

Introduction to Genetic Engineering: *Bacterial Transformation with Green Fluorescent Protein (pKiwi)*

Genetic engineering is an umbrella term that encompasses many different techniques for moving DNA between different organisms. **Transformation** the process by which an organism acquires and expresses a whole new gene. In this activity, you will have the opportunity to genetically transform bacteria cells; altering them so that they can make an entirely new protein. This procedure is used widely in biotechnology laboratories all over the world, enabling scientists to manipulate and study genes and proteins in exciting new ways.

Adding a new gene to bacteria cells is a very simple procedure. You will add a gene that codes for Green Fluorescent Protein (GFP). This protein was discovered in the bioluminescent jellyfish called *Aequorea victoria*, a jellyfish that fluoresces and glows in the dark (Figure 1).

The gene for GFP was isolated in 1994 and was quickly used in laboratories as a way to brightly label proteins in a living cell. This “tagging” of proteins allowed researchers to visualize specific proteins to learn more about their biological functions in exciting new ways. The discovery of GFP proved to be so important that the Nobel Prize in Chemistry in was awarded to Osamu Shimomura, Marty Chalfie and Roger Tsien in 2008 for their work. Since then, Roger Tsien’s laboratory at UCSD has altered the GFP gene to make a full rainbow of proteins. Figure 2 shows how bacterial expressing many different colored fluorescent proteins can be grown together on one plate.

Figure 1: Aequorea Victoria glowing under UV light
Retrieved from:
<http://voices.nationalgeographic.com/2012/04/03/love-and-war-the-essence-of-luminosity/>

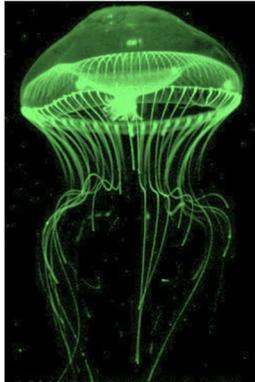
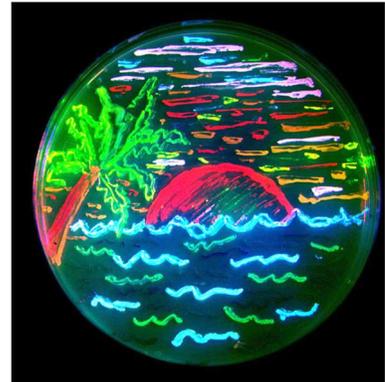
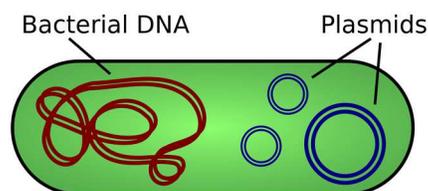


Figure 2: A rainbow of fluorescent growing on a n agar plate.
Retrieved from:
<http://www.tsienlab.ucsd.edu/Images.htm>



Bacteria are commonly used for genetic transformation experiments because they are simple, single-celled organisms that grow and reproduce very quickly. Bacteria cells store their DNA on one large, circular chromosome. But they may also contain one or more small circular pieces of DNA called plasmids. Because bacteria reproduce asexually, plasmid DNA allows for the addition of new traits into a cell. Plasmids replicate independently of the large bacterial chromosome, and can transfer easily between cells. Figure 3 shows the circular DNA chromosome and plasmid DNA inside of a cell.

Figure 3
Genetic material in bacteria takes 2 forms
Retrieved from: Figure 3 – Wikipedia.
<https://en.wikipedia.org/wiki/Plasmid>



Bacterial evolution and adaptation in the wild often occur via plasmid transfers from one bacterium to another. An example of bacterial adaptation is resistance to antibiotics via the transmission of plasmids. This natural process can be modified by humans to increase our quality of life. In agriculture, genes are added to help plants survive difficult climatic conditions or damage from insects, and to increase their absorption of nutrients. Toxic chemical spills can often be bioremediated (cleaned-up) by transformed bacteria specifically engineered to do the task. Currently, many people with diabetes rely on insulin made from bacteria transformed with the human insulin gene. Scientists use transformation as a tool to study and manipulate genes all the time.

