

## Lab Exercise #6 Purification and Restriction Analysis of Plasmid DNA

In the previous lab exercise, you transformed *E. coli* with the pGlo plasmid and selected for transformed cells on LB plates containing ampicillin. The pGlo plasmid has a gene ( $amp^r$ ) that confers resistance to ampicillin antibiotic, thus we assumed that amp-resistant colonies must contain the plasmid. Today we will prove that assumption to be correct by re-isolating the pGlo plasmid back out of an overnight culture of transformed cells. Furthermore, we will digest the plasmid with several restriction enzymes, creating a restriction map that will positively identify the isolated plasmid as pGlo. The lab exercise will require 2 lab periods. Today we will purify the plasmid from *E. coli* and digest it with restriction enzymes. Next week we will size fractionate the resulting DNA fragments by electrophoresis, determine their relative sizes in kilobases (kb), and then use that information to order the restriction enzyme sites on the plasmid.

The plasmid DNA will be isolated from cells by the combined methods of Alkaline Lysis and Ion Exchange Chromatography. The general process is as follows: Transformed cells containing the plasmid are grown to high density in the presence of selective antibiotic. Cells are resuspended in a buffer that contains ethylenediaminetetraacetic acid (EDTA) and RNase. EDTA binds divalent cations ( $Ca^{2+}$ ,  $Mg^{2+}$  and others) required for membrane stability. RNase digests RNA that would otherwise contaminate our plasmid preparation. This mixture is treated with sodium dodecyl sulfate (SDS) and sodium hydroxide. SDS is a detergent that dissolves the plasma membrane and denatures cell proteins causing cell lysis. Sodium hydroxide denatures plasmid DNA and chromosomal DNA into single strands. Further treatment with potassium acetate and acetic acid forms an insoluble precipitate of chromosomal DNA/cell proteins/lipids. Unlike chromosomal DNA, plasmid DNA reanneals completely and remains in solution, separate from the precipitate. Centrifugation of this mixture separates the precipitate (pellet) from the solution containing the plasmid.

The plasmid DNA can be purified further by several methods. We will use an ion exchange column to purify our plasmid. DNA is negatively charged due to the presence of phosphate groups in the DNA backbone. The Ion Exchange column contains beads that have a positive charge. When the DNA preparation is put onto the column under high salt conditions, the negative DNA binds tightly to the positive beads of the column while other cell components (lipids, proteins, and carbohydrates) pass through the column. The column is washed several times with various buffers to remove contaminants. Finally DNA is eluted from the column with a low salt buffer.

## Day 1

### Preparation of Plasmid DNA by Alkaline Lysis and Ion-Exchange Chromatography

#### Procedure:

1. Obtain 10 ml of an overnight culture of pGlo-transformed E. coli.
2. Transfer the culture into 2 falcon tubes, 5 ml into each tube.
3. From this point forward perform the same procedures for each tube.
4. Spin the falcon tube for 4 minutes at setting 4 in a bench-top centrifuge.
5. Decant the supernatant from the tube into a bacterial-waste container. Be certain to remove all of the supernatant.
6. Resuspend the pellet in 250  $\mu$ l of buffer P1. Pipet up and down to completely resuspend the pellet.
7. Transfer the cell suspension to a microcentrifuge tube.
8. Add 250  $\mu$ l of buffer P2 and mix thoroughly by inverting the tube 5 times.
9. Add 350  $\mu$ l of buffer N3 and mix immediately and thoroughly by inverting the tube 5 times.
10. Spin tube for 10 minutes at 13,000 rpm in a microcentrifuge. Be certain to balance your tubes before spinning. Ask neighboring groups if they wish to spin at the same time.
11. Apply the supernatant to the ion exchange column by pipetting.
12. Spin the column for 30-60 seconds at 13,000 rpm in the microcentrifuge. Discard the flow-through.
13. Add 500  $\mu$ l of buffer PB and spin for 30-60 seconds at 13,000 rpm in the microcentrifuge. Discard the flow-through.
14. Add 750  $\mu$ l of buffer PE and spin for 30-60 seconds at 13,000 rpm in the microcentrifuge. Discard the flow-through.
15. Spin for an additional 60 seconds to remove residual PE buffer. Discard the flow-through.
16. To elute the DNA from the column, place the column in a clean microcentrifuge tube. Add 50  $\mu$ l of buffer EB to the center of the column. Let stand for 1 minute. Spin for 1 minute at 13,000 rpm in the microcentrifuge.
17. Transfer the eluted DNA from both preps to a clean microcentrifuge tube and label as pGlo DNA and with your initials. You should have approximately 100  $\mu$ l of DNA. Check the volume with a micropipettor and note it on the tube.

