INTRODUCTION TO RESTRICTION ENZYMES

Ref.ER1 (4 Practices)

1. EXPERIMENT OBJECTIVE

The goal of this experiment is to develop an understanding of restriction enzymes, see how these are able to cut the lambda phage at certain points, and perform a complete electrophoresis to check that.

2. INTRODUCTION

2.1 RESTRICTION ENZYMES

Restriction enzymes are also called restriction nucleases, restriction endonucleases, and sometimes restrictases. A large diversity of endodeoxyrinucleases, discovered as enzymes specific to different bacteria, belong to this group. A great number of them with high specificity, stability, and purity are now commercially available.

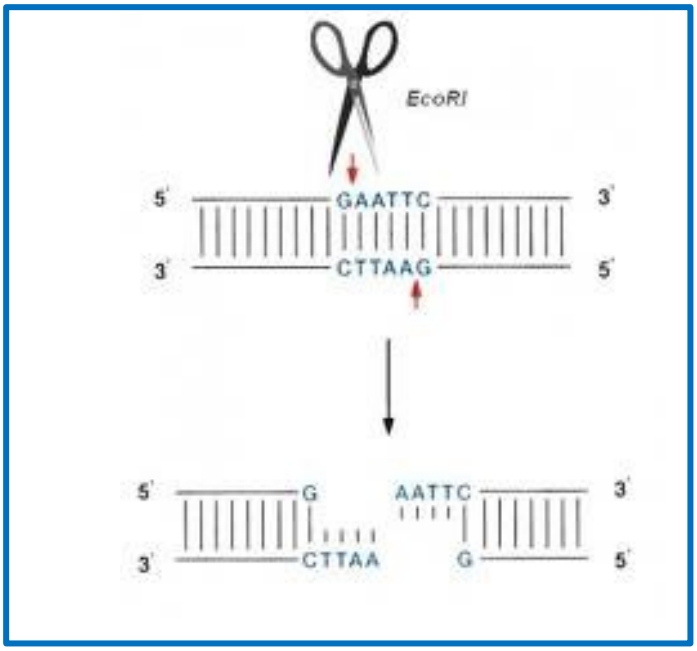
Restriction enzymes are nucleases that cut the double-stranded DNA when they recognize a specific sequence pattern. They generate DNA fragments known as restriction fragments. Restriction enzymes are essential tools in molecular biology, genetic engineering, and biotechnology.

They are named with three letters from the genus and species of the bacterium from which they were originally isolated, sometimes followed by an additional letter that identifies the serotype (antigenic variant of the bacterium) and, finally, by a roman number that identifies them when several enzymes with different specificity are found in the same variant.

The importance of restriction enzymes lies in their large specificity to recognize a short double-stranded DNA sequence and hydrolyze a phosphodiester bond on each strand, always at the same position. Each enzyme is thus characterised for its **recognition or restriction site or sequence, or target sequence**. The resulting DNA fragments are useful for initiating cellular or acellular cloning, for DNA analysis, for elaborating physic maps of restriction, or for polymorphism detection.

The diverse restriction enzymes are grouped into three families, according to their properties: Type I, Type II, and Type III.

We will focus on the study of type II enzymes, as they are used as tools in genetic engineering because they cut at a very defined point within their target sequence.



They were discovered in the 1960s and their use in molecular biology began in the 1970s enabling the development of recombinant DNA technologies. Restriction enzymes have endonuclease activity and cut phosphodiester bonds on the two DNA strands. They recognize determined DNA sequences. Type I and III restriction enzymes recognize a specific sequence but cut at a variable distance from the recognition site (restriction site). Type II restriction enzymes recognize a specific sequence known as the target sequence and always cut between the same nucleotides. Hence, they always generate the same restriction fragments. The target sequence is of variable size, mostly 4 and 6 nucleotides, and is often partially palindromic. Some restriction enzymes cut creating the so-called blunt ends while others generate so-called sticky ends.

