

Restriction Analysis of pARA and pKAN-R

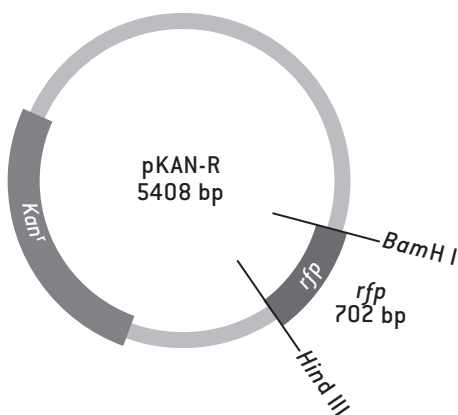
Plasmids are circular pieces of DNA that are found naturally in bacterial cells. The plasmids used in molecular biology have been modified through genetic engineering to facilitate gene cloning and protein production (*gene expression*) in bacteria. Antibiotic resistant genes have been engineered into these plasmids and function as *selectable markers*—that is to say, these genes allow us to *select* between bacteria that harbor the plasmids from those that do not. If a bacterium carries a plasmid with an antibiotic resistant gene, the bacterium will be able to grow and reproduce in the presence of that antibiotic; those bacteria without the plasmid will not be able to grow. Thus, antibiotics can be used to select bacteria that are resistant and presumably carry a plasmid with the resistant gene from those bacteria that do not carry the plasmid. Two plasmids will be used in this laboratory: **pARA** contains a gene for ampicillin resistance, *amp^r*, and **pKAN-R** contains a gene for kanamycin resistance, *kan^r*.

The purpose of this laboratory is threefold: 1) to introduce a method commonly used to analyze the genetic elements of plasmid DNA; 2) to examine the role and nature of restriction enzymes; and 3) to take the first steps in producing a *recombinant* DNA molecule.

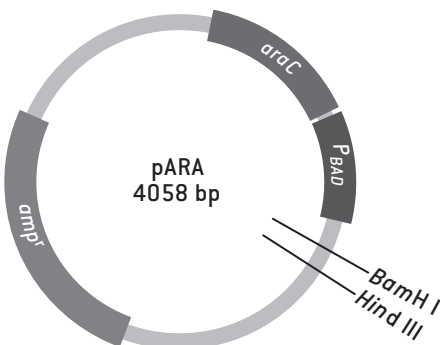
The plasmid pARA is 4058 base pairs (bp) in size. A “base pair” would be adenine:thymine or guanine:cytosine and is the common method used to express the size of DNA molecules. The plasmid carries the *amp^r* gene, which encodes the protein beta lactamase, an enzyme that destroys the antibiotic ampicillin. Beta lactamase, then, enables bacteria to reproduce in the presence of ampicillin. In addition, pARA carries a gene for the AraC protein, a protein that helps the bacterium make proteins encoded by genes inserted into this plasmid. A gene, even a foreign one, can be expressed (produced) if it is inserted into a specific location in this plasmid. The region of pARA labeled *p_{BAD}*, in the plasmid map, indicates

the site where RNA polymerase needs to bind to initiate transcription. The sites labeled “*Bam*H I” and “*Hind* III” represents restriction sites for these two restriction enzymes. Study the plasmid map below and locate these plasmid components.

The plasmid pKAN-R carries the kanamycin resistant gene, *kan^r*, which encodes a phosphotransferase, an enzyme that transfers a phosphate group to the kanamycin molecule destroying its antibiotic effects. Kanamycin is an antibiotic that kills bacteria by preventing them from making proteins. If a cell cannot synthesize proteins, it will die. The *kan^r* gene confers resistance to kanamycin for bacteria that have taken up this gene. In addition to *kan^r*, the plasmid carries the gene for mutated Fluorescent Protein, mFP, called red fluorescent protein or “*rfp*.” The pKAN-R plasmid is approximately 5,408 bp in size.



The *fluorescent* protein gene was originally isolated from *Discosoma* sp, a sea anemone found in the Indo-Pacific ocean. The wild-type gene has been mutated, through a process called directed evolution, to produce colors that are several times brighter than the wild-type protein. The term “wild type” refers to the original gene, the one that you would find in nature. The *rfp* gene has been engineered into the plasmid pKAN-R. Note that the *rfp* gene for mFP has both *Bam*H I and *Hind* III restriction sites on either side. A “restriction site” marks the specific location where an enzyme will cut the DNA plasmid. If pKAN-R is digested with *Bam*H I and *Hind* III, the *rfp* gene will be physically cut from the plasmid. During this laboratory, then, you will remove the *rfp* gene from pKAN-R and remove the small 40bp fragment from the pARA plasmid using the same enzymes. During the next laboratory, you will insert the *rfp* gene into pARA producing a recombinant DNA molecule.



Materials

REAGENT

pARA (80 ng/ μ L)
 pKAN-R (80 ng/ μ L)
 Restriction enzymes
 (*Bam*H I + *Hind* III)
 2.5x restriction buffer
 Distilled water, dH₂O

EQUIPMENT & SUPPLIES

P-20 micropipette and tips
 1.5 mL microfuge tubes
 Minicentrifuge
 37°C water bath
 Permanent marker

Methods

Preparing the pARA-R restriction digest

This laboratory protocol uses the restriction enzymes *Bam*H I and *Hind* III to digest the plasmids pARA and pKAN-R. This is the first step in making a recombinant DNA molecule.

