TRANSFORMATION OF *E. coli* WITH PLASMID DNA

Introduction

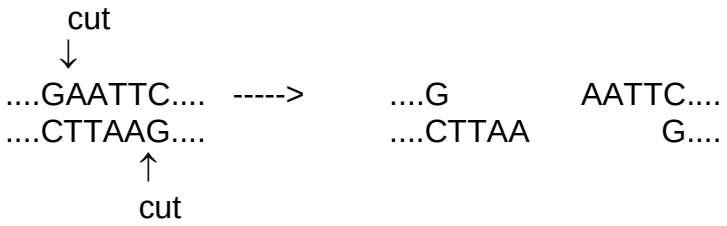
The field of **molecular genetics** has resulted in a number of practical applications that have been of tremendous benefit to us. One such benefit is the ability to produce large quantities of biological materials that were previously difficult to obtain. These new production methods involve isolating the gene for the needed product and placing that gene into a rapidly reproducing organism such as a **bacterium**, which will then manufacture large amounts of the desired substance in a relatively short period of time. This process of altering the production capabilities of living organisms by the introduction of new genes into those organisms is commonly referred to as **genetic engineering**. The most common way of introducing these genes and producing these altered organisms is through the use of **plasmids**.

Plasmids are small circular molecules of DNA that exist apart from the single chromosome present in many bacterial species. Such plasmids are usually not essential to the survival of the bacteria, but may contain genes that encode products that are useful to the bacteria. Many plasmids carry one or more genes that confer resistance to various antibiotics. A bacterium carrying such a plasmid can live and multiply in the presence of that antibiotic. Plasmids can be introduced into bacteria if the bacteria are first treated with calcium chloride. Treatment with calcium chloride renders the bacteria competent to take up foreign plasmid DNA. This process (uptake of plasmid DNA) is referred to as **transformation**. As the bacterial population increases, the number of plasmid molecules increases. Following growth of the bacteria in the presence of the antibiotic, the plasmid DNA can be easily isolated from the bacterial culture.

Plasmids are useful tools for the molecular biologist because they can serve as gene-carrier molecules, or **cloning vectors**. A gene of interest is joined to this vector plasmid to from a **hybrid** or **recombinant plasmid** that is able to replicate and be expressed in bacteria. In order to prepare recombinant plasmids, it is necessary to cut the plasmid, and the gene of interest to be inserted, at precise locations and then to join (or ligate) the plasmid and the gene together.

The cutting is accomplished by the use of enzymes called **restriction endonucleases** which recognize a specific sequence of 4-8 nucleotides (a **restriction site**) in the target DNA molecule and cut at specific locations within that site. The restriction site must be present in both the plasmid and the gene to be inserted. The

restriction site of one such enzyme (*Eco*RI) is given below:



Since any given enzyme recognizes and cuts at a unique sequence, the number of cuts in any given DNA molecule is limited. Typically, the restriction sites for a given enzyme are hundreds to thousands of base paris apart. The staggered cut of the enzyme illustrated above generates **sticky, cohesive, single-strand ends**. These are important in recombinant DNA work because they enable any two DNA fragments to be linked together by complementary base pairing at their ends, provided they were generated with the same restriction enzyme. One basic procedure for generating recombinant DNA molecules is illustrated on the next several pages.

A plasmid that carries a gene that renders its host bacterium resistant to some antibiotic is cleaved at a single restriction site by *Eco*RI (Figure 1). The gene to be inserted is also cut with the same enzyme (Figure 2). The resulting fragments are then reannealed by complementary base pairing and the newly formed joints are sealed by the action of another enzyme, namely **DNA ligase**. This enzyme normally performs this fragment sealing operation during DNA replication. Because a mixture of fragments exists, it is possible that some of the plasmids will reanneal without the gene being inserted, while others will contain the foreign gene (Figure 3). The recombinant molecules thus formed are introduced into a suitable bacteria, such as *Escherichia coli*, by **transformation**. The bacteria are then grown in the presence of the designated antibiotic. Those bacteria containing the plasmid (e.g. transformed) will grow, while those not containing the plasmid (untransformed) will be killed by the antibiotic.

In this experiment you will use the plasmid **pUC18** (Figure 1) as a cloning vector in order to amplify segments of the genome of the bacteriophage lambda. Plasmid pUC18 is a small plasmid (2686 bp) that contains an ampicillin-resistance gene. This plasmid also contains part of the **lac Z gene** that encode for the first 146 amino acids of the enzyme **beta-galactosidase**. If the bacteria used for transformation contain the remainder of the gene, **complementation** occurs and the complete enzyme is produced. When the complete enzyme is produced, the bacteria are able to metabolize the enzyme's substrate, **X-gal**. If the nutrient agar on which the bacteria are grown contains X-gal, the bacterial colonies will be **blue**. However, if the inserted lambda gene happens to be inserted in the middle of the lac Z gene, then the active enzyme cannot be produced by complementation, the bacteria will not be able to use X-gal, and the resulting colonies will be white instead of blue (Figure 4).

