

EXPERIMENT 1: CREATING RECOMBINANT DNA MOLECULES

DAY ONE: INTRODUCTION TO EQUIPMENT AND RECOMBINANT DNA

OBJECTIVES:

Today's laboratory will introduce you to some of the basic equipment used in molecular genetics research (i.e. micropipettors, microcentrifuges, and vortexers) and how to create a recombinant DNA molecule. By the end of today's laboratory you should be able to:

- Accurately use a micropipettor to measure and dispense volumes ranging from 1 μ L to 1 mL.
- Safely operate a microcentrifuge and vortexer.
- List some of the practical applications for recombinant DNA molecules
- List the qualities that make a good recombinant DNA vector
- Explain how DNA vectors are "cut open" to receive foreign DNA molecules
- Explain how recombinant DNA vectors are "pasted shut" once foreign DNA molecules have been incorporated
- Perform a ligation reaction (plasmid vector and insert DNA) to create recombinant DNA

INTRODUCTION:

1. EQUIPMENT

Molecular geneticists (and molecular biologists in general) often work with very small volumes, and special equipment and supplies have been created specifically for this purpose. A whole new set of equipment and plasticware has grown out of molecular biology and biotechnology explorations. These include **micropipettors**, which deliver down to 1/1000 of a ml (1 μ L) and **microcentrifuge tubes**, which hold a total volume of 0.2- 2.0 mL for reactions and centrifugation. Other commonly used equipment includes **vortexers**, for rapid mixing of small volumes, and **microcentrifuges** for rapid separation of small quantities of cells and large molecules.

Below is some information that you may find useful as a reference when pipetting small volumes of liquid with micropipettors.

1. VOLUME CONVERSIONS:

$$1\text{L (liter)} = 10^3 \text{ mL (milliliter or "mil")} = 10^6 \mu\text{L (microliter)}$$

2. VOLUME RANGE OF MICROPIPETTORS:

Micropipettor Size	Volume range
p20	1-20 microliters
p200	20-200 microliters
p1000	200-1000 microliters

2. RECOMBINANT DNA TECHNOLOGY

Recombinant DNA technology is a set of tools that allows molecular biologists to create novel DNA molecules that do not exist in nature, and to produce large quantities of such "recombinant DNAs" for research and therapeutic purposes. The basic tools, which include bacterial **plasmid "vectors"**, **restriction enzymes**, and **ligases**, were developed by Stanley Cohen and Herbert Boyer in the early 1970s. Boyer (who is now a multi-millionaire) then went on to become one of the founders of Genentech, the first company to apply recombinant DNA technology, and the company that is credited with starting the biotechnology "boom". In 1978, Genentech made the first important contribution of recombinant DNA technology to medicine: the company succeeded in cloning the human insulin gene into a bacterial plasmid vector that allowed the human insulin protein to be "grown up" in *E. coli*. This accomplishment provided an important breakthrough in treating diabetes, since isolating insulin protein from the pancreases of slaughtered cows was laborious and gave rise to a product that had to undergo significant modifications before it could be injected into patients without subsequent immune rejection. In 1982, recombinant human insulin, or *humulin*, became the first recombinant DNA product to receive FDA approval.

The basic process by which recombinant DNA molecules are made is quite simple. First, it is important to have a **vector** for carrying the gene of interest. The vector allows the foreign gene to be replicated, transcribed, and translated into a protein by bacteria (usually *E. coli*). Once the bacteria have produced the protein, it is usually an easy matter to lyse open the cells and isolate the protein of interest in large quantities and in a pure form.

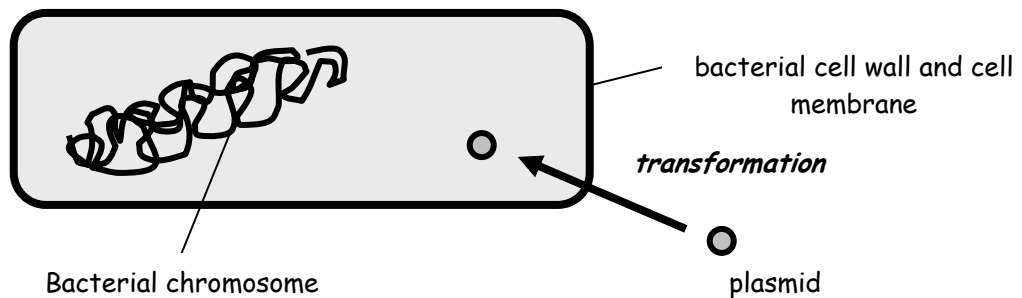
Escherichia coli is an ideal organism for growing up recombinant DNA molecules because it is easy to culture. As a prokaryotic cell, *E. coli* represents the simplest free-living assemblage capable of metabolizing, synthesizing and reproducing independently of other organisms. It is a rod-shaped cell with about 1/500th the volume of a typical eukaryotic cell. Like other bacteria, it has an outermost wall of carbohydrate and protein. The specific composition of some bacterial cell walls allow them to be stained (Gram-positive) or not (Gram-negative) by a combination of compounds formulated by the microbiologist Gram. Both Gram-positive and Gram-negative bacteria have a plasma membrane of lipid and protein inside the cell wall, and surrounding the cytoplasm. *E. coli* is a Gram-negative bacterium.

