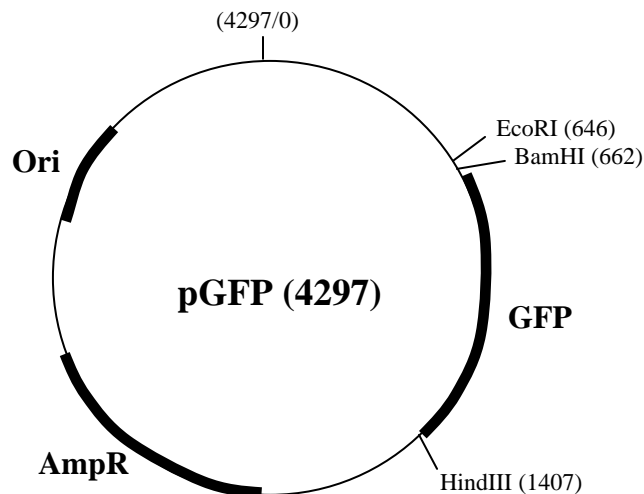


GFP LAB

INTRODUCTION

This lab will take you through the process of introducing a plasmid into bacteria, isolating the plasmid after multiplying it, and restriction analysis. These are fundamental steps in recombinant DNA technology that you will be performing over the next three lab periods.

The plasmid we are using for transformation is called pGFP. A map of the plasmid is shown below. GFP stands for “green fluorescent protein” and has been isolated from jellyfish. It glows bright green under most light conditions, and especially under UV light. GFP is one of the compounds that give many marine organisms their fluorescence. The GFP plasmid is routinely used in research. A gene of interest may be cloned in front of the GFP gene. Once your gene of interest is expressed it will have GFP linked to it. This will then make your protein of interest glow green and can be used to track its location in a cell/organism. pGFP also contains the ampicillin resistance gene, AmpR, for selection. Because GFP is always expressed from this plasmid, it will serve to confirm that cells have been properly transformed. Transformed cells will glow green!



First Step: Transformation

In nature, plasmids are usually transferred from one cell to another by a process called conjugation. Conjugation involves the transfer of DNA through cell to cell contact and requires a specific set of genes, usually carried on the plasmid itself. Molecular biologists have discovered other means of getting cells to take up plasmid DNA from their surroundings. The uptake and expression of foreign DNA is called transformation.

It was discovered in 1970, that *E. coli* cells become competent to take up foreign DNA if they are suspended in cold calcium chloride solution and subjected to a brief heat shock at 42°C. It was also found that cells in early to mid-log growth can be rendered more competent than can cells in the stationary growth phase. Subsequent work has shown that treatment with other divalent cations such as magnesium (Mg^{++}) produce comparable results.

Although the exact mechanism of plasmid DNA uptake by competent *E. coli* cells is unknown, a favored hypothesis is that the high concentrations of divalent cations and low temperature cause channels, called adhesion zones, to form between the inner and outer membranes. The plasmids can then diffuse through these channels before they disappear as the cells are warmed up.

Second Step: Miniprep

The miniprep is a simple and efficient procedure for isolating plasmid DNA from living bacterial cells. After transformation, your bacteria will be given 24 h to grow on plates, after which you will pick green colonies to grow overnight liquid cultures. You will use these overnight cultures to isolate pGFP. The reagents you will use in the miniprep protocol are described below.

Glucose-Tris-EDTA: The Tris buffers the cells at pH 7.9. EDTA binds divalent cations in the lipid bilayer and weakens the cell envelope.

SDS-sodium hydroxide: This alkaline mixture lyses the cells. SDS is a detergent and dissolves the lipid components of the cell envelope as well as cellular proteins. The sodium hydroxide denatures the chromosomal and plasmid DNA into single strands. The intact circles of plasmid DNA remain intertwined.