This exercise will allow you to perform transformation and cloning using materials that have already been prepared for you. *E. coli* cells will have been made competent by treatments with $CaCl_2$. You will receive a sample labeled A, B, or C. One of these will contain the plasmid pUC18, one will contain the plasmid pUC18 into which a foreign gene has been inserted (a fragment of lambda genome), and one will contain no plasmid. Samples will be incubated with the competent *E. coli* and plated onto nutrient agar containing the antibiotic ampicillin and the substrate X-gal. Observation of the colonies which grow on these plates will enable you to correctly identify the three samples. Your identification will be verified by **electrophoresis** in a subsequent lab exercise.

Figure 1. The *Eco*RI Restriction Site in the pUC18 Plasmid Lies Within the Lac Z Gene

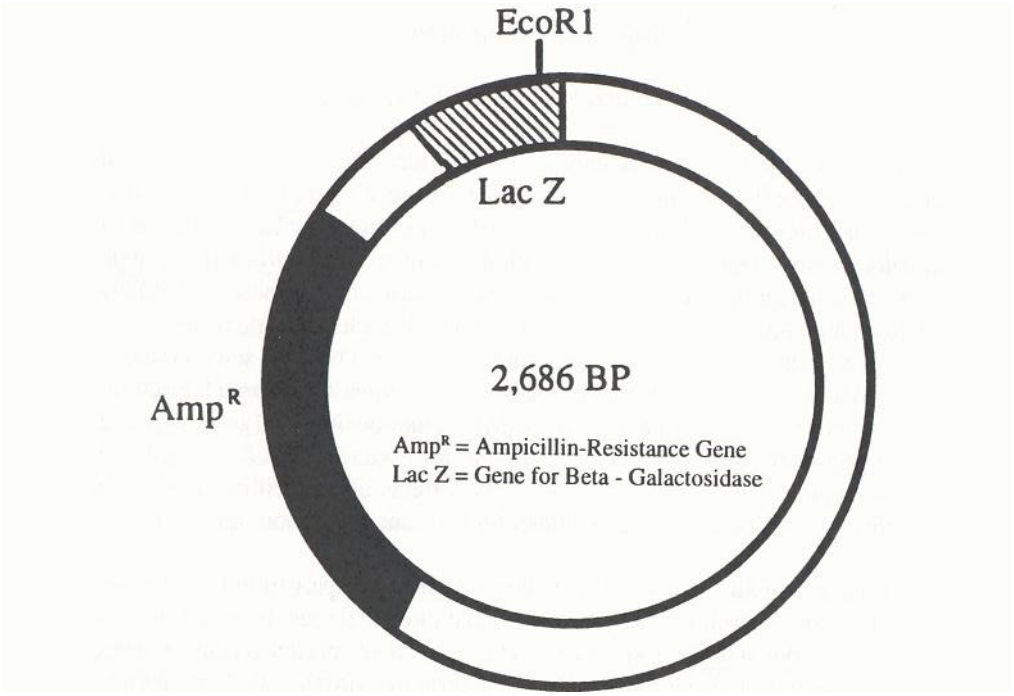


Figure 2. The Five *Eco*RI Restriction Sites in the Lambda Genome and the Resulting DNA Fragments