Within the cytoplasm there are no membrane-bound organelles; the relatively small volume in which cellular molecules interact makes internal organizing membranes dispensable. Yet, all the basic life machinery is present. Most noteworthy, there is a single, circular, double-stranded DNA molecule, which carries the bacterium's genetic information, there are thousands of ribosomes, which serve as the workbenches for protein synthesis, and there are infoldings of the plasma membrane, which organize the components of a cellular respiration system. Aside from these common structures, different species of bacteria differ in what proteins they can produce and what nutrients and other aspects of the environment they can utilize. *E. coli* is a rather versatile bacterium that can grow at a range of temperatures (optimum is body temperature, 37 degrees C), utilize a variety of sugars as carbon sources, and manufacture most of the other organic molecules it requires if provided with sugar, salts, and water.

At first *E. coli* was used in studies because, as a normal inhabitant of human intestines, it was easy to isolate and not especially infectious or pathogenic (although, *E. coli* is the most common cause of "food poisoning" and should be handled with caution). Later it was found to be suitable for genetic

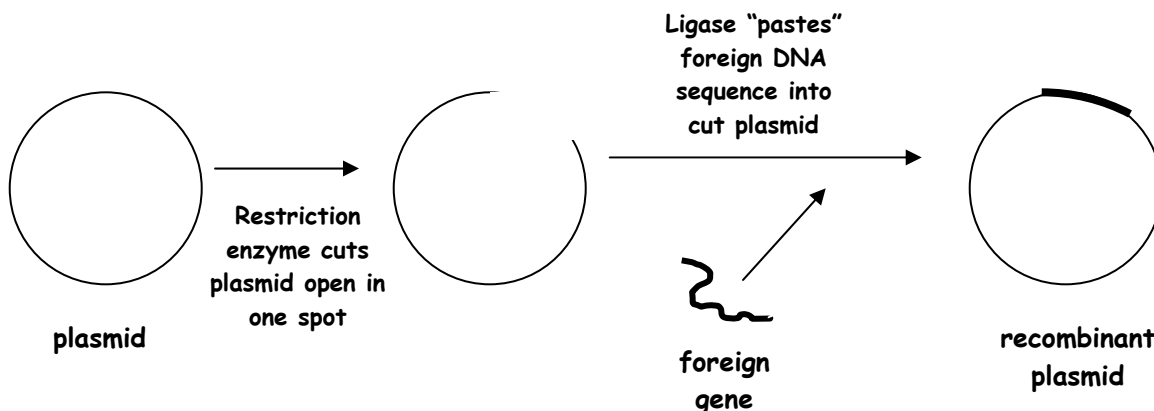
studies because it is easily grown through asexual cycles in as little as 20 minutes, generally has just one gene per trait, can have colonies representing over 100 individuals displayed on a single Petri plate, can participate in gene exchange by conjugation, transformation, and transfection, and serves as host to numerous viruses and plasmids. As it turns out, plasmids (as well as viruses) make excellent vectors for carrying foreign genes into bacteria, so *E. coli* was an obvious choice as an experimental organism to Cohen and Boyer.

Plasmids are small double-stranded circles of DNA, which are carried by some bacteria in addition to their larger circular genome. In nature, plasmids carry extra information not normally required for survival of the cell, such as genes for antibiotic resistance or toxic proteins that enable the bacterium to better invade its host. In addition, they are **self-replicating**. Moreover, many bacteria will "take up" plasmid DNA from the surrounding environment through a process called **transformation**. Together, these characteristics of plasmids make them ideal as vectors for recombinant DNA technology.



Producing a recombinant protein like humulin involves several steps:

1. **A bacterial plasmid is manipulated with enzymes outside the cell in a test tube.** First, a double stranded break in the plasmid DNA molecule is made using a **restriction enzyme**. Next, the gene of interest (e.g. human insulin) is introduced to the test tube, along with an enzyme called **ligase** that seals the DNA molecule back together. The resulting molecule is called a **recombinant plasmid**.



2. **The recombinant plasmid is introduced into *E. coli* cells by transformation.** Some bacterial cells take up plasmids naturally, but *E. coli* must be "coaxed" to do so. Treatment of the cells

