Module I: RESTRICTION DIGESTION

This module will teach you a basic understanding of restriction enzymes, restriction digest, and plasmids.

Upon completion of this lab, you will be able to:

- Know what restriction enzymes are, where they come from, and what they can be used for.
- Set up and analyze a restriction digest
- Make a gel and run an agarose gel for fragment resolution

Restriction digestion

Background:

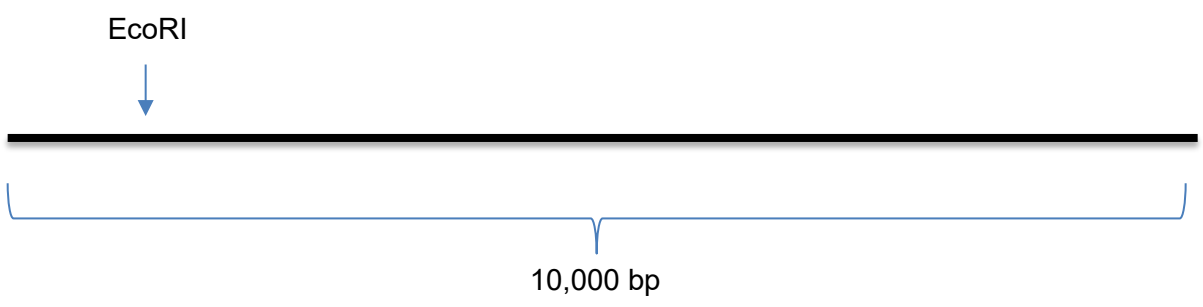
Restriction enzymes are enzymes that cleave the phosphodiester bonds between nucleotides. Restriction enzymes are a form of 'nucleases', specifically endonucleases. Endonucleases are enzymes that degrade polynucleotides at internal sites, within a continuous sugar-phosphate backbone. Bacteria contain a special type of endonuclease, called a restriction endonuclease that functions to restrict the invasion of parasites (primarily viruses) by destroying their DNA. Rather than destroying DNA at random, restriction endonucleases degrade DNA at specific base sequences. Each type of restriction endonuclease degrades at a different sequence, called its "recognition sequence". These naturally occurring enzymes are also commercially produced, and, as they allow specific DNA sequences to be targeted and manipulated, they have proven extremely useful in many areas of recombinant DNA technology.

The recognition sequences of restriction enzymes are generally 4, 5, or 6 base pairs (bp) in length and are palindromic (i.e., the sequence is the same on both strands, reading in opposite directions). For example, the recognition sequence for the enzyme *Eco*RI is GAATTC. For a different enzyme, called *Hin*d III, the sequence is AAGCTT. The palindromic nature of each sequence is seen by writing down the sequence of the double-stranded DNA at the cut site:

EcoR I 5'-G AATTC-3'
3'-CTTAA G-5'

Hind III 5'-A AGCTT-3'
3'-TTCGA A-5'

The restriction enzyme thus cuts a DNA molecule into shorter pieces. For example, suppose a piece of DNA that is 10,000 bases long is treated with the *Eco*RI enzyme, and the enzyme finds one recognition sequence (restriction site) 500 bases from one end of the molecule. Please also note that the name of the restriction enzyme is derived from the bacteria it was first isolated from including the bacterial strain. Roman numerals are used to designate if this was the first, second, third, fourth, etc. restriction enzyme isolated from that particular bacterial and strain. Because of this nomenclature, the first three letters of the restriction enzyme name are always italicized as they designate a genus and species. For example: *Eco*RI is the first (roman numeral I) restriction enzyme isolated from strain 'R' of *Escherichia* (the *'E'* stands for the genus) *coli* (the 'co' stands for the species) of *Eco*. Also notice that when in the case of *Eco*RI or *Hin*dIII cut their target restriction site, this results in jagged or puzzle piece kinds of ends called 'sticky' ends. They are called 'sticky' because the overhangs resulting from the cuts make it easy for the ends to 'stick' to complementary regions of DNA when sealing back together. Some restriction enzymes are blunt end cutters meaning that when they cut, the ends are 'blunt' and can be ligated to any other blunt end DNA molecule. The restriction enzyme *Sma*I is an example of a blunt end cutter with the restriction site: CCC GGG
GGG CCC



If the products of the restriction reaction (termed a restriction digest) are then separated on an agarose gel, two fragments will appear, one 9,500 bases long and one 500 bases long for the *Eco*RI example above (Fig. 1).

