Bacterial Transformation

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Directions for completing this assignment:

Please pay attention to the following when completing this assignment:

- Progress through this lab from top to bottom. Do not skip ahead.
- Read all directions carefully. If you have questions about something you've read, ask your instructor.
- Be sure to answer all the questions and type all of your answers in blue text.
- Read the questions carefully.
- When asked to "follow these tips," please do so.
- Answer only what is being asked.
- If the directions ask for you to start a paragraph with a specific **prompt**, please do so.
- Be precise and concise.
- Answer questions in full sentences and in paragraph form. No bullets please.

Activities in this lab:

- ✔ Make the bacterial strain, DH5α, competent for transformation using the calcium chloride method.
- ✔ Transform the plasmid pBR322 into the competent DH5α cells.
- ✔ Plate the transformations on agar plates containing ampicillin and without ampicillin.
- ✔ Analyze and discuss the results on agar plates after 24+ hours incubation

A. Introduction

DNA (deoxyribonucleic acid) is housed in the nuclei of eukaryotic cells in discrete linear structures called chromosomes. Prokaryotes such as bacteria do not have nuclei. They contain a single circular chromosome that is present in the cytoplasm. Eukaryotic and prokaryotic cells of all types can harbor DNA that is not part of their chromosome. These small, circular, extrachromosomal DNAs are called **plasmids**. Plasmids are very small (many fewer base pairs) than the chromosomes in the cell. Plasmids, like chromosomes, are kept in the nuclei of Eukaryotic cells and in the cytoplasm of

prokaryotic cells. Natural plasmids, like the chromosomes contain an origin of replication and some sequence that confers selective advantage for the cell. In this lab, you will be working with a plasmid that has been constructed by scientists for the purpose of propagating foreign pieces of DNA in the bacteria, E. coli. In order for the E. coli to replicate and maintain this plasmid, the first two DNA sequences are required:

An origin of replication- a plasmid must be able to replicate itself and be distributed to the offspring of the cell. In this experiment, you will be using bacteria, which divide by binary fission. Plasmid replication must occur so that each of the daughter bacteria can potentially receive one or more plasmids.

Selectable marker- Antibiotic resistance genes are cloned into man-made plasmids. The process of transformation results in a small subset of bacteria that take up the plasmid. When the bacteria are grown in media that have an antibiotic, all non-transformed bacteria die. The antibiotic selects for transformed bacteria because only these bacteria can survive on the antibiotic. A common antibiotic resistance gene that is cloned into plasmids is the β-lactamase gene (often referred to as amp^R). Ampicillin is an antibiotic that disrupts bacterial cell wall formation. If bacteria have the amp^R gene, it produces the enzyme β-lactamase. This enzyme hydrolyzes ampicillin making it harmless to the bacteria.

Poly-cloning site: An area of the plasmid that contains many sequences that can be cut by a variety of restriction endonucleases - enzymes that cut DNA in specific places. This is where your gene of interest will be inserted when making a recombinant plasmid.

Plasmids are used to carry cloned genes that researchers want to study. They allow for large scale purification of those genes and their gene products, which is necessary for various experiments researchers will perform on them.

The plasmid you will be using today is called pBR322. It is a commercially available plasmid that scientists use to carry genes of interest in E. coli. The location labeled "pMB1ori" is the DNA sequence that serves as an origin of replication. The location labeled "amp^r" is the DNA sequence corresponding to the β-lactamase gene.

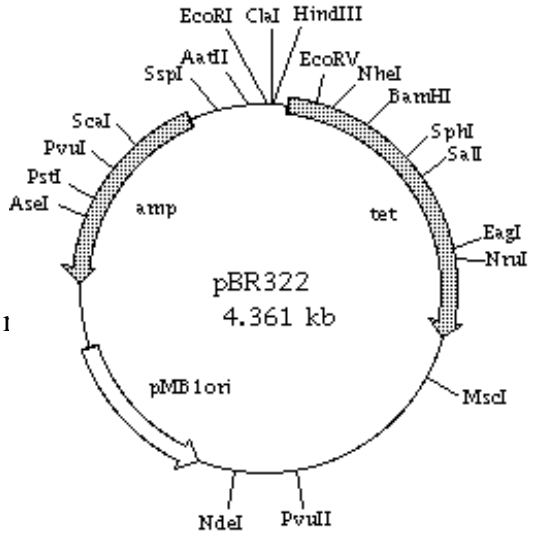


Figure 1. The plasmid pBR322.