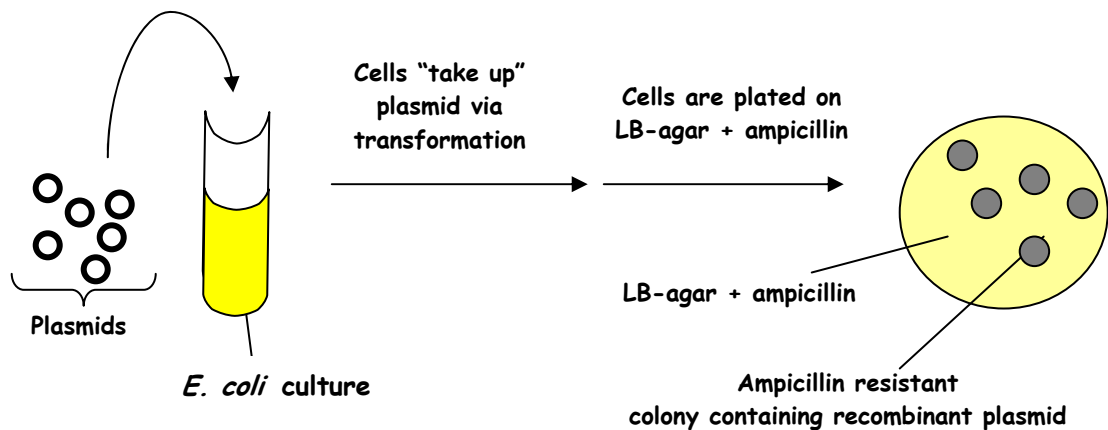
with calcium chloride followed by a brief heat shock usually leads to acceptable levels of transformation.

3. **As the transformed *E. coli* cells replicate, the plasmid is also replicated and the recombinant protein is produced.** Thus, a large amount of the desired protein is produced as the *E. coli* culture grows.
4. **The cells are lysed open and the protein of interest is purified.** Usually, this step involves some type of affinity or column chromatography that allows a pure preparation of the protein to be obtained.

There are many plasmid vectors that are commercially available. All are derived from plasmids that were originally isolated from bacterial cells in nature. However, commercial cloning plasmids differ from natural plasmids because they have been modified in clever ways that make them particularly useful for the creation of recombinant DNA molecules.

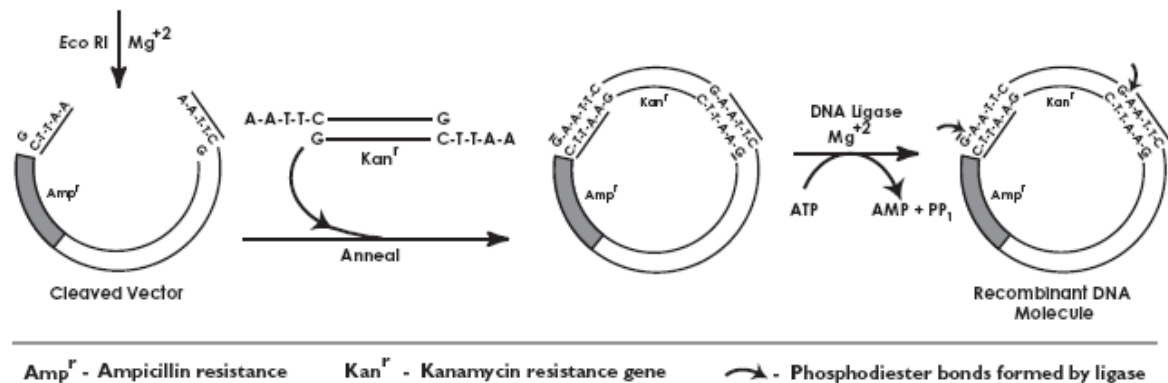
All commercial cloning plasmids have at least 3 components in common:

- a. **An origin of replication (*ori*).** This DNA sequence ensures that the plasmid will be recognized by the bacterial replication machinery and replicated along with the bacterial chromosome. The plasmid remains an autonomous unit and generally replicates to very high copy number within a single bacterial cell (1,000 or more copies/cell).
- b. **A selectable marker.** Even though the efficiency of transformation can be greatly increased by pre-treating the cells (as described above) the efficiency is still low. The best methods yield approximately one transformant per 1,000 cells. Because of this limitation, the plasmid must carry a *selectable marker* that allows only those cells that actually pick up the plasmid to be *selected* for against the background of all the cells that did not. The most common selectable markers are antibiotic resistance genes. For example, many commercial cloning plasmids contain the **amp^r (ampicillin resistance) gene**. After the bacterial cells are transformed with the plasmid, the cells are grown on media containing ampicillin. All cells that failed to take up the plasmid (the vast majority of cells) will die. Only those cells that were actually transformed will grow (i.e. are *selected*) by this method.



- c. **A multiple cloning site.** Restriction enzymes will only cut DNA at specific sites. For example, the restriction enzyme "*Eco RI*" cuts DNA only at the sequence: 5'-GAATTC-3'. (Note the complementary strand will have the same sequence with the opposite polarity.) A multiple cloning site contains the sequences for several different restriction enzymes, giving the researcher flexibility in his/her experimental strategy.

In today's experiment, you will be creating a recombinant DNA molecule by performing a ligation using a "cut" plasmid (previously digested with the restriction enzyme *Eco RI*) and insert DNA (with complementary *Eco RI* "sticky ends"). The insert DNA contains a kanamycin resistance gene which will enable us to select for bacteria that have been transformed with the recombinant molecule by identifying colonies that are resistant to the antibiotic kanamycin.



