Check out this SICK Science video by Steve Spangler!!
(https://www.youtube.com/watch?v=A0b6_kg2oMc)

College of the Canyons Biology Outreach Program

UNIT 3: TRANSFORMATION (GENETIC ENGINEERING)

Version A

****Watch the video above and answer the following questions before starting the lab.****

1. What objects had the most bacteria growing on their plates? Why do you think that is?

2. How effective was the disinfectant on each of the three household objects? Did it eliminate ALL of the bacteria? Do you still think disinfectant is important? Why?

3. Complete & review page 5 for a guideline on the upcoming lab.

For more information on College of the Canyons Biology program, contact Associate Professor of Biology Jim Wolf & Patrick Allen at (661) 362-3092 or email: jim.wolf@canyons.edu

LAB UNIT 3
TRANSFORMATION LAB
Version A

OBJECTIVES:

1. To illustrate the advantages and destructiveness of antibiotic resistance.

2. To understand how bacteria acquire new DNA through Conjugation and Bacterial Transformation.

3. To understand the mechanism of recombinant DNA or gene transfer using plasmid vectors.

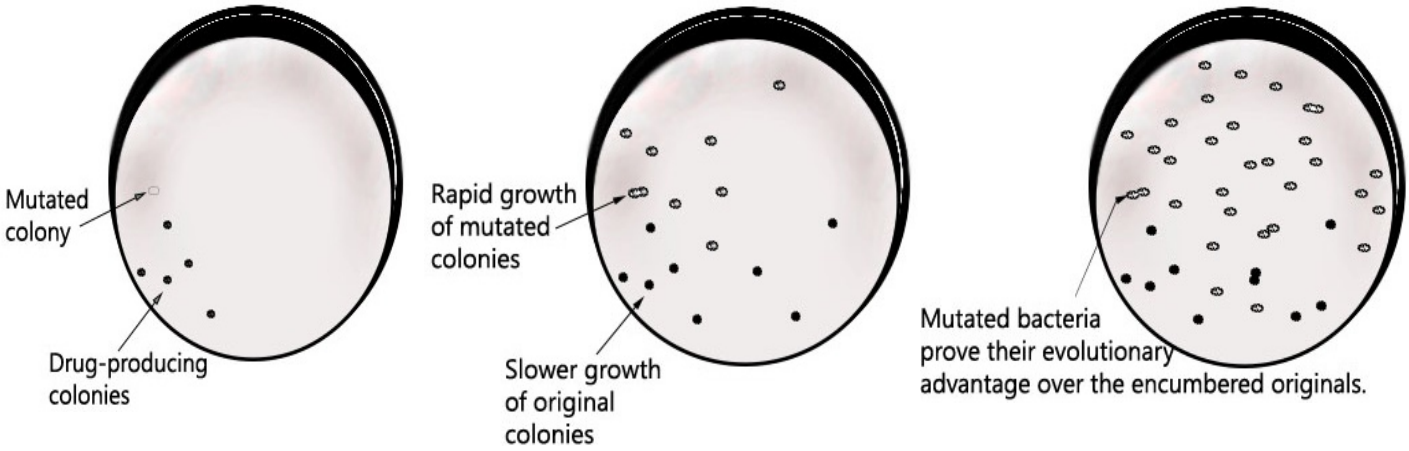
Background:

Humans can get new genes from mutation, but that's usually the only option we have. Bacteria are much more interesting and can take DNA from other bacteria. So if you and everyone in your class were bacteria you could have genes that other people have, right now. If you wanted to be taller you could find the tallest person in your class and get their "tall gene", or if you didn't want to wear braces you could find someone with a "straight teeth gene". However, like bacteria, you won't always know what you will be getting. You could end up with a peanut allergy, or have to give up ice-cream because you are now lactose intolerant. It's a dice roll whenever bacteria share DNA, but what is important is that Humans can't do this. We can only acquire new genes when an egg is fertilized, but not during our lifetimes.

