

Appendix A: Protocols for Student Experiments

*From "Using Fluorescence in Biotechnology Instruction to Visualize Antibiotic Resistance & DNA", American Biology Teacher, Aug. 2021
Christopher W Hamm, Sarah F Winburn, and Matthew T Cabeen*

Experiment 1: Transformation of *Bacillus subtilis* PY79 with Green Fluorescent Protein (GFP)

Theory

This transformation protocol uses MC medium to promote *Bacillus subtilis*' natural competence (ability to uptake DNA from the environment and then incorporate it into its own DNA) to add a segment of DNA encoding GFP and an antibiotic resistance marker (chloramphenicol at 5 µg/mL concentration) for selection. After transformation of *B. subtilis*, only cells that incorporated the added DNA will gain new traits: they will fluoresce green under blue light and will grow in the presence of the antibiotic (chloramphenicol). Hence, this demonstration illustrates the principle of how DNA encodes characteristic traits, in accord with NGSS PE HS-LS3-1.

Day 1:

1. Make 20 mL 10X MC Medium (See 10X Medium Recipe).
2. Streak out *B. subtilis* strain PY79 from a freezer stock with sterile loop on a LB Lennox agar plate and incubate at 37°C overnight (o/n) for isolates.

*Note: It may be possible to obtain both MC medium and a plate of *B. subtilis* PY79 from a local university laboratory. If growing *B. subtilis* yourself, be sure to use LB Lennox, not LB Miller, medium. LB Lennox has less NaCl, and in our experience *B. subtilis* often does not grow well on LB Miller.*

Day 2:

1. Make 1X MC Media (See 1X MC Medium Recipe) from the 10X stock just prior to using it.

1X MC Medium: (for 10 mL, can be scaled down or up as needed)

- 1 mL 10X MC medium
- 9 mL sterile H₂O
- 100 µL sterile 1M MgSO₄

Mix everything together under sterile conditions (on a bench top with a flame nearby or in a biological safety cabinet).

2. Inoculate one colony (take the entire colony with a sterile inoculation loop) into 3 mL of sterile MC medium and grow at 37°C for 4.5 hours in a sterile glass or plastic test tube, shaking at a moderate speed (150-200 RPM).

3. Add 1 μ L of GFP/chlor chromosomal DNA (at 100 ng/ μ L) to 500 μ L of the cells from step 2 in a separate sterile test tube. The culture in the original test tube from step 2 should be cloudy when taking the 500 μ L.

**Modification: This step can also be done with transfer pipettes if micropipettes are unavailable. Prepare a 4 ng/mL stock of chromosomal DNA and add 2 drops with a transfer pipette to 500 μ L. In our tests, the transfer pipette droplet size is approximately 17.5 μ L in size. Volumes may be adjusted, but the total amount of chromosomal DNA added should be around 100 ng.*

***The chromosomal DNA can contain GFP with chloramphenicol resistance as a selective marker or RFP with chloramphenicol resistance as a selective marker, or with other proteins and antibiotic resistance markers as available. Chlor-marked GFP and RFP are available from the Cabeen lab. Add the appropriate DNA for desired fluorescence isolated using a genomic extraction protocol or kit for gram positive genome extraction. For genomic extraction, we typically use the Wizard® Genome DNA Purification Kit from Promega, following the protocol for gram-positive bacteria.*

4. Incubate for 2 hours at 37° C with shaking.
5. Plate 200 μ L of cells with a sterile glass spreader or sterile glass beads on LB-Lennox agar plate with antibiotic selection (chloramphenicol at 5 μ g/mL concentration).

-If micropipettes are unavailable, plate 12 drops with a transfer pipette and spread with sterile glass beads or sterile glass spreading rod.

6. Incubate at 37°C overnight or at room temperature for 1-2 days, until visible colonies arise.

Alternative if no 37°C incubator is available: incubate plates at room temperature for 1-2 days or until visible colonies arise.

Day 3

1. The next morning, check for transformants by checking to see that colonies are now green fluorescent using the GFP light and filter combination. Only colonies that were successfully transformed should grow on the selective plate (antibiotic plate), and nearly all should be green fluorescent.