In this experiment you will be given pBR322 at a specific concentration that you will serially dilute. You will transform the DNA into *E. coli*. Although Griffith described the process of bacterial "transformation" in 1928 in what is now called *Streptococcus pneumoniae,* the practical application of this concept would not be used for decades. When single-celled organisms, such as bacteria, are transformed with DNA they must at minimum 1) take DNA across a cell wall, 2) take DNA across a cell membrane, 3) faithfully maintain the DNA as part of their genome or as a plasmid, and 4) survive the process. Bacteria become transformed in nature at a very low frequency, too low for any practical application in the laboratory. However, bacteria can be made "competent" for transformation by incubating them with chemicals. There are several popular methods for this, but the most common way is to incubate the bacteria with a solution of calcium chloride. It is thought that the charges on the calcium ion shield the negative charges on the phospholipids in the plasma membrane of the bacteria. This reduces the charge/charge repulsion of the negative phosphate groups on the plasmid DNA. The heat-shock step creates a temperature gradient that "sucks" the plasmid into the bacteria. If the bacteria survive this process, they will be selected for on antibiotic media because the plasmid contains an antibiotic resistance gene. All bacteria that do not take up the plasmid will be killed by the antibiotic.

In today's lab, you will be using a commonly used laboratory strain of *E. coli* called DH5α. You will transform this bacterial strain with the various concentrations of the plasmid pBR322 and select for transformants (cells that have taken up the plasmid) on plates containing the antibiotic, ampicillin.

B. Day 1 Procedure

Treating cells with $CaCl_2$

PLEASE READ: The success of your transformation rests on your ability to keep the cells COLD. Keep cells on ice at all times. DO NOT let them warm up. $CaCl_2$ is poisonous to metabolizing cells. This means that if the cells warm up past 4°C, they begin to die very rapidly. The most common mistake is to hold the tube in your hands, inadvertently warming up the cells, while resuspending the pellet. If you must remove the tube to resuspend, keep returning the cells to the ice bucket intermittently to keep

the cells chilled. If you do not get any transformants, it will be because you let the cells warm up.

1. The instructor will prechill the 4x250 centrifuge rotor with 50 ml adaptors by centrifuging at low speed for 15 minutes at 4°C.
2. Sterile technique must be used throughout this procedure. All tubes and pipettes have been sterilized. Remember, any organism that lands in your preparations may contaminate your bacteria. Tubes should be opened ONLY for the minimum time required to do procedural steps. Do not talk when the tubes are open.
3. Close and label 7 sterile microfuge tubes "1-7" *with a sharpie directly on the top of the tube.* Place tubes on ice.
4. Transfer the culture into the provided sterile 50mL conical tube using as sterile technique as possible up to the 50mL mark on the tube.
5. Pipet 250µl of the bacterial culture into tube 1 and close the tube. The bacteria in this tube will NOT be treated with calcium chloride. It will serve as a control to show the importance of calcium chloride treatment. Store on ice until later in the experiment.
6. Centrifuge the remaining bacteria in the conical centrifuge tube in the prechilled rotor at 4000 RPM at 4°C for 5 minutes.
7. Decant supernatant into the marked disposal flask.
8. Using a prechilled serological pipet (in the refrigerator), add 2.5 ml ice-cold 100 mM $CaCl_2$ to the pellet. Immediately plunge the tube into your ice bucket.
9. Using the same serological pipet, pipet the cells up and down to mix the cells. Close the cap.
10. Let cells incubate on ice for a minimum of 10 minutes. Return the bottle of $CaCl_2$ to the right refrigerator. While you are waiting, make your serial dilutions.
11. Pipet 250 µl of your competent cells into each of the chilled microfuge tubes labeled 2-7. Close tubes and keep on ice.
12. **Stop**. **Notify your instructor** that you have reached this step.

Making serial dilutions of your plasmid:

1. Label a new empty microfuge tube 0.5 ng/µL.

2. Label another new empty microfuge tube 0.05 ng/µL.

3. *Obtain a bottle of dH_20 from the right refrigerator.* Using a p200, add 90 µL of dH_20 to each of the two microfuge tubes.

4. *Obtain from your instructor* a microfuge tube containing **5 ng/µL pBR322** (the plasmid).

5. Using a p10 (white top), pipet 10µL of the **5 ng/µL pBR322** plasmid into the tube labeled 0.5 ng/µL. Flick it a few times with your finger to mix. Do not discard the rest of the **5 ng/µL pBR322.** You'll need it again later.

6. Using a p10 (white top), pipet 10µL of the 0.5 ng/µL tube (the tube you just mixed) into the tube labeled 0.05 ng/µL. Flick it a few times with your finger to mix.
7. You now have 3 tubes, each with different concentrations of plasmid: **5 ng/µL pBR322, 0.5 ng/µL**, and **0.05 ng/µL**.
8. Label a new microfuge tube dIH_2O and transfer 500µl of dIH_2O to this tube to be used for transformations later.
9. **Stop** and wait for your instructor to describe the next step.