One of the reasons why it is so common for bacteria to become resistant to antibiotics is because they can acquire these genes directly from other bacteria, which is called Conjugation **[1]** . Bacteroides, which are helpful bacteria in our intestines can become anti-biotic resistant through mutation when anti-biotics are abused **[2]**. However, harmful bacteria, such as *Salmonella typhi* (Typhoid) can acquire these same antibiotic resistance genes from the bacteria in your own body! In fact, from 1989-2002 there was an epidemic of *Typhoid* in Bangledesh caused by a drug resistant strain of *Salmonella typhi*. It was resistant to three different drugs, one of which was Ampicillin. The strain was believed to have gotten the genes from the Bacteroides in the human gut**[3]**. Don't blame your body's bacteria, they just don't know any better.

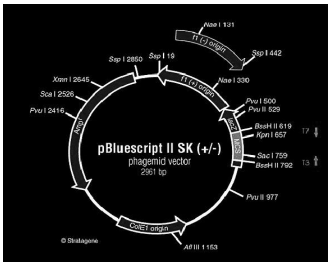
In today's lab you will create your own Ampicillin-resistant *E. Coli*, in a process called Bacterial Transformation. Now if drug resistance is so terrible, why would you want to make such a super bacteria? To take over the world? To make money? No to the first, but yes to the second. Since we can change bacterias' DNA during its lifetime we can change them to work for us. Since bacteria grow so quickly (20 minutes to divide once), people can use them as little medical factories instead of making the drugs themselves. Before Bacterial Transformation, many drugs, such as insulin, were harvested from animals' organs. Bacteria, such as *E.Coli*, can actually save time AND prevent animal cruelty. So where does the anti-biotic resistance come in?

Imagine you have transformed a bacteria strain to make the cure for cancer. They start by making a lot of your cancer cure, and soon you will be rich. However, after a few days your bacteria have slowed down, and after a week they have stopped making the drug. What happened? Are they on strike because you didn't give your microscopic employees benefits or livable wages? Actually, what likely happened is that your bacteria began by making your drug and dividing at first. Then a mutation caused some colonies to stop producing your costly drug. Those colonies didn't have to waste time and food making your cure, and could now breed faster than your drug-making colonies. It's like racing up a mountain; your drug-making colonies are well prepared and have a compass, extra water, extra food, and a water-proof jacket. They're just very slow, because all that stuff is heavy. The mutated and "lazy" colonies didn't bring anything, but can obviously run faster up the mountain. How can you make sure the colonies that you want to survive win the race? The answer lies in the antibiotic-resistance genes inside plasmids.



Plasmids are circular DNA pieces that contain genes for bacteria. This is what they trade during Conjugation and how you will Transform bacteria today (by artificially inserting a plasmid). In science, plasmids can contain multiple genes, one of which can be an antibiotic resistance gene such as Ampicillin resistance. So if you surround your bacteria with Ampicillin, then the only colonies that will survive will have to have that gene. What you can place in a plasmid is both the antibiotic resistance gene AND your cancer cure gene. So in our mountain race analogy, surrounding the bacteria with antibiotics is like covering the mountain with bears. The Amp resistance gene give the selected colonies a bear spray to scare off the bears, while the mutated colonies that can normally run fast will get eaten. Now your your slow drug-making colonies will be the only ones to survive. As long as you pair up antibiotic resistance genes with your drug-making genes on your plasmids, then your bacteria can continue producing medicine for you. This is one of the many useful technologies used by scientists in the field of Genetic Engineering.

In this lab, instead of pairing Ampicillin resistance with a "cancer cure gene", it will accompany a pGLO plasmid. This particular plasmid will allow transformed bacteria to express a green fluorescent protein. In addition to making "glow-stick like" bacteria with UV light, it is an indicator that those bacteria colonies took up the plasmid. So if a colony glows, it will have the antibiotic resistance gene. If the colonies aren't fluorescent green, then they will not have the Amp resistance.



Sources *(Always cite your sources)*-
1. Grohmann, E., G. Muth, and M. Espinosa. "Conjugative Plasmid Transfer in Gram-Positive Bacteria." Microbiology and Molecular Biology Reviews 67.2 (2003): 277-301. Web.

2. Okeke, Iruka. "Socioeconomic and Behavioral Factors Leading to Acquired Bacterial Resistance to Antibiotics in Developing Countries." Emerg. Infect. Dis. Emerging Infectious Diseases 5.1 (1999): 18-27. Web.