Potassium acetate-acetic acid: The acetic acid returns the pH to neutral, allowing DNA strands to renature. The large, disrupted chromosomal strands cannot rehybridize perfectly, but instead collapse into a partially hybridized tangle. At the same time, the potassium acetate precipitates the SDS from the cell suspension, along with proteins and lipids with which it is associated. The renaturing chromosomal DNA is trapped in the SDS/lipid/protein precipitate. Only smaller plasmid DNA and RNA molecules escape the precipitate and remain in solution.

Isopropanol: The alcohol rapidly precipitates nucleic acids, but only slowly precipitates proteins. Thus, a quick precipitation preferentially brings down nucleic acids.

Ethanol: A wash with ethanol removes some remaining salts and SDS from the preparation.

Tris-EDTA (TE): Tris buffers the DNA solution. EDTA protects the DNA from degradation by DNAses by binding divalent cations that are necessary cofactors for DNAase activity.

Third Step: Restriction Analysis

The DNA isolated from your miniprep should contain pure pGFP. However, you will need to confirm this since much can go wrong in these procedures! Contaminants and mutations could give you incorrect results. You will perform restriction digests on your miniprep DNA and compare them to control pGFP to make sure you have the correct plasmid isolated.

The procedures and timeline are shown in the grid below. Note that you will be required to come in for a brief time on the day following the first lab period.

1 st Lab Period	Transformation and plating
Next Day	Pick transformants and set up overnight cultures
2 nd Lab Period	Miniprep
3 rd Lab Period	Restriction digest of miniprep and gel electrophoresis

TRANSFORMATION

MATERIALS

2 microfuge tubes containing mid log culture of JM109 cells
 CaCl₂ solution
 0.075 µg/µl of pGFP plasmid
 LB broth
 2 LB agar plates
 2 LB/amp agar plates
 micropipets and micropipet tips
 spreader in beaker of ethyl alcohol
 Bunsen burner
 beaker filled with crushed ice

PROCEDURE

- All procedures must be carried out sterilely. Contamination will ruin the experiment.
- Obtain two culture tubes of JM109 cells. Label one "pGFP" and the other "control," and with your initials so you can identify them.
- Place culture tubes in microfuge (be sure it is balanced!) and spin at high speed for 1 minute.
- Pour off supernatant into **biohazard waste**. Make sure the pellet remains in the tube.
- Thoroughly drain the tubes by gently tapping upside-down on a small piece of clean paper towel (be sure to discard towel in biohazard container and disinfect bench area).
- Add 250 µl of cold CaCl₂ to each tube. Immediately resuspend each cell pellet by repeatedly pipetting in and out. Be careful not to create bubbles or splash the solution up the sides of the tube. Check to be sure there are no visible clumps of cells remaining.
- Return tubes to ice bath when cells are resuspended.
- Add 10 µl of the 0.075 µg/µl of pGFP plasmid directly to the "pGFP" tube, and suction in and out to mix. Return tube to ice bath.
- Incubate both tubes in the ice bath for an additional 15 minutes.
- Arrange the 2 LB and 2 LB/amp agar plates as shown in the matrix below and label them as indicated in Table 1.

LB	pGFP	Control
LB/amp	pGFP	Control
- When the 15 minute ice bath incubation is complete, carry the ice bath beaker with the tubes still in it to the 42°C water bath.
- Heat shock the cells by transferring the tubes from the ice water immediately into the 42°C water. Hold the tubes at 42°C for 90 seconds.
- Immediately return the tubes to the ice bath for at least one minute.
- Add 250 µl of LB broth to each tube. Gently tap tubes to mix.
- Place in 37°C water bath for 15 minutes.
- Use the matrix in Table 2 as a guide for inoculating the spread plates

	Transformed cells (+pGFP)	Control cells
LB	100 µl	100 µl
LB/amp	100 µl	100 µl
- Add 100 µl of cell suspension to the appropriate plate

18. Sterilize the spreader, cool it by touching the agar surface, and spread the cell suspension evenly over surface of plate.
19. Repeat for the remaining three plates.
20. Allow the plates to sit right side up for several minutes to allow liquid to be absorbed into agar.
21. Place plates in 37°C incubator for 12-24 hours.

