pARA-R Restriction Digest:

An Introduction to Plasmids and Restriction Enzymes

Two powerful but fundamental tools used in biotechnology are restriction enzymes and bacterial plasmids. Restriction enzymes allow molecular biologists to cut DNA molecules from different organisms and recombine the molecular pieces to produce *recombinant* DNA molecules. Plasmids are circular pieces of DNA that are naturally found in bacteria. Through recombinant DNA technology and restriction enzymes, recombinant DNA plasmids can be engineered to clone genes or to express proteins encoded by genes.

Restriction enzymes were first observed by Werner Arbor in 1962. Arbor discovered that some bacteria appeared to use a primitive immune system that prevented viral DNA from replicating within the infected host cell. Some years later, it was revealed that this immune mechanism involved a class of proteins now known as restriction enzymes. The name is derived from the enzyme's ability to *restrict* the growth of viruses in the bacterial cells. Restriction enzymes accomplish this by breaking a bond in the sugar-phosphate backbone of the viral DNA- the enzymes cut the viral DNA into small fragments.

The restriction enzymes that were first identified appeared to digest the DNA molecule randomly. Later, restriction enzymes were found and purified that would cut the sugar-phosphate backbone at a specific location or within a specific nucleotide sequence, commonly four to six nucleotides in length. Table 1 identifies some of these *specific* restriction enzymes, their source and the nucleotide sequences each recognizes. In 1978, Daniel Nathans (Johns Hopkins University), Hamilton Smith (Johns Hopkins University) and Werner Arbor received the Nobel Prize for Medicine for their work with restriction enzymes.

Source	Restriction enzyme	Recognition sequence
Bacillus amyloliquefaciens	*Bam*H I	5' G↓GATCC 3' 3' CCTAG↑G 5'
Escherichia coli	*Eco*R I	5' G↓AATTC 3' 3' CTTAA↑G 5'
Haemophilus influenzae	*Hin*d III	5' A↓AGCTT 3' 3' TTCGA↑A 5'

Table 1. Restriction enzymes used in this laboratory. ↓↑ indicate sites where the sugar-phosphate backbone is cut or cleaved.

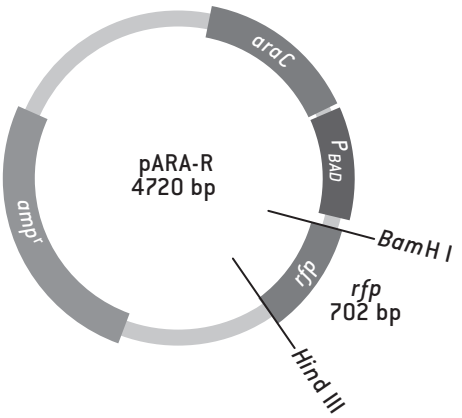
When restriction enzymes cut or digest DNA, the fragments that result—called *restriction fragments*—have several unpaired bases extending from their cut ends. These are called "sticky ends." If DNA molecules from different sources are digested using the *same* restriction enzyme, the unpaired bases from each piece should be able to join (or anneal) together as the unpaired bases at the sticky ends will be complementary—A:T and G:C. It is this unique attribute of restriction enzymes that enable genetic engineers to combine DNA fragments from different organisms to produce recombinant DNA molecules.

Figure 1. (a) DNA molecule with *Bam*H I and *Hin*d III restriction sites (bold). The arrows indicate sites where enzymes will cut the sugar-phosphate backbone of the DNA molecule. (b) The lower DNA molecule indicates the location of the "sticky ends" (bold).

Bacterial plasmids are relatively small, circular pieces of DNA that bacteria can carry in addition to their genomic DNA (single chromosome). In nature, the plasmid DNA frequently carries one to several genes that help the bacterium survive—perhaps by providing resistance to an antibiotic. Bacteria can pass along plasmids during conjugation (mating). The bacteria we use in the laboratory have been mutated, so they cannot exchange plasmids during sexual reproduction.