Background Information and Scientific Theory

The Central Dogma of Molecular Biology

A basic tenet of biology, from single-celled bacteria to eukaryotes, is the mechanism of coding, reading and expressing genes. The central dogma of molecular biology states that: **DNA > RNA > PROTEIN > TRAIT**. This curriculum is an example of the central dogma in action. The instructions for GFP production are encoded in the DNA. When transcription is turned on, the cell turns those instructions into an mRNA transcript. This transcript is then translated into protein, which provides the trait of fluorescence.

The Transformation Procedure

In order to increase the chances that your *E. coli* will incorporate foreign DNA, you will need to alter their cell membranes to make them more permeable. This is a three-step process.

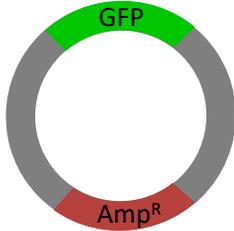
1. First you place your cells and pKiwi together in a **transformation solution** (which contains calcium chloride) to neutralize the charge.
2. Second, you quickly **heat shock** them with a temperature change (42°C). This hot temperature permeabilizes (loosens) the bacterial cell wall, making it easier for pKiwi to cross it.
3. This process can be harmful to the cells, so you want to give them a nutritious broth to restart their growth as soon as you're done. **Luria Broth (LB)** is a liquid that contains proteins, carbohydrates and vitamins so that the *E.Coli* can rapidly recover and thrive. They will then be placed on an **agar medium**, a jello-like substance containing LB, with or without antibiotic or sugar, to grow overnight.

In this lab, you will be using non-pathogenic *E. coli* bacteria and pKiwi, a plasmid modified with two genes. The pKiwi plasmid contains the genetic codes for (see Table 2):

1. a **green fluorescent protein (GFP)** from the bioluminescent jellyfish, *Aequorea victoria*
2. ampicillin or antibiotic resistance (amp^R)

If pKiwi transformation is successful and the bacteria are growing, the colonies will appear green under natural light, neon green under UV light. These green bacteria must contain the plasmid with the GFP. For this reason, the **green fluorescent protein (GFP)** gene is often used as a "reporter gene" to identify expression of other genes of interest. Note: the p in pKiwi stands for plasmid while the protein is Green Fluorescent Protein (GFP.)

Table 1: pKiwi plasmid and its two important genes.

Type of gene	pKiwi plasmid with inserted genes
Ampicillin resistance gene forming beta-lactamase, which inactivates ampicillin in media	 pKiwi Plasmid
Kiwi gene produces the green fluorescent protein (GFP)	

In order to successfully transform bacteria, you will need to add CaCl_2 , to neutralize both the bacterial cell wall and membrane charges, then, quickly shock them with a temperature change in order for them to uptake the pKiwi plasmid. After the stressful event, you will provide nutritious broth to restart their growth. Deviating from the protocol listed below may decrease your success in obtaining transformants.

General Lab Skills Required for Success

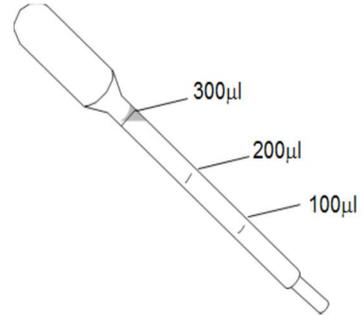
Using Sterile Technique

Students should wash their hands before starting lab, after handling recombinant DNA organisms/containers, and before leaving the lab area. All lab surfaces should be **decontaminated** at least once a day during each class section and following spills. Students should avoid touching the tips of the pipettes or inoculating loops onto any contaminating surfaces, unless instructed in the protocols.

Using Transfer Pipettes

A transfer pipette works just like an eye-dropper. Observe the 100 μ l, 200 μ l and 300 μ l marks on the transfer pipette. You will need to transfer a volume of 250 μ l, found between 200 μ l and 300 μ l. You will also need to transfer a volume of 150 μ l. Where do you think that volume will be found?

When using, bring the pipette up to eye level to confirm that liquid has been transferred correctly. For practice, get a feel for the pipette by transferring water from one container to another. Success of the lab depends on the proper use of tools and reagents required for the protocol.