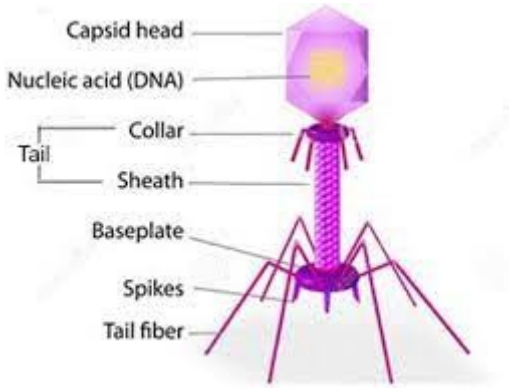
Restriction enzymes are a basic tool in molecular biology: PCR labs, creation of probes for hybridization, DNA sequencing labs, genetic engineering, cloning processes, gen library management, and many other genomic areas.

ENZIMA DE RESTRICCIÓN	ORGANISMO DE DONDE SE EXTRAE
EcoRI	Escherichia coli
EcoRII	Escherichia coli
HindII	Haemophilus influenzae
HindII	Haemophilus influenzae
HaeIII	Haemophilus aegyptius
HpaII	Haemophilus parainfluenzae
PstI	Providencia stuartii
MayI	Serratia marcesens
BamI	Bacillus amyloliquefaciens
BglII	Bacillus globiggi

2.2 LAMBDA BACTERIOPHAGE

A bacteriophage is a virus that exclusively infects bacteria. As with many other viruses that infect eukaryotic cells, bacteriophages or phages also have a protein capsid that is mainly used to house the genetic material that they will propagate to the cells they infect.

Most phages possess variable-length DNA as genetic material. Attending to the morphology they present, they can be classified as icosahedral heads without a tail, viruses with a contractile tail, with a non-contractile tail, and filamentous.



Phages are ubiquitous and it can be safely said that there are phages in almost any environment where there exist bacteria. It is also often said that, for any bacterium species, there is with full probability a corresponding phage that can infect it.

The most known phage, since it has served as a model of study in molecular biology, is the lambda phage. It also has a structure that we could call typical: an icosahedral capsid that encloses the genetic material, a contractile tail, and a series of spicules that are used for the contact with the cell to infect.

The binding to the bacterium is done through the union to specific receptors in the bacterial surface, which determines the specificity of the virus infection to a particular bacterial species.

Phages can follow two possible types of infectious cycles: **the lytic cycle and the lysogenic cycle**. In the lytic cycle, the phage infection causes the lysis of the host bacterium and the release of new phage particles. In contrast, in the lysogenic cycle, the phage inserts its genetic material into the bacterial genome, or it remains as an independent plasmid, replicating in any case at the same time as the bacterial genome, but without producing bacterial lysis. Lysogeny could be considered as a kind of latency applied to phages.

In general, very few phages can accomplish both cycles. Those who do so enter in one or the other version depending on the external conditions.