Naturally occurring plasmids have been engineered to perform specific functions: typically, gene *cloning* and gene *expression*. This laboratory examines pARA-R, a *recombinant* DNA plasmid that has been engineered to express the *rfp* gene to produce a mutant Red Fluorescent Protein (mFP). The plasmid contains various control elements that allow a bacterium carrying this plasmid to express this foreign gene. The gene was originally obtained from the genome of *Discosoma* sp., a sea anemone from the Indo-Pacific Ocean. The plasmid map below indicates some of the important control regions,

*ara*C and P_{BAD}, and the location of the *rfp* gene. In addition, the map indicates the location of two restriction sites: one for *Hin*d III and one for *Bam*H I. How might you go about cutting out the *rfp* gene? Also note, the plasmid carries an antibiotic resistance gene, *amp*^{r}. This gene will enable a bacterium carrying this plasmid to live in an environment containing the antibiotic ampicillin.



Materials

REAGENTS

pARA (70 ng/ µL)
Restriction enzymes (*Bam*H I + *Hin*d III)
2.5x restriction buffer
Distilled water (dH_2O)

EQUIPMENT & SUPPLIES

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

Methods

Restriction Digest of pARA-R

The purpose of this laboratory is twofold: 1) to examine the role of restriction enzymes and their importance in genetic engineering; 2) to examine a bacterial plasmid and how it is used in biotechnology.

This laboratory protocol uses the restriction enzymes *Bam*HI and *Hin*d III to digest the recombinant plasmid, pARA-R. The restriction digest will isolate from pARA the *rfp* gene from the larger fragment of the plasmid that containing *ampr, ara*C and P_{BAD}. The protocol uses a control, undigested pARA-R, along with a DNA size marker or ladder that will help you identify and confirm the sizes of the restriction fragments.

Preparing the pARA-R restriction digest

1 Obtain the following three 1.5 mL microfuge tubes from your teacher: **pARA-R, enzyme mix** and **2.5x restriction buffer.**

2 Obtain two clean 1.5 mL microfuge tubes and use a marker to label the tubes as follows: "**A+**" and "**A-**." **Include your group number and class period on each tube, so that you can locate them for the next lab period.**

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	H_2O	pARA-R	Enzyme mix	Total volume
A+	4µL	–	4µL	2µL	10µL
A-	4µL	2µL	4µL	–	10µL

4 Use a fresh tip and add 4μL of 2.5x restriction buffer to **both** tubes.

5 Add 2μL of dH_2O to tube labeled A-.
What is the purpose of this step?

6 Use a fresh tip and add 4μL of pARA-R to tubes labeled A+ and A-.

7 Bring the A+ tube to your teacher, who will dispense the enzyme mix into the tube, or if you were given this enzyme mix, carefully add 2μL of the enzyme mix **directly** into the solution in tube A+ containing plasmid and buffer. After the addition of the enzymes, cap the tube and gently flick the lower portion of each tube to mix the contents.

8 If there is a minifuge 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.

9 Place both tubes into the 37°C water bath, and incubate for at least **60 minutes.**

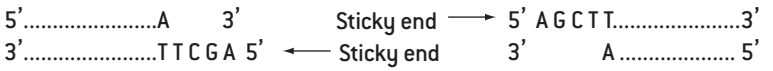
10 Following the 60-minute incubation, your teacher may place the tubes into the freezer until you are ready for electrophoresis (Lab 4a).

Digested plasmids can be kept at -20°C indefinitely.

Conclusions

Review the restriction map of the pARA-R plasmid. *Bam*H I and *Hin*d III are *specific* restriction enzymes and will consistently cut the double-stranded DNA wherever they encounter their respective six-base *recognition sequence* given in the table on page 2a.1. These locations cut are called *restriction sites*. 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 stands that makeup a DNA molecule are said to be "anti-parallel." For convenience, we can say that one strand in oriented in a 5' ("five prime") to 3' ("three prime") direction while the other strand is oriented 3' to 5'. Careful examination of the *Bam*H I and *Hin*d III restriction sequences will reveal that the nucleotide sequences are palindromes; that is to say, they read 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 are the *recognition sequences* for *Hin*d III and for *Bam*H I?

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

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2a Examine the pARA-R plasmid map and fill in the following:
How many restriction fragments will result from the digestion of pARA with *Bam*H I and *Hin*d III?

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2b What will be the approximate lengths, in base pairs, of these restriction fragments?

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2c Which restriction fragment will carry the *amp*^{r} gene?

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2d Which restriction fragment will carry the *rfp* gene?

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3 Assume your teacher gave you a culture of bacteria. The culture could be one containing bacteria carrying the plasmid pARA-R or a culture containing bacteria without the plasmid. Design a simple experiment that you could use to determine which of these cultures you were given.

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