Using Inoculating loops

You can measure precisely 10 μ l with a 10 μ l inoculation loop. Dip the loop into the tube containing liquid. A noticeable film will form around the ring due to surface tension (like a bubble wand). Swirl the loop into the tube using your index finger and thumb. Note that the loop may not fit into the narrow bottom end of most microtubes.



UV Safety

Ultraviolet radiation can cause damage to eyes and skin. If possible, use UV-rated safety glasses or goggles if looking directly at UV light.

Using Experimental Controls

In this lab, it is important to confirm which cells have received the plasmid, and under which conditions the green fluorescent proteins are being produced. You will need to prepare **experimental controls** to be able to interpret your results correctly. These controls are designed to minimize the effects of factors other than the single concept that you are testing. Therefore, each student group will perform 2 different reactions: one with pKiwi plasmid (+DNA) and one without it (- DNA). Each reaction then, will be plated on LB only and LB/AMP plates.

Laboratory Activity

The protocol outlined next describes the procedure for bacterial transformation. Follow the steps very carefully for higher likelihood of success. Make sure you work aseptically and accurately.

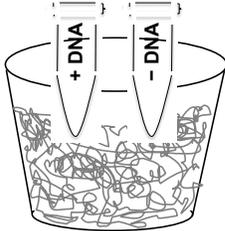
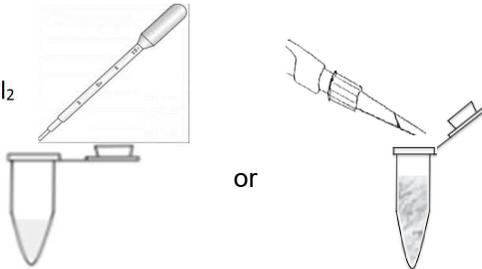
Reminders:

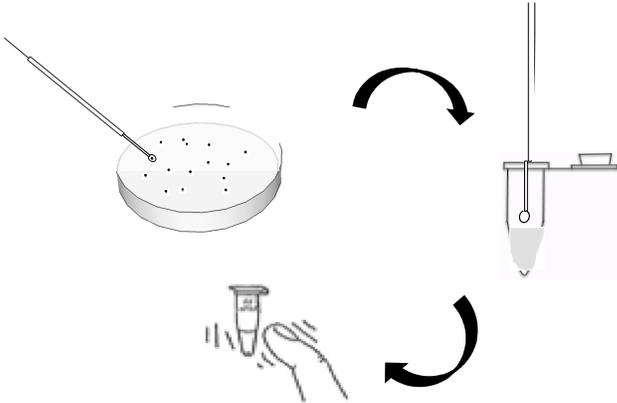
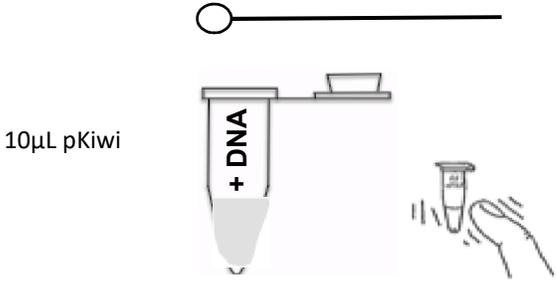
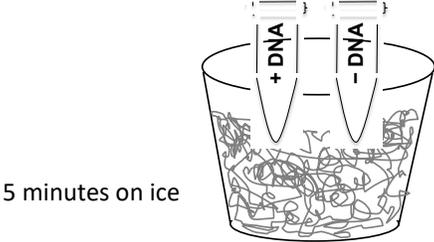
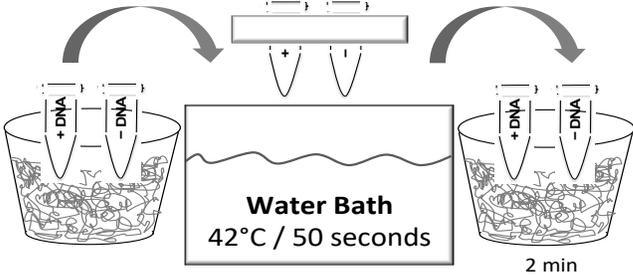
1. Do not open anything until needed.
2. Do not touch the ends of the instruments.
3. Always work from **negative (-) to positive (+)**. Why?
4. Make good use of your time. While waiting for short incubations, read and get ready for the next step.