THINGS TO DO:

Part I. Equipment

1. Practice using the micropipettors as directed by your instructor. The steps are listed below for your reference.
 - a. Place a tip of the appropriate size on the end of the micropipettor. The p20 and p200 micropipettors use the smaller (yellow or white) tips. The p1000 uses the larger (blue) tips.
 - b. Use the dial on the micropipettor to set the desired volume.
 - c. Depress the plunger until initial resistance is met.
 - d. Place the tip below the surface of the liquid being pipetted.
 - e. Slowly release the plunger, *making sure that the tip remains below the surface of the liquid.*
 - f. Remove the tip from the liquid. The volume of liquid being pipetted should now be inside the tip. (Always check visually to make sure this is the case.)
 - g. Place the tip in the container (e.g. microfuge tube, plate) into which the liquid is to be transferred.

7. Add 2 μL of 6x gel loading buffer to your "V/I" tube for agarose gel electrophoresis on Day 4 for us to examine the pre-ligation mixture (Why do we consider this a pre-ligation mixture?). Your instructor will store this sample in the freezer.

DAY TWO: TRANSFORMATION OF RECOMBINANT DNA INTO HOST BACTERIA

OBJECTIVES:

Today we will be transforming *E. coli* bacteria with the ligation mixture that was prepared last lab and plating these *E. coli* on kanamycin agar plates to select for transformants that contain recombinant DNA molecules. By the end of today you should:

- ❑ Know how to carry out a transformation of *E. coli* cells
- ❑ Know how to use sterile technique to spread cells onto agar plates

THINGS TO DO:

1. Label a new 1.5 mL microcentrifuge tube as "DLR DNA" (=Diluted Ligase Reaction DNA). Add 18 μL of distilled H_2O to this "DLR DNA" tube.
2. Take your T4 DNA Ligase reaction tube ("Lig") that was incubating in the refrigerator and remove 2 μL and dispense into "DLR DNA" tube. Mix contents in the "Lig" tube by gently pipetting up and down and then briefly pulse in a microcentrifuge (~5 sec) to collect the solution at the tube bottom.
 - ✓ The ligase reaction is diluted prior to transformation because transformation works best when the concentration of plasmid is relatively low. At high concentrations of plasmid, the efficiency of transformation decreases for reasons that are not yet entirely clear.
3. Add 2 μL of 6x gel loading buffer to "Lig" tube. Mix and set aside for agarose gel electrophoresis on Day 4 for us to examine the post-ligation mixture. Your instructor will store this sample in the freezer.
4. Obtain two 1.5 mL microcentrifuge tubes from the ice bucket located at the instructor's lab bench. Each of these tubes contains 250 μL of ice cold 0.05M CaCl_2 . Label one of the tubes "LigT" (for ligation transformation) and the other "ConT" (for control transformation). Be sure to also label each tube with your initials.
5. With a sterile loop, transfer 2 well isolated colonies from the plate labeled "HB101" to the tube "LigT". Be sure to twist the loop vigorously between your fingers to dislodge cells. Pipet up and down gently to resuspend and mix cells (should not see clumps).
6. With a new sterile loop, repeat step 5 above with the "ConT" tube.
7. Add 5 μL of diluted ligase reaction DNA from "DLR DNA" to the tube labeled "Ligation". Tap the tubes with your finger to gently mix.

8. Add 5 μL of supercoiled control DNA ("scDNA") to the tube labeled "ConT". Tap the tube with your finger to gently mix. The control DNA is a supercoiled plasmid that contains the kanamycin resistant gene. (Is this a "positive" or a "negative" control? Why do we include it?)
9. Incubate the tubes on ice for 15 min.
10. Place tubes in a 42°C waterbath for 90 seconds to heat shock the cells. This step facilitates the incorporation of the recombinant DNA into the bacterial cells.
11. Immediately plunge tubes into ice and incubate for 2 minutes. Then add 250 μL of Recovery Broth ("RB"; found in the 37°C water bath) to each tube and mix. Incubate tubes for 15 min in the 37°C waterbath for a recovery period.
12. Microcentrifuge the tubes for 2 min at high speed to pellet the cells. Remove and discard 400 μL from each tube and resuspend pellet in the remaining 100 μL solution.
13. Obtain 2 kanamycin agar plates and label them "Ligation" and "Control", and also with your lab section and initials. Pipet 100 μL of solution from the transformation tubes to the corresponding plates. Using a sterile spreader (blue) for each plate, spread the solution containing the cells evenly and thoroughly over the entire surface. Turn the plate 90° and thoroughly spread again.
14. After covering the plates, allow the liquid to be absorbed (do not turn upside down). The instructor will place your plates into the walk-in incubator set for 37°C for overnight incubation. Afterwards the plates will be stored at 4°C .

