

# GENETIC ENGINEERING



## OVERVIEW

Adapted from Philip Jardim, CCSF

### Introduction

During the next three labs, we will construct a recombinant DNA molecule and insert it into bacteria in a complex process involving a number of steps. If successful, when these bacteria replicate their genetic material and pass it onto succeeding generations, all the progeny will be replicating our recombinant DNA molecule as well.

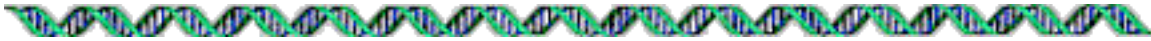
In the first lab, we will conduct a restriction digest using the restriction enzymes BamHI and HindIII to cut the ampicillin resistance gene from a bacterial plasmid named pAMP (size 4539 bp). This gene encodes the information for the enzyme  $\beta$ -lactamase that cuts the ampicillin ring thus rendering it harmless to bacteria. The same restriction enzymes will be used to cut the kanamycin resistance gene from a different bacterial plasmid named pKAN (size 4207 bp). This gene codes for an enzyme, a protein kinase, which phosphorylates the kanamycin molecule making it harmless to the bacteria.

In the second lab, we will confirm the success of our digestion by using agarose gel electrophoresis to separate the resulting DNA fragments by size. We will then incubate the remaining fragments with DNA ligase to link the fragments together to produce recombinant DNA molecules. At least one should contain a fragment with the ampicillin resistance ( $\beta$ -lactamase) gene ligated to a fragment containing the kanamycin resistant gene.

In the third lab, we will attempt to "transform" a mutant strain of *E. coli* by making them "competent" to take up the recombinant molecules. After the bacteria have been transformed, we will be able to select bacterial cells that have the desired recombinant DNA molecule by plating out the treated bacterial cells on agar containing the antibiotics. Any bacterial cell containing the genes that confer resistance to both antibiotics will grow and reproduce a "clone" of genetically identical cells, which we will recognize as a single colony.



# GENETIC ENGINEERING I



## RESTRICTION DIGEST

### A. Before coming to lab

1. Read this handout;
2. Write a concise protocol for this lab in your notebook;
3. This must include a TABLE with the amount of reagents you will add to each tube. If a particular reagent will not be added to a tube, mark the space with an "X".

### B. Materials

sterile microfuge tubes	p10 micropipettor	mini ice bucket
37° C water bath	box of sterile tips	microfuge
pKAN (0.2 µg/µ L)	pAMP (0.2 µg/µL)	λ DNA (0.2 µg /µL)
2X restriction buffer	sterile dH <sub>2</sub> O	BamHI (10 U/µL)
HindIII (10 U/µL)	vortexer	

### C. Introduction

Restriction enzymes are the molecular biologists' precision scalpels in working with large DNA molecules because they will cut the DNA at specific points known as "restriction sites". The enzymes are purified from bacteria where they function to protect the cells from viruses. The names of these enzymes are derived from the species and strain of bacteria from where they were isolated and a reference number. For example, BamHI was the first restriction enzyme isolated from *Bacillus amyloliquifaciens* strain H, while HindIII was the third from *Haemophilus influenzae* strain d.

The region where the DNA is cut by a restriction enzyme is called the recognition sequence. The length of this sequence can be four, six, or eight base pairs (bp) depending on the enzyme. The recognition sequences for the restriction enzymes BamHI and HindIII are diagrammed below. Notice that where the enzymes cut, they leave one end of the double helix longer than the other -- these are called "sticky ends" where different fragments cut with the same restriction enzymes will combine due to complementary base pairing. DNA ligase can be added to seal the recombined ends to make a stable circular DNA molecule.

BamHI	– G <u>↓</u> GATC C –	HindIII	– A <u>↓</u> AGCT T –
	– C CTAG <u>↑</u> G –		– T TCGA <u>↑</u> A –

Plasmids are small circular DNA molecules found in bacteria. They contain a few thousand base pairs and a few dozen genes. The names of the plasmids are derived from their genes. For example, the plasmid with the gene that protects the bacteria from the antibiotic ampicillin is called pAMP, while the plasmid with the gene that confers resistance to kanamycin is called pKAN.

In molecular biology, we use concentrated buffers when setting up reactions. They are designated with an "X" to denote the prepared concentration as opposed to the desired final concentration. For example, a 10X buffer is ten times as concentrated as the desired final concentration. To obtain the appropriate final concentration, this buffer needs to be diluted 1:10. Let's say you wanted to restrict 3  $\mu\text{L}$  of DNA with 1  $\mu\text{L}$  of enzyme. This gives a total of 4  $\mu\text{L}$ . If you added 1  $\mu\text{L}$  of 10X buffer, you would need to add 5  $\mu\text{L}$  of sterile  $\text{dH}_2\text{O}$  to arrive at the final concentration of 1X. As you can see, a 1X buffer solution could not be used since after adding enzyme and substrate, the 1X buffer would be diluted to less than the desired final concentration. These calculations must be done beforehand. You always want to add your enzyme last. Adding an enzyme to a too concentrated buffer solution or to an unbuffered solution could damage it and greatly reduce its activity. A good laboratory practice is to add substrate, diluent (usually sterile  $\text{dH}_2\text{O}$ ) if needed, concentrated buffer, and lastly enzyme(s).

In this lab you will prepare a HindIII digest of  $\lambda$  (lambda) DNA, a linear DNA molecule of approximately 48,000 bp (48 kb - kilobase pairs) obtained from the bacteriophage  $\lambda$ . Since the entire  $\lambda$  genome has been sequenced, the number of HindIII sites as well as the fragment sizes generated are known. HindIII digestion of  $\lambda$  DNA will generate 8 fragments that can be used as size standards. When these fragments are electrophoresed on an agarose gel along with your experimental fragments, their positions on the completed gel can be used as standards to determine the size of the unknown pieces.

Lastly, here are a few tips to help you successfully accomplish your goal. Before withdrawing buffers or DNA, pulse all your tubes in the microfuge to pool any contents sticking to the sides to the bottom of the tube. If you do not do this, some of your tiny sample will stick to the wall of the tube and your pipette will not pick up enough to work with. There are NOT enough plasmids for you to get another tube. Also, when working with DNA and enzymes, always keep all tubes on ice until you are ready to incubate them at the appropriate temperature. Tubes should only be removed from ice when withdrawing or adding reagents, pulsing, and vortexing. You SHOULD remove your tube from the ice bath when adding or withdrawing reagents. When using such small quantities, you should never go blindly into a tube.