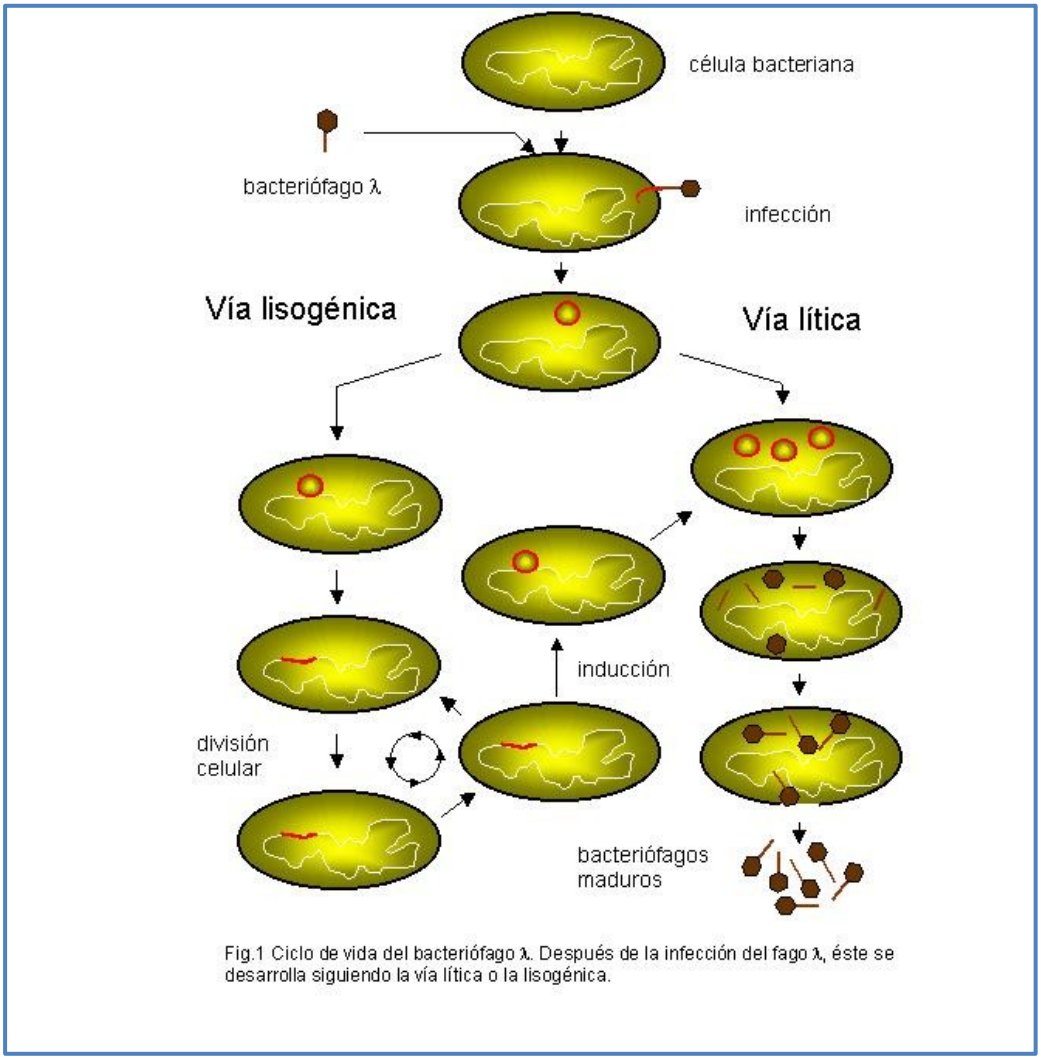
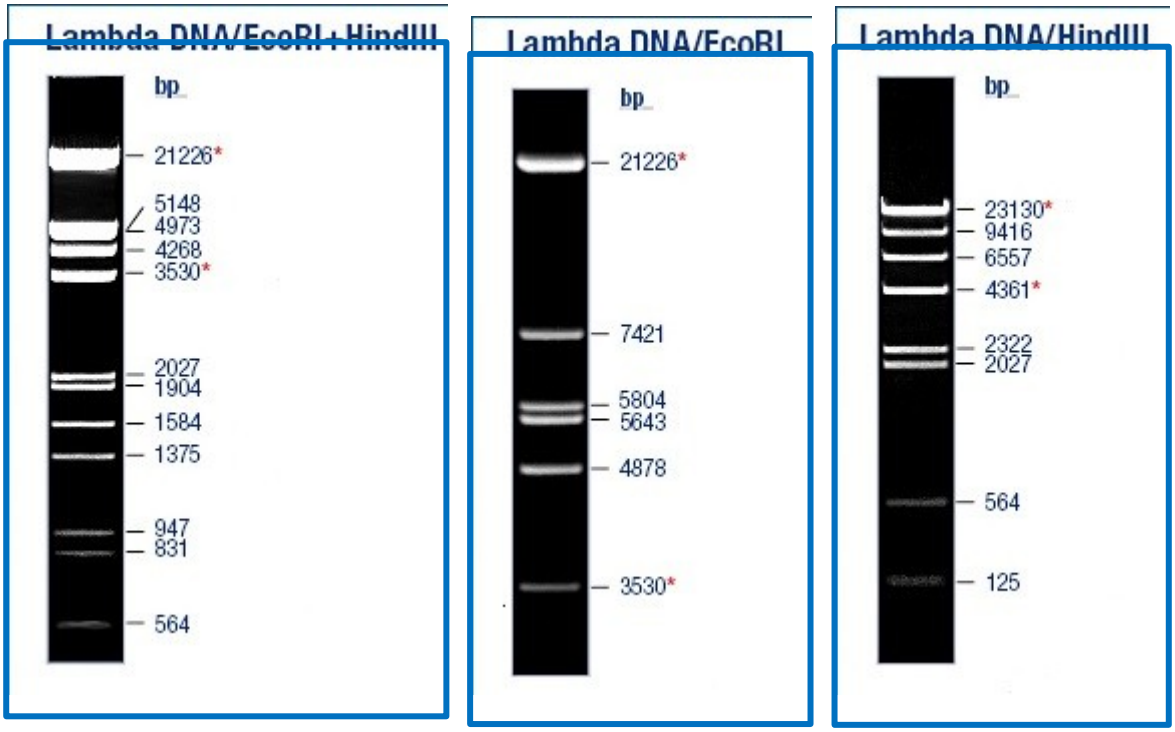