DAY THREE: EXTRACTION OF RECOMBINANT PLASMIDS FROM TRANSFORMANTS

OBJECTIVES:

Today we will be extracting the plasmid DNA from the transformed *E. coli* bacteria so that we can analyze whether we created a recombinant plasmid in the next lab period. By the end of today you should:

- Know how to extract plasmid DNA from *E. coli* cells
- Know how to ethanol precipitate DNA from aqueous solutions

THINGS TO DO:

1. A single, well isolated colony from the kanamycin agar plates was inoculated into medium yesterday and grown overnight for you to extract the plasmid DNA today in lab. The instructor will provide you with a tube of cultured transformed bacteria that has been pelleted and resuspended in 100 μL of a glucose/Tris/EDTA solution (GTE).
2. Resuspend the cells in the culture tube by tapping. Add 200 μL of fresh NaOH/SDS cell lysis solution. Close cap and mix solutions by rapidly inverting tube 5 times. Incubate on ice for 5 min.

3. Add 150 μ L of ice-cold potassium acetate solution. Close the cap, and mix solutions by rapidly inverting tube 5 times. A white precipitate should appear. Incubate on ice for 5 min.
4. Microcentrifuge for 5 min at full speed (Note- balance centrifuge with another lab group's tube). Transfer 400 μ L of the supernatant to a new 1.5 mL microcentrifuge tube (**label** the tube). Be sure to avoid pipeting the white precipitate and wipe off any precipitate clinging to outside of tip prior to expelling supernatant. Discard old tubes containing precipitate.
5. Add 400 μ L of isopropanol to the supernatant tube. Close the cap, and mix solutions by rapidly inverting tube 5 times. *Let stand at room temperature for only 2 min* (proteins may precipitate with the DNA if allowed to incubate longer).
6. Microcentrifuge for 5 min (14,000 rpm). Align tube in rotor so that cap hinge points outward (remember to balance the centrifuge). The nucleic acid residue, visible or not, will collect on the tube side under hinge during centrifugation.
7. Pour off supernatant from the tube (be careful not to disturb the nucleic acid pellet). Invert the tube and tap gently on surface of a clean paper towel to drain thoroughly.
8. Add 200 μ L of 100% ethanol to tube and close tube. Flick tubes several times to wash pellets.
9. Centrifuge the tube at full speed for 5 min. Pour off supernatant from the tube (be careful not to disturb the nucleic acid pellet). Invert the tube and tap gently on surface of a clean paper towel to drain thoroughly.
10. Keep the lid off your tube and place it in the rack provided by the instructor to allow the pellet to air dry. Instructors will add 15 μ L of TE buffer (Tris-EDTA) to dissolve pellet before the next lab period.

DAY FOUR: RESTRICTION ENZYME ANALYSIS AND GEL ELECTROPHORESIS

OBJECTIVES:

Today we will be performing a restriction enzyme digestion on the plasmid DNA you extracted from the transformed *E. coli* and then analyze the plasmid DNA by agarose gel electrophoresis to determine if your experiment worked. By the end of today you should:

- Know how to perform a restriction enzyme digest
- Understand the basis of agarose gel electrophoresis and ethidium bromide staining of DNA
- Know how the molecular weight of the DNA on a gel can be estimated
- Know how to safely carry out electrophoresis and detection of a DNA sample
- Understand why restriction digestion and ligation will change the mobility of a DNA molecule on a gel

INTRODUCTION:

Another important piece of equipment used by molecular biologists is the **electrophoresis apparatus** (commonly called a "gel rig"). Electrophoresis refers to any separation of molecules in an electrical field; the molecules are usually placed in a solid supporting matrix, like acetate strips, paper, or gels. Gels of agarose and polyacrylamide are used for the separation of nucleic acid molecules because they contain microscopic pores that differentially impede molecules of different sizes in the size range of nucleic acids. Polyacrylamide forms smaller pores than agarose, and higher concentrations of each substance create smaller pores than lower concentrations. As the size of the pores gets smaller, molecules of smaller size (containing fewer nucleotides) can be separated.

Very small volumes of DNA samples are placed in wells at one end of the gel. The gel is submerged in a buffer, which carries the electric charge and dissipates heat around the gel. When an electric charge is applied, the molecules migrate through the gel based on size and charge. Since nucleic acids have a net negative charge from their phosphate groups and a uniform negative charge distribution along the entire molecule, they will move toward the positive electrode based primarily on size. Larger molecules will be impeded more by the gel's pores and will thus migrate more slowly. Circular molecules of the same molecular weight (i. e., same number of nucleotides) may migrate at different rates if they are supercoiled (twisted; imagine "winding-up" a rubber band by holding one end and rolling the other end between your thumb and fingers) or relaxed (somewhat uncoiled due to a break in one of the two strands).