3. Rahman, M., A. K. Siddique, S. Shoma, H. Rashid, M. A. Salam, Q. S. Ahmed, G. B. Nair, and R. F. Breiman. "Emergence of Multidrug-resistant *Salmonella Enterica Serotype Typhi* with Decreased Ciprofloxacin Susceptibility in Bangladesh." Epidemiol. Infect. Epidemiology and Infection 134.02 (2005): 433. Web.

4. Rowe, B., L. R. Ward, and E. J. Threlfall. "Multidrug-Resistant *Salmonella Typhi*: A Worldwide Epidemic." Clinical Infectious Diseases 24.Supplement 1 (1997): n. pag. Web.

5. Salyers, Abigail A. "Human Intestinal Bacteria as Reservoirs for Antibiotic Resistance Genes." Vol.12 No.9. TRENDS in Microbiology, Sept. 2004. Web.

Transformation Lab
Version A Checklist

Be careful not to contaminate anything designated sterile!

- ☐ 5 sterile plastic inoculating loops (share 1 bag of 10 with another group)
  8 sterile plastic transfer pipettes (share 1 bag of 20 with another group)

- ☐ 2 sterile culture tubes with caps
  Insulated cup 3/4 full of crushed ice
  Vial of sterile Luria broth marked "L"
  Vial of 50 mM calcium chloride solution marked “Ca”
  Vial of purified ampicillin-resistance plasmid solution (0.005 mg/µl) marked “pGLO” (might be dosed out by your instructor)

- ☐ Waterproof marking pen
  2 sterile culture plates containing Luria broth agar marked "LB" (has a black stripe)
  2 sterile culture plates containing Luria broth agar, ampicillin, and Arabinose marked "LB+A" (has 3 stripes; black, red, and blue)
  Culture plate with colonies of ampicillin-sensitive *E. coli* marked "Starter culture".

- ☐ Biohazard bag
  Plastic beaker A(used to collect paper trash)
  Plastic beaker B(used to collect loops, pipettes, tubes, and other bacteria-contaminated materials)

- ☐ Water bath set at 42^{O}C
  Water bath set at 37^{O}C
  Incubator set at 37^{O}C

****Use this page to follow the lab instructions. BEFORE starting the lab, write down the times of each step, and identify & write which steps are the; *Weakening the cells step/ Introducing the plasmid step/ Effecting the Transformation Step/ Regaining strength step/ Recovery step/ and Plating the cells step*. Write the times wherever you see "__min" and write the step names above the pictures.**

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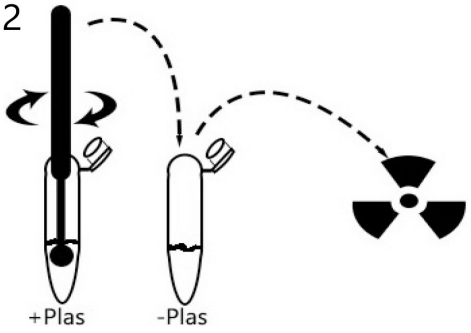

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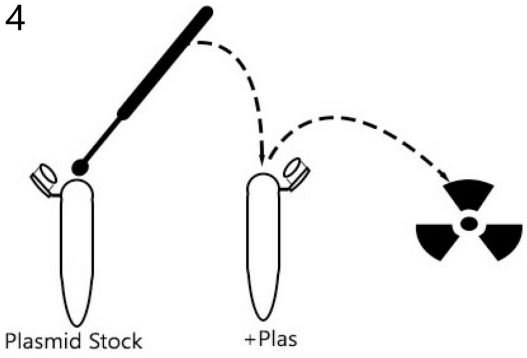

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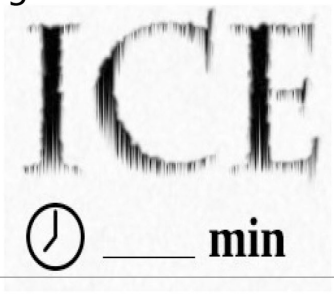