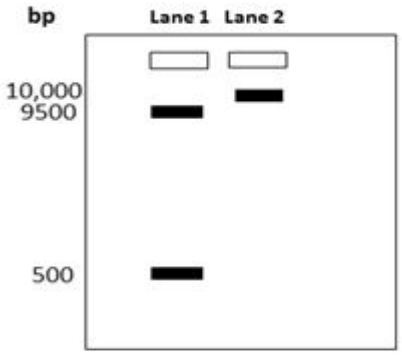


Figure 1. Gel electrophoresis of a restriction digest. Lane 1 fragments produced from the restriction digest, lane 2 undigested control.

Methods: Part A

Cut out the DNA sequence below (or copy to a piece of paper, if you don't have a printer). Pretend that you are the enzyme *Eco*RI, and cut the sequence, using a pair of scissors. Take a picture of your cut sequence and **paste it below the original sequence in your notebook.**

5' -ATGGGCGAATTCCCAACTGGCGGCGGAATTCGACAGATTTAGACCCATAGACGGTCACATACGATA-3
3' -TACCCGCTTAAGGGTTGACCGCCGCCTTAAGCTGTCTAAATCTGGGTATCTGCCAGTGTATGCTAT-5'

Answer the questions below in your research notebook.

1. How many *Eco*RI cut sites were in the sequence above? _____
2. How many fragments would an *Eco*RI digest form? _____
3. By counting nucleotides, what were be the sizes of each fragment resulting from the *Eco*RI digest of the sequence above? _____

4. What is the purpose of the undigested control?_____

Notes of interest: If you had a circular piece of DNA, cutting once would result in only one fragment. To make sure you understand this concept, please take a rubber band (we will imagine that this is a circular double-stranded piece of DNA). Now cut it once – you will have one linear fragment (no need to show your work, but please make sure that you understand this concept!). If you cut a circular piece of DNA twice, it will result in two fragments, and so on.

Part B

Methods

1. **Per a pair**, set up a restriction digest and an undigested control for pDEST17 plasmid DNA. pDEST17 is a plasmid, an extrachromosomal **circular** DNA, in this case, man-made but based on naturally occurring bacterial plasmids. pDEST17 has a genome of 6,354 bp. You will use one of the six restriction enzymes listed below for your digest. You will not know which enzyme from the list below you have. This is the 'mystery' that you need to solve.

   Prepare a tube with the following ingredients *in the order listed!* Make sure to add the enzyme last and keep the enzyme on ice. Your enzyme tube will have a number on it.

	Digest	undigested control
pDEST17 DNA	4.0 µl	4.0 µl
10 X restriction buffer	2.0 µl	2.0 µl
Sterile water	13.0 µl	14.0 µl
Restriction enzyme	1.0 µl	0.0 µl
Total volume of the reaction:	20 µl	20.0 µl

2. Place your digest and undigested control in the heating block in the back of the room set at 37 ^{o}C. for 15 minutes.
3. Place your completed reactions in the rack at the front of the room which will be placed at -20 ^{o}C for storage until next week.

4. **Bioinformatics:** Look at the image of the pDEST17 plasmid below.

5. Determine the number of fragments produced by the *Bam*HI restriction enzyme.

6. Determine the fragment sizes in bp produced by the *Bam*HI restriction enzyme and then draw these fragments on the gel image below. You determine the size of fragments but subtracting cut positions. For example, *Bam*HI has two restriction sites on the pDEST17 plasmid and cuts at positions 337 and 1040 bp. This will result in two fragments. One fragment will be 1040 -337 = 703 bp. The other fragment will be the remaining pDEST17 plasmid, so 6,354 bp - 703 bp = 5651 bp.