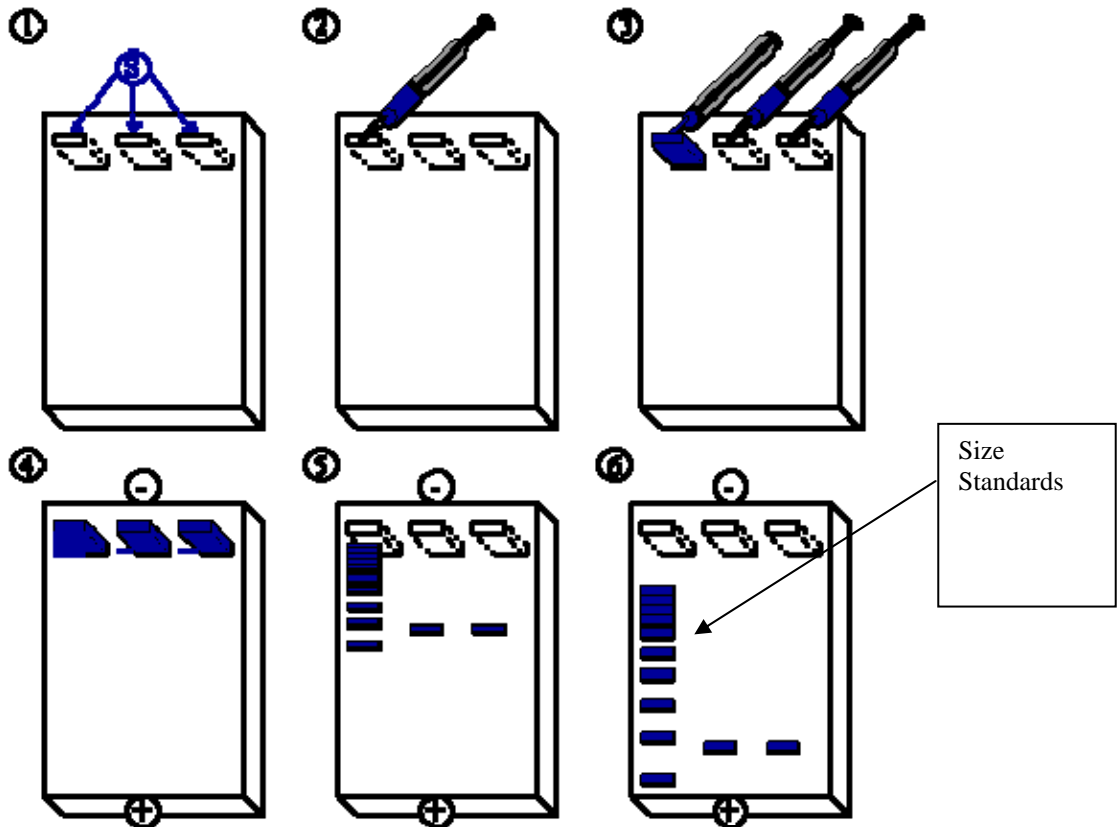


Figure 3-1. Agarose gel electrophoresis of DNA. (1) Wells are created in the agarose slab by the gel comb as the agarose cools and solidifies. (2-4) DNA samples are loaded into the wells sequentially using a micropipettor. (5-6) When the power supply is turned on, the DNA samples move into the gel toward the positive end and separate from one another according to size. During the actual gel run, the DNA fragments will not be visible, but they can be seen after ethidium bromide staining of the gel when exposed to UV light.

When the gel is removed from the electric field (the electrophoresis chamber), the molecules will be present in a lane extending from the sample well, but they will not be visible. One way to make nucleic acids visible is to treat the gel with an intercalating dye that fluoresces when exposed to ultraviolet (uv) light. When a dye like ethidium bromide (EtBr) is used, the DNA molecules show up as orange fluorescent bands crossing the lanes.

THINGS TO DO:

1. Obtain two Eco RI reaction buffer tubes (4 uL in each) from the instructor's lab bench. Label one tube "D" for digestion and the other "C" for control (label with your initials also). Obtain your resuspended recombinant plasmid DNA (from last lab period) and then add 5 uL recombinant DNA to each tube.
2. Add 1 uL of Eco RI enzyme from the ice bucket located at the instructor's lab bench to your tube "D". Add 1 uL of distilled H₂O to your tube "C". Mix contents in the tubes by pipetting up and down and then briefly pulse in a microcentrifuge (~5 sec). Incubate tube for 20 min at 37°C.
3. Prepare an agarose gel by performing the following steps (Note: wear gloves and be cautious since you will be working with Ethidium Bromide- a known mutagen):
 - a. Get a gel casting tray, a gel comb, and a gel electrophoresis rig and rig cover.
 - b. Insert the comb into the casting tray as demonstrated by your instructor. Be sure to insert the comb using the thicker side down to create larger wells.
 - c. Place the casting tray into the gel rig so that the rubber washer strips are pressed up against the sides of the rig to create a tight seal. Make sure the rig is on a flat surface before you add the agarose.
 - d. In the area designated for gel casting, pour about 50 ml of melted 0.8% (8g/100 ml) agarose (Hazard: contains ethidium bromide mixed into agarose) into a graduate cylinder. Return the agarose stock to the water bath and pour the measured agarose into the casting tray. In about 15 minutes, the agarose will turn grey and translucent signifying that it has hardened.
 - e. When the gel is firmly set, remove the comb by gently lifting it *straight up* (avoid breaking the walls of the sample wells).
 - f. Pour agarose gel buffer (TBE) over the gel until the buffer chambers are filled and the buffer covers the gel with a little room to spare. The wells should be completely filled with buffer when you are done.