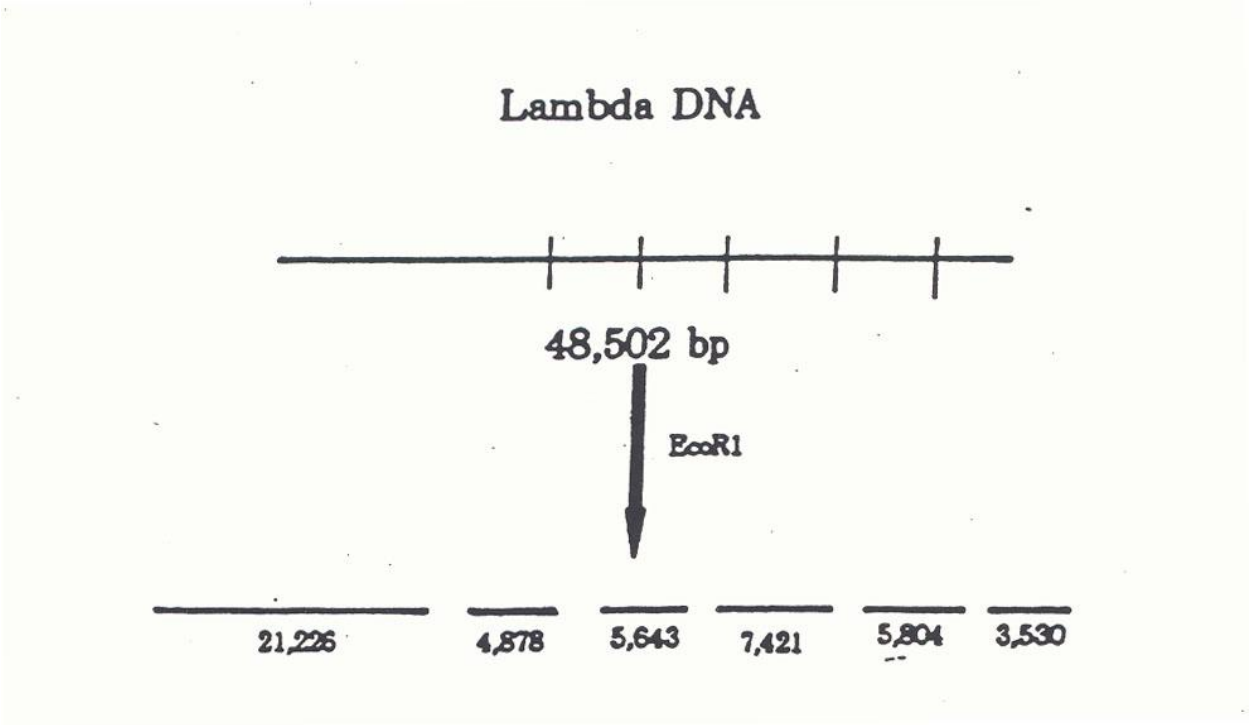


Figure 3. Ligation of Lambda DNA Into the pUC18 Plasmid

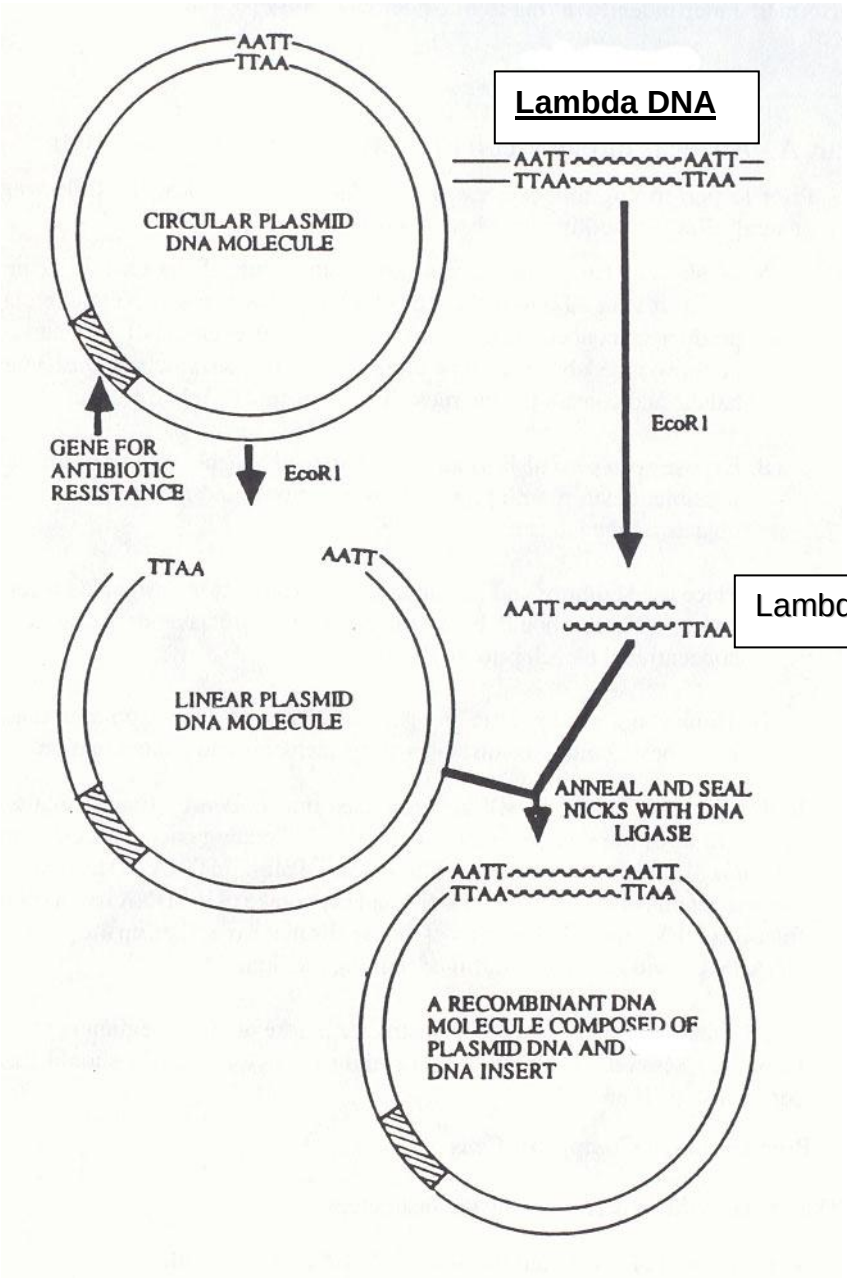
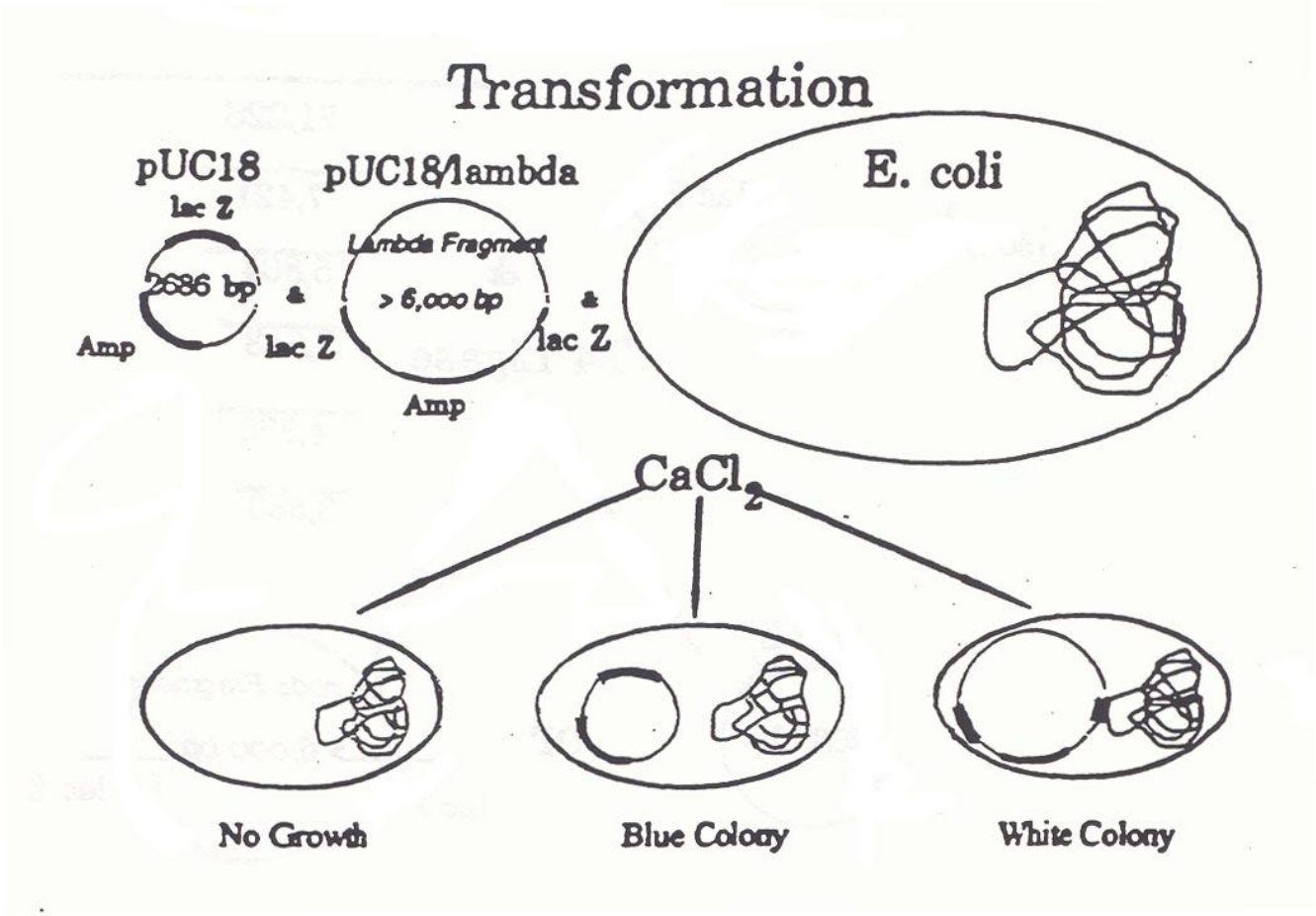


Figure 4. Transformation of *E. coli*



The entire protocol is provided for your reference. **YOU WILL ACTUALLY BEGIN WITH STEP B-3 (**).** Gloves and protective glasses should be worn at all times. **Dispose of all used materials that have come in contact with or may be contaminated by the plasmids and/or *E. coli* (e.g. pipet tips, pipets, microfuge tubes, gloves) in biohazard bags.**

A. Preparation of Competent Cells (*performed prior to lab by your instructor or a lab assistant*)

Procedure

1. Place the vial of $CaCl_2$ and the tube of *E. coli* in an ice bath and invert the tube several times to mix the *e. coli*.