#### D. Procedure

In this investigation, **you will work in pairs** and set up three restriction digests: one for the plasmid pAMP, one for pKAN, and one for  $\lambda$  DNA. In addition you will have a control tube for each plasmid. The plasmid DNA is already aliquoted for you. You'll get a tube with 8.5  $\mu\text{L}$  of pKAN and a separate tube with 8.5  $\mu\text{L}$  of pAMP.

For each plasmid digest, add 1.1  $\mu\text{g}$  of DNA to two labeled microfuge tubes - one for each plasmid. See concentration of plasmids to figure out the volume you need to add. Remember that there are 0.2  $\mu\text{g}$  plasmids per  $\mu\text{L}$  of plasmid solution. Add the appropriate amount of 2X buffer. To calculate the proper amount of buffer to add, you must determine the final reaction volume. You usually want to have the most concentrated reaction possible since it is easier to dilute the products later than to concentrate them. Thus, you want the smallest final volume possible. In this case you need not add  $\text{dH}_2\text{O}$  since you can dilute the concentration of the 2X buffer with the volume of the reactant and enzymes. Your instructor will give you 1.0  $\mu\text{L}$  of BamHI and 1.0  $\mu\text{L}$  of HindIII (total of 2  $\mu\text{L}$  of enzymes) for each of your experimental plasmid digests once all reagents have been added. Before getting the restriction enzymes for your plasmid digests, place tubes on ice and set up control tubes and the  $\lambda$  DNA digest.

After removing plasmids for your experimental restriction digests, you should have 3.0  $\mu\text{L}$  of plasmids left in both the pAMP and pKAN tubes. To conserve plastic, use these same tubes as your undigested controls. Add 2.0  $\mu\text{L}$  of  $\text{dH}_2\text{O}$  instead of restriction enzymes (add the same volume of  $\text{dH}_2\text{O}$  as your total restriction enzyme volume in your experimental tubes). Add the appropriate amount of 2X buffer. Be sure to relabel these tubes appropriately.

NOTE: We are cheating here!! Usually a control differs in ONLY ONE variable. The control tubes we are asking you to set up differ in two variables. What are the two? We are doing this to conserve precious plasmid which is time-consuming and expensive to isolate (Bio 65, the Recombinant DNA class here at CCSF, isolates it for you since it would be prohibitive for the Biology Department to purchase it from a vendor). Forgive us on this! We are making the assumption that it still works as a control based on prior experience!

The tube labeled " $\lambda$ " contains 4.0  $\mu\text{L}$  of  $\lambda$  DNA. To conserve plastic, use this same tube to perform your restriction digest. Your instructor will give you 1  $\mu\text{L}$  of HindIII (no BamHI). Before obtaining the enzyme, add the appropriate amount of 2X buffer.

Once your restriction digests and control tubes have been set up, bring your tubes on ice to the instructor to obtain the appropriate restriction enzymes. Pulse all reagents to the bottom of the tubes in the microfuge, vortex the tubes, then pulse again. Place tubes in the 37<sup>o</sup> C water bath for two hours. Once restricted, the fragments can be stored at 0<sup>o</sup> C indefinitely. Label your tubes so that you can distinguish them from the tubes of other teams and place them in the rack provided by your instructor for freezing until the next laboratory session.

## E. Review questions

### 1. Define

- a) Restriction enzyme
- b) Endonuclease
- c) Sticky end
- d) Plasmid
- e)  $\beta$ -lactamase
- f) size standard

11. What kind of molecules are BamHI and HindIII? What is their function?

12. Which genes did we want to cut out of pKAN and pAMP?

13. Why do we add water to the tubes?

14. What is the function of the restriction buffer?

15. Why do we digest bacteriophage lambda DNA?

16. What will the pAMP control tell us?

17. What will the pKAN control tell us?

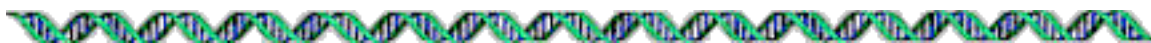
18. Hypothesize about how  $\text{kan}^r$  and  $\text{amp}^r$  work.