4. Retrieve the samples listed below from the lab instructor's bench:
 - λ HindIII DNA standard fragments (labeled "Std")
 - Supercoiled Recombinant plasmid (labeled "scDNA") [positive control]
 - Your tube "V/I" from Day 1 (= pre-ligation control)
 - Your tube "Lig" from Day 1 (= ligation rxn)
5. Heat the λ HindIII DNA standard fragments at 65°C for 2 minutes and then allow to cool to prevent the "sticky ends" of this digested DNA from sticking to each other.
6. When the Eco RI digestion is finished from step 2, add 2 uL of 6x gel loading solution to the two reaction tubes ("D" and "C").
7. Briefly centrifuge all DNA samples (~5 sec). Load the samples listed below into the wells of the agarose gel in consecutive order. To load samples into the wells, brace your forearm so that you can get the micropipette tip down to just above the well, without bumping its sides or bottom. As you slowly dispense, the sample will sink into the well because it is heavier than the buffer. Record any leakage or contamination of wells.

Lane

1	12 uL	Standard DNA Fragments
2	12 uL	supercoiled recombinant plasmid
3	12 uL	tube "V/I" from Day 1 (= pre-ligation control)
4	12 uL	tube "Lig" from Day 1 (= ligation rxn)
5	12 uL	tube "C" (Eco RI enzyme Control of recombinant plasmid)
6	12 uL	tube "D" (Eco RI enzyme Digest of recombinant plasmid)

8. Put the rig cover onto the gel rig and plug the leads into the power supply (red to red and black to black). Check again to make sure that the black lead is closest to the end of the gel containing your samples. Then turn on the power supply to 100 Volts. Bubbles should appear from the platinum electrodes and the tracking dye should begin to move once the power is on. Electrophoresis will continue until the tracking dye reaches the middle of the gel (45-60 min).
9. Your instructor will take a picture of your gel and provide you with an image to interpret in the next laboratory.

DAY FIVE: ANALYSIS OF GEL ELECTROPHORESIS

OBJECTIVES:

Today we will be examining the image of the gel electrophoresis performed during last laboratory period to determine if your experiment worked. By the end of today you should:

- Know how to determine the size of DNA by comparison to a standard
- Know difference between supercoiled, nicked circular, linear, catenanes and multimeric DNA
- Know how to analyze your gel to determine if your ligation and restriction digest worked

INTRODUCTION:

As mentioned in the previous section, DNA molecules move towards the positive pole during gel electrophoresis since the net charge on DNA is negative from their phosphate groups. The agarose gel acts like a molecular sieve and separates the DNA according to their size, whereby smaller sized DNA moves through the gel faster than larger DNA. The effective size of DNA is influenced by two factors: 1) the base pair (bp) content of the DNA and 2) the conformation or shape of the DNA. For example, the linear plasmid DNA in our experiment is 3000 bp and will move more slowly through the gel than the linear insert DNA (Kanamycin resistance gene) which is 1300 bp.