2. Transfer the bacteria to the $CaCl_2$. Transfer 0.5 ml from the vial to the tube and back again.

3. Mix by gentle tapping.

4. Incubate 1 hour or more on ice.

B. Transformation and Incubation *(*you will start at step 3*)

Procedure

1. Obtain three sterile microcentrifuge tubes and label them A, B and C.

2. Using a sterile micropipet, place 10 μl of the sample (A, B or C) into the appropriate tube.

3. Prepare an ice bath using one of the beakers at your bench. Place the tubes containing sample in the ice bath.

4. Using a micropipet with a sterile disposable tip, add 100 μl of competent *E. coli* to each tube. ***Use a new sterile tip for delivery to each tube.*** This will prevent contamination of the *E. coli* stock with plasmid samples as well as contamination between sample tubes. **Dispose of pipet tips in a biohazard bag.**

5. Mix the contents of each tube by gently tapping the tube with your finger. Store tubes on ice for 30 minutes. This allows the plasmid DNA to adsorb to the surface of the *E. coli.*

6. Incubate @ 42 °C in a waterbath (or heating block) for 40 seconds (minimum). This allows the adsorbed plasmid DNA to be transported

across the membrane into the *E. coli*. Then transfer tubes to ice for 2 minutes.

7. Using a sterile 1 ml pipet, add 0.7 ml of nutrient broth (without ampicillin) to each tube and incubate at 37 °C for 30 minutes. Use a new sterile pipet for delivery to each tube. This incubation period allows the bacteria to recover from the $CaCl_2$ treatment and also allows them to begin to express the ampicillin resistance gene (if present). **Dispose of pipets in a biohazard bag.**

8. Obtain 3 plates of nutrient agar containing ampicillin and X-gal. Label the bottom of each plate with date, group ID, and the transformed *E. coli* sample they will receive.

9. Using a micropipet with a sterile disposable tip transfer pipet, transfer 100 μl of sample from one tube onto one of the nutrient agar plates and spread the drop evenly over the agar surface using a bent sterile transfer needle. Repeat this step for the remaiing two samples using a new sterile pipet tip and transfer needle for each sample. **Dispose of used tips and transfer needles in biohazard bags.**

10. Incubate the plates at room temperature for a few minutes until the liquid has been absorbed. **Gloves and any remaining *E.coli* suspension can be discarded in the biohazard bags. Do not throw the glass test tube containing the nutrient browth away – this can be washed and reused.**

11. Invert plates and incubate @ 37 °C for 24-48 hours. Your instructor will place plates in the refrigerator until the next lab period.

12. Examine plates for bacterial growth. Growth will appear as small circular spots, or colonies. Record observations (e.g. note if a plate has bacterial growth; if there is bacterial growth, note the color of the colonies). **Dispose of plates in a biohazard bag.**

ANALYSIS OF PLASMID DNA BY RESTRICTION DIGESTION AND GEL ELECTROPHORESIS

Introduction

Large organic molecules can be separated by a variety of techniques. **Chromatography** makes use of either paper or specially coated glass plates to separate proteins, amino acids, carbohydrates or other molecules with the aid of organic solvents. **Electrophoresis** is a technique that relies on differences in size and/or overall electrical charge to separate molecules in an electric field. It is most commonly used to separate either proteins or nucleic acids (both DNA and RNA). Support media most often used include cellulose acetate, starch agarose, and polyacrylamide. A liquid solution composed of one of these chemicals, when properly treated, will solidify to form a semi-solid support matrix in the shape of a slab known as a gel.

In this exercise, a separation procedure involving agarose gel electrophoresis will be performed to separate DNA fragments. Since all DNA has basically the same overall charge, the gel electrophoresis used here will essentially separate fragments based on differences in size. The agarose gel, in this case, acts like a molecular sieve. Smaller molecules will be able to travel more quickly through the gel. As electrophoresis continues, smaller molecules will be located towards the bottom of the gel, while larger ones will be found closer to the loading wells.

The fragments to be analyzed by agarose gel electrophoresis are the following: 1) the plasmids (A, B, and C) used in the Transformation and Cloning exercise that have been digested with the restriction enzyme *Eco*RI, 2) lambda genome DNA that has been digested with *Eco*RI, and 3) a mixture of DNA fragments of known size (known as molecular weight standards). Electrophoresis of these samples will allow you to confirm your tentative sample identification from the previous week's lab exercise, as well as identify the specific lambda fragment contained in the recombinant plasmid. For example, the sample with no plasmid should produce no DNA fragments upon digestion, whereas the sample containing only pUC18 should produce a single DNA fragment of 2686 base pairs because the plasmid has a single cut site for *Eco*RI. The closed circular form will be converted to a linear form by enzymatic cleavage. Identification of a plasmid prep as buffer, the pUC18 plasmid, or a recombinant pUC18 plasmid can be based on the number of digestion products/fragments obtained and their size. By including in the electrophoretic run a set of molecular weight standards, it should be possible to determine the size of any fragment detected. The specific lambda fragment contained by the recombinant plasmid can be determined by comparing the observed size of this fragment to the six that are possible when the lambda genome is digested by *Eco*RI (see **Figure 1**). Identification of this lambda fragment can also be determined digesting and electrophoresing lambda DNA and comparing the mobility of the cloned fragment to those in the Lambds digest. The fragment contained in the recombinant plasmid should co-migrate with only one fragment in this lambda digest (see **Figure 2**).