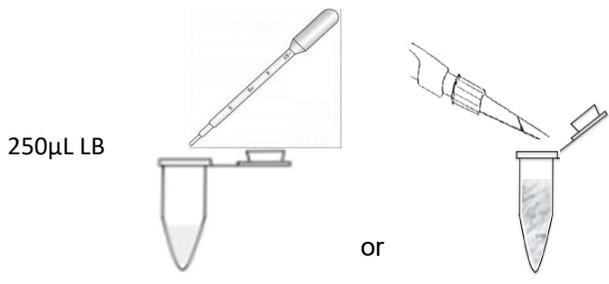
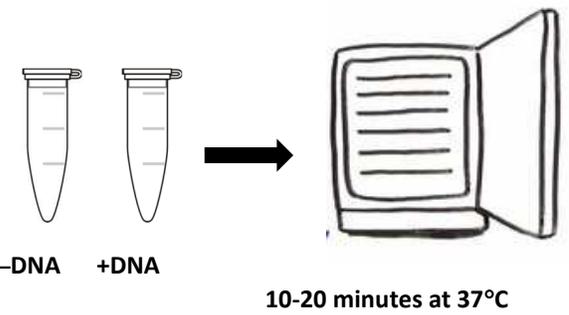
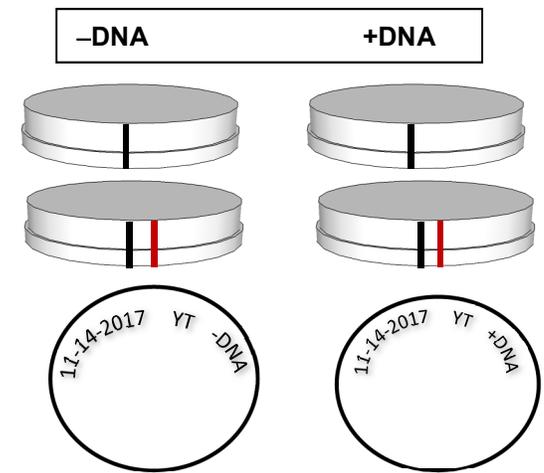
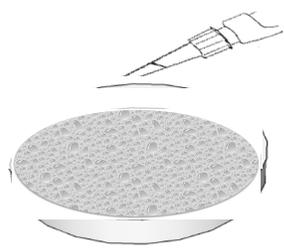
Before your start, make sure your group has the following items:

- Cup of crushed ice
- 1 tube labelled CaCl₂
- 1 tube labelled LB
- 2 empty/blank tubes
- Tubes should be in a rack
- 4 transfer pipettes (DO NOT OPEN UNTIL NEEDED)
- 1 Permanent marker (may have to share with the whole class)

Place a check mark in the box as you complete each step.

pKiwi Transformation Protocol	
<p>1. STERILIZE lab surfaces and wash hands before beginning the lab. (Why?)</p>	
<p>2. LABEL TUBES: Using a permanent marker, label one empty microfuge tube +DNA and the other -DNA. Put your team name on the side of the tube.</p> <p>Label each tube twice, on the lid and on the side. Place these tubes into a Styrofoam cup containing crushed ice.</p>	
<p>3. ADD 250µL of CaCl₂ to each tube using a sterile transfer pipette.</p> <p>Or a micropipettor. If using a P200 micropipettor, use 125µL twice.</p> <p>Note: TS contains calcium chloride (CaCl₂), which helps neutralize both the bacterial cell wall membrane and DNA charges. Keep your tubes on ice.</p>	<p>250µL CaCl₂</p> 