19. How many fragments do you get restricting pKAN with BamHI and HindIII?

20. How many fragments do you get restricting pAMP with BamHI and HindIII?



# Genetic Engineering II



## AGAROSE GEL ELECTROPHORESIS AND LIGATION OF RESTRICTION FRAGMENTS TO MAKE RECOMBINANT DNA

**Note:** you will need goggles for this week's experiment.

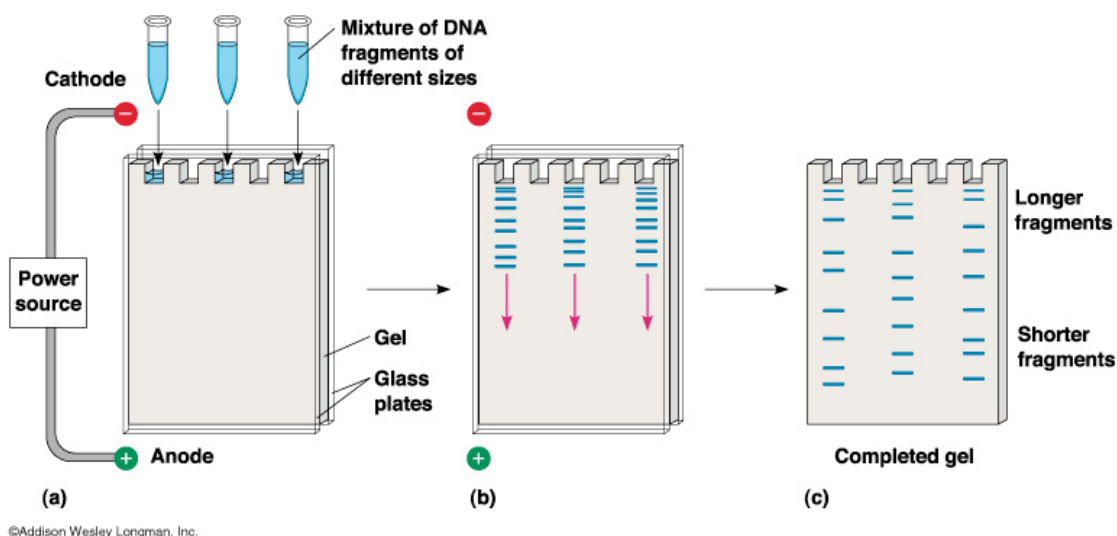
### A. Before coming to lab

1. Read this handout;
2. Read Reed et. al, pp.
3. Write a concise protocol for this lab in your notebook

### B. Introduction

#### Horizontal agarose gel electrophoresis

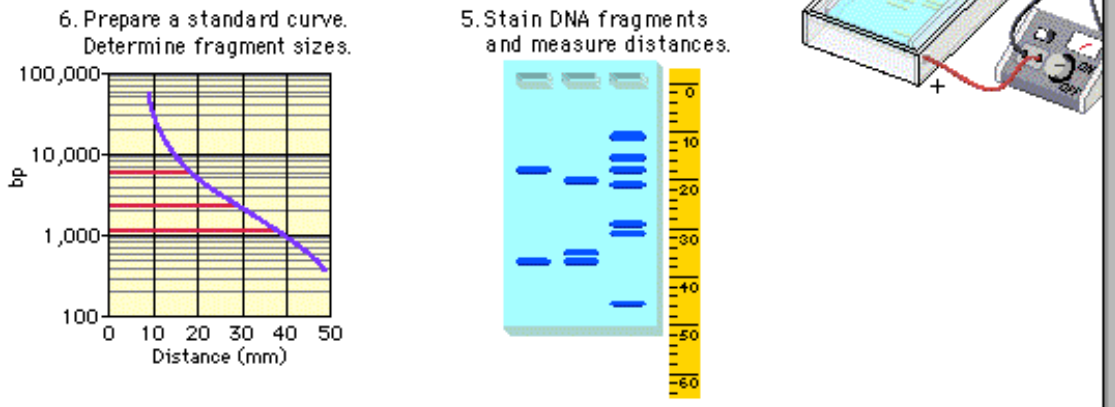
To determine the success of the restriction digest, a sample will be electrophoresed through an agarose gel (see Figure1). This will separate the fragments by size. Electrophoresis literally means "to carry with electricity". A power supply applies a current to electrodes at both ends of a chamber creating an electric field across the gel. The buffer within the electrophoresis chamber contains the ions necessary to conduct electricity. DNA is an organic acid that is negatively charged at neutral pH due to the phosphate groups which alternate with deoxyribose sugars forming the "backbones" of both chains of the double helix. When placed in an electric field, DNA molecules migrate toward the positive pole (anode). The intertwining polysaccharide agarose molecules form pores and act as a molecular sieve through which smaller molecules can move more easily and thus faster than larger molecules.



**Fig. 1:** Electrophoresis procedure

Since the size of the pAMP and pKAN fragments are unknown, known sizes must be run on the same gel to be used as molecular size standards. These can be plotted on a graph that can be

used to determine the size of the unknowns since the distance of migration of the unknowns can be compared with the knowns (Fig. 2).



**Fig. 2:** Preparation of a standard curve on semi-log paper from electrophoresis size standard

The size standards utilized in this exercise are the HindIII digest fragments of  $\lambda$  NA, a linear molecule that consists of approximately 48,000 base pairs. This molecule contains all the genes (the entire genome) of the bacteriophage  $\lambda$ . The amount of  $\lambda$  DNA we used in this digest is approximately 1 billion molecules. Since  $\lambda$  DNA is linear with seven HindIII restriction sites, this digest, if complete, will yield 8 DNA fragments - 23.1 kb, 9.4 kb, 6.6 kb, 4.4 kb, 2.3 kb, 2.0 kb, 0.56 kb and 0.125 kb. (1 kb is 1,000 base pairs of DNA.) To visualize the DNA, the gel is stained with ethidium bromide which intercalates between the base pairs in the interior of the helix. Ethidium bromide will fluoresce when exposed to ultraviolet light thus allowing us to "see" the DNA. Each individual band is the result of millions of fragments of the same size fluorescing at the same position in the gel. Since a greater number of base pairs will show increased fluorescence, you should think about which variables in the bands will influence the differences in brightness. Because of its small size, the 0.125 kb fragment will most probably not be seen.

Linear DNA fragments migrate at rates inversely proportional to the  $\log_{10}$  of their molecular weights. Using semi-log graph paper, the molecular weight of the known fragments can be plotted on the log scale (Y axis) against the migration distance on the linear scale (X axis). This should generate a best-fit straight line which can be used to determine the size of the pAMP and pKAN fragments. The graph cannot be used to determine the size of the uncut plasmids. (Why not?)

When generating your standard curve, you must also take into account the concentration of the agarose in the gel. A low concentration of agarose (0.3%) will produce large pores which can accurately separate large fragments (23,000 bp, 20,000 bp, 14,000 bp) however small fragments (for example - 3,000 bp, 2,300 bp, 560 bp) will travel unhindered and will result in one band. Likewise, a high concentration (2%) will accurately separate small fragments (350 bp, 1,000 bp, 210 bp) however larger fragments (8,000 bp, 23,000 bp, 12,000 bp) will have the same difficulty getting through the small pores and result in one band. In this exercise, we will use a 0.8% agarose gel. See table 1 below to determine on which of the HindIII  $\lambda$  DNA fragments you should base your standard curve (hint: There are two fragments you shouldn't use since they are not accurately separated in a 0.8% gel).

Loading dye which is added to the sample before electrophoresis has two main functions. It contains two negatively charged visible dyes, bromophenol blue and xylene cyanol. They do not interact with the DNA, but migrate independently toward the anode. Bromophenol blue will migrate at a rate equivalent to a 300 bp DNA molecule. Thus visible movement of the dye allows one to monitor the relative migration of the unseen DNA bands. Loading dye also contains 50% sucrose. This dense sucrose solution weights the DNA sample, helping it to sink and displace the buffer when loaded in the well.

