Name _____ Date_____

Isolation and Electrophoresis of Plasmid DNA

Prior to lab you should be able to:

- Explain what "cloning" a gene accomplishes for a geneticist.
- Describe what a plasmid is.
- Describe the function of the three essential features of all cloning plasmids.
- Explain how electrophoresis of DNA works.
- Explain how electrophoresis can be used to determine the size of a fragment of DNA.

I. Objectives:

- **Isolate a plasmid containing a cloned soybean gene.**
- **Use restriction enzymes to release the soybean gene from the plasmid.**
- **Use gel electrophoresis to determine the size of the soybean gene.**

II. Background:

Plasmids

Bacteria have a single large circular chromosome which contains the all the genes necessary for metabolism, growth and cell division. In E. coli, this chromosome is about 4.6 million base pairs. In addition to this large chromosome, many bacteria also contain small "extra-chromosomal" circular DNA called plasmids. The small circular plasmids may be just a few thousand base pairs. Typically, these natural plasmids do not contain genes necessary for normal growth, but instead have genes for specialized functions such as bacterial sex or antibiotic resistance.

Scientists clone DNA in order to generate millions of identical copies of a single fragment of DNA. In a cloning experiment, the fragment of DNA is introduced into a cell and the cell replicates the DNA producing the millions of copies of it. One technical problem in cloning is that most fragments of DNA lack an origin of replication. Therefore, when they are introduced into a cell, the cellular replication machinery will not copy the DNA. To overcome this problem, fragments of DNA to be clone are linked to a piece of DNA called a cloning vector. The cloning vector is a piece of DNA that includes an origin of replication. When the cloned fragment is linked to the cloning vector and they are introduced into a cell, the cell copies both of them, producing millions of copies of both the cloning vector and the DNA that is attached to it. Because natural plasmids have their own origin of replication, they have been modified to function as cloning vectors. Cloning plasmids have been engineered so that they contain the minimum DNA to allow the cell to replicate the plasmid and any fragment of DNA linked to it.

Cloning Plasmids

All cloning plasmids have three essential features (Figure 1). They have an origin of replication so that the host cells can copy the plasmids. When a plasmid is introduced into a bacterial cell, the cell will produce up to 200 copies of the plasmid. Additionally, bacterial cells reproduce rapidly. A single cell can divide multiple times, producing 10 million daughter cells overnight. Because each daughter cell contains 200 copies of the plasmid, 20 billion copies of the plasmid are generated. It is easy to analyze this large amount of DNA. The second feature that all cloning plasmids have is an antibiotic resistance gene. This gene allows the bacterium that harbors the plasmid to grow in the presence of the antibiotic. The plasmid in today's exercise contains a gene for ampicillin resistance. *E. coli* cannot grow in the presence of ampicillin unless it contains this plasmid. By growing the cells in ampicillin, we ensure that every cell in a culture contains the cloning vector. The third feature on all cloning vectors is the cloning site. A cloning site is a unique restriction enzyme recognition site. When the vector is digested with the restriction enzyme it "opens up" the plasmid (Figure 2). This allows a foreign fragment of DNA to be ligated within it.

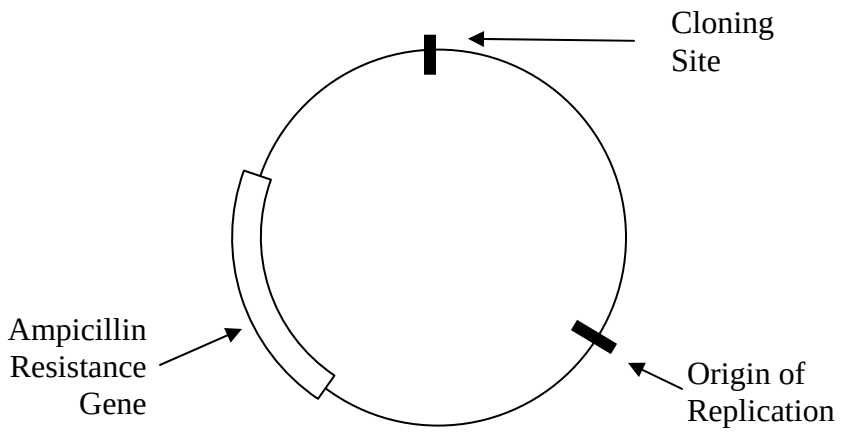

Figure 1: Three features of a basic cloning vector.