-This is an extra step to check for contaminants and verify results

-The GFP-bearing DNA also carries an adjacent chloramphenicol resistance marker on it, allowing for selection of GFP colonies with antibiotics (chloramphenicol at 5 μ g/mL concentration)

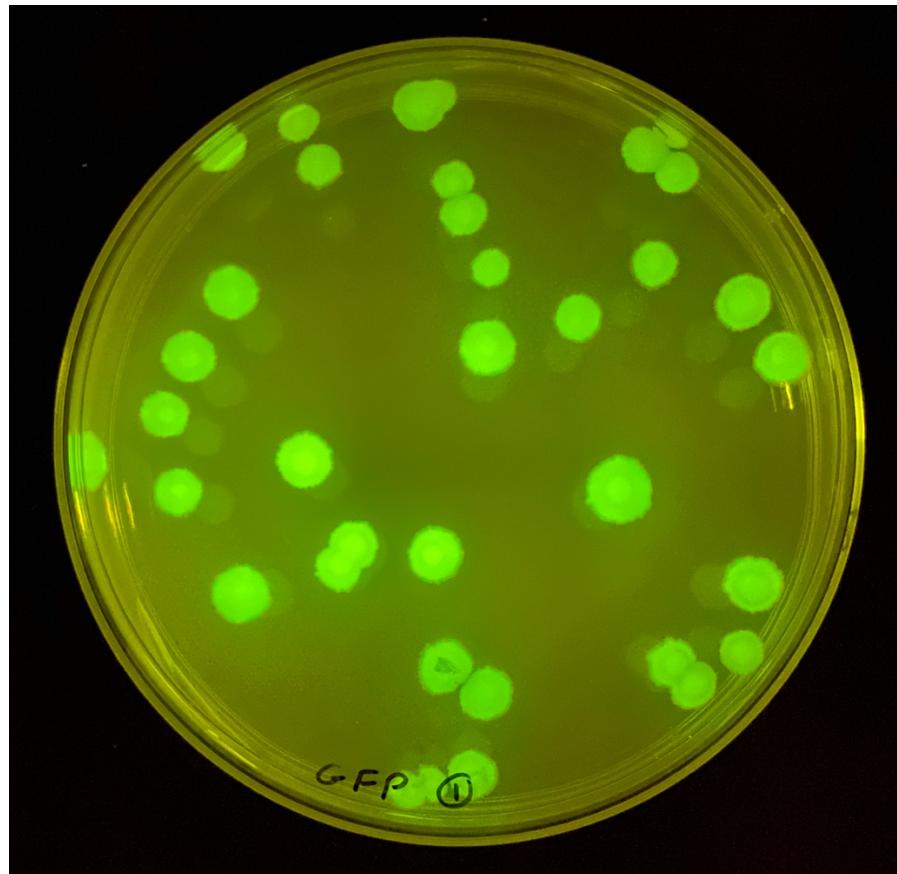
**This step can be performed later (up to several days later), but plates should be removed from the incubator after 24 hrs and kept in a cool room or in a refrigerator until they can be checked for GFP fluorescence*

Expected Results

Expected results from transformation are below. The image, taken with GFP illumination, shows plated cells on a LB 1.5% agar plate with 5 $\mu\text{g}/\text{mL}$ of chloramphenicol. We typically obtain approximately 10-50 colonies on the plate, with all or nearly all displaying green fluorescence, as shown below. The plate shown in the image was incubated for 2-days in a 37°C incubator; if taken out after 1 day, colonies will appear smaller.

This plate contains approximately 35 bacterial colonies. But we placed many thousands or millions of bacterial cells on the plate. Why so few, and why did these cells grow into colonies? When we spread the cells on the plate, we spatially separated them across the surface of the agar. Most (more than 99.99%) of the cells in the test tube died when we put them on the agar plate because they were NOT able to grow in the presence of 5 $\mu\text{g}/\text{ml}$ of the antibiotic chloramphenicol. These cells either did not take up DNA (only some of the cells in the culture even take up the DNA we added) or did take up DNA but did not incorporate into their chromosome the part of the DNA encoding GFP and the chloramphenicol-resistance gene. Only a tiny, lucky fraction of the cells took up DNA and incorporated the GFP and chloramphenicol-resistance genes into their chromosome. These are the cells that gave rise to the colonies.