Fig.1 Ciclo de vida del bacteriófago λ. Después de la infección del fago λ, éste se desarrolla siguiendo la vía lítica o la lisogénica.

Phages played and play a very important role as biotechnological tools. Their genome can accept the inclusion of extra genetic material till some point, reason why they are used as cloning vectors creating the so-called phage libraries. In these libraries, a population of phages contains, fragmented and distributed between the different phages that form the population, a genome or transcriptome of interest. This way is really easy to manage gene collections, since phages are easy to reproduce and preserve. The libraries often include systems that allow amplifying or releasing the genetic fragment of interest. This possibility, combined with that of controlled recombination and the ability of determined phages to show exogenous proteins in their coat is the basis of the phage display technique.

2.3 RESTRICTION MAP LAMBDA BACTERIOPHAGE

In the nucleotide sequence of the lambda phage that has 48502 base pairs, there can be found different sites where the restriction enzymes cut. In this practical, we will use the **Eco RI** and the **Hind III** enzymes to see which strand pattern provides the lambda phage digestion. These digestions are commercially prepared under the name of **known or standard molecular-weight markers** and are used to determine the size of the DNA fragments in a agarose gel electrophoresis.



We can observe in the above images 3 of the molecular-weight markers most used in molecular biology works and, as we mentioned, they have as basis the digestion of lambda phage.

These images represent 1% agarose gel electrophoresis of the DNA of the lambda phage digested with diverse restriction enzymes (the ones we will use in this experiment). We have to take into account that the DNA visualisation was performed with ethidium bromide which has a greater sensitivity than our NON-TOXIC method (used in this kit). This means that we will not observe the strands of the smallest fragments in our experiment, unless your lab has a DNA detection method with a sensitivity similar to the ethidium bromide's, such as our NON-TOXIC method of **GELSAFE** that requires the use of a UV light transilluminator like the ethidium bromide.

3. COMPONENTS

Concentrated Electrophoresis Buffer 10X (2 bottles 50ml)	2 x 50 ml	Room Temperature
Agarose	1.75 gr	Room Temperature
Sample microtubes	5	Preserve at -20ºC
DanaBlue 0.1 %	400 µl	Room Temperature
FashBlue 0.75X	125 ml	Room Temperature

SAMPLES	COMPOSITION	AMOUNT
WHITE	**LAMBDA DNA WITH LOAD BUFFER**	100 microliters
BLACK	**ECORI LAMBDA DNA**	100 microliters
YELLOW	**HINDIII LAMBDA DNA**	100 microliters
PURPLE	**ECORI + HINDIII LAMBDA DNA**	100 microliters
RED	**StyI LAMBDA DNA**	100 microliters

Add 450 ml of distilled water to each bottle of Electrophoresis Buffer 10X to elaborate 2 x 500 ml of Electrophoresis Buffer 1 X which is the working Buffer.

