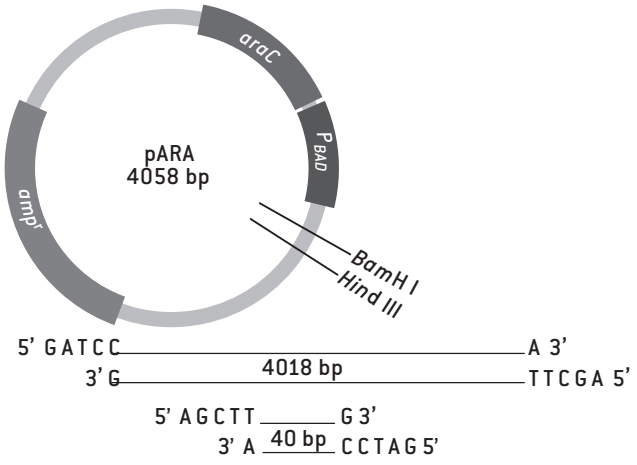
Ligation of pARA/pKAN-R Restriction Fragments
Producing a Recombinant Plasmid, pARA-R

In this laboratory the restriction fragments produced during Lab 2 will be ligated, or bonded together, using DNA ligase, making new recombinant plasmids. These newly formed plasmids will represent *recombinant* DNA molecules because the four restriction fragments have been recombined in different ways to produce new constructs. For example, assume that the four plasmid fragments were represented by the letter **A, A', K** and **R,** where **A** and **A'** represent the pARA fragments and **K** and **R** represent the two fragments resulting from the pKAN-R digest. Plasmids could be represented by any combination of two letters, such as AK or A'R, and any combination of even numbered fragments, such as AKA'R or ARAAKK and so forth. As you can see, there are many kinds of recombinant molecules that could result from mixing together these restriction fragments.

As you will remember, the restriction enzymes we are using are *Bam*H I and *Hin*d III. Cutting the plasmids at the *Bam*H I and *Hin*d III restriction sites leave "sticky ends." The sticky ends on the cut DNA can be ligated to any other fragment of DNA with a complementary sticky end. Examine the pARA plasmid map, below, to see the locations of the *Bam*H I and *Hin*d III restriction sites and the sticky ends that form on the 5'-ends of its restriction fragment.

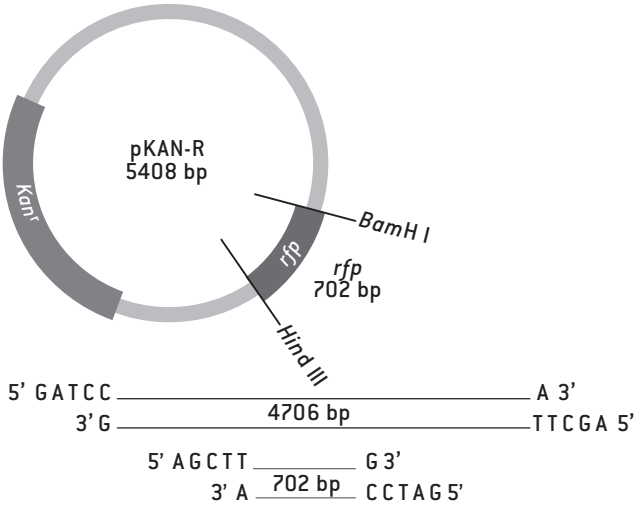
Because pARA has one *Bam*H I and one *Hin*d III restriction site, the digest will leave two fragments. The restriction fragments are depicted below. It is important to remember that the large restriction fragment carries the *amp*^{r} gene, the gene that provides resistance to ampicillin. The smaller fragment does not carry any genes.



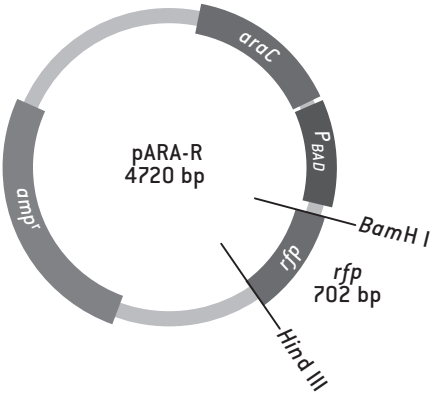
The plasmid pKAN-R has one *Bam*H I and one *Hin*d III restriction site that flank the *rfp* gene. The digestion of pKAN-R will leave two fragments, one will be 4706 bp and the other will be 702 bp.