**Table 1:** Range of separation in gels containing different amounts of agarose

Amount of agarose in gel (% [w/v])	Efficient range of separation of linear DNA molecules (kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

### Ligation

To make a recombinant DNA molecule, the restriction fragments of the two digests must be mixed together. Since they were both digested with the same enzymes, they contain single stranded overhangs that are complementary to each other. Fragments with BamHI ends will reanneal to other BamHI ends regardless of which plasmid they are derived from. The same applies to fragments with HindIII ends. (You should be able to explain why HindIII ends will not reanneal to BamHI ends.) Once you have added the digests together, it is important to thoroughly vortex the mixture so that the fragments of different plasmids have a maximum chance of reannealing with each other instead of merely reannealing with fragments of the same plasmid - reforming the same plasmid that you started with. Once vortexed, remember to pulse any DNA sticking to the sides of the tubes to the bottom.

The "sticky ends" are only joined by the hydrogen bonds of the complementary base pairs. How many base pairs per sticky end are joining the individual fragments together? To make a stable molecule, the nicks of the phosphodiester linkages between the fragments must be restored as covalent bonds.

The ligation of restriction fragments into a stable recombinant DNA molecule involves the action of the enzyme DNA ligase. This enzyme is used during DNA replication to seal the phosphodiester bond between Okasaki fragments on the lagging strand once the RNA primers have been replaced by DNA nucleotides by the action of DNA polymerase I.

Restriction enzymes do not require an outside source of energy because the breaking of the phosphodiester bonds at the restriction sites is energetically favorable. However, resealing the breaks is not. Thus DNA ligase requires ATP to accomplish its task as well as Mg<sup>++</sup> ions as cofactors. These are provided in the ligation buffer.

## C. Materials

gel casting tray	0.8% agarose	TAE buffer
6-well comb	electrophoresis chamber	power supply
10X loading dye	p10 micropipettor	staining box
plastic gloves	ethidium bromide	camera set up
spatula	UV transilluminator	2X ligation buffer w/ ATP
vortexer	sterile dH <sub>2</sub> O	65° C water bath
T4 ligase		

## D. Procedure

### 1. Agarose gel electrophoresis procedure

#### a. Pouring the gel

Your instructor will place a gel solution of 0.8% agarose in the microwave to liquefy it. Let the agarose cool until the glass is comfortable (but not too comfortable!) to touch. While waiting for it to cool, set up your gel casting tray - loosen the plastic screws, raise the gates and retighten the screws to a snug fit. DO NOT OVERTIGHTEN or the plastic screws will shear or the threads will strip. Place the gel casting tray on a paper towel on a level area of your bench. Place a 6-well comb into the notches at the end of the fixed sides. Notice the middle notches on the fixed sides. You will pour your gel so that it reaches the bottom of the notch on the lowest fixed side. CAUTION: If you pour your gel too hot, you will warp the tray. If you let it cool too much it will begin to gel and cause an uneven matrix. When pouring the gel, do it rapidly so that it will cool evenly. Watch for air bubble formation. Bubbles can interfere with the movement of the DNA molecules if they are in the path. Have a pipette tip ready. If bubbles form, simply move them to the outermost edges of the gel with the pipette tip. Be especially vigilant for bubbles around the comb.

Once your gel is poured, DO NOT move it. Let it cool to solidify. You will know when it is ready because it will turn opaque. While waiting for your gel to solidify, prepare your samples.

#### b. Preparing the samples

Retrieve the tubes of your restriction digest. Pulse all the tubes to make sure there is no solution sticking to the sides of the tubes. You will run samples of the experimental tubes on the gel to see if the restriction enzymes were working and to determine the sizes of your fragments. You should add 5.0  $\mu$ L of each digest to a new tube to run on your gel. The remainder of the digest (in the original tubes) will be used for cloning so you should put it back on ice in a safe place. DO NOT GET THESE TUBES CONFUSED! The control tubes and the lambda digest tube will not be used for cloning and the entire contents will be run on the gel.

Add 1  $\mu$ L of loading dye to all tubes you will run on your gel. Pulse to mix. DO NOT add loading dye to the tubes you will use for cloning.

#### c. Loading the gel

Once the gel has solidified, loosen the screws, lower the gates, and retighten the screws to ensure that the gates remain down. The gel was prepared in the same buffer solution (TAE

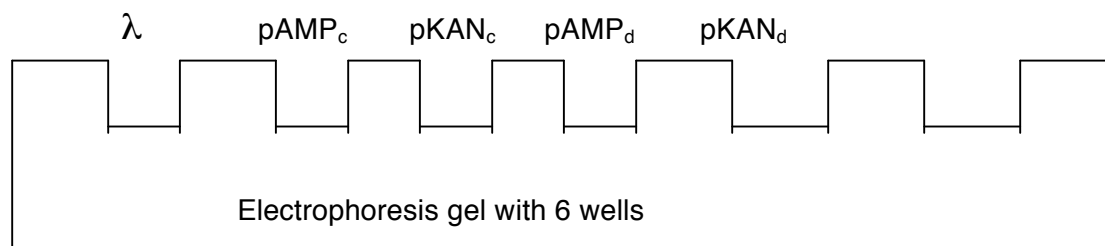
buffer) that your electrophoresis chamber contains so that it will carry an electrical current. If the gates are up, the current will not be able to pass through the gel.

If the electrophoresis chamber is empty, add TAE buffer until it just covers the gel platform (~ 300 mL). If it has buffer in it used by a previous class, rock the chamber back and forth to make sure that the ions are evenly distributed. Place the tray on the platform of your electrophoresis chamber. Remember that DNA has a phosphate backbone on both strands so that it is highly negatively charged. Make sure your gel is pointing in the correct direction for how you want the DNA molecules to migrate! You need not be afraid if you dip your fingers in the TAE buffer since it is merely a harmless salt solution.

The TAE buffer in the chamber should barely cover the gel. Make sure that there is some TAE buffer around the comb to act as a lubricant. With two fingers securing the gel casting tray, gently wiggle the comb and pull straight out at the same time to remove the comb. Do this firmly but carefully to not tear the gel. This will form six wells in which you can load your samples. Look for "dimples" in the wells. This means you do not have enough TAE buffer covering your gel. Obtain a beaker with TAE buffer (label it!!) and slowly pour TAE buffer into one of the chambers until the "dimples" disappear.