OVERNIGHT CULTURES

1. Obtain your plates and arrange them according to the grid in Table 3.

2. Count the number of green colored colonies in each plate and record in Table 3. If the green color is not apparent yet, you may incubate the plates until the next lab

	Transformed cells (+pGFP)		Control cells	
	# Green	Total	#Green	Total
LB				
LB/amp				

period after picking colonies (see below). UV lights will be provided to help visualize the green color in the next lab period. You can record your results then.

3. Record the **total** number of colonies (green + white) on each plate. There will probably be too many to count in the two LB plates; record such as a "lawn."
4. If the transformation worked well, you should observe 50-500 colonies in the pGFP LB/amp plate.

MAKE OVERNIGHT CULTURES OF TRANSFORMED CELLS FOR NEXT LAB

1. Obtain two microfuge tubes with 1,000 µl of sterile LB/amp broth. Label each with your team name.
2. Select one colony from the LB/amp plate. This colony should consist of a clone of transformed cells. Your colonies may or may not look green yet. If they are not green, pick one anyway, and re-incubate the plates until the next lab period.
3. With the inoculating loop, transfer part of one colony to one of the tubes and part of the same colony to the other tube (don't forget to flame the loop for a sterile transfer). You do not have to scrape up the entire colony, a mere touch with your loop will transfer thousands to millions of cells! After transferring, mark the back of your plate with sharpie to indicate the colony you picked.
4. Make sure the tubes are appropriately labeled and place in the rack in the 37 degree C incubator for 24 h. Due to lab scheduling, you may not have a lab period the next day. If this is the case, the lab technician will move your cultures into the refrigerator after the 24 h incubation. Your plates and cultures will be perfectly fine for reading and miniprepping the next period.

THE MINIPREP

MATERIALS

- 2 microfuge tubes with 1ml of overnight culture of *E. coli* transformed with pGFP plasmid in the previous lab.
- Glucose/Tris/EDTA (GTE)
- SDS/NaOH
- Potassium acetate/acetic acid (KOAc)
- Isopropyl alcohol (isopropanol)
- 95% Ethyl alcohol (ethanol)

Tris-EDTA (TE)
Micropipets and tips
1.5 ml microfuge tubes
Beaker of crushed ice
Biohazard bag for waste and used tips

PROCEDURE

1. Disinfect workspace.
2. Label the two microfuge tubes of overnight culture of *E.coli* with your name.
3. Close caps and centrifuge both tubes for 1 minute.
4. Pour off supernatant into biohazard waste. Make sure the pellet remains in the tube.
5. Thoroughly drain the tubes by gently tapping on a small piece of clean paper towel (be sure to discard towel in biohazard container and disinfect bench area).
6. Add 100 μ l of GTE solution to each tube. Resuspend pellets by pipetting solution in and out several times. Hold tubes up to light to check that suspension is homogeneous and that no visible clumps of cells remain.
7. Add 200 μ l of SDS/NaOH solution to each tube. Close caps and mix by rapidly inverting tubes five times.
8. Stand tubes on ice for 5 minutes. Suspension should become relatively clear as the cells lyse.
9. Add 150 μ l of ice-cold KOAc solution to each tube. Close caps, and mix solutions by rapidly inverting tubes 5 times. A white precipitate will immediately appear.
10. Stand tubes on ice for 5 minutes.
11. Centrifuge tubes for 5 minutes to pellet the precipitate.
12. Transfer 400 μ l of supernatant from each tube into two clean microfuge tubes. (**Save the supernatants**)
13. Add 400 μ l of isopropanol to each tube of supernatant
14. Close caps and mix vigorously by rapid inversion five times.
15. Stand at room temperature for **exactly 2 minutes**. Isopropanol preferentially precipitates nucleic acids, but with longer exposure proteins also begin to precipitate.
16. Centrifuge tubes for 5 minutes to pellet the precipitate. Position the hinge of microfuge tube up in the microfuge so you can tell where to expect the pellet will appear (Do this for all subsequent spins).
17. Pour off supernatant from both tubes. Be careful not to disturb nucleic acid pellets. (**Save the pellets**)
18. Invert tubes over a paper towel and tap gently to drain thoroughly.
19. Rinse the pellets by adding 200 μ l of 100% ethanol to each tube. Flick the tubes several times.
20. Centrifuge tubes for 2 minutes.
21. Pour off supernatant from both tubes. **Save the pellets**. Invert tubes over paper towel and drain thoroughly.
22. Close caps and pulse tubes in centrifuge. **Carefully** remove any remaining ethanol with 2-20 μ l pipette.
23. Leave caps open and allow pellets to dry at room temperature for 10 minutes. All ethanol must be evaporated before proceeding.
24. Add 15 μ l of TE buffer to each tube. Resuspend pellets by smashing them with the pipette tip and pipetting in and out. Focus your efforts where the pellet should have formed during centrifugation (on