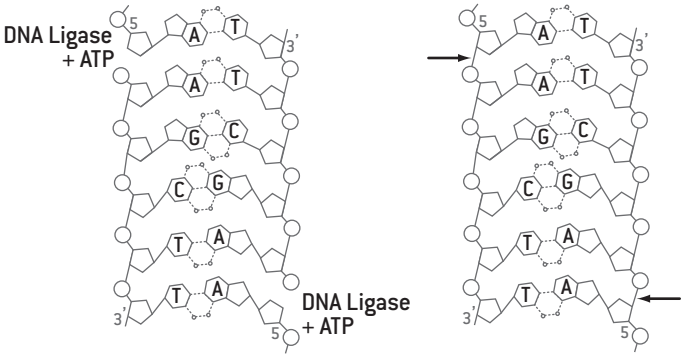
Ligation will bond any two *Bam*H I sticky ends together and any two *Hin*d III sticky ends together. You should be able to see that many different combinations of fragments are possible. The combination of interest to us is the 4018bp pARA fragment recombined (containing the *amp*^{r} gene) with the 702bp pKAN-R fragment (*rfp gene*). The combination of these two fragments will yield a recombinant plasmid we will call **pARA-R**.

The ligation of the 702bp pKAN-R fragment will place the *rfp* gene into the plasmid at a location that will allow a bacterium to synthesize (*express*) the mutant Fluorescent Protein, mFP.



The restriction fragments are initially held together by the hydrogen bonding between the nucleotide bases that makeup the sticky ends. You may recall that adenine and thymine share two hydrogen bonds while cytosine and guanine share three. This helps to ensure that only complementary sticky ends will match up.





Hydrogen bonds are weak chemical bonds, and they are inadequate to hold the sticky ends together permanently. The enzyme DNA ligase, with energy supplied by ATP, will form covalent bonds between the sugar and phosphate groups of the DNA backbone. In the diagram below, you can see the positions of these bonds on each side of the DNA molecule. When the covalent bonds are formed, the bonds complete the phosphodiester linkage between the two sugars and the phosphate group on each strand. The resulting chemical bonds are a relatively strong bond.

Materials

REAGENTS

Digested pARA (A+ from Lab 2)
Digested pKAN-R (K+ from Lab 2)
5x Ligation buffer with ATP
T4 DNA ligase in "lig" tube
Distilled water

EQUIPMENT & SUPPLIES

P-20 micropipette and tips
70°C water bath
Plastic microfuge tube rack
Permanent marker

Methods

1 Obtain your A+ and K+ tubes from the rack at the front of the class. **Place the two tubes in the 70°C water bath for 30 minutes.** This heat exposure will denature (inactivate) any *Bam*H I and *Hin*d III that might be active.
Why is this important?

2 While your tubes are in the water bath, obtain the 5x buffer and a **Ligase** tube from the instructor. The ligase tube contains 2μL of DNA ligase. Label this tube with your initials.

3 After the 30-minute, 70°C-incubation step, add 4μL of A+ directly into the DNA ligase at the bottom of the *Ligase* tube.

4 Using a new tip, add 4μL of K+ to the solution in the *Ligase* tube.

5 Using a new tip, add 3μL of 5x ligation buffer directly into the solution at the bottom of the *Ligase* tube. Discard the buffer tube.

6 Add 2μL of dH_2O to the *Ligase* tube, using a clean tip. **Gently and slowly** pump the plunger in and out to mix the reagents. Do this without splashing the solution onto the sides of the microfuge tube. The table below summarizes the contents of the *Ligase* tube.

A+	K+	5x ligation buffer	dH_2O	Ligase	Total volume
4µL	4µL	3µL	2µL	2µL	15µL

7 If you have droplets of liquid clinging to the sides of the tube, briefly centrifuge the tube to pool the reagents.

8 Place your ligase, A+ and K+ tubes in the microfuge racks at the front of the room. Your ligase tube will be kept overnight at room temperature.

Conclusions

1a Why was it important to place the A+ and K+ tubes in the 70°C water bath before setting up the ligation reaction?

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1b What do you think might have happened if this step was omitted?

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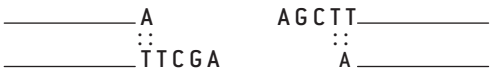
2 Make a diagram to show how the following sticky ends would join together. (: = hydrogen bonding) See page 3.2 for base pairing example.



3 Although many recombinant plasmids are possible, draw *three* possible recombinant plasmids. Include as one of the three the combination in which we are most interested—the one that combines pARA with the pKAN-R fragment carrying the *rfp* gene.

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4 Could two *rfp* fragments join together and circularize in the Ligase tube?

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5 In the DNA molecule, there are two kinds of chemical bonds: covalent chemical bonds and hydrogen bonds. Briefly describe how these bonds differ in strength and where, in the DNA molecule, you would find them.

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6a During ligation, which of the bonds (hydrogen or covalent) form first?
Where do they form?
Which bonds form next and where do they form?

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6b DNA ligase is required to form which bond?

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