gfp (gene for green fluorescent protein) is as follows: Cells are grown in the presence of iPTG which binds and releases the bound *lac* repressor. The release of the repressor allows the RNA polymerase to be produced from the *E. coli* genome. The RNA polymerase, in turn, recognizes the promoter on the plasmid enabling production of large quantities of the fluorescent GFP protein. A strong promoter, combined with an active RNA polymerase, allows for very high levels of gfp mRNA (and thus GFP protein expression) in the transformed cells.

Answer the following questions:

11) What conditions increase the competency of bacterial cells?

12) Suggest reasons for why the conditions above increase competency.

13) Some bacteria are naturally resistant to antibiotics, but others are not. How could you use two LB/agar plates, some *E. coli*, and some ampicillin (an antibiotic) to determine how *E. coli* cells are affected by ampicillin?

14) What would you expect your experimental results to indicate about the effect of ampicillin on the *E. coli* cells?

15) Do you think that exposure to ampicillin will cause the *E. coli* cells to evolve resistance to ampicillin? Why or why not?

16) How will you be able to tell if host *E. coli* cells have been genetically transformed?

Learning Objectives

- To demonstrate the universality of DNA and its expression
- To explore the concept of phenotype expression in organisms
- To explore how genetic information can be transferred from one organism to another
- To investigate how horizontal gene transfer is a mechanism by which genetic variation is increased in organisms
- To explore the relationship between environmental factors and gene expression
- To investigate the connection between the regulation of gene expression and observed differences between individuals in a population of organisms.

Laboratory Safety

Important READ ME!

Transformation experiments contain antibiotics which are used for the selection of transformed bacteria. Students who have allergies to antibiotics such as penicilllin, ampicillin, kanamycin or tetracycine should not participate in this experiment.

1. Gloves and goggles should be worn routinely as good laboratory practice.

2. Exercise extreme caution when working with equipment which is used in conjunction with the heating and/or melting of reagents.

3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.

4. The *E. coli* bacteria used in this experiment is not considered pathogenic. Although it is rarely associated with any illness in healthy individuals, it is good practice to follow simple safety guidelines in handling and disposal of materials contaminated with bacteria.

5. Properly dispose materials after completing the experiment:

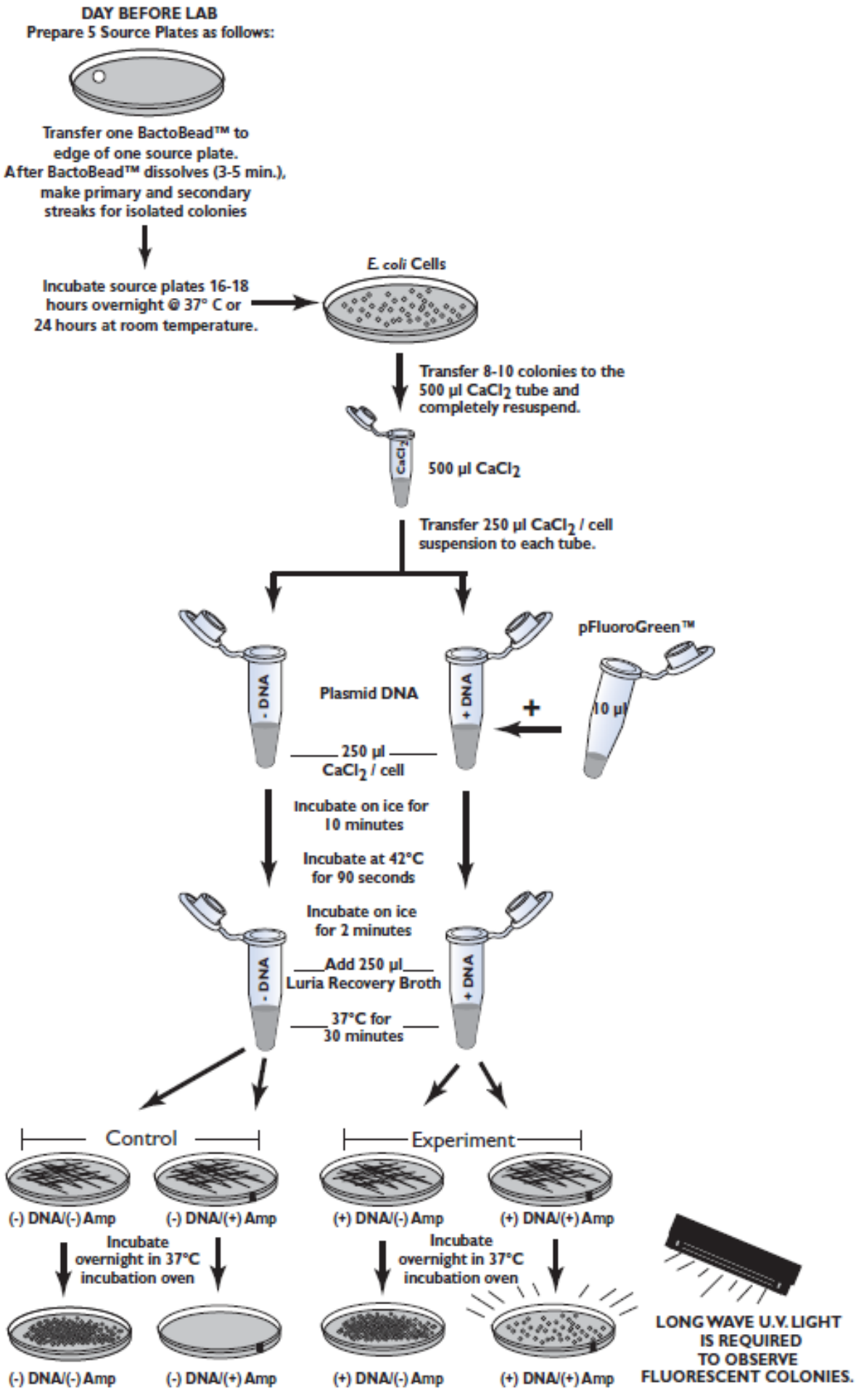
   A. Wipe down the lab bench with a 10% bleach solution or a laboratory disinfectant.