the side of the cap hinge). Check that all DNA is dissolved and that no particles remain in tip or on the side of tube.

25. Pool the DNA solution into one tube. You will now have a total of 30 μ l of DNA.
26. Freeze the DNA/TE solution at -20°C until you are ready to proceed with the restriction analysis of the plasmid DNA.

RESTRICTION ANALYSIS

MATERIALS

Miniprep DNA/TE from previous period
 0.075 $\mu\text{g}/\mu\text{l}$ pGFP
 0.075 $\mu\text{g}/\mu\text{l}$ λ phage DNA
*Bam*HI/*Hind*III restriction enzymes
 5X restriction buffer/RNAase
 Loading dye
 0.8% agarose
 1X Tris/Borate/EDTA (TBE) buffer
 Micropipets and tips
 Microfuge tubes

PROCEDURE

1. Label 4 microfuge tubes:
 - mini-** this tube will have the miniprep DNA without restriction enzymes
 - mini+** this tube will have the miniprep DNA with restriction enzymes
 - pGFP-** this tube will have pGFP DNA (supplied) without restriction enzymes
 - pGFP+** this tube will have pGFP DNA (supplied) with restriction enzymes
 - λ marker** this tube will have λ phage DNA (supplied) with *Hind*III.
2. Use the grid below as a guide to prepare the tubes.

Tube	Miniprep DNA	pGFP	λ DNA	Buffer/RNAase	<i>Bam</i> HI	<i>Hind</i> III	H ₂ O
mini-	5 μ l	-	-	3 μ l	-	-	7 μ l
mini+	5 μ l	-	-	3 μ l	2 μ l	2 μ l	3 μ l
pGFP-	-	5 μ l	-	3 μ l	-	-	7 μ l
pGFP+	-	5 μ l	-	3 μ l	2 μ l	2 μ l	3 μ l
λ marker	-	-	5 μ l	3 μ l	-	2 μ l	5 μ l

3. Add 5 μ l of miniprep DNA, pGFP DNA, λ phage DNA to the appropriate tubes. Use a fresh tip for each different DNA transferred.
4. Use a fresh tip to add 3 μ l of restriction buffer/RNAase to each reaction tube.
5. Use a fresh tip to add 2 μ l of enzyme to the appropriate tube.
6. Use a fresh tip to add proper volumes of DI H₂O to each tube.
7. Each tube should now have 15 μ l total. Visually confirm that you have about this amount in each tube.
8. Close tubes. Pulse in centrifuge to mix reagents.