Required material and not provided

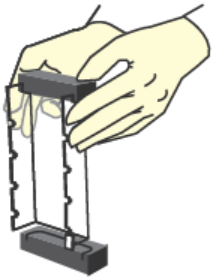
- Automatic micropipettes and tips (5-50 microliters)
- Erlenmeyer or beaker to make the agarose gel
- Electrophoresis device and power
- Scale
- Microwave or heating plate
- Distilled water

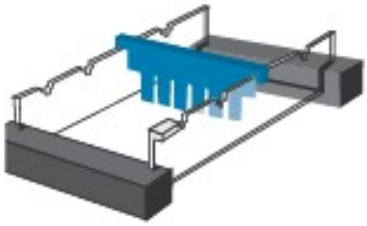
4. PRACTICAL

4.1 AGAROSE GEL PREPARATION

A) Preparing the gel bed

Take the gel bed and close the open ends with the casting dams so that the agarose does not come out. Afterwards, place the necessary combs to create the wells.





B) Preparing the agarose gel

Depending on the available time to carry out the practice, the agarose gel can be prepared another day and preserved in a refrigerator at 4ºC. If there is enough time, it can be done the day of the practice before performing the reactions so that there is time for it to solidify and that the wells do not break when extracting the comb.

1.b) Use a beaker or erlenmeyer of 100 ml to prepare the gel solution:

2.b) **For 7 x 7 cm casting trays:** add **32 ml of Electrophoresis Buffer 1 X plus 0.32 gr of agarose plus 80 µl of 0.1 % DanaBkue**, shake the mix to dissolve the agarose clumps.

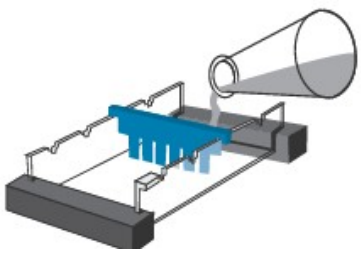
For 7 x 10 cm casting trays: add **42 ml of Electrophoresis Buffer 1 X plus 0.40 gr of agarose plus 100 µl of 0.1 % DanaBkue**, shake the mix to dissolve the agarose clumps.

Assure that the 450 ml of distilled water has been added to the Electrophoresis Buffer 10 X and we are working with the Buffer 1 X.

3.b) Heat the mix to dissolve the agarose powder, the fastest method is the use of a microwave, but a heating plate can be used as well, in both cases, the **solution** has to be taken to **boiling** state so that the agarose dissolves. The final solution should appear clear without any particles.

4.b) **Cool** the agarose solution to about 55ºC (you can place the glass under a tap of water and shake it to speed up the process). If a lot of evaporation of the liquid occurs, add electrophoresis buffer.

5.b) Add the agarose solution to the gel bed.



6.b) Let the gel solidify. You can place the gel in a refrigerator to speed up the process **(if the electrophoresis will be executed the next day, store the gel at 4ºC)**.

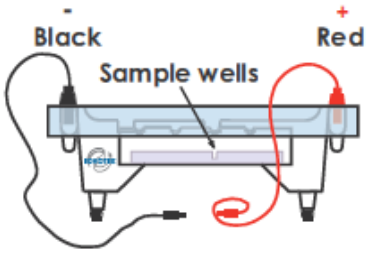
4.2 GEL LOADING AND ELECTROPHORESIS

Note: If you are unfamiliar with loading samples in agarose gels, it is recommended that you practice sample delivery techniques before conducting the actual experiment, or carry out the complete experiment before realizing it with the students.