<p>4. PICK UP BACTERIA FROM PLATES: With a sterile inoculation loop that the teacher gives you (<i>Hold the loop in the middle. DO NOT TOUCH THE ENDS</i>), pick up a small smear of bacteria from the starter plate, using the small end.</p> <p>Dip and swirl the loop into the -DNA tube to evenly disperse the colony in the solution and release it from the loop. With the cap closed, flick the tube with your finger to mix.</p> <p>Repeat but put the bacteria into the +DNA tube. Return tubes to ice.</p>	
<p>5. ADD the pKiwi DNA: With the 10μL inoculation loop, dip the big loop into the stock plasmid tube. A noticeable film will form around the ring due to surface tension (like a bubble wand). Swirl the loop into tube labelled +DNA only.</p> <p>Micropipette: Use a p-20 and put 10μL into the tube. What should the dial window look like?</p> <p>**DO NOT add plasmid to the -DNA tube.</p> <p>Close the cap and flick the tube to mix. Quickly, place the tubes on ice.</p>	
<p>6. INCUBATE ON ICE: Incubate both tubes on ice for 5 minutes, making sure the tubes are in contact with the ice.</p> <p>Note: This step allows the charges to neutralize so the cells can take up the plasmid DNA in the next step.</p> <p>While waiting, read through the next step as it is CRUCIAL for success.</p>	
<p>7. HEAT SHOCK your bacteria by transferring both tubes to a foam rack and placing them into a water bath set at 42°C for 50 seconds.</p> <p>Make sure the tubes are pushed down as far as they can go in the rack to contact the hot water.</p> <p>After 50 seconds, quickly place both tubes on ice for another 2 minutes. It is VERY important to watch the time and speed of the transfers.</p>	

<p>8. FEED THE CELLS WITH FOOD: Return your tubes to a tube rack. Using a sterile transfer pipette, add 250µL of LB broth to each of the tubes. Close the tubes. Mix each tube by flicking it several times with your finger.</p> <p>Or use a micropipettor, If using a P200, use 125µL twice. What should the dial window look like?</p>	
<p>9. LET THE CELLS RECOVER: Incubate the tubes for 10-20 minutes at 37C. You can use the bacterial incubator or other warm place like the top of a refrigerator for this step.</p> <p>Note: This process allows the transformed bacteria to recover from the “shock” by providing nutrients for their growth.</p>	
<p>10. READY YOUR PLATES by LABELLING ALL PLATES: While you’re waiting, pick up: 2 LB and 2 LB/AMP PLATES</p> <p>On the bottom of the plate (non-lid side and the edge of the plates), write the date, your initials or your team initials, and -DNA or +DNA onto the LB and the LB/amp plates. Use the picture on the right to guide you.</p>	
<p>11. PLATE CELLS. Using a transfer pipette, transfer 150µL of the -DNA to each plate labeled -DNA. Slowly pipette/dribble directly onto the agar (bottom plate with agar, not the lid!)</p> <p>Then do the same for the +DNA, using the same transfer pipette. <i>By going from -DNA to +DNA, you are using good lab practices and saving on resources.</i></p> <p>Swirl to coat the agar evenly. <i>Allow bacteria to saturate (and dry) into the agar plate for a few minutes before the next step.</i></p>	

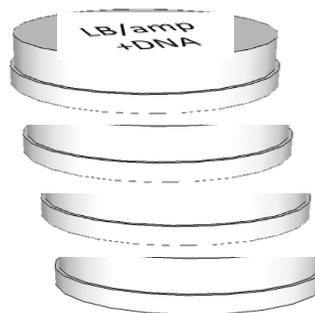
12. **INVERT, TAPE, INCUBATE.** Invert your plates (so lid is again on the bottom). Then stack and tape them together.

Place plates into an incubator oven set at 37C until the next day or when colonies are visible.

Alternatively, stack the plates into a warm spot in the classroom. It may take 2-3 days for bacterial colonies to appear.

After the colonies have appeared, you can observe and analyze your results! Take pictures!

For storage, you may keep the plates by wrapping them in parafilm or tape and storing them in the refrigerator.



14. **CLEAN UP, WASH UP:** When done with all lab activity, clean up you trash and instruments. Decontaminate all lab surfaces with dilute disinfectant and wash hands!