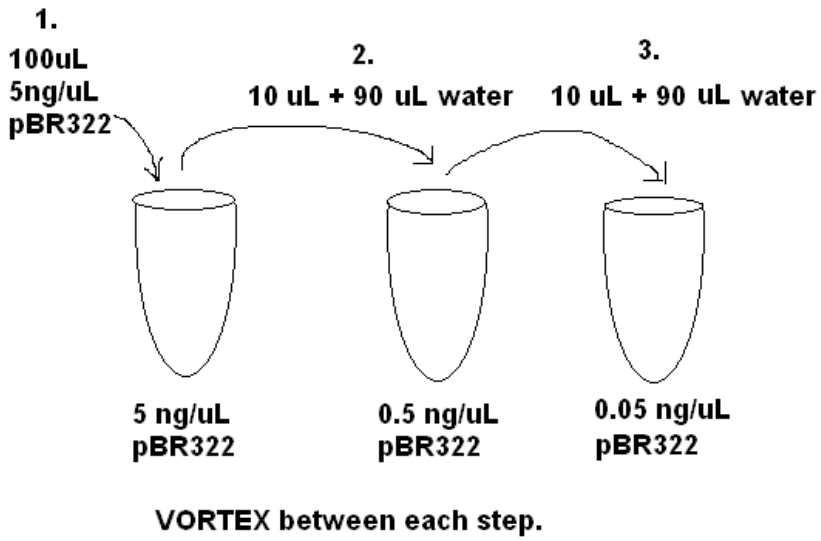
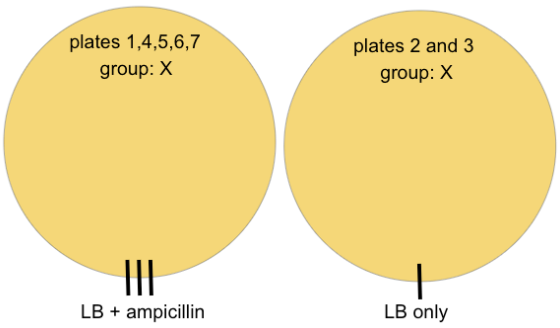


Figure 2. Making serial dilutions of plasmid pBR322.

Labeling Plates:



1. *Ask your instructor to give you **5** LB+Ampicillin plates and **2** LB-only agarose plates*.

2. Note that the three stripes on 5 of the plates mean they have ampicillin, and the single stripe on the other two plates mean they **do not** have ampicillin.
3. Use Sharpie to write **directly** on the bottom of each plate (not the lid). Label each of the plates as shown in the figure above. Write the day and time of lab class on the bottom of the plate. *Be sure you are labeling the correct plate with the correct number.*

Transformation of *E. coli* with the plasmid pBR322:

At this point you have 7 tubes of *E. coli* on ice. **Tube 1** was NOT treated with calcium chloride. **Tubes 2-7** all received Calcium chloride treatment. The next step is to add plasmid DNA to each tube.

10. You are now ready to set up your bacterial transformations. Add **50 µl** of the pBR322 plasmid or dH_20 as indicated in Table 1 below.

Tube #	pBR322 plasmid or dH_20	Plate on plate#...
1	5 ng/µL pBR322	1 LB + ampicillin
2	5 ng/µL pBR322	2 LB only
3	dH_2O	3 LB only
4	dH_2O	4 LB + ampicillin
5	0.05 ng/µL pBR322	5 LB + ampicillin
6	0.5 ng/µL pBR322	6 LB + ampicillin
7	5 ng/µL pBR322	7 LB + ampicillin

Table 1: The component to be added to each transformation and the plates each transformation will be plated on.

11. Heat shock the *E. coli* by incubating the tubes at 42°C for 2 minutes.
12. Immediately add 500 µL, at the front of the room, of LB to each tube.
13. Incubate tubes at 37°C for 5 minutes. While you are waiting, obtain 7 plates (2 LB-only and 5 ampicillin) from your instructor and label them.
14. **Centrifuge** the tubes in your tabletop centrifuge for **5 minutes**. *Be sure to properly balance them with an 8th tube containing the same **volume** of water (800uL) as your other tubes.* Watch this video to learn how to spread cells onto plates while you are waiting.

15. Using a 1mL micropipette set to 800µL, remove the supernatant, being careful not to suck up the bacterial pellets! Discard the supernatant into the waste beaker at your bench. **Save the pellet**. Discard the contents of the beaker into the waste flask in the back of the room when you are done.