A) Preparing the gel for the electrophoresis

1) After the gel is completely solidified, carefully and slowly remove the casting dams.

2) Place the gel into the electrophoresis chamber, properly oriented, with the wells nearer to the negative pole (black color).



3) Fill the electrophoresis apparatus chamber with **300 ml of Electrophoresis Buffer 1 X**. ***The electrophoresis buffer can be used for 2 electrophoresis experiments, once the electrophoresis is finished you can store the buffer used, in a packaging different to the supplied, to use it in a new electroforesis.***

4) Assure the gel is completely covered with buffer.

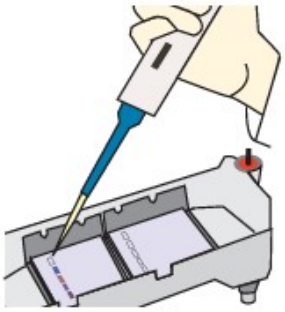
5) Remove the comb or combs that created the wells. Be very careful not to break any well.

6) Proceed to the samples loading and electrophoresis.

B) Electrophoresis samples: *check the samples' volume. Sometimes small sample drops can be in the microtubes walls. Make sure that the entire sample volume is uniform before loading the gel. Briefly centrifuge the sample microtubes, or tap them on a table to keep the whole sample at the bottom of the microtube.*

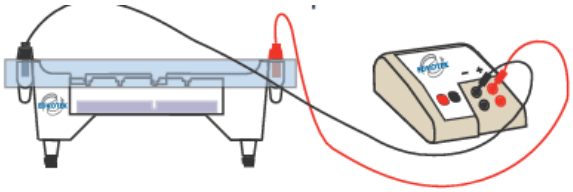
Well	Sample	Description	Volume
1	**WHITE**	**LAMBDA DNA WITH LOADING BUFFER**	**20 microliters**
2	**BLACK**	**ECORI LAMBDA DNA**	**20 microliters**
3	**YELLOW**	**HINDIII LAMBDA DNA**	**20 microliters**
4	**PURPLE**	**ECORI + HINDIII LAMBDA DNA**	**20 microliters**
5	**RED**	**StyI LAMBDA DNA**	**20 microliters**

1) Load the stated microliters of each sample. Use a micropipette with its tip.



2) After the samples are loaded, carefully snap the cover down onto the electrode terminals.

3) Insert the plug of the black wire into the black input of the power source (negative input). Insert the plug of the red wire into the red input of the power source (positive input).



4) Set the power source at **75 volts (30 min) or 150 volts (20 min)**. Keep an eye on the development of the loading buffer dye, which indicates how the electrophoresis runs.

5) After approximately 10 minutes, you will begin to see the separation of the samples.