Figure 1. The Five *Eco*RI Restriction Sites in the Lambda Genome and the Six Resulting DNA Fragments

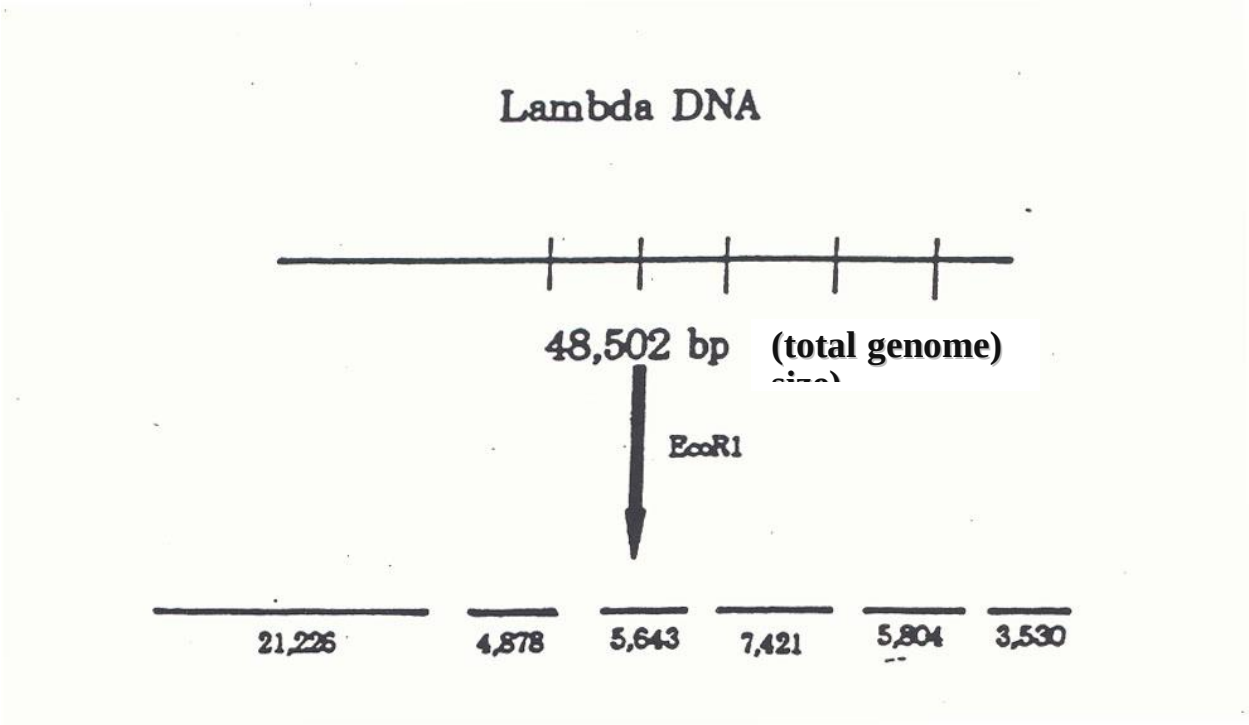
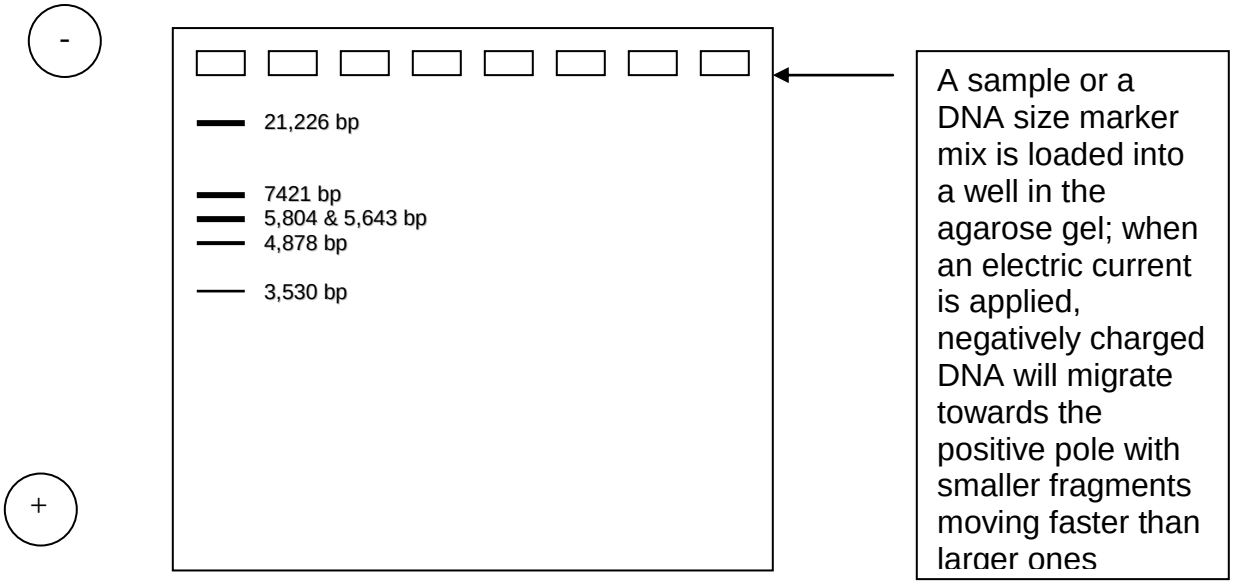


Figure 2. Electrophoretic Analysis of *Eco*RI Digested Lambda DNA. Note that six fragments are generated, but usually 5 are detected. The 5,804 and 5,643 bp fragments often can not be resolved from one another under the conditions used.