Add a little more buffer (to ~ 2 mm above the surface) to make sure that evaporation does not lower the level of buffer below the gel surface. DO NOT add excessive buffer or too much current will pass over the gel instead of through the gel. Pour your extra buffer back into the carboy. (NOTE - we usually do not pour reagents that we use for experiments back into stock solutions - in this case, this is an exception since it is only a buffer needed to conduct a current and does not enter into or contaminate a reaction.)

Load the entire contents of EACH SAMPLE TUBE carefully into a separate well of the agarose gel according to the diagram below. (Remember, not all tubes have the same volume.)



Note that the gels are customarily viewed with the wells on top, so your lambda sample should be at the top left. BE CAREFUL - not all the tubes will have the same volume! The loading dye will weigh down your DNA samples and displace the TAE buffer occupying the wells. If there is a little bit of residue left in the micropipettor tip after trying to load the well, DO NOT try to force it out. By moving the plunger back and forth, you risk displacing what you have put into the well up into the TAE buffer of the chamber. The small amount of residue left in the tip will most likely not affect the results you are trying to "see". Displacing the DNA into the buffer definitely will!

Once your samples are loaded, slide the lid of the electrophoresis chamber and connect the electrical leads to the power supply. Set the power supply to constant voltage and increase the voltage to 100 volts (40 - 80 milliamps).

Record the starting time in your laboratory notebook and leave a space for recording the ending time. Ensure that your gel is running by looking for gas formation at the cathode (negative pole)

and anode (positive pole). If you do not see bubbles forming, make sure to check with your instructor.

**NOTE: Though this is usually not done in the real world, because of time implications you should now set up your ligation reaction. See the next part of this exercise for the protocol.**

Run the electrophoresis to obtain a good separation of the purple and blue-green dyes (about 40 - 60 minutes). These bands are simply visual indicators for the progress of migration since the DNA bands won't be visible until they are stained. The leading purple band should migrate to one half to three quarters the length of your gel. Record the ending time in your notebook.

Shut off the power supply. Carefully slide out the chamber cover. **DO NOT** hold onto the electrodes or you can detach them from the cover. Rather grab the plastic edge of the cover and gently slide it off. Do not allow the gel to sit in the buffer for a long time once you've shut the power off or the DNA will begin to diffuse.

#### **d. Viewing the gel (REGULAR GOGGLES (not UV) REQUIRED)**

To view the DNA fragments within the gel you will stain them with ethidium bromide and place the gel onto a UV transilluminator which will cause the stained DNA to fluoresce under ultraviolet light.

Carry the gel tray from the chamber to the staining station - be careful that the gel does not slide off the tray! Your instructor will stain it for you in ethidium bromide for **15-20** minutes. The gel will then be rinsed three times with tap water to wash off any unbound ethidium bromide. Remember to wear gloves and take precautions before rinsing the gel. See note below.

**CAUTION: Ethidium bromide is a known mutagen and suspected carcinogen. ALWAYS wear gloves on both hands before handling a stained gel. You must also wear goggles to protect your eyes from splashing. Keep stained gels and anything that touches them in a contained area of the lab (where the protective paper is).**

Your instructor will place the stained gel on the UV transilluminator. After the UV blocking lid is lowered, you can safely observe the DNA fragments on your gel.

**CAUTION: Ultraviolet light is hazardous. It can damage your eyes and cause severe burns on your skin in a very short amount of time of exposure. ALWAYS wear SPECIAL UV PROTECTIVE GOGGLES and cover your skin with clothing before exposing yourself. (The transilluminator used in Bio 101A has a UV protective shield which will not allow ultraviolet rays to pass through.)**

Once the gel is determined to be satisfactorily stained, your instructor will take a picture of it so that you can have the results of the experiment. Keep your picture and include it in your lab report. Don't forget to treat the picture like a figure with a number, a descriptive title, and a reference in the text. Dispose of your gel in the proper biohazard receptacle.

## **2. Ligation procedure**

Your original restriction digest tubes contain restriction fragments of pKAN and pAMP as well as restriction enzymes. You must first inactivate the restriction enzymes before ligating the

fragments together. To do this, place the tubes in a 65<sup>o</sup> C water bath for 10 minutes. This will denature the restriction enzymes.

Label a sterile microfuge tube for the ligation reaction so that you can easily recognize it in the next lab period. The label should include your initials and a clear indication of what's in the tube. Remember that it is always a good idea to label microfuge tubes on the top as well as the sides in case one of the labels rubs off. Add 3  $\mu$ L of digested pAMP and 3  $\mu$ L of digested pKAN to your ligation tube. Your instructor will give you 10  $\mu$ L of 2X ligation buffer and 1  $\mu$ L of T4 ligase. Before you receive the buffer and enzyme from your instructor, you should add sterile dH<sub>2</sub>O so that the final volume of your reagents will include 50% buffer. How much water should you add? Check with your instructor to make sure that the amount you calculated is correct before adding the water to your ligation tube.

Once you've added the digested pAMP, pKAN, and sterile dH<sub>2</sub>O to your tube, pulse them to the bottom and vortex the tube VIGOROUSLY so that you do not have pKAN fragments in one part of the solution and pAMP fragments in a different part slowly diffusing toward each other. Pulse again and bring the tube to your instructor on ice. Once you receive the ligation buffer and enzyme put your tube back on ice. (ATP is not very thermally stable so you want to minimize temperature change.) Pulse the contents to the bottom, vortex again, pulse again and bring your tube on ice to the instructor. Your instructor will incubate the tubes overnight at 16<sup>o</sup> C then place the tubes in the -20<sup>o</sup> C freezer for the next lab period.

### **E. After lab**

Measure the distance each band has migrated. Measure the distance from the lower edge of the well to the leading edge of the band. Record these distances in a table. Develop a standard curve using the known sizes of the lambda DNA fragments so that you can determine the unknown sizes of your pKAN and pAMP digests. Please use semi-log paper (provided) to generate your graph. To make best use of your Y-axis, mark the point where the x-axis and y-axis intersect 10<sup>2</sup>. Accordingly, your y-axis will go to 10<sup>5</sup>. This graph will eventually be part of the report for this lab, so **do not** glue it into your lab notebook.