   B. All materials, including petri plates, pipets, transfer pipets, loops and tubes, that come in contact with bacteria should be disinfected before disposal in the garbage. Disinfect materials as soon as possible after use in one of the following ways:

   - Autoclave at 121° C for 20 minutes.
     Tape several petri plates together and close tube caps before disposal. Collect all contaminated materials in an autoclavable, disposable bag. Seal the bag and place it in a metal tray to prevent any possibility of liquid medium or agar from spilling into the sterilizer chamber.

   - Soak in 10% bleach solution.
     Immerse petri plates, open tubes and other contaminated materials into a tub containing a 10% bleach solution. Soak the materials overnight and then discard. Wear gloves and goggles when working with bleach.

6. Wear gloves, and at the end of the experiment, wash hands thoroughly with soap and water.


DAY BEFORE LAB
Prepare 5 Source Plates as follows:
Transfer one BactoBead™ to edge of one source plate. After BactoBead™ dissolves (3-5 min.), make primary and secondary streaks for isolated colonies
Incubate source plates 16-18 hours overnight @ 37° C or 24 hours at room temperature.
E. coli Cells
Transfer 8-10 colonies to the 500 µl CaCl2 tube and completely resuspend.
CaCl2
500 µl CaCl2
Transfer 250 µl CaCl2 / cell suspension to each tube.
- DNA
Plasmid DNA
+ DNA
pFluoroGreen™
10 µl
250 µl CaCl2 / cell
Incubate on ice for 10 minutes
Incubate at 42°C for 90 seconds
Incubate on ice for 2 minutes
Add 250 µl Luria Recovery Broth
37°C for 30 minutes
Control
Experiment
(-) DNA/(-) Amp
(-) DNA/(+) Amp
(+) DNA/(-) Amp
(+) DNA/(+) Amp
Incubate overnight in 37°C incubation oven
LONG WAVE U.V. LIGHT IS REQUIRED TO OBSERVE FLUORESCENT COLONIES.

SETTING UP THE TRANSFORMATION AND CONTROL EXPERIMENT

1. Label one microcentrifuge tube "+ DNA". This will be the transformation tube with plasmid DNA.
2. Label a second microcentrifuge tube "– DNA" . This will be the experimental control tube without plasmid DNA.
3. Using a sterile 1 ml pipet, add 0.5 ml of ice cold 0.05 M $CaCl_2$ solution into the "– DNA" tube and place on ice.
4. With a sterile loop, transfer a group of 8-10 single, well-isolated colonies from the plate labelled *E. coli* source plate to the "– DNA" tube. Twist the loop vigorously between your fingers to dislodge the cells. Vortex the cells to mix and fully suspend the cells in the $CaCl_2$.
5. Transfer 250 µl of this cell suspension to the tube labeled "+ DNA".
6. Place both the "– DNA"and the "+ DNA"tubes on ice. At this point, each tube should have 250 µl of the $CaCl_2$ suspended cells.
7. To the tube labeled "+ DNA", add the following:
   - 10 µl of pGFP (from tube labeled "pGFP")

8. Incubate the two tubes on ice for **10 minutes.**
9. Place both transformation tubes at **42° C for 90 seconds.**

   This heat shock step facilitates the entry of DNA in bacterial cells.