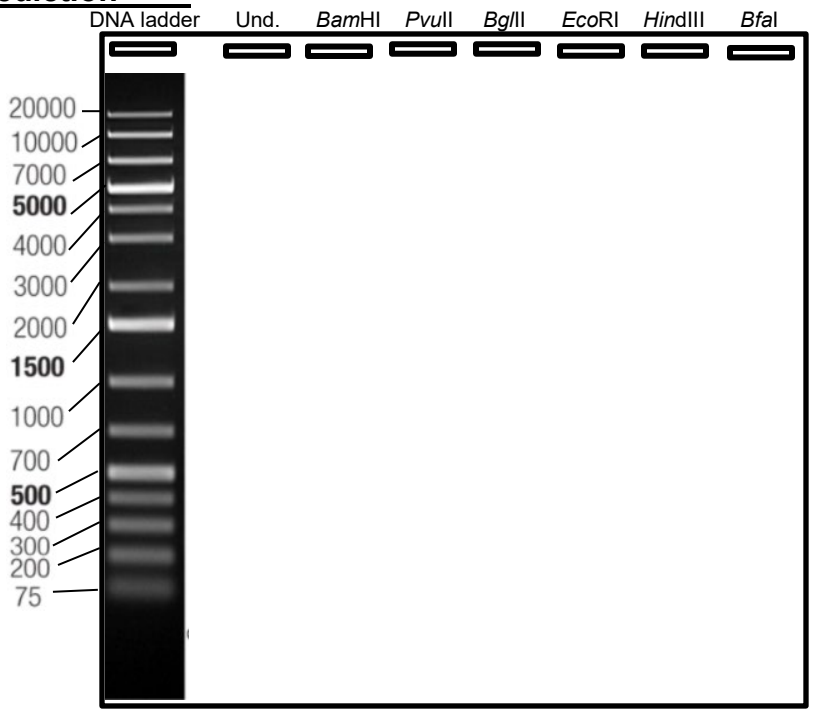
7. Repeat steps 5-6 for each of the six enzyme choices. Next week you will resolve your restriction digest and undigested control on a 1% agarose gel. You will then compare the fragment sizes on your actual gel with the fragments on the predicted gel image to determine which enzyme you had for your digest. An example of how to analyze your gel results is at the end of this lab module on the last page of the Module I worksheets.

8. **Make sure to place your predicted gel picture in your research notebook this week.**

The four possible enzymes that could have been used are the following:

1. *Bam*HI 2. *Pvu*II 3. *Bgl*II 4. *Eco*RI 5. *Hin*dIII 6. *Bfa*I

Gel prediction



Und.= undigested control

*The DNA ladder we will be using is the 1kb plus DNA ladder by Thermo Scientific. A picture of this ladder is given in the prediction gel image above along with the sizes of the fragments in the DNA ladder.

**The undigested control is just that, an identical reaction with the same DNA but not digested. Therefore, on one band is expected at 6,354 bp.

