

[www.dnagoestoschool.org](http://www.dnagoestoschool.org)

*Currently, there are more than 1000 restriction enzymes described*

*Check **REBASE**, the Restriction Enzyme data **BASE**, a collection of information about all restriction enzymes. The database was created by Dr. Richard J. Roberts and Dana Macelis. It contains restriction sites and other information about restriction enzymes. Available at <http://rebase.neb.com/>*

## Second Module

Content:

- Introduction to Restriction Enzymes
- Introduction to DNA electrophoresis
- Introduction to restriction maps and genetic tests

### What are Restriction Enzymes?

Endonucleases or restriction enzymes are proteins that cut DNA in small fragments. They recognize a very specific sequence of DNA called restriction site, such as AGCT. Wherever the particular sequence of a restriction site occurs in a DNA molecule, the restriction enzyme will cleave the DNA at that specific location. These enzymes are isolated from bacteria and named according to the organism. It usually has 3 or 4 letters followed by a number. The first letter designates the genus from which the enzyme was isolated while the second two letters comes from the species. For example, HindIII was isolated from Haemophilus influenzae while EcoRI was isolated from Escherichia coli. The numbers refer to the the order that the enzyme was isolated from the same organism, i.e, HindIII was the third enzyme isolated from Haemophilus influenzae. Different bacterial speeces have different restriction enzymes.

But why bacteria would produce an enzyme that cuts DNA in a very specific fashion and according to a specific DNA sequence? At first, it seems very unique that a bacteria would produce such an enzyme. These enzymes - discovered during the '60s when the field of molecular biology was just blooming- comprises the bacteria's main

defense system and their function is to cut the DNA of foreign organisms that penetrates the bacteria cell, such as bacterial virus called bacteriophages (phages for short). Restriction enzymes prevent replication of phage DNA inside the bacterial cell by cutting its DNA into small pieces. With their DNA in pieces, these organisms become harmless to the bacteria. To protect its own DNA from being cut by its own restriction enzyme, bacteria make use of a protection system called methylation in which an enzyme called methylase adds methyl groups ( $-\text{CH}_3$ ) to adenine or cytosine bases within the recognition sequence of the restriction enzyme. When methyl groups cover the backbone of the bacteria DNA, the access to the bacteria's DNA is blocked, precluding the action of the restriction enzyme. Phages cannot count on a methylation system to protect their DNA, although once inside the cell the bacteria methylase can add methyl

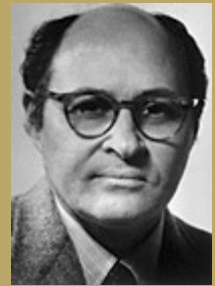
groups to the DNA of these organisms as well. But different from restriction enzymes, methylase is not a very fast-acting enzyme and before the methylase can mistakenly add methyl groups to the phage DNA, the restriction enzyme has already cut it.

The bacteria methylation system is considered a primitive immune system, since its main function is to protect the bacteria from invaders. More evolved organisms do not present such a system as a way to protect its DNA. Although methylation is also found in all superior organism,

*The first restriction enzyme was isolated in 1968 by Werner Arber, Hamilton O. Smith, and Daniel Nathans while working at the Johns Hopkins University. In 1978 they shared the Nobel Prize in Physiology or Medicine. To know more about this event, visit the Nobel e-museum at <http://www.nobel.se/medicine/laureates/1978/>*



W. Arber



D. Nathans



H.O. Smith



*The phage lambda is a type of virus that infects the bacteria cell*



## What is DNA electrophoresis?

DNA fragments generated due to the action of restriction enzymes can be analyzed through a technique called DNA electrophoresis, which separates DNA fragments according to their molecular weight. The molecules are separated across a span of gel, motivated by an electrical current. Activated electrodes at either end of the gel chamber produces the electric field. Because DNA is a negatively charged molecule due to the presence of phosphate groups, it moves toward the positive pole. The separation of fragments occurs according to each fragment's properties, which determines how fast the electric field can move the molecule across the gel. The bigger the fragment, the slower it will move toward the positive pole. Smaller fragments reach the bottom of the gel first. Most gels are made by agarose. Purified agarose is provided in powdered form, and it stays insoluble in aqueous solutions at room temperature. In order to dissolve, agarose needs to be melted. When it cools down, it starts the polymerization process and the gel is formed. The more agarose is added -or the more concentrated the gel is- the firmer it will be.