[Type here]

[Type here]

Name: _____ Period _____

Date: _____

PreLab Activity: Student Learning Outcome & Pre-Lab Predictions For Laboratory Activity

Student Learning Outcomes – at the end of this laboratory, students will be able to:

1. Explain the process of bacterial transformation.
2. Relate the use of bacterial transformation in biotechnology.
3. Differentiate transformed from non-transformed cells.
4. Calculate transformation efficiency and compare with the class data.

Predict the results by filling in the Pre-Lab before you see the results of the experiment.

Lab Predictions: Answer the lab prediction questions below by filling in the table.

Make your prediction and fill out the table below:

Item	Prediction growth on LB	Prediction: growth on LB/Amp	Will it be green?	Explanation of Your Prediction
Untransformed bacteria (– DNA tube)				
Transformed bacteria (+ DNA tube)				
Plasmid only (DNA only)				

[Type here]

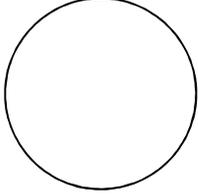
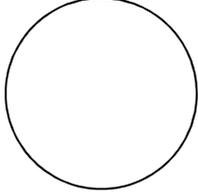
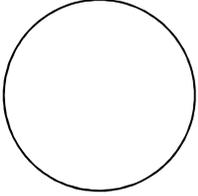
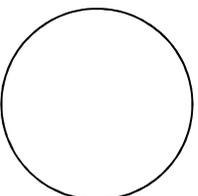
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Name: _____ Period _____

Date: _____

Post-Lab Questions

1. Post-Lab Results. Put your results here by

	-DNA on LB	-DNA on LB/Amp	+DNA on LB	+DNA on LB/Amp
Illustration of Results				
Description of results				

2. Compare your predictions with your actual lab results. Describe how close your predictions were to your actual results and explain possible reasons for any differences.

3. Explain the purpose of these processes or substances during transformation.

	Process or Substance	Purpose
a.	LB agar	
b.	<i>Ampicillin or antibiotic</i>	
c.	Calcium chloride	
d.	Heat shock	

[Type here]

[Type here]

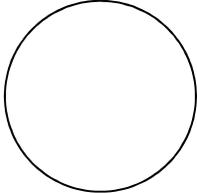
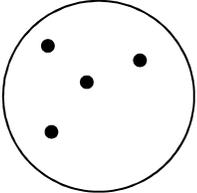
Name: _____ Period _____

Date: _____

4. Describe 2 differences and 2 similarities between these bacteria.

Condition	- pKiwi DNA bacteria	+ pKiwi DNA bacteria
Difference		
Similarity		

5. Explain what may have occurred to produce these results. (• = colony)

Contents	LB -DNA	LB/amp -DNA
Illustration of Results		
Description of Results		
Possible explanation for results		

[Type here]

[Type here]

Name: _____ Period _____

Date: _____

- 6. If growth appeared on the LB/amp +DNA plate, would these bacteria be transformed? Explain.

- 8. Provide an example of how transformation can be beneficial and an example of how it can be potentially harmful to humans. You may look up information on the web.

	Condition	Transformation example
a.	Beneficial	
b.	Harmful	

- 7. Provide a rational or benefit of adding DNA sequences coding for fluorescent proteins such as GFP, to tag genes of interest in plasmids used for transformation.

- 9. Using another sheet of paper, draw a model of the phenomom of bacterial transformation. Think about the steps you used to for this process to take place what happened at those steps (eg. adding (TS) CaCl₂, heat shocking, cooling, etc).

- 10. Bacterial transformation is a process that scientists use under the large term of genetic engineering. Did you think that you were doing genetic engineering? What other "genetic engineering" have you heard or thought about? Did you think YOU were able to do this type of experiment? Why/Why not?