10. Return both tubes **<u>immediately</u>** to the ice bucket and incubate for **two (2) minutes.**

NOTE:

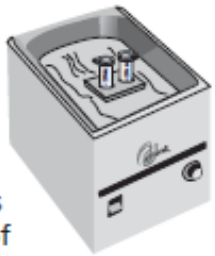
Remember to resuspend the cells thoroughly by vortexing or vigorous mixing by hand (flicking the tube of cells). It is very important that the cell suspension is homogenous and no clumps are visible. The cell suspension must appear somewhat cloudy.

NOTE:

Avoid scraping up agar when transferring the cells from the source plate to the tubes with calcium chloride solution. It is important that the cells are resuspended in the calcium chloride solution and is not left on the toothpick or on the wall of the tube.

Transformation of *E. coli* with pGFP

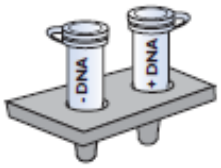
11. Using a sterile pipet, add **250 µl** of Luria Recovery Broth to each tube and mix.



12. Incubate the cells for **30 minutes** in a 37° C waterbath for a recovery period.

13. While the tubes are incubating, label 4 agar plates as indicated below. Write on the bottom or side of the petri plate.

    - Label one unstriped plate: (-) DNA/(-) Amp
    - Label one unstriped plate (+) DNA/(-) Amp
    - Label one striped plate: (-) DNA/(+) Amp
    - Label one striped plate: (+) DNA/(+) Amp
    - Put your initials or group number on all the plates.



14. After the recovery period, remove the tubes from the water bath and place them on the lab bench. Proceed to plate the cells for incubation.

Quick Reference:

DNA and competent cells are combined in a suspension. After the cells have incubated with the DNA, growth medium (recovery broth) is added. Bacterial cells continue to grow through the recovery process, during which time the cell wall is repaired. Cells recover and begin to express the antibiotic resistance gene.

PLATING THE CELLS

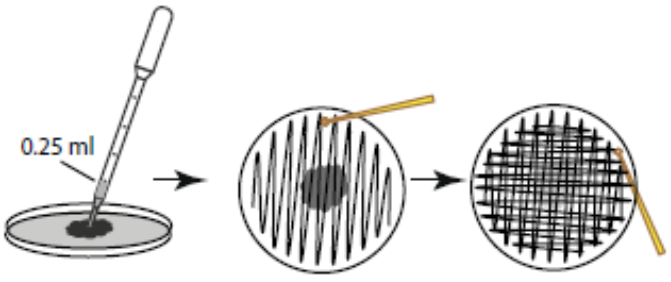
Plating cells from the tube labeled "- DNA" (Control Experiment):

15. Use a sterile 1 ml pipet to transfer recovered cells from the tube labeled " – DNA " to the middle of the following plates:

    - 0.25 ml to the plate labeled (-) DNA/(-) Amp
    - 0.25 ml to the plate labeled (-) DNA/(+) Amp

16. Spread the cells over the entire plate with a sterile inoculating loop (see Figure at left).

17. Cover both plates and allow the liquid to be absorbed.



NOTE: Ensure the entire plate has been completely streaked over with the inoculating loop.

To avoid contamination when plating, do not set the lid down on the lab bench -- Lift the lid of the plate only enough to allow spreading. Be careful to avoid gouging the loop into the agar.

Reminder:

Follow proper procedures for disposal of contaminated materials.

Important:

Do not allow the plates to incubate for longer than 20 hours at 37° C.

IPTG induced expression in the cells is very high and cell lysis can occur with extended incubation time resulting in low GFP yields.

WEAR SAFETY GOGGLES

Do not use short U.V. light, which can cause burns and serious damage to the eyes.

Plating cells from the tube labeled "+ DNA"

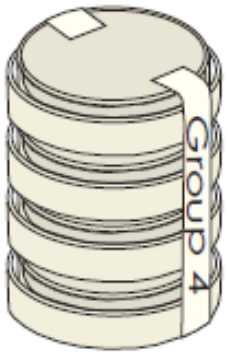
18. Use a sterile 1 ml pipet to transfer recovered cells from the tube labeled "+ DNA" to the middle of the following plates:
    - 0.25 ml to the plate labeled (+) DNA/(-) Amp
    - 0.25 ml to the plate labeled (+) DNA/(+) Amp
19. Spread the cells with a sterile inoculating loop in the same manner as step 16 .
20. Cover the plate and allow the liquid to be absorbed (approximately 15-20 minutes).