16. Add 100µL LB to each pellet and resuspend by pipetting up and down. It is important that all the pellets are resuspended into the LB.

17. Using a p200 micropipetter set to 150µL, plate all of the transformed bacteria from each tube on to the appropriate plate (*example: Tube 1 on to Plate 1, and Tube 2 on Plate 2, etc.*)

18. Bring your plates to your instructor. They will be incubated at 37°C overnight.

19. Throw away all tubes and tips into the red bag in the back of the room.

20. Wipe down your bench with disinfectant (on your bench in a squirt bottle). Ask your instructor if you don't have paper towels at your bench. **Answer questions 1-10 below.**

C. Experimental Predictions:

Complete this section on Day 1 of lab

- Complete this section on Day 1 (the day that you do the transformations).
- Submit the entire assignment to Canvas (Questions 1-10 completed, questions 11-24 blank)

Conditions of transformations and the platings.	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7
Bacteria cells treated with $CaCl_2$?	**No**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**
Is there ampicillin in the plate?	Yes	no	no	yes	yes	yes	yes
Concentration of plasmid DNA used in transformation? (ng/uL)	5.0	5.0	No DNA used.	No DNA used.	0.05	0.5	5.0
Volume of DNA used in transformation (uL)	50	50	No DNA used.	No DNA used.	50	50	50

Table 2: Conditions used for each of the seven transformations.

Is the antibiotic, ampicillin, effective at killing <u>non-transformed</u> bacteria?

1. Which two plates should you compare to answer the question? (*Hint: They should be identical to one another except for the variable being tested.*)
   We should be comparing plates 3 and 4, because they both are non-transformed by a plasmid, but differ in the presence of ampicillin.

2. Based on what you have learned about ampicillin, how do you predict these two plates will compare to one another? Why do you predict this?
   Plate 3 will have a normal amount of colony growth, while plate 4 will have little to no colonies. This is because plate 4 has ampicillin treatment, disrupting cell wall formation and thus killing them, while plate 3 does not.

In the process of transformation, does every $CaCl_2$ treated bacterial cell become transformed?

3. Which two plates should you compare to answer the question? (*Hint: One plate will tell you how many bacteria cells there are, the other will tell you how many have been successfully transformed.*)
   We should compare plates 2 and 7, because they are both treated with $CaCl_2$ and transformation, but plate 2 does not have ampicillin, and thus does not select for the presence of B-lactamase that comes with transformation.

4. Hypothetically, assume every single bacterial $CaCl_2$ treated cell DOES get transformed. How do you predict these two plates will compare to one another? Why do you predict this?
   Assuming every single bacterial $CaCl_2$ treated cell does get transformed, the two plates should be identical because plate 2 has no ampicillin selection, and plate 7 has selection, but if every bacteria is transformed, then they should be unaffected.

Does the treatment of bacteria with $CaCl_2$ increase their competence?

5. What does the term **competent** mean in regards to bacteria that will eventually be transformed with plasmid DNA? What does it mean if bacteria are made more competent? What process was used to make bacteria more competent in your experiment?
   The term competent with regards to bacteria refers to a bacterium's ability to transform, or take in foreign DNA. If bacteria are made more competent, then they are more able to take up foreign DNA. In the experiment, $CaCl_2$ treatment was used to make the bacteria more competent.

6. Which two plates should you compare to answer the question? (*Hint: They should be identical to one another except for the variable being tested.*)
   Plates 1 and 7 should be compared, because they are both transformed, but only plate 7 is treated with $CaCl_2$.

7. Assume $CaCl_2$ does increase the competence of bacteria. How do you predict these two plates will compare to one another? Why do you predict this?
   Plate 7 will have more colony growth than plate 1, because $CaCl_2$ decreases electrostatic hindrance between the cell membrane and DNA, thus allowing for bacteria a higher affinity for extracellular DNA.

What effect does plasmid DNA concentration have on bacterial transformation efficiency?

8. Which plates (3 of them) represent the experimentals for this question?
   - Plates 5, 6, and 7 represent the experimentals.
9. What do you predict the relationship to be between the number of colonies seen on a plate (***transformants***) and the amount of DNA added to the transformation?
   - We predict that the plate with the highest concentration of plasmid DNA will produce the most colonies, whereas the plate with a low concentration of plasmid DNA will produce the least colonies.
10. What do you predict the relationship to be between the transformation efficiency (***transformants/microgram of DNA added***) and the amount of DNA added to the transformation?
    - We predict that the higher the concentration of DNA added to the transformation, the greater the transformation efficiency.