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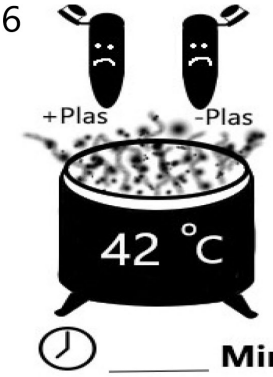

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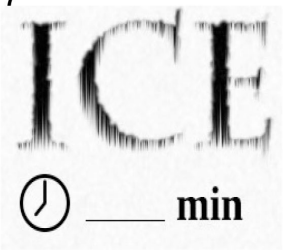

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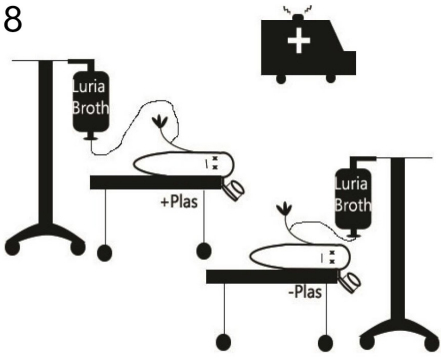

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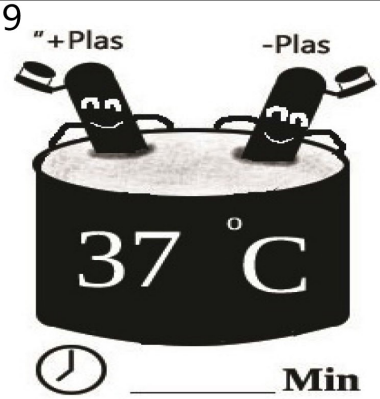

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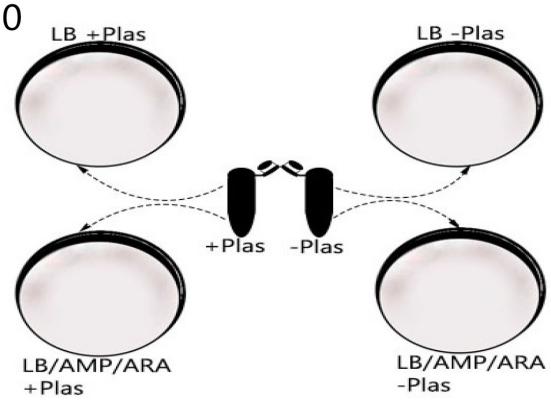

Version A BIOLOGY TRANSFORMATION PROCEDURE

Check off each sterile tool as it is used

1. **WEAKENING THE CELLS SO THEY MORE READILY TAKE IN "FOREIGN" DNA**

A. Label the two test tubes with team name. Label one tube "+ plasmid" and the other "- plasmid".

B. Add 250 µl ice-cold 50 mM $CaCl_2$ to each test tube using sterile transfer pipette. Replace caps. Put tubes on ice. ☐ *Pipette 1*

C. Transfer one or two 3mm diameter colonies of *E. coli* cells from starter culture to "+ plasmid" tube as follows:

i. Using sterile inoculating loop, gently scrape off the cells but be careful not to scrape off any agar. A 3mm diameter colony is the size of this capital O. ☐ *Loop I*

ii. Submerge the loop in the $CaCl_2$ solution in the "+ plasmid" tube and twirl and tap vigorously to dislodge the mass of cells. Hold tube up to the light to make sure cells come off the loop.

iii. Quickly suspend cells by pipetting solution in and out several times with sterile transfer pipet. Hold tube up to light to check for clumps. ☐ *Pipette 2*

iv. Replace cap on "+ plasmid" tube and return it to ice.

D. Transfer *E. coli* cells to "- plasmid" tube using same steps as those described in C i-iv. ☐ *Loop 2/ Pipette 3*

E. Keep both test tubes on ice for at least 1 minute.

Questions:

1. Why must sterile technique be used even after bacteria are introduced to the tube?

2. What makes the $CaCl_2$ solution quite different from the normal conditions inside the large intestine enjoyed by *E. coli*? Be specific about at least three differences.
   - {
   - {
   - {

3. In what ways could $CaCl_2$ interact with the cell membrane of the cells? Give at least two specific examples of possible interactions.