These colonies have new properties compared to their parental cells, thanks to the new DNA that they have. The new DNA has 1) made them glow green under blue light and 2) has given them the ability to grow in the presence of chloramphenicol, which would normally make them die. This is a simple example of a related (but more complex) situation in humans, where differences in our DNA sequences (i.e., genotypes) give rise to new properties (i.e., phenotypes). The way that our maternal and paternal DNA sequences come together in different combinations can give rise to new traits, which is why we might have a different hair color, skin tone, or height than our parents or our siblings.



Experiment 2: Competition Experiment

Theory

This experiment illustrates how two organisms with traits that confer different fitness advantages in a particular environment will change in their relative populations, with the fitter strain expanding in population size and the less-fit strain contracting in its population size. The experiment takes advantage of the dissimilar fitness of 1) a wild-type PY79 strain of *B. subtilis*, which is sensitive to the antibiotic chloramphenicol, and 2) a PY79 derivative containing genes for chloramphenicol resistance and for GFP. By virtue of its chloramphenicol resistance gene, strain 2 is fitter in the presence of low concentrations of chloramphenicol that slow the growth of strain 1. When these strains are grown together in the same culture vessel in the presence of a low, sub-lethal concentration of chloramphenicol, both strains continue to grow, but strain 2 grows much faster, so that its relative population expands. As the strains grow in competition, samples of the culture are taken, diluted, and grown on agar plates. The two populations can be distinguished by the GFP fluorescence of the chloramphenicol-resistant strain. The expectation (and students can come up with their own hypotheses) is that the relative population of the green-fluorescent strain will expand, while the population of the non-fluorescent strain will contract.

This experiment is a clear, microbiology-based demonstration in support of NGSS PE HS-LS4-3, helping to explain how organisms with an advantageous heritable trait tend to increase in proportion. The experiment also supports AP Biology topics 7.2-3 (Natural and Artificial Selection), as we are selecting (artificially in this case) for antibiotic-resistant bacteria. The setup of this particular competition experiment also illustrates principles relating to antibiotic use. The presence of low concentrations of antibiotics, either if a patient does not correctly take his medication or if antibiotic residues are present in the environment, selects for antibiotic-resistant bacteria by slowing the growth of antibiotic-sensitive bacteria, just as in the experiment. Hence, antibiotic-resistant bacteria can outcompete antibiotic-sensitive bacteria and become more abundant. This is one reason why the United States requires that antibiotics be taken under the care of a physician (they are prescription drugs)—to help ensure that they are used appropriately.

Day 1 (Day 3 if performed together with experiment 1)

1. Inoculate one transformed colony (positive for GFP fluorescence) into a sterile test tube containing 3 mL of LB Lennox using a sterile wooden applicator or sterile inoculation loop. In a separate test tube containing 3 mL of LB, inoculate one colony of the wild-type PY79 (non-fluorescent donor colony from Day 1 Step 1). Incubate at 30°C o/n in a shaking incubator. (The cultures can also be inoculated in the morning and grown at 37°C in a shaking incubator for approximately 6 hours until the cultures appear cloudy).

Day 2 (Day 4 if performed together with Experiment 1)

1. Make or obtain LB-Lennox agar plates (may be obtained from a local university ahead of time or prepped by the instructor in advance. These should be kept in the fridge until 1-3 hours, when they should be removed to allow time for the agar surface to dry).

If this experiment is being performed on its own and not as a follow-up to Experiment 1, the GFP-producing and wild-type strains will need to be obtained before starting. See supply list.