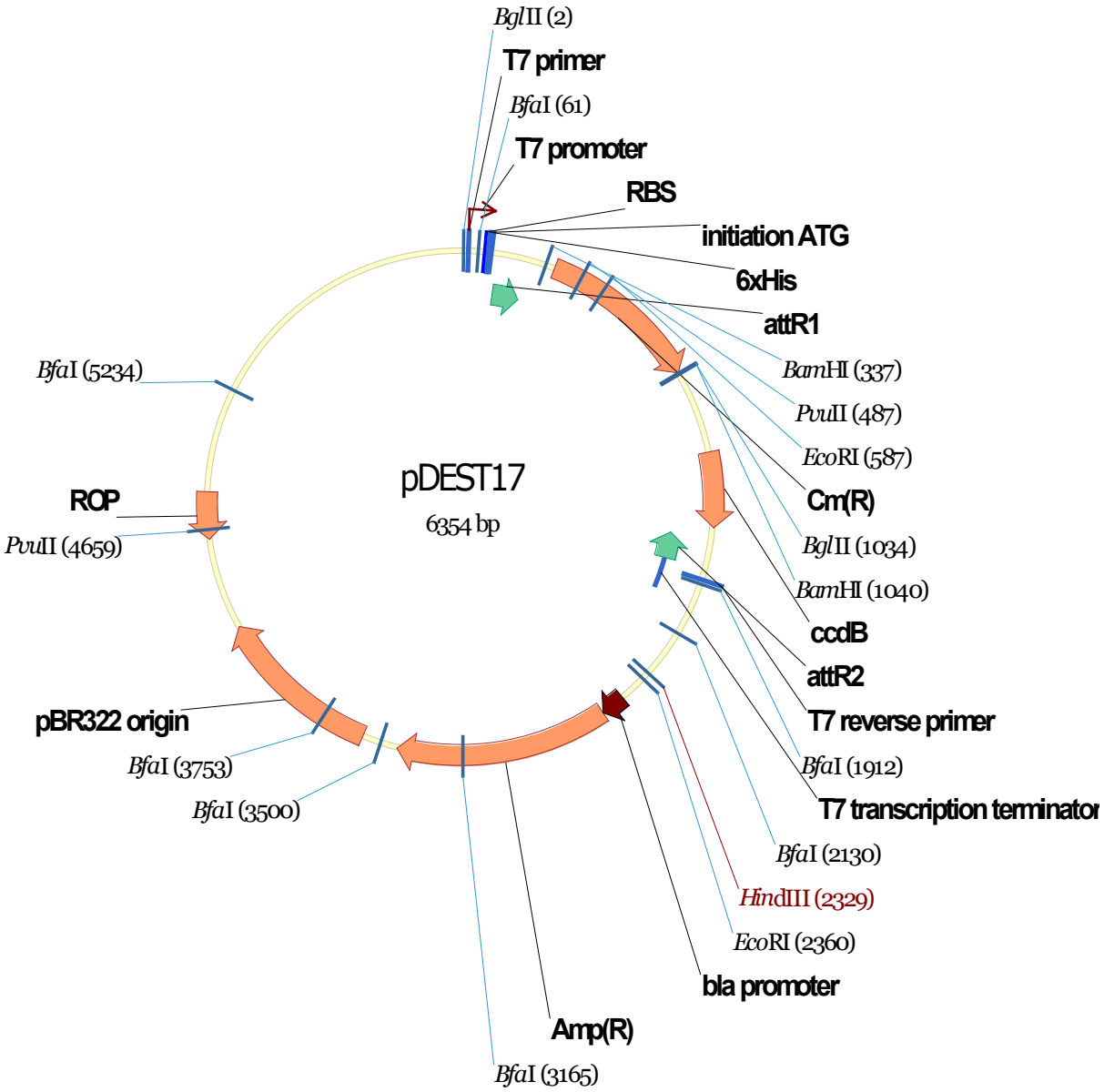


Figure 1: pDEST17 plasmid for mystery digest. Restriction enzymes and nucleotide position of restriction sites on plasmid are shown.

Part C: Gel electrophoresis

Now we need to run an agarose gel to resolve the restriction fragments from the digest and undigested control reactions performed last week.

Before coming into lab:

I. Watch the following YouTube video, so you get a general idea of how to make a gel. Also, the electrophoresis units used in this video are the same that are being used in our labs, so if you will ever run a DNA gel in a subsequent lab in Swain, you know what you are doing. https://www.youtube.com/watch?v=1_CE4cY_vpM

II. Now complete this virtual lab: https://learn.genetics.utah.edu/content/labs/gel/ and answer the questions below:

1. During gel electrophoresis the DNA is separated according to what?
2. Describe the purpose of an agarose gel.

3. Agarose gel preparation. The amount of agarose in a certain volume of buffer determines the pore size of your gel. The more agarose, the more ‚solid' your gel will be, with smaller pores for DNA to travel through. The less agarose, the larger the pores for DNA to travel through. The, percentage' of agarose of the gel indicates the amount of agarose that was used to prepare it. Percentages as low as 0.5% can be used, and as high as 2-3%.