*As the voltage applied to a gel is increased, larger fragments migrate proportionally faster than small fragments. For that reason, the best resolution of fragments larger than 2 kb is obtained by applying no more than 5 volts per each cm of the distance between the two electrodes positioned at both ends of the electrophoretic chamber.*

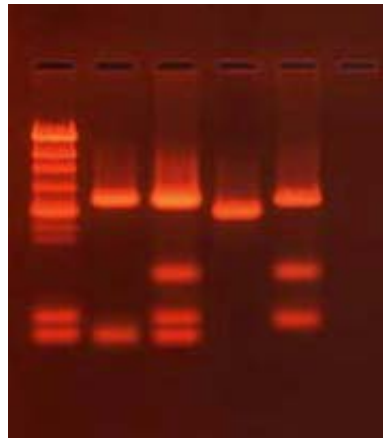
*The most common DNA electrophoresis gel is made by agarose, a neutral polysaccharide extracted from the cellular walls of certain algae. Its chemical structure gives agarose the ability to form gels that are very resilient even at low concentrations. Other polymers such as polyacrilamide are also used for DNA electrophoresis.*

Agarose is melted in an electrophoresis buffer solution and while still hot, it is poured into a template called a casting tray. A comb is placed in one end of the gel in order to produce small slots when the gel is solidified. These slots will be used to load the DNA samples. When the gel is solidified the comb is carefully removed. The gel is then placed in the electrophoresis chamber and covered with electrophoresis buffer. DNA samples are mixed with a special dye that allows the sample to *dive into* the slot (otherwise the samples would be floating on the surface of the buffer) and loaded into the slots. The lid and power leads are placed on the electrophoresis apparatus, and the current is applied. After running the gel for the desired time -running time can vary from 15 minutes to 48

hours-DNA can be visualized by different ways. The most common dye used for many years as a nucleic acid stain is called ethidium bromide. This compound intercalates with DNA and emits a red-orange color under ultraviolet light. But the ability of ethidium bromide to intercalate with DNA also makes this compound a potent mutagen. Methylene blue is an alternative stain used to visualize nucleic acids. It is much safer than ethidium bromide and does not require ultraviolet light for visualization.

*The polymerization process observed with agarose is the same process that occurs when we prepare Jell-O.*

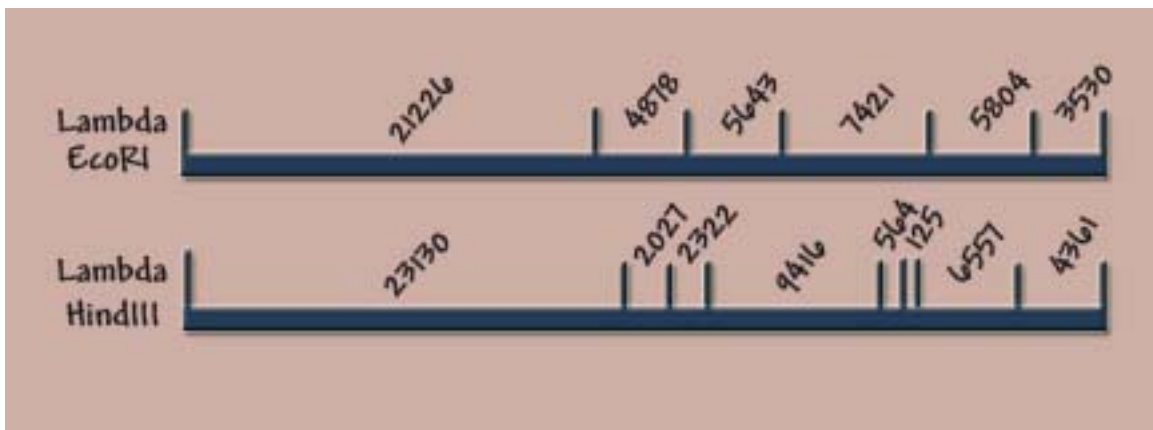
*Several different buffers have been recommended for electrophoresis of DNA. The most commonly used for duplex DNA are TAE (Tris-acetate-EDTA) and TBE (Tris-borate-EDTA). DNA fragments will migrate at different rates in these two buffers due to differences in ionic strength.*



*To see a simulation of how agarose electrophoresis gels work, check the Simulation of Electrophoresis & Sequence Building of DNA developed by Dr. Paul Craig at the Rochester Institute of Technology at <http://www.rit.edu/~pac8612/electro/dna/DNA.html#>*

*An agarose gel showing DNA fragments of different sizes. The small fragments reach the bottom of the gel first.*

Restriction sites  
EcoRI G/AATTC  
HindIII A/AGCTT



*Two restriction maps of Lambda DNA, one cut with ECORI and the other with HindIII. The numbers correspond to the sizes of the fragments generated by the action of each enzyme.*

## Lab Activity

In this activity you will load 4 different DNA samples into an agarose gel to observe the different restriction fragments. First you will prepare an agarose gel according to the specifications provided by your instructor.

### Protocol 1: Preparing an agarose gel

- 1) Weight \_\_\_\_\_ gr of agarose
- 2) Pour the wighted agarose in a Beaker
- 3) Add \_\_\_\_\_ml of the electrophoresis buffer
- 4) Using the microwave, let the mixture melt. When the solution is totally melted and the agarose cannot be detected in the solution, carefully remove the beacker from the microwave.
- 5) Let the solution sit for aproximately 10 minutes to chill a little bit. Do not let it sit for a long period as the agarose will solidify in the Beaker
- 6) While the agarose is cooling down, prepare the gel casting. If necessary use scotch tape to seal the ends of the casting tray. Add the comb to one of the ends of the casting tray.
- 7) When the solution has reached aproximately 60°C, pour the gel into the castingtray.
- 8) Let it solidify for aproximately 15 minutes
- 9) When the gel is hard, remove the comb and the scotch tape from the ends of the casting.
- 10) Place the gel in the electrophoresis cast
- 11) Add electrophoresis buffer to the chamber until it covers the surface of the gel.