## Restriction Mapping of pGlo Plasmid DNA

Restriction Endonucleases (restriction enzymes) cut DNA at specific nucleotide sequences. They are the equivalent of molecular scissors. Restriction enzymes are derived from microorganisms (mostly bacteria) that use the enzymes as a defense against bacteriophage. Once injected into the host cell, phage DNA is cleaved by restriction enzymes, thus the host cell is saved. Host DNA is spared from restriction enzyme cleavage by methylation of the enzyme's DNA recognition sequence. Over 1200 restriction enzymes from various species have been identified and over 70 are commercially available. DNA recognition sequences can be as short as 4 nucleotides and as long as 8 nucleotides. Enzymes that have 4-nucleotide recognition sequences (4-cutters) are predicted to cut DNA on average every 256 nucleotides ( $4^4$ ). Enzymes that have 8-nucleotide recognition sequences (8-cutters) are predicted to cut DNA on average every 4096 nucleotides ( $4^6$ ). A common restriction enzyme is EcoR1. This enzyme, which was derived from *E. coli*, recognizes the DNA sequence GAATTC. This sequence is a palindrome (reads the same 5' to 3" on both strands) as are the recognition sequences of most restriction enzymes. EcoR1 cleaves this sequence at the phosphodiester bond between the G and the A nucleotides, creating staggered (sticky) ends.

### Procedure for Digesting DNA:

1. Label 7 microcentrifuge tubes as 1-7.
2. Obtain an ice bucket with water, buffer, and Restriction enzymes EcoR1, EcoRV, and ScaI.
3. Add water, buffer, DNA and enzymes to each tube in that order, according to the table shown below.
4. Incubate the tubes for 2 hours at 37<sup>0</sup>C in a water bath.
5. Store your DNA digests at 4<sup>0</sup>C until the next lab period.

Tube #	Water	Buffer	pGlo DNA	Enzyme	Total Volume
1	7 µl	2 µl	10 µl	1 µl EcoR1	20 µl
2	7 µl	2 µl	10 µl	1 µl EcoRV	20 µl
3	7 µl	2 µl	10 µl	1 µl ScaI	20 µl
4	6 µl	2 µl	10 µl	1 µl EcoR1, 1 µl EcoRV	20 µl
5	6 µl	2 µl	10 µl	1 µl EcoR1, 1 µl ScaI	20 µl
6	6 µl	2 µl	10 µl	1 µl EcoRV, 1 µl ScaI	20 µl
7	5 µl	2 µl	10 µl	1 µl EcoR1, 1 µl EcoRV, 1 µl ScaI	20 µl

## Day 2

### Electrophoresis of DNA Digests:

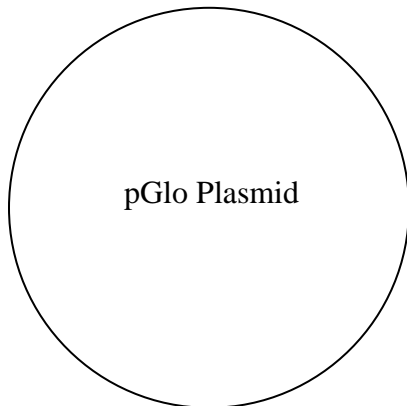
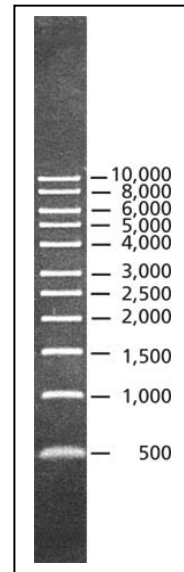
#### Procedure:

1. Put on a pair of vinyl gloves to protect yourself from exposure to EtBr
2. Pour an 0.8% agarose gel (already containing EtBr) with an 8-well comb
3. Place the solidified gel into the electrophoresis chamber and cover with 1X TBE solution
4. Obtain your pGlo DNA digests from the instructor.
5. Incubate your digests in a 37°C water bath for 10 minutes.
6. Add 2 µl of loading dye to each of your pGlo DNA digests and mix.
7. Add 1 µl of loading dye to the tube labeled DNA Ladder and mix.
8. Spin your samples in the microcentrifuge to ensure that the entire sample is in the bottom of the tube.
9. Load 10 µl of your samples left to right in the following order: ladder, 1,2,3,4,5,6,7.
10. Connect the electrophoresis chamber to the power supply and run the gel at 100 Volts for approximately 60 minutes.
11. Turn the power supply off, put latex gloves on and then carefully transfer the gel to the UV light box.
12. Take a digital picture of the gel.
13. Stable a Xerox copy of the photograph to your lab.
14. While still wearing gloves, dispose of the electrophoresis buffer by filtering it through charcoal, as demonstrated by the instructor

## Mapping Restriction Enzyme Sites on the pGlo Plasmid

1. Determine the relative sizes of the restriction fragments in each digest by comparing them to the fragments in the 1 Kb Ladder. The figure below indicates the sizes of fragments in the 1 Kb ladder.
2. Enter your data in the table below.
3. Use this information to estimate the overall size of the plasmid and to order the restriction sites on the plasmid.
4. Label the plasmid drawing below with the restriction sites in their relative positions and the distance (in Kb) between them.

Lane	Enzyme	Fragment sizes in Kb
3	EcoR1	
4	EcoRV	
5	ScaI	
6	R1, RV	
7	R1, ScaI	
8	RV, ScaI	
9	R1, RV, ScaI	



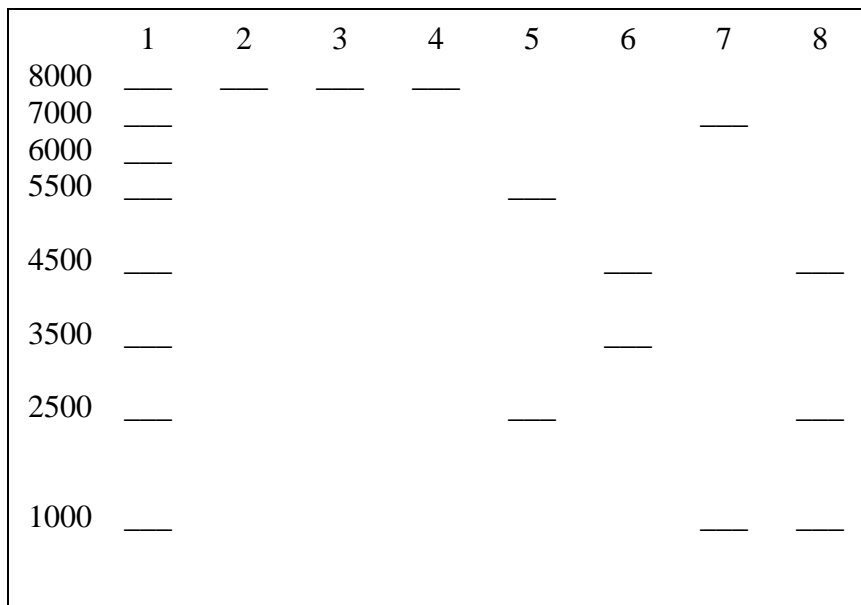
## Questions

1. We extracted plasmid DNA from cells using the Alkaline Lysis Method. State the role of the following reagents in that process

- SDS
- Sodium Hydroxide
- RNase
- EDTA
- Potassium acetate / acetic acid

2. How does the ion-exchange column function to purify DNA away from other molecules?

3. What is the substrate for restriction enzymes?
  
4. What organisms naturally contain restriction enzymes and what do they use them for?
  
5. You have digested the pJim plasmid with 3 restriction enzymes: EcoR1, HindIII and PVUII, then size fractionated the resulting DNA fragments on an agarose gel. The figure below shows this gel after staining with EtBr. Use the information in the gel complete the table below and then order the restriction sites on the plasmid (shown as a circle).



Lane	Sample	Size of DNA fragments (Kb)
1	Size marker ladder	
2	pJim-EcoR1	
3	pJim-PVUII	
4	pJim-HindIII	
5	pJim-EcoR1, HindIII	
6	pJim-EcoR1, PVUII	
7	pJim-HindIII, PVUII	
8	pJim-EcoR1, HindIII, PVUII	