2. Inoculate 3 μ L of o/n-grown culture (GFP and non-GFP strain, from Step C2 above) into separate test tubes, each containing 3 mL sterile LB. Incubate at 37°C in a shaking incubator for approximately 3 hours until slightly cloudy.
3. While cultures are growing prepare 10 sterile microcentrifuge tubes or sterile test tubes by adding 900 μ L sterile LB to each.
4. Prepare one sterile test tube containing 5 mL of LB.
5. Inoculate 3 μ L of each culture (GFP and non-GFP strain from step 2, after growing for 3 h) into the test tube containing 5 mL of LB.
6. Shake in an incubator or by hand for one minute. (Optional: record the optical density at 600 nm if a spectrophotometer is available.)

The goal here is to make an approximately 1:1 ratio of GFP and non-GFP strains in the liquid culture, so that after plating there will be roughly an equal number of fluorescent and non-fluorescent colonies at the beginning of the experiment (time 0, $t=0$) (see 1.0 μ g/mL chlor experiment below for expected results). If a perfect 1:1 ratio is not achieved, it is okay, as the point here is to see that the GFP strain has a higher level of fitness than the non-GFP strain. Therefore, even if the starting ratio is off (2:3 for example), we should still see a trend that changes over the course of a few hours as GFP has a higher fitness level (see 0.5 μ g/mL experiment results for an example of an imperfect starting ratio).

7. Make dilutions for plating by adding 100 μ L of the culture to 900 μ L of sterile LB in a sterile microcentrifuge tube (prepared in step 3). Mix thoroughly by shaking or vortexing. (This is a 10^{-1} dilution.)
8. To make further serial dilutions, add 100 μ L of the 10^{-1} culture to 900 μ L of sterile LB in another microcentrifuge or test tube, thus making a 10^{-2} dilution. Make sure to label microcentrifuge tubes for dilutions. Continue this process until as many dilutions as desired are made.
9. We recommend plating 10^{-1} and 10^{-2} dilutions at time 0 by spreading 100 μ L of each dilution (*This can also be done by spreading 6 drops from a transfer pipette of each dilution*). This may have to be adjusted for the density of culture for a countable number of colonies. To plate the dilutions, spread the culture using sterile glass beads or sterile plate spreader ("hockey stick") until the agar surface is dry.

-Make sure to label plates for time sample was taken from experiment start, and dilution factor. See below for example of plates.

10. Add chloramphenicol to the test tube to start competition experiment.

-We recommend running the selective competition experiment at a concentration of chloramphenicol at 1.0 μ g/mL, but it can also be run at a concentration of 0.5 μ g/mL. To easily add chloramphenicol to test tube, you can prepare a dilute stock of chloramphenicol at 0.2 mg/mL, and then when adding the antibiotic for

competition to the test tube add 25 μ L of 0.2 mg/ml chloramphenicol stock to 5 mL of LB for a 1.0 μ g/mL concentration.

**1.0 μ g/mL is a sublethal concentration of chloramphenicol, slightly inhibiting growth, but not killing B. subtilis cells.*

11. Return test tube to 37°C incubator with shaking for 1 hour.
12. At 1 hour repeat Steps 7-8 above, plating one further dilution that at time point 0. (For example, if you plated 10^{-1} and 10^{-2} dilutions at $t=0$, now plate 10^{-2} and 10^{-3} dilutions at $t=1$ h.)
13. Return test tube to 37°C incubator with shaking for 1 hour.
14. At 2 hours repeat Steps 7-8 above, plating one further dilution that at $t=1$ h. (For example, if you plated 10^{-2} and 10^{-3} dilutions at $t=1$ h, now plate 10^{-3} and 10^{-4} dilutions at $t=2$ h.)
15. Incubate all inoculated plates overnight in 37°C incubator.
16. Image all plates with GFP illumination and filter and under white light. Count colonies comparing # of GFP and non-GFP colonies (example below of plates with counts).

Expected Results

Experiment 2 is designed to demonstrate competition between two different strains of bacteria as occurs in nature every day. Bacteria live in communities in soil, water, and within our own bodies competing for resources and survival of their progeny. In nature, the dominant strain in a community often has an advantage (antibiotic resistance, ability to utilize a specific nutrient, or some ability to survive an environmental stressor) over other strains of bacteria within the community. The strain that survives or grows better has a higher ‘fitness’ than the other strains. Fitness is a key concept in evolution, as these cells evolve over time to become better and adapt to survive.