Next, you will add the DNA samples to the slots in the gel. You will add one sample per slot. You will work with the following samples:

Sample #1- Lambda DNA not digested by restriction enzymes

Sample #2- Lambda DNA digested by EcoRI

Sample #3- Lambda DNA digested by HindIII

Sample #4- Lambda DNA digested by EcoRI AND HindIII

#### Protocol 2- Loading the samples

- 1) Add \_\_\_ul of loading buffer to each sample
- 2) Add \_\_\_ul of each sample to each slot in the gel

#### Protocol 3- Running the gel

- 1) Close the lid of the chamber
- 2) Turn on the power supply and set the voltage to \_\_volts
- 3) Verify that the gel is really running by watching the bubbles being formed in the electrophoresis buffer. If no bubbling is seen it means that the gel is not running. In this case you should (a) make sure the electrophoresis buffer is covering the gel; (b) make sure the scotch tape was removed from both ends of the casting tray; (c) make sure the chamber lid is securely closed ; (d) verify if there are any loose wires inside the electrophoresis chamber.
- 4) Let the gel run for \_\_\_hours

#### Protocol 4- Staining the gel

- 1) Place the gel on a plastic tray and add a few drops of electrophoresis buffer
- 2) Place the blue side of the Blue sheet on the gel
- 3) Run your finger firmly on the top of the blue sheet several times
- 4) Place the gel casting and a small Beaker over the blue sheet
- 5) Let it sit for 15 minutes
- 6) Remove the blue sheet and submerge the gel in distilled water
- 7) Change the water 3 times until the bands are visible

Write it down the sizes of the fragments generated by each enzyme individually and by the combination of both enzymes.

	EcoRI	HindIII	EcoRI+HindIII
24K			
23K			
22K			
21K			
20K			
18K			
17K			
16K			
15K			
14K			
13K			
12K			
11K			
10K			
9K			
8K			
7K			
6K			
5K			
4K			
3K			
2K			
1K			

## Questions:

- 1) Why does the first sample in the gel show only one big band?
- 2) Why do the DNA fragments generated by each enzyme separately have different sizes?
- 3) Why does the DNA sample submitted to both enzymes have more fragments than the sample submitted to only one enzyme?
- 4) Look at the lambda DNA map. Can you calculate the size of each fragment when the Lambda DNA is cut by both enzymes?

## Reading a restriction map

Now that you understand how restriction enzymes work, let's see how they can be used to distinguish two alleles. The two alleles shown below differ by only one nucleotide. By analysing the restriction map of each allele it is possible to check if a specific enzyme acts on only one of the alleles. The combination of all existing restriction sites in a specific DNA sequence is called a restriction map because it indicates every single site in the sequence that can be recognized and cut by a specific enzyme. By comparing restriction maps of slightly different sequences (sequences that differ in only few nucleotides) we can look for enzymes that cut one sequence but not the other. When a specific enzyme cuts sequence A but doesn't cut sequence B, after exposing the two to the enzyme, only sequence A will be cut. This effect can be easily tested on an agarose electrophoresis because sequences A and B will separate during the electrophoresis according to their sizes. Sequence A will be smaller than sequence B and therefore will run faster on the gel. By knowing the original size of the sequence before cutting it with the enzyme and also the specific spot where the enzyme will cut the sequence, we can calculate the final sizes of the fragments generated.

## Exercise

Analyse the two alleles shown below and find the spot where these two sequences differ.

### Normal Allele

Eam1105I

AhdI

```
aaactttgagtgacatttagtccatttatggttgatattaggt  
ttgaaactcactgtaaatcaggtaaataccaactataatcca
```

EclHKI

AspEI

### Mutant Allele

```
aaactttgagtgatatttagtccatttatggttgatattaggt  
ttgaaactcactataaatcaggtaaataccaactataatcca
```

Let's suppose that this nucleotide change that you just found is responsible for a recessive genetic disease called the Z Syndrome. Let's suppose that you are a genetic doctor who needs to perform a family study to detect which individuals have the normal allele and who have the mutant allele.

### Questions:

- 1) Which restriction enzymes could you use to analyze the DNA of each individual?
- 2) Which fragment sizes should you expect to obtain in each situation? To answer this question, check the restriction sites of the enzymes below
- 3) What are the sizes of the DNA fragments you will obtain in the following situations:
  - (a) Normal homozygote individual
  - (b) Normal heterozygote individual
  - (c) Sick homozygote individual

Enzyme	Restriction Site
AhdI	gacnnn/nngtc
AspEI	gacnnn/nngtc
Eam1105I	gacnnn/nngtc
EclHKI	gacnnn/nngtc

*Note: The n in the sequence of the restriction site means that the sequence contains ambiguous nucleotides and any nucleotide (A, T, C or G) can be filling that specific position. Different restriction enzymes that have the same recognition site are called isoschizomers. In some cases isoschizomers cut identically within their recognition site, but sometimes they do not.*