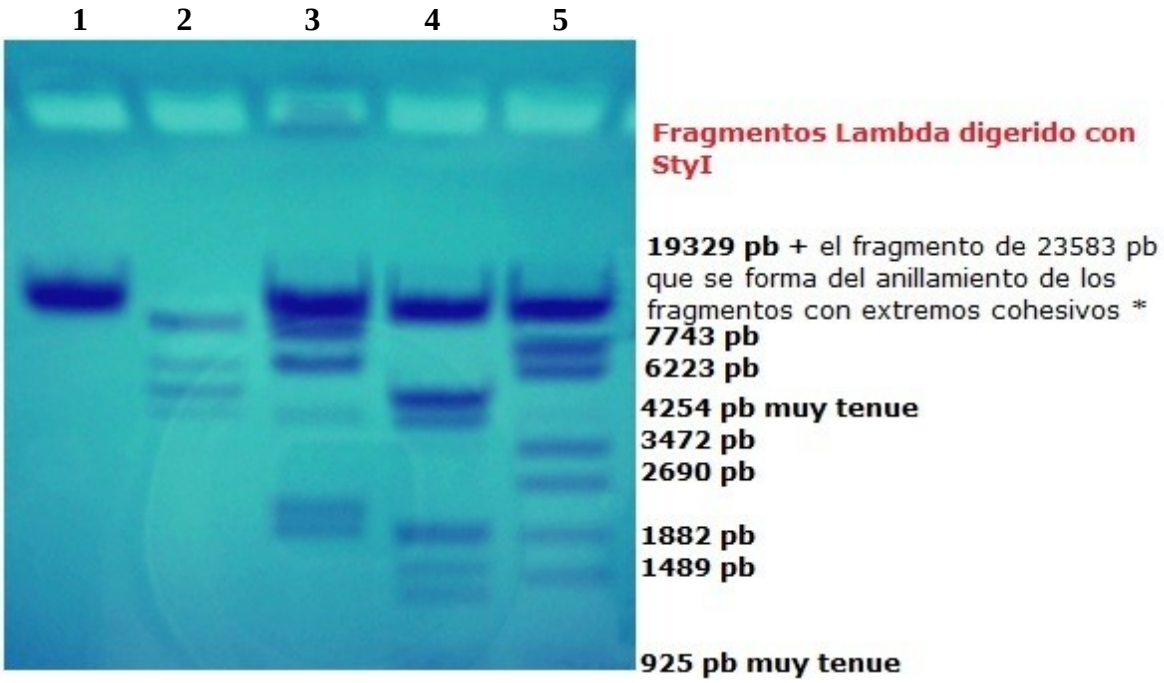
6) After the electrophoresis is completed, **turn off the power**, unplug the power source, disconnect the leads, and remove the cover.

7) Place the gel in a white light box (a piece of white paper can also be used). For a correct visualisation of the bands, go to the next section, which is the gel staining to visualise all the bands.

4.4 AGAROSE GEL STAINING

1. Do not stain the gels in the electrophoresis device.
2. Place the gel in a container with **125 ml of FlashBlue 0.75 X** so that it is completely covered.
3. Incubate for 15 minutes. Increasing the staining time will lead to a higher number of water washes.
4. Store the 125 ml of FlashBlue 0.75 X for another staining.
5. Place the container with the gel under a water tap and let the water flow until blue color does not come out. Hold the gel to not lose it. Fill up the container with water.
6. Carefully take the gel out of the container and examine it under a white light transilluminator (a piece of white paper can also be used).
7. Perform several water washes shaking it if possible. It will be observed how every time the bands are more visible and the blue color in the background decreases.
8. If the color is still intense blue, it is allowed to leave it under water all night.

5. RESULTS



Well 1: Undigested Lambda Phage.

Well 2: EcoR I MARKER 21226* bp; 7421 bp; 5804 bp; 5643 bp; 4878 bp; 3530*bp.

Well 3: Hind III MARKER 23130* bp; 9416 bp; 6557 bp; 4361* bp; 2322 bp; 2027 bp; 564 bp; 125 bp.

Well 4: EcoRI + HindIII MARKER 21226* bp; 5148 bp; 4973 bp; 4268 bp, 3530* bp; 2027 bp; 1904 bp; 1584 bp; 1375 bp; 974 bp; 831 bp; 564 bp.

Well 5: StyI MARKER 19329* bp; 7743 bp; 6223 bp; 4254* bp; 3472 bp; 2690 bp; 1882 bp; 1489 bp; 925 bp; 421 bp; 74 bp.

IMPORTANT: Fragments marked with a * present sticky ends, so they tend to band together and create larger fragments. Fragment 3530 does not usually appear in the gel and fragments 4361 and 4254 are tenuous. These sticky fragments can be separated by heating them at 65ºC for 5 minutes and then placing them on ice for 3 minutes before the loading.

1. Probably we will not observe the smaller bands (under 900 bp), unless a more sensitive method with ethidium bromide or other fluorescent dyes is used. Another option is to use our GELSAFE, NON-TOXIC approach but that uses a UV light transilluminator.

2. The undigested lambda phage shows up at the same height as the larger band of 23Kb. This also happens when human genomic DNA is isolated, the band appears at the 23Kb height.