### **F. Review questions**

#### **Electrophoresis of digested pKAN and pAMP**

1. Define
  - a ) Semi-log paper
  - b) Standard curve
2. What are the two functions of loading dye?
3. How does ethidium bromide work? How does this relate to the need to minimize exposure to humans?

4. Why do DNA fragments migrate through the gel?
5. Why do different DNA fragments migrate at different rates?
6. What would happen if you filled the gel box with water instead of TAE buffer?
7. What would happen if you reversed the electrodes?
8. Examine the photograph of your gel. Compare the expected number of bands per tube with your results. Account for all bands you see.

	Expected # of bands	# of bands
pKAN <sub>d</sub>		
pAMP <sub>d</sub>		
Control pKAN		
Control pAMP		
Lambda		

9. Why are some bands fainter than others?

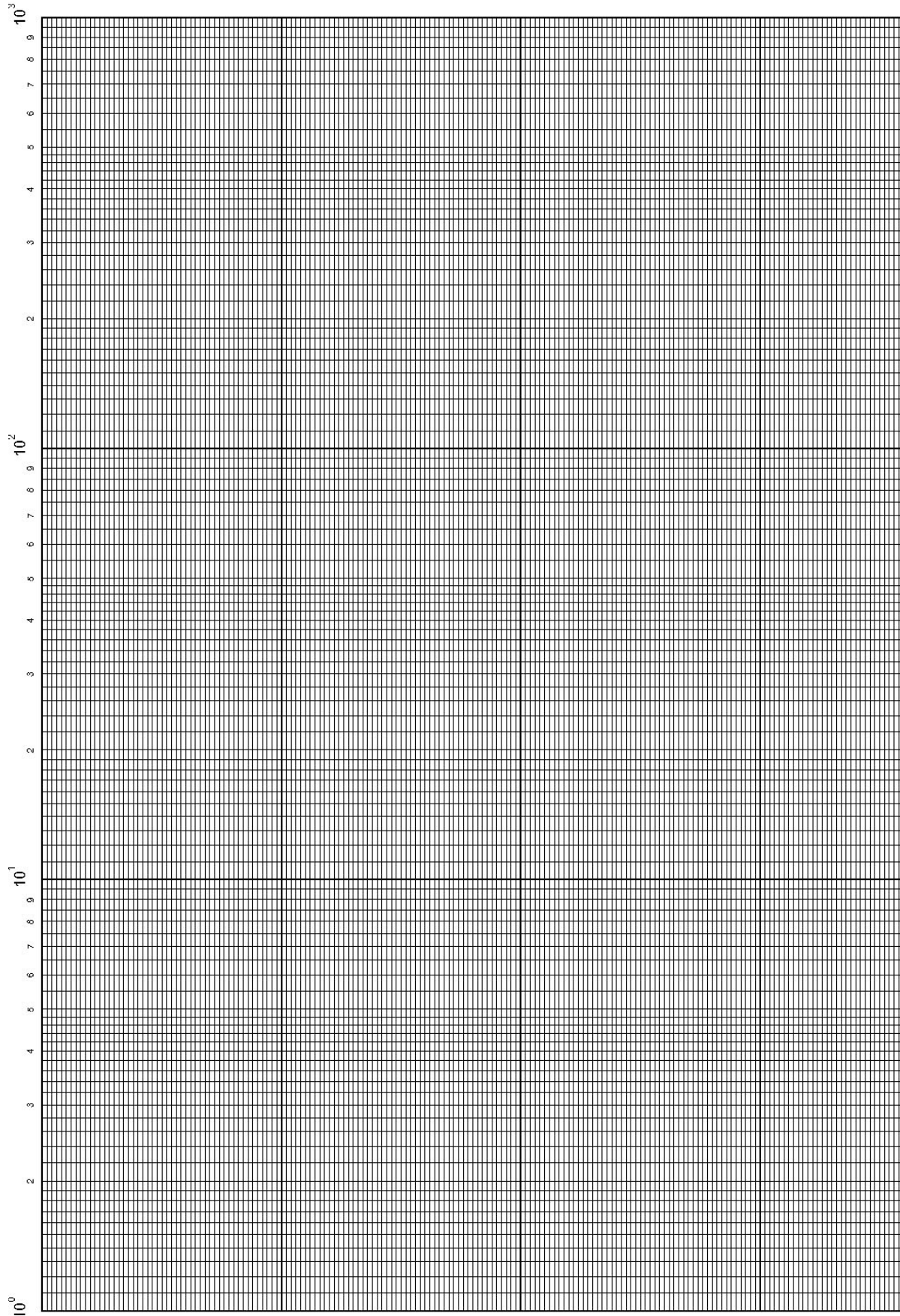
### **Ligation**

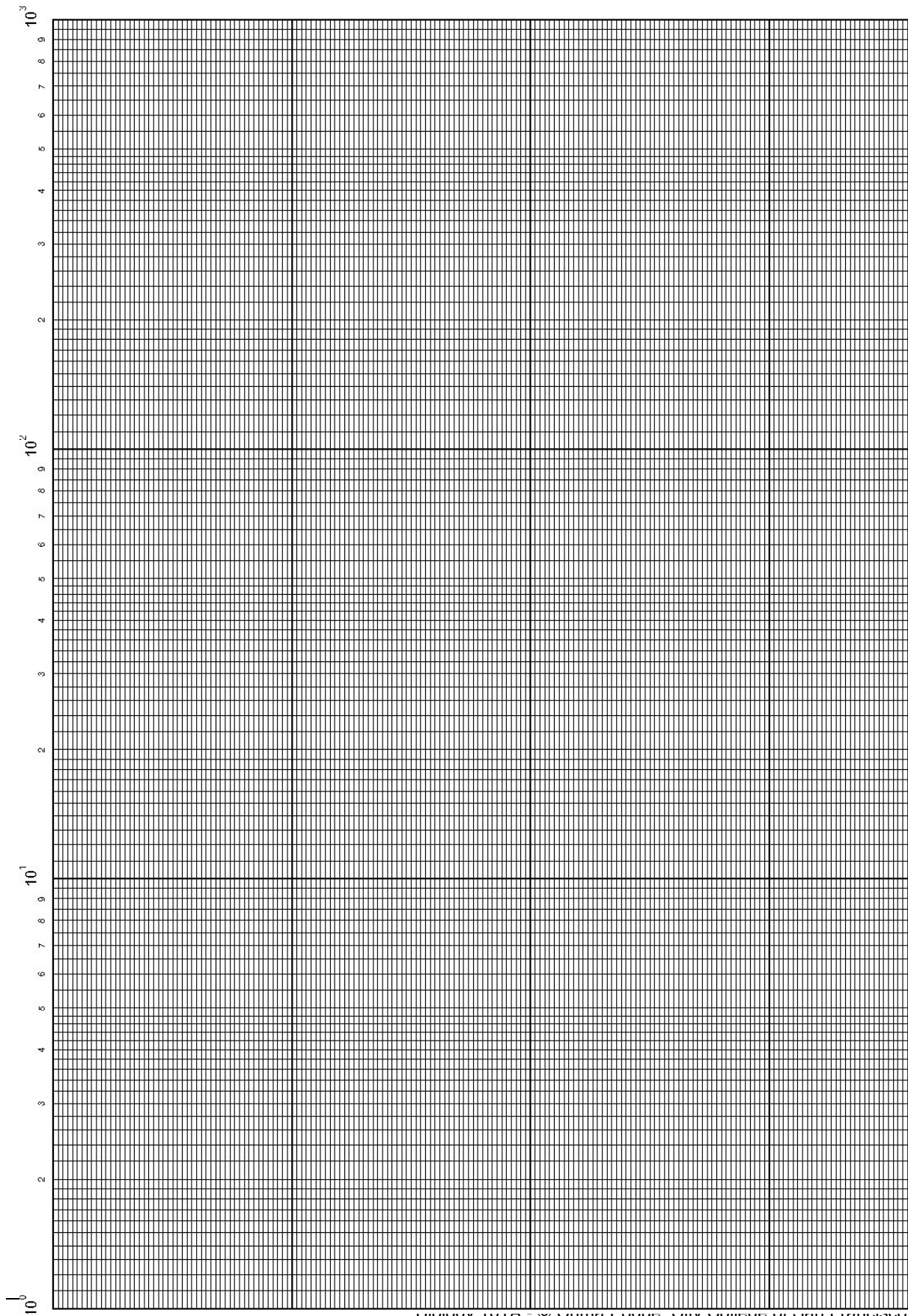
10. Define "recombinant DNA"
11. How many different fragment combinations can we get?
12. How many of these possible combinations can replicate?
13. How many of these possible combinations can express resistance to both kanamycin and ampicillin?
14. What are sticky ends? Why are they so useful in creating recombinant DNA molecules?

15. Based on your bands and the calculation of DNA sizes, draw one recombinant plasmid that contains a fragment each of pKAN and pAMP. Include fragment sizes and location of *Bam*HI and *Hind*III restriction sites.

16. Why is ATP essential for the ligation reaction?

**Note that you have to re-label the log scale of the y-axis in the following graph paper to accommodate data from 560 to 23,000 base pairs.**





# Genetic Engineering III



## TRANSFORMATION OF COMPETENT CELLS WITH RECOMBINANT DNA

### A. Before coming to lab

1. Read this handout;
2. Write a precise protocol for this laboratory;
3. Generate a list of materials you will need for this exercise

### B. Introduction

The host cell we will use to clone our genes is a highly mutated strain of *E. coli*. We use this strain to ensure that it will not be able to escape from a laboratory environment and carry with it possible harmful genes to wild type populations.

*E. coli* do not naturally take up foreign DNA. They must be made “competent” (able to take in extraneous DNA). Once they have taken up, accepted, and replicated the foreign DNA, we say they have been “transformed”, cloning the desired genes and passing them on to each generation.

The bacterial cell membrane is made up of phospholipids, and thus carries a negative charge all around the interior and exterior surfaces of the cell. DNA, likewise, has a highly negatively charged surface due to the phosphate sugar backbones of the two strands. Thus DNA is repelled from the bacterial membrane. To counteract the repulsion, the cells and the DNA are incubated with a divalent cation ( $\text{Ca}^{++}$ ). The cation will stick to the negative charges and neutralize the surfaces. In addition, the incubation must be done at  $0^{\circ}\text{C}$  to stabilize the membrane, disrupting its fluidity and causing it to contract. This is what makes the cells “competent”.