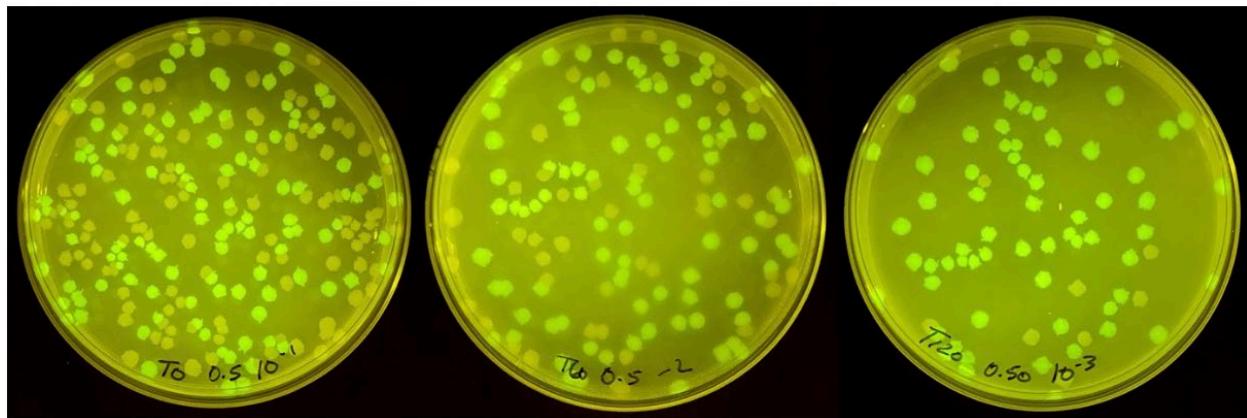
In this case, we show how adding a gene (GFP) conveys a higher fitness to the strain by providing the cells with the ability to survive in the presence of chloramphenicol (an antibiotic). This not only allows the fluorescent cells to survive, but also the ability to grow faster compared to cells without this resistance (the non-GFP strain). The only difference between these two strains is the genetic info the GFP strain took and incorporated into its own genome using its natural competence. Competence is a key factor allowing bacterial cells to uptake DNA in the environment in hopes of finding advantageous DNA allowing them to obtain a higher level of fitness to survive.

Below are two different experiments, one with 0.5 μ g/mL chloramphenicol and one with 1.0 μ g/mL chloramphenicol. The 0.5 μ g/mL chloramphenicol is an example of an ideal starting point with a 1:1 starting ratio (ratio obtained by counting number of GFP and non-GFP cells on plate to compare) prior to adding any antibiotics. If no antibiotics had been added, we would expect their growth rates to remain relatively the same with some minor fluctuations. The 0.5 μ g/mL chloramphenicol concentration is ideal as it shows the fitness advantage of the GFP/chloramphenicol resistance DNA and is very forgiving. At 1.0 μ g/mL chloramphenicol, this

protocol is even more forgiving. In the 1.0 $\mu\text{g/mL}$ chloramphenicol experiment below, the initial ratio of populations was not even close to 1:1 but heavily favored the non-fluorescent strain at a 3:7 ratio. However, once antibiotics were added, the greater fitness of the GFP-labeled strain became evident, as we obtained a near 1:1 ratio after 1 hour, and ended with a 2:1 ratio in favor of the GFP-labeled strain after 2 hours. This shows the clear trend and greater fitness of the strain with GFP, even though there was not a perfect 1:1 starting ratio.