9. Place reaction tubes in 37°C water bath and incubate for 30 minutes.
10. While incubating, cast agarose electrophoresis gel. Pour to a depth of about 5 mm. Let stand for at least 15 minutes.
11. When incubation is complete, remove tubes from water bath.
12. Add 2 µl of loading dye to each tube and pulse in centrifuge.
13. Load entire reaction mixture from each tube into appropriate well in your gel.
14. Load them in the same order they are in column 1 of the table above. Avoid using lanes next to the edge of the gel.
15. Close gel box and connect to power supply. Run at about 100 volts until the smallest dye marker has migrated about 2/3 of the way across gel.
16. Turn off power and remove gel from box. Take the gel to the lab technician for staining with Ethidium Bromide. You will stain for 5 minutes followed by destaining with water for 5 minutes.
17. The lab technician will photograph your gel.

ANALYSIS

The data you recorded in Table 3 will allow you to calculate your *transformation efficiency*. Transformation efficiency gives you an idea of how well your plasmid inserted into the bacterial cells. It is simply the number of transformed cells produced per mass of plasmid used in the procedure. We will use the formula:

$$\text{Transformation efficiency} = \# \text{ colonies}/\mu\text{g DNA}$$

Be sure to count green colonies from your LB/amp plate only and to calculate exactly how much plasmid DNA was plated onto the plate. You will need to use the starting concentration of pGFP and calculate by how much it was diluted in the transformation and plating procedures.

As you have done in a previous lab, you will use your λ HindIII lane to produce a standard curve. This curve will be used to calculate estimated sizes and you will compare them to the actual fragment sizes you expected. Use Table 4 to record all relevant bands from your gel.

λ HindIII		Mini uncut	Mini cut	pGFP uncut	pGFP cut
Dist. (mm)	Actual Base Pairs	Dist. (mm)	Dist. (mm)	Dist. (mm)	Dist. (mm)
	23,130				
	9,416				
	6,557				
	4,361				
	2,322				
	2,027				
	564				
	125				

Plasmid DNA can assume several forms that affect their migration in electrophoresis gels. This combined with impurities that can contaminate samples, make interpreting miniprep gels somewhat confusing and tricky.

On the photograph of your gel locate the cut and uncut lanes for the miniprep plasmids and the pGFP control. The miniprep lanes can usually be distinguished by the following:

A background "smear" running much of the length of the miniprep lane. The smear is composed of partially digested chromosomal DNA, plasmid DNA, and RNA. A heavy background smear, along with high molecular-weight DNA near the well, indicates the miniprep is contaminated with large amounts of chromosomal DNA.

Frequently, undissolved material and high-molecular weight DNA are seen "trapped" at the front edge of the well. These anomalies are not seen in commercial preparations, where plasmid DNA is separated from degraded nucleic acids by density-gradient centrifugation.

A "cloud" of low molecular weight RNA is often seen in both the cut and uncut miniprep lanes at a position corresponding to 100-200 bp. This represents RNA remnants that were only partially digested by the RNAase.

The appearance of extra bands may indicate incomplete digestion. This is usually due to contaminants in the miniprep that inhibit restriction enzyme activity or may occur when the miniprep solution contains a very high concentration of plasmid DNA.

One would expect to see only one band in the electrophoresis of purified, uncut plasmid DNA. Inspection of the uncut lane shows that this is not the case. This is because plasmid DNA can exist in one of three major molecular forms:

- **Relaxed circle:** This is the classic open circle structure that would be obtained if the two ends of the plasmid were joined together, with no extra twisting on either strand. Relaxed circles are formed when single strand nicks allow the supercoiled form (see below) to unwind. Physical shearing and enzymatic cleavage during the miniprep introduce such nicks. This is the slowest migrating form of plasmid DNA.
- **Linear:** Linear plasmid DNA is produced when a restriction enzyme cuts the plasmid at a single recognition site, or when damage results in strand nicks directly opposite each other on the DNA helix. Under most conditions linear plasmid DNA migrates at a rate intermediate between the supercoiled and relaxed circle. The presence of linear DNA in a miniprep indicates contamination with nucleases or sloppy lab procedure.
- **Supercoiled (Fig 2):** One of the topoisomerases (DNA gyrase) nicks one strand of the DNA and rotates it around the other. This introduces a torsional force that results in the plasmid DNA wrapping around special protein molecules to make them more compact in the bacterial cell. The miniprep process strips away the supporting proteins and leaves the DNA molecule to coil about itself. Under most conditions, the supercoiled plasmid is the fastest migrating form. Therefore, the fastest moving band in uncut, plasmid DNA is assumed to be the supercoiled form.