Enough DNA is added to cover the membranes of the cells. After a 20-minute incubation period, the cells are then carried on ice to a  $42^{\circ}\text{C}$  water bath and “heat shocked”. One hypothesis for the heat shock is that the membrane will regain movement and expand from the added heat, allowing the neutralized DNA to be sucked through some of the transmembrane channels. After the heat shock, the cells are immediately put back on ice to prevent them from ejecting the unwanted DNA. They are then fed LB broth so that they will begin replication. Since the recombinant DNA has a sequence recognized by the bacterial replicative enzyme complex, they will accept it as “self” and will clone the recombinant molecule passing it on to the daughter cells. The bacterial cell machinery is thus used to clone millions of copies of your gene of interest.

Bacterial cells are most easily made competent when they are in the mid-log phase of growth. To achieve this growth phase, 10 mL of LB broth is inoculated (using aseptic technique!) with  $100\ \mu\text{L}$  of an overnight (18-hour) culture 2 to 3 hours before you plan to induce competency. This already has been taken care of for you by our laboratory technicians.

The next step in cloning is finding out which of the billions of bacteria in your transformation tube contain the recombinant plasmid you are interested in. One must “screen” the bacteria for those who have the desired sequence of DNA. To begin with, most of the bacteria, even with the best of laboratory technique, will not take up any DNA. In addition, when you ligated your plasmid fragments, there were many possible combinations that could have joined together. In this exercise, we are only interested in the combination of fragments that has the kanamycin-resistant gene spliced to the fragment of the ampicillin-resistant gene. To find the bacteria (if any) which have incorporated the desired sequences, we will plate them on “selective” media. This media will select for those bacteria that took up and accepted our desired recombinant plasmid. Those who did not will not be able to survive.

In this exercise, it is important to keep everything ice cold that will come into contact with your bacteria (DNA tubes, culture tubes, etc.). Even adding a small amount of heat (i.e. from your 37 °C hands) will reduce competency exponentially. However, this does not mean you shouldn't make sure that your cells are in suspension because you are afraid to hold the tube in your hand and finger vortex (!); as in any laboratory exercise involving living organisms, balance is of utmost importance! In addition, since the success of the experiment depends on growing these cells, your best aseptic technique must always be utilized. Thirdly, remember to dispose of any materials that have come in contact with live bacteria in the appropriate biohazard receptacle.