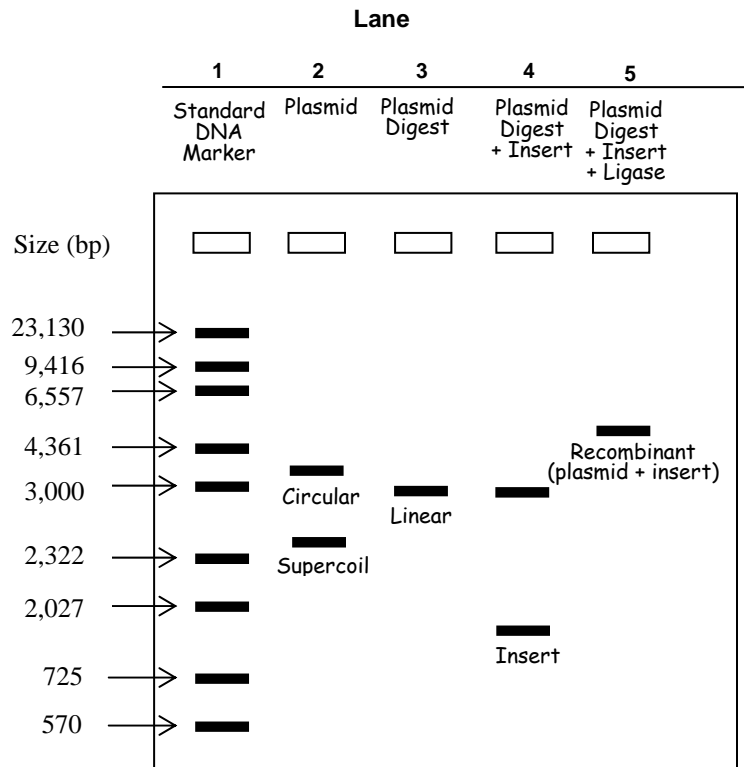
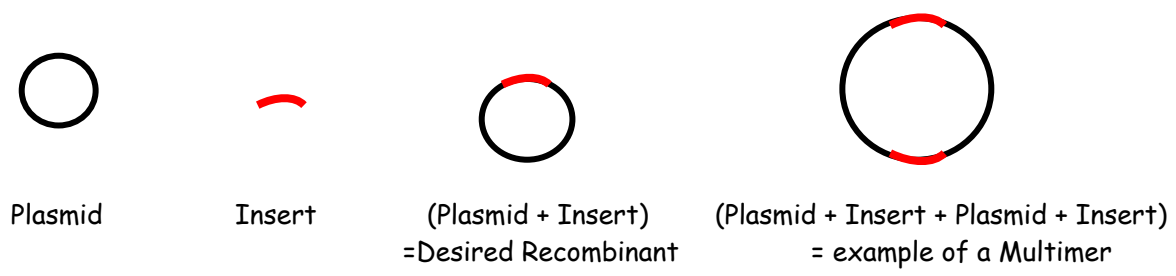
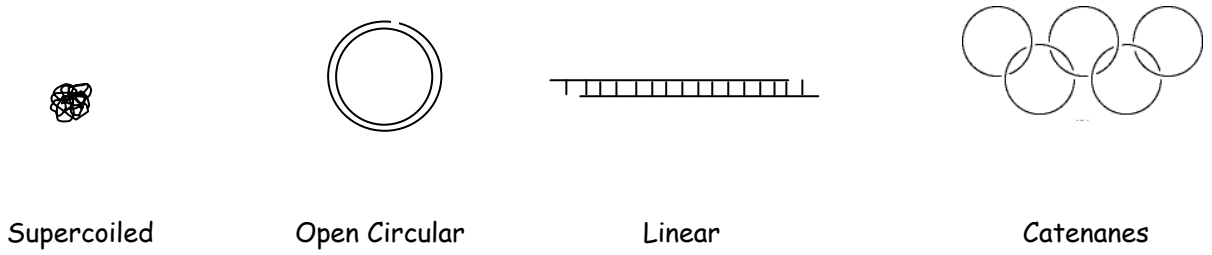
However, plasmid DNA in the transformed host *E. coli* exists in a compact circular form termed supercoiled DNA. Supercoiling is performed by topoisomerase enzymes in the host bacteria that create superhelical turns by either overwinding (positive supercoiling) or underwinding (negative supercoiling) the DNA strands which then twist upon themselves forming a compact ball (imagine "winding-up" a rubber band by holding one end and rolling the other end between your thumb and fingers). In the process of purifying plasmid DNA from bacterial cells, some of the supercoiled DNA is nicked (covalent bond broken) and thereby causes a relaxation of DNA into an open circular form. Supercoiled DNA is very compact whereas the open circular form has more surface area exposed causing more friction when passing through the pores of the gel (imagine the supercoiled DNA as a bullet and the open circular form as a floppy hula hoop). The linear form of the plasmid DNA does not exist until both strands of the plasmid DNA are broken or cut, such as by restriction enzymes. The linear form of the plasmid DNA will migrate slower than the supercoiled DNA, but faster than the open circular DNA since it has less frictional force and can slither its way through the pores. Note that the supercoiled, linear and open circular forms of the plasmid DNA contain the exact same base pair content but rather the shape (or conformation) change of the DNA caused the migration differences.

In addition, plasmids replicating within *E. coli* remain attached as linked circles until topoisomerase separates the plasmids from each other (imagine the Olympic symbol as the independent plasmids that need to be disentangled). These linked forms are termed catenanes and might be observed in your gel image of purified plasmid. The catenane forms will migrate much more slowly since they contain two or more plasmids linked together.

The ligation of two linearized fragments of DNA that contain complementary "sticky ends" (e.g. Eco RI ends) will result in a larger circular molecule proportionately (i.e. more base pairs). Optimally, one plasmid will ligate to one insert DNA fragment resulting in the desired recombinant DNA molecule. However, it is also possible to get more than one plasmid and/or insert ligating together to form even larger recombinant molecules. Since there are many different combinations possible, these larger recombinants are termed multimers (or concatamers). The various recombinant forms will separate on the gel according to their base pair size differences. However, *E. coli* is preferentially transformed by the smaller recombinants since they are easier to maintain and replicate within the cell.

The size of an unknown piece of DNA can be deduced by its position in the lane compared to the position of standards of known size that are also loaded on the gel. A standard DNA marker was loaded into lane 1 on our gel that contains linear fragments of DNA (fragment sizes are listed in the figure below). Linear DNA in our experimental lanes can be estimated by comparison to the standard DNA by plotting the distance migrated for each standard DNA fragment by the \log_{10} of its molecular size (bp). Although much less accurate, you can get a reasonable estimate of the size of the experimental band by visual comparison to the size markers (e.g. determine the markers it is closest to and then estimate). However, the size of circular DNA forms can not be accurately estimated by this method (why? Hint: think about the migration differences of the plasmid conformations).

FORMS OF DNA



THINGS TO DO:

1. Your instructor will provide you with an image of the gel electrophoresis that was performed last laboratory period. Label all the lanes for your gel image. Mark the sizes of the "standard DNA markers" next to the appropriate bands.
2. Explain the banding pattern for each lane. For example, how many bands can you detect for the recombinant plasmid (lane 2)? What are these bands? Repeat this exercise for each lane so that you understand the resulting banding pattern.