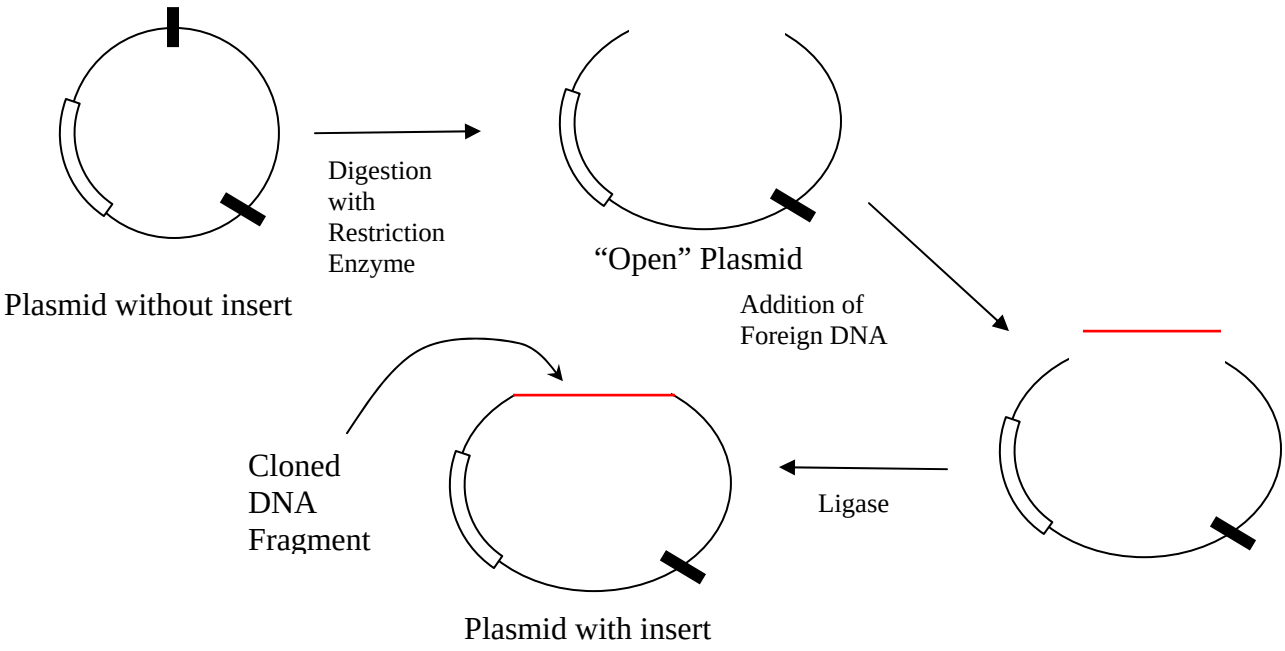

Figure 2. Basic steps to generate a recombinant plasmid. The plasmid DNA starts off as a closed circular molecule. To insert a foreign piece of DNA, the plasmid is digested with a restriction enzyme that cuts the double stranded DNA at the "cloning site". This opens up the plasmid. Foreign DNA is mixed with the plasmid and the plasmid is treated with the enzyme ligase. Ligase joins the fragment of foreign DNA to the plasmid. This recombinant molecule can then be introduced into a bacterial cell by transformation. The cell will replicate this plasmid as it divides. As stated earlier a single colony of cells may contain 10 billion copies of the plasmid.

Isolation of Plasmid DNA

To analyze the cloned fragment of DNA, the plasmid DNA must be isolated from the other components of the cell. In the procedure today you will break open the cells in an alkaline detergent solution. The detergent will denature and bind to all the protein in the cell. The high pH conditions will cause the large chromosome to denature and shear apart. The small plasmids are resistant to the high pH conditions. After the cells are broken apart, a high concentration acidic salt solution is added. This salt solution will neutralize the pH and cause the detergent to precipitate. The detergent/denatured proteins form a matrix that captures the broken chromosomal DNA. The mixture is centrifuged at high speeds. The denatured protein along with the large chromosomal fragments pellets in the bottom of the tube and are discarded. The supernatant contains plasmid DNA and other small cellular molecules. The plasmid DNA can be precipitated by adding ethanol to the supernatant. When the sample is centrifuged a second time, the precipitated plasmid DNA pellets leaving the other small molecules in solution. The supernatant is discarded and the pelleted plasmid DNA can be dried, and then dissolved in a buffer for further analysis.

Restriction Enzymes and Electrophoresis of DNA

To analyze the cloned insert, it must be separated from the plasmid. The insert can be released from the plasmid using the same restriction enzyme that cut the cloning site.