- 1 Obtain the following four microfuge tubes: **pARA**, **pKAN-R**, ***Bam*H I** and ***Hind* III** (enzyme mix) and **2.5x buffer**.

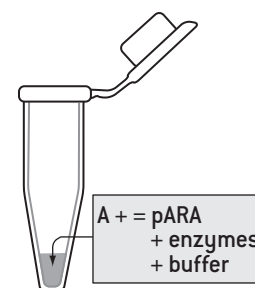
- 2 Obtain four clean 1.5 mL microfuge tubes and use a marker to label a set of four 1.5 tubes as follows:

A + = pARA + *Bam*H I and *Hind* III

A - = undigested pARA (pARA without enzyme)

K + = pKAN-R + *Bam*H I and *Hind* III

K - = undigested pKAN-R (pKAN-R without enzyme)



- 3 The reaction matrix summarizes the reagents used in the restriction digest. To set up the digest, follow the specific directions beginning at step 4.

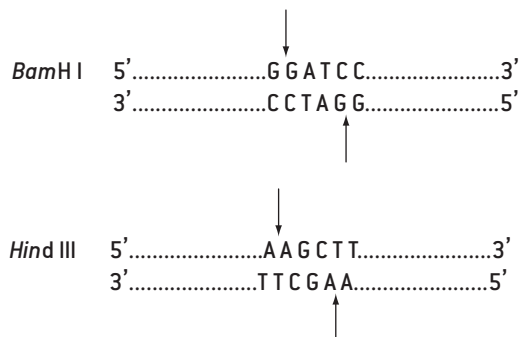
Tube	2.5x Buffer	dH ₂ O	pARA	pKAN-R	Enzymes	Total Volume
A+	4 μ L	—	4 μ L	—	2 μ L	10 μ L
A-	4 μ L	2 μ L	4 μ L	—	—	10 μ L
K+	4 μ L	—	—	4 μ L	2 μ L	10 μ L
K-	4 μ L	2 μ L	—	4 μ L	—	10 μ L

- 4 Use a fresh tip and add 4 μ L of 2.5x restriction buffer to **all** four tubes.
- 5 Add 2 μ L of dH₂O to tubes labeled A- and K -. What is the purpose of this step?
- 6 Use a fresh tip and add 4 μ L of pARA to tubes labeled A+ and A-.
- 7 Use a fresh tip and add 4 μ L of pKAN-R to tubes labeled K + and K -.
- 8 Add 2 μ L of enzyme mix, containing *Bam*H I and *Hind* III, to the A+ and K + tubes. Add the enzymes directly into the solution at the bottom of the microfuge tube. Be certain to use a new tip for each tube to avoid contamination. After the addition of the enzymes, gently pump the solution in and out with the pipette to mix the reagents and cap the tubes .
- 9 If there is a minicentrifuge available, set the tubes into the rotor, being certain the tubes are in a balanced configuration, and spin the tubes for four seconds. This brief spin will pool all of the reagents at the bottom of each tube.
- 10 Place **all four tubes** into the 37°C water bath, and incubate for at least **60 minutes**.

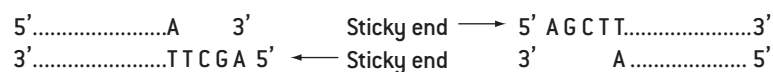
Following the 60 minute incubation, the digest can be kept frozen, at -20°C, until time is available for electrophoresis.

Conclusions

Review the restriction maps of plasmids, pARA and pKAN-R. Because *Bam*H I and *Hind* III are specific restriction endonucleases, they will consistently cut DNA wherever it encounters the six-base *recognition sequences* indicated below. The precise location that is cut is called a *restriction site*. The DNA molecule consists of two strands of nucleotide building blocks. These building blocks are oriented in the opposite direction on each strand. Thus, the two strands that makeup a DNA molecule are said to be “anti-parallel.” For convenience, we can say that one strand is oriented in a 5’ (“five prime”) to 3’ (“three prime”) direction while the other strand is oriented 3’ to 5’. Careful examination of the restriction sequences will reveal that the sequence of nucleotides is a palindrome; that is to say, it reads the same on both strands when read in a 5’ → 3’ direction.



Therefore, whenever *Hind* III encounters this six-base sequence, it will cut the DNA helix between the adjacent adenine bases. This leaves four unpaired bases forming a “sticky end.”



1a What is the *recognition sequence* for *Bam*H I?

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1b In a 5' → 3' direction, what sequence of bases represents the “sticky-ends for each?”

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Examine the pARA and pKAN-R plasmid maps and fill-in the following:

2a pARA digestion will yield fragments and will be base pairs in length.

2b pKAN-R digestion will yield fragments and will be bp and bp in length.

3 Assume you were given a culture of bacteria carrying one or both of these plasmids. Design a simple experiment that you could use to determine which of these plasmids, pARA or pKAN-R, the bacteria in the culture were carrying.

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