## **C. During lab**

### **1. Prepare competent cells**

Put all tubes you will use on ice! Have ice baths ready for all procedures you will do when making cells competent. Remove a 50 mL conical centrifuge tube containing 10 mL of mid-log *E. coli DH5* cells from the 37 °C shaking water bath. Balance with a water blank. (Remember to use shields and caps when balancing.) Pellet cells in the clinical centrifuge at the highest setting for 10 minutes. While your cells are in the centrifuge, we will need three teams to volunteer to prepare controls: one pAMP control (undigested pAMP), one pKAN control (undigested pKAN, and one negative control (buffer only, no DNA).

After centrifugation, make sure that your cell pellet is stuck to the side and quickly pour off supernatant into proper waste receptacle containing disinfectant. Make sure that all broth has been removed. You should not rock your tube back and forth. Once you pour, have faith in your pellet. Keep your tube tilted so that the liquid does not flow back and dislodge it from the side.

Add 5 mL ice-cold 50mM calcium chloride. Vortex the pellet until the cells are in suspension. Do this rapidly, but thoroughly. Incubate for 20 minutes at 0 °C. Respin the cells in the clinical centrifuge for 5 minutes at setting #6. Don't forget to balance the tubes! Pour out supernatant and add 1mL of ice-cold CaCl<sub>2</sub>. Finger vortex to resuspend the cells. The cells at this point are extremely fragile so the mechanical vortex mixer should NOT be used. Again, do this rapidly, but thoroughly. Swirl and transfer 200 μL of your cells to a 15 mL culture tube. If you volunteered to be a control, prepare an additional 15 mL tube with 200 μL of cells.

Add 10 μL of your ligation mixture directly into your cell suspension (NOT down the side of the tube). If you are also doing a control, obtain the proper DNA from the instructor and add 10 μL of control DNA or buffer (negative control) to the control tube you prepared. Finger vortex well so that every cell is coated with DNA. Incubate for 20 minutes, again at 0 °C.

### **2. Transform *E. coli* cells**

Carry cells to 42 °C water bath on ice. Heat shock by immersing cells in water for exactly 90 seconds. (Control groups should also do the same with your control tube.) You can immerse both control and experimental tubes at the same time.) Immediately return tubes to ice for at least 2 minutes. Add 800  $\mu$ L of LB broth to each tube. At this point cells should be taken off ice. Place in 37 °C shaking water bath for 40 – 60 minutes (the longer the better).

### 3. Plate the cells

Obtain one each of four different media plates: LB (LB agar only), LB amp (LB agar + ampicillin), LB kan (LB agar + kanamycin), and LB amp/kan (LB agar + ampicillin + kanamycin). Controls will need two sets of plates. After incubation, plate 250  $\mu$ L of cells on each plate using the spread plate technique previously described in the Aseptic Technique laboratory. Plate LB plate last. Remember to let hockey stick cool for 15 seconds before spreading bacteria. It is best to spread as soon as possible after adding cells to prevent agar from soaking up the liquid placed in the middle of the plate. After spreading, allow the broth to seep into the agar for 5 minutes, then turn plates over and incubate at 37 °C overnight. After 24 hours, your plates will be stored in the refrigerator (4 °C) to retard growth until you have a chance to record your results.

### D. After lab

Make sure you know the answers to the following questions.

1. Define: Competence, transformation, heat shock
2. What did we try to accomplish by incubating certain tubes in a water bath at 37° C in the first and third lab sessions?
3. What did we try to accomplish by putting certain tubes in a water bath at 42° C for 90 seconds?
4. How can you tell whether your experiment was successful?
5. One semester, none of the students had growth on either ampicillin, kanamycin, or the ampicillin/kanamycin plates. The controls, however, came out as expected. Additional information: all gels showed two bands each for pAMP and pKAN of the expected sizes. Suggest an explanation.

Prepare a lab report for the three Genetic Engineering labs. Your laboratory report should include

#### **Descriptive title**

- Think of the overall objective here

#### **Introduction**

Objectives of Genetic Engineering I (Restriction Digest)

- Why did we digest pAMP and pKAN with identical restriction enzymes?
- Why did we digest lambda?

- What purpose did the controls serve?

#### Expectations

- How many bands and of what size do you expect in the gel electrophoresis picture? Discuss all lanes.

#### Objectives of Genetic Engineering II (Ligation)

##### Expectations

- Why would you get plasmids? Discuss the significance of sticky ends, buffer, ATP, and ligase.
- Analyze possible fragment combinations. Which of your fragments might combine? Which will be replicated inside *E. coli*?

#### Objectives of Genetic Engineering III (Competency, Transformation, Plating)

##### Expectations

- Why would *E. coli* become competent?
- Why would *E. coli* become transformed?
- On which plates do you expect bacterial colonies?

### Results

- Report calculated size of DNA for **all** bands. Submit standard curve on semi-log paper with **all** bands plotted as well as the photo of your gel.
- Report result of plating, including controls.

### Discussion

- Identify and interpret electrophoresis bands. Note that sizes for circular DNA are inaccurate. Report whether the results match your expectations. Interpret **both** matching of expectations and results and discrepancies.
- Interpret whether digestion, ligation, and transformation were successful (**note**: you might have had success in both ligation and transformation even if you do not have growth on the kan/amp plates).
- Hypothesize what might have led to discrepancies between expectations and results (you might want to consult the “field guide to electrophoresis results”).
- Interpret your results in light of the results of your classmates. You **must** ask at least two other groups for their results.
- If you had discrepancies between expectations and results, what did you learn from it? What would you do differently if you had to do it again?

### Conclusion

In this section, only report whether your digest, your ligation, and your transformation with recombinant plasmids (plasmids containing both  $amp^r$  and  $kan^r$  genes) were successful, not successful, or whether your results were inconclusive.

#### Include

- Photo of your gel
- Your size standard graph on semi-log paper
- Table with fragment sizes versus distances migrated.
- Report evaluation chart.

Remember that all these figures and tables need to be referenced in the text, and need to have numbers and descriptive titles; species names are italicized.