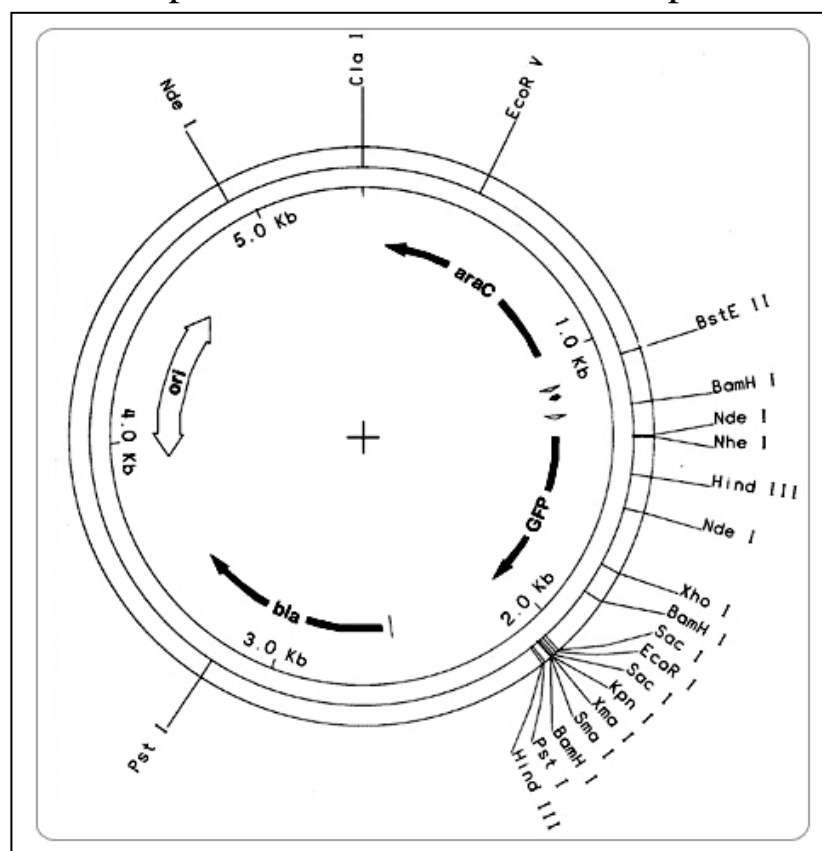


Lab # 8 Mutagenesis of pGlo Plasmid by Restriction Digest

Scientists routinely introduce mutations into cloned genes in order to further understand the function of the gene and its protein product. Through site-directed mutagenesis, scientists can alter any nucleotide within the gene. We will be performing a mutagenesis experiment in which we use a restriction enzyme to introduce a mutation in the pGlo plasmid. This plasmid has several important genes and DNA elements (refer to the diagram below). The *ori* region is an origin of replication element required for the plasmid to efficiently replicate in cells. The GFP region encodes the green fluorescent protein. The *araC* region encodes a gene regulatory protein (AraC) that binds to the arabinose promoter, which is located just upstream of the GFP gene. Ara C protein acts as an activator of GFP gene expression, depending on the absence or presence of arabinose sugar in the growth medium. When arabinose is present, arabinose binds to the AraC protein and changes its conformation in a way that allows AraC to bind to the arabinose promoter and recruit RNA polymerase to the promoter, thus stimulating expression of GFP.

Referring to the restriction map of the pGlo plasmid (shown below), note that digestion of the plasmid with restriction endonuclease Hind III should delete a portion of the GFP gene from the plasmid. Electrophoresis of this digest will yield 2 DNA fragments. The larger of the two fragments can be gel-purified and ligated to yield a plasmid that has an origin of replication (*ori*) and a functional ampicillin resistance gene (*bla*), but lacks a functional GFP gene. Subsequent transformation of this plasmid into *E. coli* should yield amp-resistant colonies that fail to express GFP.

pGlo Plasmid Restriction Map



Day 1: Gel-purify and Ligate Hind III-digested pGlo plasmid

Procedure:

1. Label a microcentrifuge tube as pGlo DNA
2. In this tube, combine the following reagents in the order listed: 10 μ l of pGlo plasmid, 6 μ l water, 2 μ l NEB buffer #2, and 2 μ l Hind III restriction enzyme (found at teacher's bench).
3. Mix the reagents well (remember that the enzyme will stratify at the bottom of the tube).
4. Incubate the digest in a 37⁰C water bath for 1 hour.
5. Pour a 0.8% agarose gel that already contains EtBr. Pour the gel thick enough to accommodate 20 μ l of sample in the wells. Be sure to wear gloves when handling EtBr.
6. After an hour's time, remove the digest from the water bath.
7. Add 2 μ l of loading dye to the digest
8. Load the entire digest in one lane of the gel (avoid far outside lanes).
9. Add 2 μ l of loading dye to the 20 μ l of size ladder found at your bench. Load 10 μ l of 1 ladder in the lane next to your digest. Save the remainder of the size ladder for later in the lab.
10. Perform electrophoresis at 100 volts until the bromphenol blue dye (dark blue) has migrated $\frac{1}{2}$ the length of the gel
11. Put on a pair of gloves.
12. Observe the gel on the UV illuminator.

Using the size ladder as a reference, estimate the sizes of the 2 bands _____

Are the sizes of the bands consistent with the restriction map of the pGlo plasmid?

If so then proceed to the next step. If not then consult with the instructor.

13. Place the gel on a glass plate on the UV illuminator. Using a razor blade, cut the larger of the 2 bands out of the gel. Carefully remove any agarose that is not stained with EtBr. Place the EtBr stained DNA into a clean microcentrifuge tube. Keep the remainder of the agarose gel intact. You will use it to run out more DNA later in the lab period.
14. Spin the tube containing the gel in a microcentrifuge at 14K RPM for 30 seconds. Estimate the volume of the gel in the tube.
15. Add 2-3 volumes of 6M NaI solution to the gel and incubate the mix in a 55⁰C water bath for 5 minutes to completely dissolve the gel
16. Vortex the mixture and then return to the 55⁰C water bath for an additional minute
17. Add 10 μ l of glassmilk suspension (silica in glassmilk binds DNA) to the dissolved gel mixture and incubate at room temperature for 10 minutes. Mix the tube very 2 minutes to make sure the glassmilk stays suspended.
18. Centrifuge the tube for 30 seconds.
19. Remove and discard the supernatant
20. Add 450 μ l of NEW buffer to the tube and resuspend the glassmilk pellet by pipeting up and down
21. Centrifuge the tube for 30 seconds.
22. Remove and discard the supernatant
23. Wash the pellet 2 more times with NEW by repeating steps 20-22 twice over
24. Make sure to completely remove all the supernatant from the pellet after the final wash
25. Allow the pellet to air dry for 10 minutes
26. Add 20 μ l of TE to the pellet and resuspend the pellet by pipeting up and down. Incubate 5 minutes at room temp with occasional mixing.
27. Centrifuge for 30 seconds.

28. Transfer the supernatant, which contains the DNA, to a new microcentrifuge tube. Label this tube as “gel pure DNA”
29. Transfer 5 μ l of your gel-purified DNA to a clean microcentrifuge tube. Add 5 μ l of water and 1 μ l of loading dye.
30. Load this sample on the gel you saved from the previous electrophoresis procedure.
31. Load 10 μ l of ladder next to the DNA sample.
32. Run the gel at 100 volts until the bromphenol blue dye is approximately $\frac{1}{2}$ way down the gel
33. Observe the gel under the UV illuminator.

Do you see DNA? Is it the size you expect?

34. While the gel is running, set up the ligation reaction. Combine the following reagents in a microcentrifuge tube in the order listed: 10 μ l of gel-purified DNA, 6 μ l water, 2 μ l ligation buffer, 2 μ l T4 Ligase (found at teacher’s bench). Mix the reagents well. Label the tube as “Ligation” and with your initials
35. Give the ligation reaction to the instructor. The reaction will incubate at room temperature overnight, and then the reaction will be stored in the freezer until the next lab period.