PREPARING PLATES FOR INCUBATION

21. Stack your group's set of plates on top of one another and tape them together. Put your initials or group number on the taped set of plates.

    The plates should be left in the upright position to allow the cell suspension to be completely absorbed by the agar.
22. Place the set of plates in a safe place designated by your instructor.
23. After the cell suspension is completely absorbed by the agar, you or your instructor will place the plates in the inverted position (agar side on top) in a 37° C bacterial incubation oven for overnight incubation (16-18 hours).

    If the cells have not been absorbed into the medium, it is best to incubate the plates upright. The plates are inverted to prevent condensation on the lid, which could drip onto the culture and may interfere with experimental results.



VIEWING PLATES AFTER INCUBATION

24. Darken the room and use a long wave U.V. light to visualize the transformed cells that will glow green due to the expression of the green fluorescent protein.

    To visualize the fluorescent colonies, the long wave U.V. light (EDVOTEK cat. # 969 recommended) can be held underneath the plates in a darkened room.
25. Proceed to analyzing your results.

Experiment Results and Analysis

Think about these questions *before* collecting data and analyzing your results.

1. On which of the plates would you expect to find bacteria most like the original non-transformed *E. coli* colonies you initially observed? **Why?**

2. If there are any genetically transformed bacterial cells, on which plate(s) would they most likely be located? Again, **why?**

3. What is the purpose of the control plates? Explain the difference between each and why it is necessary to run each.

4. Which plates should be compared to determine if any genetic transformation has occurred? **Why?**

5. What barriers might hinder the acquisition of plasmids?

6. How can the procedures described above (addition of Cl_2 and "heat shocking") help facilitate the introduction of plasmids into the *E. coli* cells?

Teacher initials_____

DATA COLLECTION

7. Observe the results you obtained on your transformation and control plates.

Control Plates:

- (-) DNA/(-) Amp
- (-) DNA/(+) Amp
-

Transformation Plate:

- (+) DNA/(-) Amp
- (+) DNA/(+) Amp

8. Draw and describe what you observe. For each of the plates, record the following:
   - How much bacterial growth do you observe? Determine a count.

   - Why do different members of your class have different transformation efficiencies?

   - If you did not get any results, what factors could be attributed to this fact?

9. Do your results support your original predictions about the "+ plasmid" transformed *E. coli* cells versus "- plasmid" nontransformed cells? Explain why or why not

10. Which of the traits that you originally observed for *E. coli* did not seem to become altered?

11. Which traits seem now to be significantly different after performing the transformation procedure?

12. What evidence suggests that the changes were due to the transformation procedures you performed?

13. What advantage would there be for an organism to be able to turn on or off particular genes in response to certain conditions?

DETERMINATION OF TRANSFORMATION EFFICIENCY

Transformation efficiency is a quantitative determination of the number of cells transformed per 1 µg of plasmid DNA. In essence, it is an indicator of the success of the transformation experiment.

You will calculate the transformation efficiency using the data collected from your experiment.

1. Each colony is the result of many genetically identical offspring from a single transformed bacterium. Count the number of colonies on the plate that is labeled: (+) DNA/(+) Amp. A convenient method to keep track of counted colonies is to mark each colony with a lab marking pen on the outside of the plate.

2. Determine the transformation efficiency using the following formula:

$$\frac{\text{Number of transformants}}{\mu\text{g of DNA}} \times \frac{\text{final vol at recovery (ml)}}{\text{vol plated (ml)}} = \text{Number of transformants per } \mu\text{g}$$

Example:

Assume you observed 40 colonies:

$$\frac{40 \text{ transformants}}{0.05\ \mu\text{g}} \times \frac{0.5\text{ ml}}{0.25\text{ ml}} = \frac{1600}{(1.6 \times 10^3)} \text{ transformants per } \mu\text{g}$$

Quick Reference for Expt. 223

50 ng (0.05 µg) of DNA is used.

The final volume at recovery is	0.50 ml.
The volume plated is	0.25 ml.

Results: Draw and show work for transformation efficiency

14. What are some possible sources of error in the transformation procedure? If you had to repeat the procedure, what are ways to minimize potential sources of error?

15. Does a bacterial cell take in a plasmid with genes the cell already possesses? If so, would this affect your calculations?