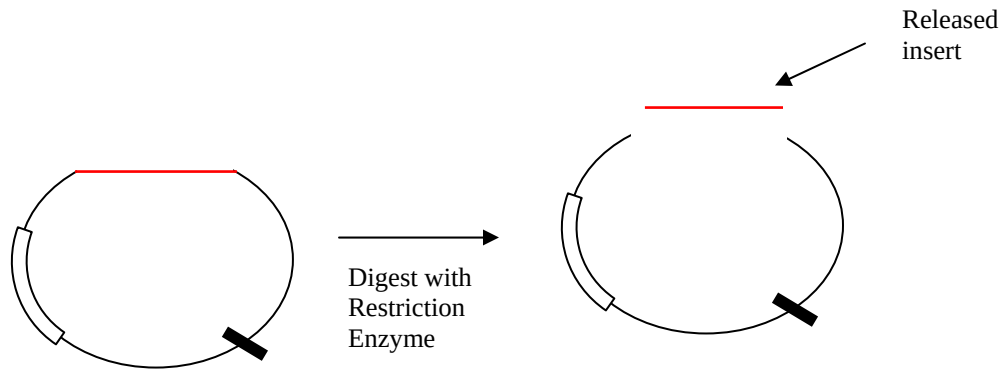

Figure 3: Restriction enzyme releases cloned insert from gene

The size of the cloned insert can be determined by gel electrophoresis. The digested plasmid DNA is applied to a gel. An electric voltage is applied to the gel. DNA is negatively charged because of its phosphate backbone. Therefore, it migrates to the positive electrode (anode). As with protein electrophoresis, small molecules migrate faster than big molecules. To determine the molecular weight of the cloned insert, it migration must be compared to a set of DNA standards using a standard curve.

Materials

Ice Bucket
Microfuge tubes
Microcentrifuge tube of bacterial cells (Note Strain _____)
NaOH/SDS solution
5MKOAc solution
Ethanol
70% Ethanol
TE buffer
Restriction Enzyme in Buffer (See TA)
Loading Dye
Agarose Gel (Ethidium Bromide in gel but not running buffer)
DNA Standards (See TA)
Photodocumentation system
Kimwipes

Procedure

Miniprep Isolation of DNA

1. Obtain a tube of E. coli cells from the TA (vol = 100µl)
    a. Note Strain # _____
2. Add 200µl of NaOH/SDS solution. Mix contents by inverting 5X vigorously, store cells on ice.
3. Add 150µl 5M KOAc. Mix contents by inverting 5X vigorously, incubate cells on ice five minutes.
4. Microcentrifuge sample on highest speed for 5 minutes.
5. Transfer 400µl of supernatant to new tube, store at room temperature. (Discard tube with pellet.)
6. Add 800µl ethanol to supernatant. Mix vigorously and incubate at room temperature for 2 minutes.
7. Microcentrifuge sample on highest speed for 5 minutes.
8. Observe pelleted nucleic acids at bottom of tube.
9. Carefully pour off supernatant
10. Add 70% ethanol to tube with pellet. Invert tube gently.
11. Microcentrifuge sample on highest speed for 5 minutes.
12. Observe pelleted nucleic acids at bottom of tube.
13. Carefully pour off supernatant
14. Use twisted kimwipe to remove remaining droplets of supernatant.
15. Air dry pellet 10mins
16. Add 40 µl of TE buffer to the dried pellet. Resuspend by vortexing vigorously.

Restriction Digest Reaction

1. Transfer 10µl of plasmid to new tube.
2. Add 10 µl of EcoRI + Buffer
3. Incubate 37º for 30min

Electrophoresis

1. Add 4µl of 6X blue loading dye to reaction- mix by vortexing
2. Microcentrifuge 30sec
3. Load 20µl onto agarose gel
4. TA will load DNA standards
5. Electrophoresis at 100 Volts 1 hour
6. Photograph gel on UV transilluminator

Short lab report.

1. Print out a photo of your gel from the web page. Label the gel showing the position of the vector DNA and the position of the soybean gene.
2. Prepare a standard curve of the standards. See size of DNA standards below.
3. Estimate the size of the soybean gene using the standard curve. The plasmid DNA is 2686 base pairs
4. What distinguishes the restriction enzyme used in this experiment EcoRI from other types of restriction enzymes?
5. How would your results have changed if the soybean gene had a recognition sequence for the restriction enzyme EcoRI?
6. Predict what might have happed in the experiment if the cells had been grown without ampicillin.