[Type here]

[Type here]

Name: _____ Period _____

Date: _____

Worksheet: Calculating Transformation Efficiency

When performing transformation experiments, you usually want to obtain as many transformants as possible. This is important because you want to make sure your conditions for transformation is at its optimum. Transformation efficiency is the efficiency whereby cells take up the introduced DNA. Many factors contribute to transformation efficiency: cell age and competency (the ability to take up DNA), the type of cells being transformed, plasmid length and quality, the method of transformation (heat shock or electroporation) and just different conditions in general. Having a low transformation efficiency may point to poorly competent cells, poor conditions, or poor techniques (not following protocol). In a research lab, it's good to have many transformants for research, just in case individual transformants may not work as well (e.g. different levels of expression), or some other unknown problems associated with transformed cells. In making a genomic library, you want as many transformants as possible to have a robust library. In cell culture, you may take a population of transformed cells for further study therefore having a high transformation efficiency allows for better study.

In this exercise, we will calculate the transformation efficiency of the E. coli bacteria by pKiwi . The data can then be gathered from each team of the class and the data compared with a different transformation technique called electroporation.

Transformation efficiency calculation: $\frac{\text{The number of colonies observed growing on an agar plate (cfu)}}{\text{Amount of DNA used (in } \mu\text{g)}}$
cfu=colony forming units

Two data are needed for this:

1. Total number of green fluorescent colonies on your LB/amp plate.
2. Total amount of pKiwi plasmid DNA used for bacterial transformation that was spread on the LB/amp plate.

1. Determine the total number of transformed green fluorescent colonies.

Place the LB/amp plate near a UV light source. Count the number of green fluorescent colonies that glow under UV light.

Enter that number here →

Total number of colonies = _____

2. Determine the amount of pKiwi DNA in the cells spread on the LB/AMP plate.

- a. Total amount of DNA: **DNA in μg = (concentration of DNA in $\mu\text{g}/\mu\text{L}$) x (volume of DNA in μL)**

In this experiment, 10 μL of pKiwi at a concentration of 0.01 $\mu\text{g}/\mu\text{L}$ was used.

Enter that number here →

**Total amount of pGFP DNA,
 μg used in this experiment = _____**

[Type here]

[Type here]

Name: _____ Period _____

Date: _____

- b. **Fraction of pKiwi plasmid DNA (in the bacteria).** For this experiment, a certain amount was spread onto each plate. To find that fraction:

Fraction of DNA used →

$$\frac{\text{Sample volume spread on LB/amp plate, in } \mu\text{l}}{\text{Total sample volume in tube, in } \mu\text{l}}$$

- **150 μl** of cells was spread from the tube containing a total volume of **500 μl** of solution.

Enter that number here →

Fraction of DNA= _____

- c. How many μg of pKiwi DNA was spread on the LB/amp plate? Multiply the total amount of pKiwi DNA used by the fraction of pKiwi DNA you spread on the LB/amp plate.

$$\text{pKiwi DNA spread } (\mu\text{g}) = \text{amount of DNA used } (\mu\text{g}) \times \text{fraction of DNA}$$

Enter that number here →

pKiwi DNA spread, μg = _____

Now, we are finally ready to calculate the transformation efficiency!

Number of colonies on LB/amp plate = _____

pKiwi DNA spread, μg = _____

Transformation efficiency calculation: $\frac{\text{The number of colonies observed growing on an agar plate}}{\text{Amount of DNA used (in } \mu\text{g})}$

Enter that number here →

Transformation efficiency = _____ transformants or
cfu/ μg

cfu=colony forming units

[Type here]

[Type here]

Name: _____ Period _____

Date: _____

1. Analysis of results: What is the transformation efficiency of each team in the class?

Team	Efficiency

a. Calculate the Mean, Median and Mode of the results from above.

i. What was the average transformation efficiency?

ii. What was the Median?

iii. Was there a Mode?

2. In past studies, this method of “heat shock” protocol that was performed by research labs usually has a transformation efficiency between 8×10^2 and 7×10^3 transformants per microgram of DNA.

a. How does your team’s result compare to this data?

b. How does the class’ result compare to your data and to the data by research labs?

[Type here]

[Type here]

Name: _____ Period _____

Date: _____

3. Another method for transformation is called electroporation. In this method, an electric field is applied to allow the cell membrane to open up and take up DNA. The transformation efficiency from electroporation may be 1×10^8 cfu/ μ g.

What fold higher is the transformation efficiency by electroporation vs. heat shock?

4. How does electroporation compare to the heat shock method?