2. INTRODUCING THE PLASMID CONTAINING AMPICILLIN-RESISTANCE TO THE SUSPENDED BACTERIA

A. Add one loopfull of pGLO Ampicillin Resistance Plasmid solution (0.005 mg/ml) to the "+ plasmid" tube using a sterile inoculating loop. One loopful (when the solution forms a "bubble" across loop opening) is approximately 10 µl of solution. Submerge the loopful of plasmid solution directly into "+ plasmid" cell suspension and twirl and swish loop to mix. Return tube to ice. ☐ *Loop 3*

B. Keep both tubes on ice for 15 minutes. Answer questions, then go to step 3 while you wait.

Questions:

1. What negatively charged functional groups are plentiful on DNA?

2. If Ca^{++} interacts with the negatively charged phosphate groups on DNA, what change(s) might take place in the overall arrangement of the plasmid in the presence of plentiful Ca^{++} ions?

3. GENERAL HOUSEKEEPING: Label the culture plates while you wait:
(Write labels on plate bottom around the perimeter so they do not obscure the center area of plate.)

A. Label "LB" plates with group name and:

i. On one: "+ plasmid cells on Luria broth agar"

ii. On the other: "-plasmid cells on Luria broth agar"

B. Label "LB+Amp+Ara" plates with group name and:

i. On one: "+ plasmid cells on Luria broth agar/ amp"

ii. On the other: "-plasmid cells on Luria broth agar/ amp/ara"

ALSO WHILE YOU WAIT:

a. Two plates will be inoculated with cells that we hope have been transformed with the pGLO-Ampicillin-Resistance Plasmid. Predict colony number by circling:

On Luria broth agar plate:

None Few Many

On Luria broth agar with Ampicillin/Arabinose plate:

None Few Many

b. Two plates will be inoculated with cells that have not been transformed (although they have gone through all the same processes except for the addition of plasmid). Predict colony number:

On Luria broth agar plate:

None Few Many

On Luria broth with Ampicillin/Arabinose plate:

None Few Many

4. EFFECTING THE TRANSFORMATION-- MAKING THE PLASMIDS ENTER THE CELLS

A. Transfer both test tubes <u>suddenly</u> from the ice bath to the 42^{O}C water bath. Leave them at 42^{O}C for 90 seconds.

B. Transfer both test tubes <u>suddenly</u> from the water bath back to the ice. Keep on ice for at least one minute.

5. ALLOWING THE CELLS TO REGAIN THEIR STRENGTH AND START TO MULTIPLY

A. Add 250 µl sterile Luria broth to the "- plasmid" tube using sterile transfer pipette. Shake and tap tube to mix. ☐ *Pipette 4*

B. Add 250 µl sterile Luria broth to the "+ plasmid" tube using sterile transfer pipette. Shake and tap tube to mix. ☐ *Pipette 5*

C. Incubate both tubes in 37^{O}C water bath for 5 minutes.

6. RETURNING RECOVERED <u>CONTROL CELLS</u> TO SOLID MEDIUM WITH AND WITHOUT ADDED ANTIBIOTIC & ARABINOSE

A. Transfer 100 µl of "- plasmid" cell suspension to plate marked "-plasmid cells on Luria broth". Use sterile transfer pipette. *Pipette 6*

B. Transfer 100 µl of "- plasmid" cell suspension to plate marked "-plasmid cells on Luria broth plus Amp/Ara". Use the same sterile transfer pipette. ☐ *Pipette 6 (again)*

C. Spread cells over surface of plates without delay.

i. Lifting the lid of the "- plasmid cells on Luria Broth agar" plate, sweep the drop of cell suspension with a loop to distribute it over the surface of the plate. *Loop 4*

ii. Repeat to spread cells on "- plasmid on Luria broth agar with Ampicillin/Ara" plate. Use the same loop. ☐ *Loop 4 (again)*

7. PLATING RECOVERED <u>TRANSFORMED CELLS</u> ON SOLID MEDIA WITH AND WITHOUT ADDED ANTIBIOTIC & ARABINOSE

A. Transfer 100 µl of "+ plasmid" tube to plate marked "+ plasmid cells on Luria broth agar". Use sterile transfer pipette. *Pipette 7*

B. Transfer 100 µl of "+ plasmid" tube to plate marked "+ plasmid cells on Luria broth agar plus Amp/Ara". Use the same sterile transfer pipette. ☐ *Pipette 7 (again)*

C. Spread cells over surface of plates without delay (same method as in 6 C.) ☐ *Loop 5*

Question:

Why did you make the transfer of control cells in step 6 before doing anything with the transformed cells in step 7?