0.5 $\mu\text{g/mL}$ chloramphenicol

Time point (dilution for plating)	Non-GFP colonies	GFP colonies	Population proportions (non-GFP:GFP)
t=0 (10^{-1})	118	116	50% - 50% (1:1)
t=1 (10^{-2})	46	79	36.8% - 63.2% (1:1.7)
t=2 (10^{-3})	10	70	12.5% - 87.5% (1:7)



1.0 $\mu\text{g/mL}$ chloramphenicol

Time point (dilution for plating)	Non-GFP colonies	GFP colonies	Population proportions (non-GFP:GFP)
t=0 (10^{-1})	250	105	70.4% - 29.6% (7:3)
t=1 (10^{-2})	76	60	55.9% - 44.1% (1.3:1)

$t=2 (10^{-3})$	16	30	34.8% - 65.2% (1:2)
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Equipment and Supply Lists

Supplies and equipment needed for Experiment 1:

- 1 mL 10X MC medium (constituent ingredients listed below; recipe follows supply lists)
 - 14.036 g $K_2HPO_4 \cdot 3 H_2O$ (or 10.71 g of anhydrous K_2HPO_4)
 - 5.239 g KH_2PO_4
 - 20 g D-glucose
 - 10 ml of 300 mM trisodium citrate dihydrate (0.88 g in 10 ml H_2O , store in dark at -20°C)
 - 1 ml 22 mg/ml ferric ammonium citrate (0.22 g in 10 ml H_2O , store in dark at -20°C)
 - 1 g casein digest (Difco)
 - 2 g L-glutamic acid, potassium salt
- 9 mL sterile H_2O
- Sterile 1 mL 10X MC medium
- 9 mL sterile H_2O
- 100 μL sterile 1M $MgSO_4$
- LB (Lennox broth, not Miller)-1.5% agar plates (for streaking of original donor colony *B. subtilis* PY79)
- Chloramphenicol stock
- LB (Lennox) 1.5% agar plates with chloramphenicol at 5 $\mu g/mL$ concentration
- 0.2 μm sterile syringe filter
- 15 mL syringes
- Sterile glass beads or cell spreaders
- 15-ml sterile polypropylene conical tubes
- Sterile glass or plastic culture tubes
- Sterile metal (flame-sterilized) or sterile disposable plastic inoculating loop
- Shaking and static incubators
- gDNA GFP or RFP for transformation (obtainable from [Cabeen Lab](#))
- *Bacillus subtilis* strain PY79 (obtainable from [Cabeen Lab](#) or a nearby university)
- Fluorescence illuminator and filter set, plus smartphone for imaging

Supplies for Experiment 2:

- LB (Lennox) sterile liquid medium (100 ml)
- Sterile culture tubes (glass or disposable plastic)
- Micropipettes (preferred) or sterile transfer pipettes
- Sterile micropipette tips
- Sterile cell spreader (glass hockey stick) along with ethanol to flame and sterilize (disposable sterile plastic versions will also work)
- Sterile polypropylene microcentrifuge tubes (can substitute larger sterile plastic test tubes)
- Sterile glass or plastic culture tubes
- *Bacillus subtilis* strain PY79 (obtainable from [Cabeen Lab](#) or a nearby university)
- GFP- or RFP-labeled, chloramphenicol-resistant *B. subtilis* PY79 strain (obtainable from [Cabeen Lab](#))
- Chloramphenicol stock
- Fluorescence illuminator and filter set, plus smartphone for imaging

MC Medium Recipe

10X MC medium: (for *B. subtilis* transformation by competence)

The following recipe is to make 100 ml:

- 14.036 g $K_2HPO_4 \cdot 3 H_2O$ (or 10.71 g of anhydrous K_2HPO_4)
- 5.239 g KH_2PO_4
- 20 g D-glucose
- 10 ml of 300 mM trisodium citrate dihydrate (0.88 g in 10 ml H_2O , store in dark at -20°C)
- 1 ml 22 mg/ml ferric ammonium citrate (0.22 g in 10 ml H_2O , store in dark at -20°C)
- 1 g casein digest (Difco)
- 2 g L-glutamic acid, potassium salt

Mix everything in 40-50 ml H_2O , then adjust with H_2O to a final volume of 100 ml.

Filter sterilize (with a 0.2- μ m syringe filter) into 10-ml aliquots (in sterile 15-ml conical tubes) and freeze at -20°C.

1X MC Medium: (for 10 mL, can be scaled down or up as needed)

- 1 mL 10X MC medium
- 9 mL sterile H_2O
- 100 μ L 1M $MgSO_4$

Mix ingredients together in a 15-ml sterile conical tube under sterile conditions (under flame).