D. Results:

Complete this section on Day 2 of lab

- To be completed on the day you count colonies.
- **Read the comments on the graded lab from Day 1 and use those corrections to answer this week's questions.**
- Answer Results questions 1-5.
- Be sure you complete the assignment, so that your answers to 1-10 from last week are present.
- Submit the entire assignment to Canvas.

Conditions of transformations and the platings.	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7
Bacteria cells treated with $CaCl_2$?	**No**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**
Is there ampicillin in the plate?	Yes	no	no	yes	yes	yes	yes

Concentration of plasmid DNA used in transformation? (ng/uL)	5.0	5.0	No DNA used.	No DNA used.	0.05	0.5	5.0
Volume of DNA used in transformation (uL)	50	50	No DNA used.	No DNA used.	50	50	50
Total mass of DNA used in transformation (ng)							
Total number of transformants		lawn	lawn				
Transformation efficiency (transformants/ug of DNA)							

Table 3. Conditions used for each of the 7 transformations, and the resulting number of colonies and transformation efficiency of each plate.

1. **Insert the labeled picture of the 7 bacterial plates below.**
   If you need a review of how to do this, go to in this file

2. Look carefully at plates 2 and 3. They look very different from the rest. Describe what you see and why they are different.

3. Count every colony (transformants) on plates 1, 4, 5, 6, and 7. Enter this information into the blue boxes in TABLE 3.

4. Calculate the total mass of DNA used in each transformation and fill in the red boxes of TABLE 3. Please show your work. ***Hint:*** *remember the relationship between volume, mass, and concentration.*

5. Use the equation below to determine the transformation efficiency of plates 5-7 and enter that information in the green boxes of TABLE 3. Please show your work. *Don't forget to first convert the total mass of DNA from ng to ug (there are 1000 ng to 1 ug).*

$$\text{Transformation efficiency} = \frac{\text{Number of colonies counted}}{\text{Total µg of plasmid DNA added}}$$

E. Discussion and Conclusions

Complete this section on Day 2 of lab

- To be completed on the day you count colonies.
- Answer Discussion/Conclusion questions 1-9.
- Be sure you complete the assignment, so that your answers to 1-10 from last week are present.
- Submit the entire assignment to Canvas.

Is the antibiotic, ampicillin, effective at killing non-transformed bacteria?

1. Describe the results. What did you predict would happen last week (refer to question C2)? Do your experimental results match what you predicted?

2. Based on the results, is ampicillin effective at killing non-transformed bacteria?

In the process of transformation, does every single $CaCl_2$ treated bacterial cell become transformed?

3. Describe the results. What did you predict would happen last week (refer to question C4)? Do your experimental results match what you predicted?

4. Based on the results, does every $CaCl_2$ treated bacterial cell of a transformation take up the plasmid? Explain your answer.

Does the treatment of bacteria with $CaCl_2$ increase their competence?

5. Describe the results. What did you predict would happen last week (refer to question C7)? Do your experimental results match what you predicted?

6. Based on the results, does $CaCl_2$ increase the competence of bacterial cells?

What effect does plasmid DNA concentration have on bacterial transformation efficiency?

7. What happened to the number of transformants (colonies) as the concentration of plasmid DNA increased? Be sure to support your answer with data. What did you predict would happen last week (refer to question C9)? Do your experimental results match what you predicted?

8. What happened to the transformation efficiency as the concentration of plasmid DNA increased? Support with data. What did you predict would happen last week (refer to question C10)? Do your experimental results match what you predicted?

9. Based on these results, can you conclude that increases in DNA concentration will always lead to increases in transformation efficiency? Explain

Checkout list

Disposal of lab materials:

1. Empty your pipet tips into the red bag (autoclave coffin).
2. Throw away your microcentrifuge tubes into the autoclave coffin.
3. Put your blue capped 50 mL centrifuge tube into the autoclave coffin.
4. Discard your ice in sink

Return material to the refrigerator:

1. Return the following to the rack in the refrigerator:
    a. Blue tube with sterile water
    b. Orange tube with plasmid (pBR322)
2. Return to the refrigerator shelf
    a. Bottle of 100mM $CaCl_2$
    b. Bottle of $CaCl_2$/glycerol

The following equipment should be on your side of the bench:

1. Beaker with ethanol and glass spreader
2. A new blue cap centrifuge tube (get from center table) in a rack
3. Yellow tips- CLOSED
4. Blue tips- CLOSED
5. Bunsen Burner
6. Microfuge centrifuge tubes in a CLOSED container

Wipe down bench and wash your hands:

1. Wipe down your bench top with disinfectant
2. Wash your hands for at least 30 seconds with soap.