8. PREPARING TO INCUBATE INOCULATED PLATES

A. Allow liquid on plates to set for 5 minutes before inverting them.

B. Invert plates (so the medium side is up).

C. Stack and tape (or rubber-band) your group's four plates.

D. Incubate plates at 37^{o}C for 24 hours.

CLEAN UP:

- ☐ BE SURE ALL USED LOOPS, PIPETTES, AND TUBES ARE IN THE BIOLOGICAL WASTE BEAKER.
- ☐ PAPER WRAPPINGS NOT CONTAMINATED WITH BACTERIA GO IN TRASH BEAKER.
- ☐ WASH DOWN LAB BENCH. THEN WASH YOUR HANDS THOROUGHLY!!
- ☐ WASH HANDS AGAIN BEFORE EATING!!

OBSERVATIONS

Observe the plates (do not open the lids) and record number of colonies on each. If colonies are extremely plentiful, only count one quarter of the plate and quadruple (x4). If cell growth is too dense to distinguish individual colonies, record "lawn".

	LB only	LB/AMP/ARA
+Plasmid		
-Plasmid		

Explain how your results compared to your predictions in Step 3 for each of the fours plates.

PUT THE PLATES AWAY, THEN WASH THE BENCH, THEN WASH YOUR HANDS!

Question:
Each colony grew from how many cell(s) present at that location on the plate before incubation?

CONCLUSIONS AND CALCULATIONS

I. Compare the number of colonies that grew on each pair of plates listed below.
   a. Are the numbers about the same or quite different for the two plates being compared?
   b. Give an explanation for the similarity or difference in colony number

      1. "- plasmid cells on Luria broth agar" compared to "+ plasmid cells on Luria broth agar"
         a.
         b.

2. "- plasmid cells on Luria broth agar with amp/ara" compared to "- plasmid cells on Luria broth agar"
   a.
   b.

3. "+ plasmid cells on Luria broth agar with amp/ara" compared to "- plasmid cells on Luria broth agar with ampicillin
   a.
   b.

4. "+ plasmid cells on Luria broth agar with amp/ara" compared to "+ plasmid cells on Luria broth agar"
   a.
   b.

CALCULATION OF TRANSFORMATION EFFICIENCY

The transformation efficiency calculates how many cells take in the PGLO plasmid versus how many cells die during the transformation process. Every time a scientist performs an experiment, there is a cost to it. It is $41.5 for 20 micrograms of PGLO (from BioRad), a custom DNA sequence is at least $0.23 per base pair AND $45 at least to place it in 4 micrograms of plasmid (from ThermoScientific). If you invented a drug that was 3,000 bp, 4 micrograms of your plasmid would cost at least $735!

a. What was the total mass of plasmid DNA added to the cell suspension? (See list of materials for concentration of original solution.)

b. What was the volume of the cell suspension at the conclusion of the recovery step (Directions-step 5, page 8)

c. What fraction of the cell suspension final volume (from b) was transferred to each plate for incubation on solid medium?

d. What was the mass of plasmid DNA that was transferred to one plate?

e. Calculate the number of colonies transformed per microgram of plasmid DNA. Use the colony count from your observations and the values computed above to solve this problem. You are calculating the transformation efficiency.

f. If you were using the custom 3,000 bp plasmid (mentioned above) with the same transformation efficiency as your PGLO ($735 per 4 micrograms), how much would it cost to perform one Bacterial Transformation experiment? How much would one experiment cost using PGLO?

Final Discussion: List and discuss the numerous factors that could influence transformation efficiency.