Procedure

A. Sample and Gel Preparation (Week 1)

Sample Preparation (restriction digestion of your sample and lambda DNA)

1. You will be given 4 microfuge tubes. Label tubes A, B, C, and lambda (or λ). Also label each tube with a group ID.

2. Add 10 µl of *Eco*RI to each tube. A single pipet tip can be used to deliver this enzyme to your tubes. When you have finished adding *Eco*RI to your tubes, discard the pipet tip into a waste beaker located at this station.

3. Add 5 µl of the corresponding DNA sample to the tubes (deliver the DNA sample *into* the EcoRI solution and mix sample and enzyme using the pipet (slowly draw liquid up and expel six times). *Use a new pipet tip for each DNA sample.* Discard used tips into a waste beaker at this station.

4. Place tubes in an incubator or heating block set at 37 °C. Incubate for 90 minutes (or longer) to ensure that all sample DNA is digested by *Eco*RI.

5. Add 5 µl of electrophoresis sample buffer (blue in color) to each tube. Gently mix tube contents by gently tapping the tube with a finger. Use a new pipet tip to deliver sample buffer to each digested plasmid sample. Discard used tips into a waste beaker at this station.

6. Immediately give your tubes to your instructor; he/she will place them in a freezer until next week.

Preparation of an Agarose Gel

1. Place your gel mold into a casting tray with the neighboring lab group. Orient the gel molds such that the open ends of the tray are facing the rubber gaskets that line the two sides of the casting tray. Turn the knob at the top of the casting tray to seal the ends of each tray with the rubber gaskets.

2. Place two combs in your mold – one at an end of the gel mold and the other in the middle of the mold. There will be groves on the gel mold for the combs to fit into.

3. Prepare 40 ml of a 0.8 % agarose solution as follows: Weigh 0.32 g of agarose into a weigh boat and transfer this to a 250 ml Erlenmeyer flask. If the available balance has a maximum weighing capacity that will allow you to do so, you can weigh the agarose directly into the flask. Use a 50 ml graduated cylinder to measure 40 ml of electrophoresis buffer (Tris-acetate-

EDTA, pH 8.0) and add this volume to the flask containing the agarose. Swirl contents briefly to mix. Note: The agarose will not dissolve.

4. Gently stuff a clean KimiWipe into the mouth of the flask. You will dissolve the agarose by heating it in a microwave. Begin by heating for 30 seconds. Remove and swirl flask contents – note that the agarose has not completely dissolved. Heat the solution again for 15 seconds. Watch flask carefully and stop the microwave when solution begins to boil violently and upward out of the flask. Remove from microwave and carefully observe solution - no solid particles should be visible. If necessary, heat again for 15 seconds. NOTE: If two flasks are heated at the same time, a *minimum* of three 30 second heating periods may be needed to melt the agarose.

5. Allow gel solutions to cool for 5 minutes. (NOTE: To determine if the melted solution is cool enough to pour, touch the bottom of the flask to inside of your wrist – when it is hot (just uncomfortable, but not yet not "warm") to touch the solution is ready to pour; Do not pour an agarose solution that has begun to solidify into a gel mold)

6. Your instructor *MAY* decide to add ethidium bromide (EtBr) to your gel solution at this time. The final EtBr concentration in the gel solution should be 0.5 µg/ml. For example, if a 10 mg/ml ethidium bromide stock is used, then 2 µl of this stock solution would be added to your 40 ml of agarose solution. Swirl flask carefully to mix. Note: Ethidium bromide is used to visualize DNA in agarose gels; it will bind to DNA during electrophoresis and allow you to visualize the DNA as fluorescence under UV illumination.

7. Pour the liquid agarose solution into the gel mold.

8. Allow the solution to cool and solidify until it is firm (about 30 minutes).

9. Remove mold with gel from the casting tray. Remove combs, rinse and leave them at your bench. DO NOT TRHOW COMBS AWAY. Your instructor will place the mold with gel into gel storage box containing a small amount of tank buffer. You will use this gel next week.

B. Analysis of DNA Digests Using Agarose Gel Electrophoresis (Week 2)

1. Your instructor will remove your digested samples from the freezer and return them to you. Allow samples to thaw on ice.

2. Place a gel tray (containing an agarose gel) into the gel tank so that the wells in the gel are oriented at the cathode end of the gel tank (the end with the black electrical connection).