For the electrophoresis units in our lab, a total volume of 50 – 100 mL can be used to prepare a gel. What volume you prepare is up to you, but depends on a few things:

a. How much sample you would like to load. If you have a lot of sample and suspect a small DNA amount present, then you want to load as much as possible. A thicker gel (100 mL) will give you a deeper loading well than a thinner gel (50 mL).
b. The thicker the gel, the longer it takes to solidify before you can load your samples. If you only want to load a small amount of DNA (up to 20 uL), a thinner gel will speed things up.

The specific buffer that is usually used is TAE buffer. It is made of a Tris-acetate (Tris base and acidic acid, your buffer system, which also delivers the ions to conduct electricity) and EDTA, which binds divalent cations that could interfere with the electrophoresis.
Back to the percentage of your gel! One (1) g of agarose in 100 mL of buffer is a 1% gel. From here you can scale up or down to find out, how to make your desired percentage of gel

Please answer the following questions:

i. Assume you wanted to separate very large DNA molecules. Would you do this on an agarose gel of a high or low percentage of agarose? Explain your answer!

ii. How would you prepare a 0.8% agarose gel with a total volume of 50 mL of buffer.

4. When boiling the agarose/buffer mixture in the microwave, one must be careful to only heat for short intervals of 10 – 15 seconds. Take the flask out in between and look at it while holding it up against a light source. Swirl it around a little, if you still see undissolved agarose, heat for another few seconds, check again. You want to avoid overboiling the mixture, as this lowers the gel percentage AND messes up the microwave. Why is heating in the microwave required?
5. What is the purpose of the loading dye?
6. What is the purpose of the DNA standard?
7. What would happen if you plugged the black power cord into the red outlet and the red power cord into the black outlet (i.e. why is the important to choose the correct outlet?).
8. What is the purpose of ethidium bromide? **Note: This online lab omits the destaining part, which involves destaining the gel twice for 5 minutes on a shaker in destilled water. Ethidium bromide can also be added to the gel during gel preparation, which is shown in the YouTube video and is the method we will use in lab. This eliminates the need for staining of the gel. The gel can then be analyzed directly after the run is finished.*

***Part D: In lab Methods**:*

1. Place the undigested control and restriction digest from last week in the heating block set to 55 ^{o}C for five minutes.

2. Make a gel. Make one 1% agarose gel **per a table**. You will use a total volume of 50 mls for your gel and we will be using 1X TAE buffer to run the gel. ***Before you pour your gel in the gel support, call me over to add the Ethidium bromide to the gel.*** **Make sure to wear gloves at all times since EtBr is a carcinogen.**

3. Loading dye was included in the restriction enzyme buffer. So you do not need to add this to your samples. Go to step 4.

4. Load 20 µl of each reaction into a well on the gel. Make sure to record which lane has what in it. Your group will be the only ones who know this information.

Things to keep in mind when analyzing your gel:

Plasmids are circular. They can run in a gel undigested in three conformations: As an open circle (runs larger than the actual size), as supercoiled (runs smaller than the actual size), and anything in between the open circle and the supercoiled (runs closer to the actual size). Because of these different 'shapes' you may see up to three bands in your gel for the undigested plasmid DNA control.

Make sure to include in your notebook your labeled gel photo including fragment sizes expected and the size of fragments observed on your gel.

<u>Example of how to analyze your mystery digest:</u> Take a look at the gel image below. Assume that this is the result of your mystery digest. First, label the ladder fragments based on the ladder pic posted on Moodle.

Which of the six enzymes was used to cut the pDEST17 plasmid DNA?