Day 2: Transformation of E. coli with Mutagenized pGlo Plasmid

1. Label 3 microcentrifuge tubes as 1-3
2. Add 250 μ l of cold CaCl_2 to each tube. And place on ice
3. Obtain a petri plate streaked with E. coli
4. Using a sterile loop, scoop up some cells and put them in the CaCl solution of tube #1 (as demonstrated by the instructor)
5. Immediately resuspend these cells so there are no clumps and the suspension appears homogeneous. Place the cells back on ice
6. Repeat steps 4-5 for tubes 2 and 3 such that all the tubes contain a cell suspension.
7. To tube #1 add all of your ligation reaction from the previous lab period and mix.
8. To tube #2 add 10 μ l of control pGlo plasmid (this is the wt plasmid that still contains the GFP gene)
9. Tube #3 receives no DNA
10. Incubate Tubes 1-3 for 15 minutes on ice
11. Heat shock tubes 1-3 for 90 seconds at 43⁰C. This will increase transcription of the bla gene.
12. Place the tubes back on ice for 1 minute
13. Add 250 μ l of LB broth to each tube and incubate for 15 minutes at room temperature.
14. During this incubation period, label your agar plates:
 - Label the LB-amp plates as Plate #1, #3 and #5
 - Label the LB-amp-ara plates as Plate #2 and #4
 - Label the LB plate as #6
15. Using a sterile glass spreader,
 - spread 200 μ l of cells from Tube #1 on to Plate #1 and 100 μ l onto Plate #2.
 - spread 200 μ l of cells from Tube #2 on to Plate #3 and 100 μ l onto Plate #4.
 - spread 200 μ l of cells from Tube #3 on to Plate #5 and 100 μ l onto Plate #6.
16. Incubate the cells on the plate for 30 minutes at room temperature. Then tape all your plates together, label with your name and incubate the plates at 37⁰C upside down overnight. The instructor will then store the plates at 4⁰C until the next lab meeting

Day 3: Analysis of Transformation Results

For plates 1-6 describe the type of growth you observe (isolated colonies, lawn or no growth) and note whether or not the cells express GFP. Provide a rationale for the results you observe.

Plate #1

Plate #2

Plate #3

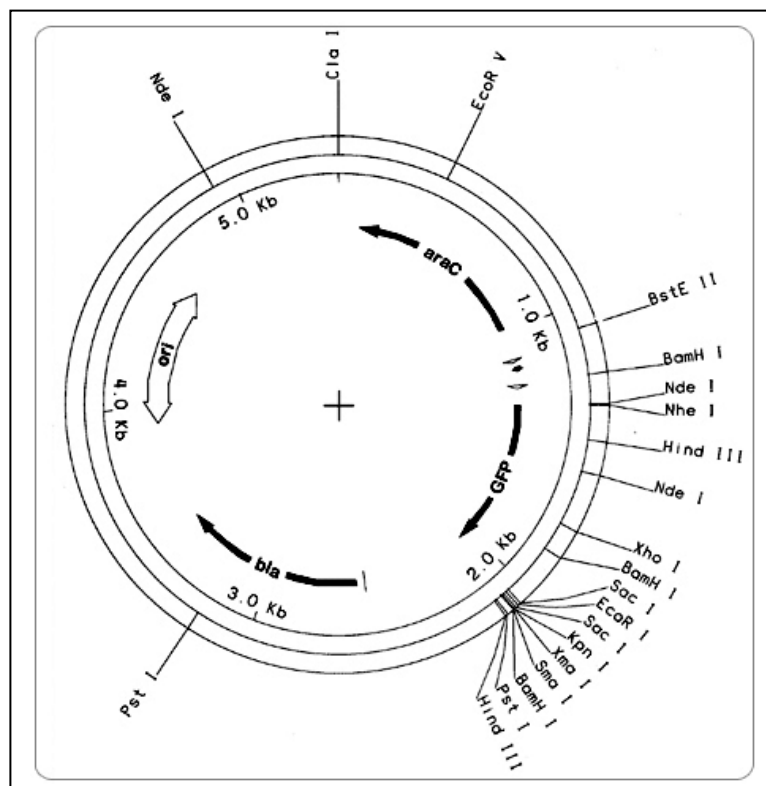
Plate #4

Plate #5

Plate #6

Questions

1. Refer to the map of the pGlo plasmid (below) while answering the following questions:
 - a. You mutagenize the pGlo plasmid by cutting the plasmid with PstI restriction enzyme. You gel-purify and ligate the larger of the 2 fragments. Then you transform E. coli with this mutagenized plasmid and plate on LB-amp-ara plates. What type of growth do you expect? Is GFP expressed? Provide a rationale for the result.
 - b. You do a similar experiment, but this time you cut the plasmid with Bam HI. What type of growth do you expect? Is GFP expressed? Provide a rationale for the result.
 - c. You do a similar experiment, but this time you cut the plasmid with ClaI and EcoRV. What type of growth do you expect? Is GFP expressed? Provide a rationale for the result.



Notes