3. Fill the buffer reservoir with TAE buffer, covering the gel to a depth of about 2 mm (i.e. the gel is *just* covered with buffer). If combs are still in the gel, remove them carefully by grasping at both sides and pulling straight up.

4. *As soon as samples have thawed*, load 15 - 20 µl of each into the wells of the agarose gel. NOTES: Use a new pipet tip to load each sample; You *should* have close to 20 µl of each digest – load the entire sample; Dispose of tips in a biohazard bag or waste collection beaker at your bench. You will load 4 different sample digests - A, B, C, and lambda (all prepared during the previous lab period). Make a note as to the well that each sample was loaded into.

5. Load 15 µl of a DNA marker mix into a separate well (use a new pipet tip). The DNA marker mix will contain several (4) different DNA fragments of known size: 3621, 2040, 1120, and 784 bp. Make a note as to the well that the DNA marker mix was loaded into.

6. Place the tank top on the gel tank and press it down in order to connect electrode wires to electrodes. NOTE: Wells of the gel should be oriented towards the black (negative) electrode; Match the black electrode wire with the black electrode)

7. Plug the gel rig electrode wires into the power unit (match electrode wire colors with receptacle color – red with red and black with black). Turn power unit on. Select constant voltage and set display to 100 volts. Press the button with the runner symbol. If the rig was set up properly, as soon as you push the "runner" button you should begin to see small bubbles arise from the thin silver wire attached to the electrodes (the wire is covered with electrode buffer and runs across the width of the gel rig at each end).

8. Perform the electrophoresis at 100 volts until the blue tracking dye has migrated to within ½ cm of the end of the gel/ next set of wells (approximately 50 - 60 minutes).

9. Wearing gloves, remove the gel from the electrophoresis unit and slip the gel onto a sheet of plastic wrap. Analyze the gel on an ultraviolet transilluminator. BE SURE TO WEAR PROTECTIVE EYEWARE WHEN USING UV LIGHT. Ethidium bromide attaches to DNA and causes it to fluoresce under UV radiation. DNA should be visible as distinct fluorescent bands. Each band represents a different DNA molecule. *NOTE: If you did not add ethidium bromide (EtBr) to your melted agarose solution when preparing the gel, you will need to soak your gel for 10 minutes in an EtBr solution prior to examination.*

10. Use a metric ruler to measure the distance migrated by each fragment in the standard mix. Do this by measuring the distance between the bottom of the loading well to the leading edge of a band. *Note: record distance in mm*.

11. Next, record the number of bands detected in samples A, B, and C. Also measure and record the distance migrated by each band you recorded for these samples.

12. Sample B should contain two fragments. Which band is likely to be the plasmid? Explain the basis for your answer. Which band is likely to the portion of the lambda genome inserted into the recombinant plasmid (i.e. which band represents the cloned Lambda insert/fragment)?

13. NOTE which band in the Lambda digest your cloned Lambda fragment (identified in step 12 above) co-migrated with. Refer to Fig. 2 on page 56 for an illustration of the digestion products/fragments you can expect to see in your Lambda digest. This information is needed to identify the fragment you cloned.

14. Clean your bench area as follows: dispose of your gel in the ethidium bromide waste container; carefully pour the electrophoresis buffer into the waste bottle provided; discard plastic wrap and gloves in the regular trash; if you haven't done so already, discard any unused plasmid sample in a biohazard bag; return any unused DNA standard mix to your instructor.

15. Construct a standard curve from the DNA fragments in the molecular weight standard sample using Microsoft Excel. Plot the distance migrated by a fragment (x axis) vs. the $\log_{10}$ equivalent of the fragment's size/length (y axis). Note: the molecular weight standard mix contains 4 fragments: 3621, 2040, 1120 and 784 bp in size. Include a trendline with line equation. Don't forget to number the figure, title the graph, include axis labels and units, and a caption. In addition, include your and your lab partner's names somewhere on the graph.

16. To use the line equation from your standard curve, you will plug in the distance migrated by a band/fragment as the x value. Note that when you solve for y, you now have the $\log_{10}$ equivalent of the fragment's size. You must take the anti-log of this value to obtain the size of the fragment in bp. Do this for each fragment detected in sample A, B, and C.

17. What is your estimate of size for the pUC18 plasmid? Does it match the expected value of 2686 bp for pUC18?

18. What is the estimated size of the lambda DNA fragment contained in the recombinant plasmid? Compare this estimated size with the sizes of the six that are possible (consult Figure 1). Based on this estimate of size alone,

which fragment in the Lambda genome was contained in the recombinant plasmid?

19. In step 13 above you were asked to compare the migration of Lambda insert (i.e. the Lambda fragment contained in the recombinant plasmid) with the fragments detected in your Lambda digest. Which band in the Lambda digest did the insert co-migrate with? How does this compare to your answer for step 15? If there is a discrepancy, which observation do you have more confidence in and why? So, which *Eco*RI digestion prodect of the Lambda genome did you clone?

20. Sample B should have contained two bands/DNA fragments, however addition bands may have ben detected. Expalin what are these additional DNA fragments could be.