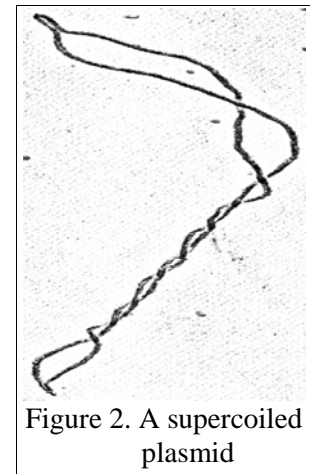


Figure 2. A supercoiled plasmid

Some strains of *E. coli* (MM294 is one of them) carry an enzyme called *recA* that promotes recombination (crossover) between homologous regions of separate plasmids. When this happens, plasmids are joined into one giant plasmid called a "super plasmid" or "concatemer." Some super plasmids may contain up to four or five copies of the plasmid.

"Super plasmids" appear as a series of slow-moving bands at the top of the gel. Since the sequence of restriction sites within each plasmid is not disrupted in super plasmids, they produce the same restriction fragments as regular plasmids.

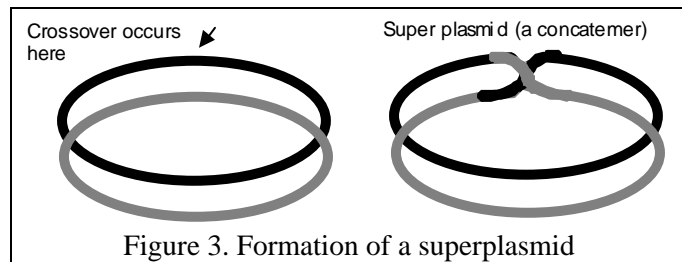


Figure 3. Formation of a superplasmid

QUESTIONS FOR YOUR REPORT

(30 points, 3 pts for each question)

1. Present an abstract of your work. Your abstract should include a brief description (1/2 page) of 1) what you set out to learn, 2) what procedures you performed, and 3) a summary of your data or discoveries.
2. Explain the significance of keeping track of the number of total colonies and green colored colonies on your plates.
3. Present your data in Table 3. Compare your transformation results to the 50-500 figure given above. Explain possible reasons for variation from the expectations.
4. What was the *transformation efficiency* for your work in this lab? *Show your calculations.*
5. Explain how the miniprep procedure captures plasmid DNA, but not the bacterial chromosomal DNA.
6. Present your photograph of the gel. Clearly label all lanes. Did you find any extra bands/smears in your miniprep lanes? If so, what do you think the bands/smears are? Label the extra bands/smears on the photograph with what you think they are. (See analysis section above for help on this question.)
7. Present your data from Table 4 and your standard curve using the λ *HindIII* lane.
8. Use the map of pGFP to calculate the actual fragment sizes you expected from your *BamHI/HindIII* digestions of pGFP. *Show your calculations.*
9. Using your standard curve and the appropriate bands from your gel, estimate the fragment sizes of your *BamHI/HindIII* digestions of pGFP and your minipreps. **Create a new table that shows estimated and actual sizes expected for each lane.** How did your estimated sizes compare to the actual sizes for each?
10. What would you expect to find on your gels if you accidentally used *BamHI* and *EcoRI* instead of *BamHI* and *HindIII* in your miniprep digests? Calculate the sizes of bands you would have gotten instead.