

Restriction Digestion of pGLO™

This was printed and adapted from “Biotechnology Explorer™: Restriction Digestion and Analysis of Lambda DNA Kit” Bio-Rad Laboratories, 2004.

Background

DNA splicing, the cutting and linking of DNA molecules, is one of the basic tools of modern biotechnology. The basic concept behind DNA splicing is to remove a functional DNA fragment – let’s say a gene – from one organism and to combine it with the DNA of another organism in order to study how the gene works. The desired result of gene splicing is for the recipient organism to carry out the genetic instructions provided by its newly acquired gene. For example, certain plants can be given the genes for resistance to pests or disease, and in a few cases to date, functional genes have been given to people with nonfunctional genes, such as those who have a genetic disease like cystic fibrosis.

This activity may be used to simulate the real world application of gene splicing. You may suggest to your students that the DNA they are working with represents a chromosome that has been cut into many fragments. Of the fragments that are produced, one particular fragment may represent a specific gene. This imaginary gene can code for any number of traits, but before it can be given to a recipient organism, your students must first identify the gene by its size using agarose gel electrophoresis.

Restriction Enzymes

The ability to cut and paste, or cleave and ligate, a functional piece of DNA predictably and precisely is what enables biotechnologists to recombine DNA molecules. This is termed recombinant DNA technology. The first step in DNA splicing is to locate a specific gene from the rest of the chromosome. This same enzyme is also used to cut the DNA of the recipient into which the fragment will be inserted.

Restriction enzymes are proteins that cut DNA at specific sites. Restriction enzymes, also known as restriction endonucleases, recognize specific sequences of DNA base pairs and cut, or chemically separate, DNA at that specific arrangement of base pairs. They were first identified in and isolated from bacteria bacteriophages – viruses that infect bacteria. Any foreign DNA encountering a restriction enzyme will be digested, or cut into many fragments, and rendered ineffective. These enzymes in bacteria make up the first biological immune system. There are thousands of restriction enzymes, and each is named after the bacterium from which it is isolated. For example:

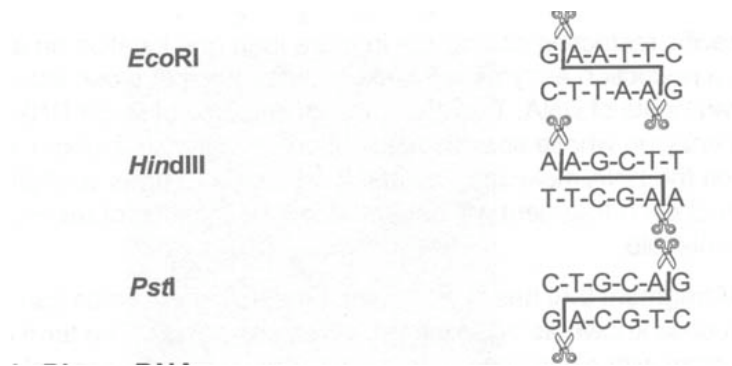
EcoRI = The first restriction enzyme isolated from *Escherichia coli* bacteria

HindIII = The third restriction enzyme isolated from *Haemophilus influenzae* bacteria

PstI = The first restriction enzyme isolated from *Providencia stuartii* bacteria

Each restriction recognizes a specific nucleotide sequence in the DNA, called a restriction site, and cuts the DNA molecule at only that specific sequence. Many restriction enzymes leave a short length of unpaired bases, called a “sticky” end, at the DNA site where they cut, whereas other restriction enzymes make a cut across both strands creating double-stranded DNA fragments with “blunt” ends. In general, restriction sites are palindromic, meaning the sequence of bases reads the same forwards as it does backwards on the opposite DNA strand.

For example, here is a list of enzymes and the sites where they cut:



A restriction enzyme acts like molecular scissors, making cuts at the specific sequence of base pairs that it recognizes. The three-dimensional structure or shape of a restriction enzyme allows it to fit perfectly in the groove formed by the two strands of DNA molecule. When attached to the DNA, the enzyme slides along the double helix until it recognizes a specific sequence of base pairs which signals the enzyme to stop sliding. The enzyme then chemically separates, or cuts, the DNA molecule at that site – called a restriction site.

If a specific restriction site occurs in more than one location on a DNA molecule, a restriction enzyme will make a cut at each of those sites, resulting in multiple fragments of DNA. Therefore, if a given piece of linear DNA is cut with a restriction enzyme whose specific recognition sequence is found at five different locations on the DNA molecule, the result will be six fragments of different lengths. The length of each fragment will depend upon the location of restriction sites on the DNA molecule.

Electrophoretic Analysis of Restriction Fragments

A DNA fragment that has been cut with restriction enzymes can be separated using a process known as **agarose gel electrophoresis**. The term electrophoresis means to *carry with electricity*. Agarose gel electrophoresis separates DNA fragments by size. DNA fragments are loaded into an agarose gel slab, which is placed into a chamber filled with a conductive buffer solution. A direct current is passed between wire electrodes at each end of the chamber. Since DNA fragments are negatively charged, they will be drawn toward the positive pole (anode) when placed in an electric field. The matrix of the agarose gel acts as a molecular sieve through which smaller DNA fragments can move more easily than larger ones. Therefore, the rate at which a DNA fragment migrates through the gel is inversely proportional to its size in base pairs. Over a period of time, smaller DNA fragments will travel farther than larger ones. Fragments of the same size stay together and migrate in single bands of DNA. These bands will be seen in the gel after the DNA is exposed to UV light.

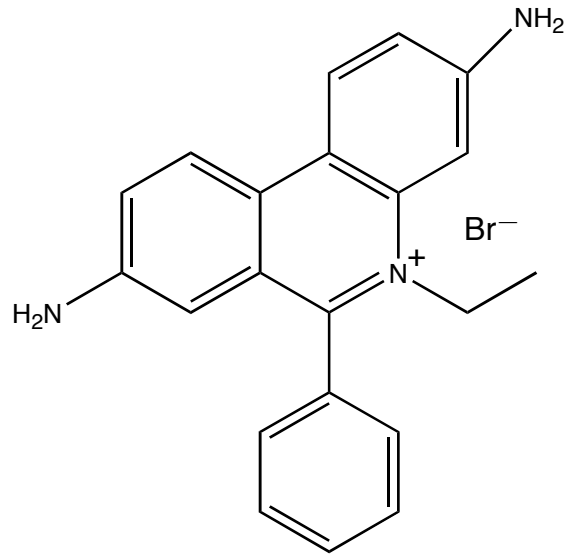
An analogous situation is one where all the desks and chairs in the classroom have been randomly pushed together. An individual student can wind his/her way through the maze quickly and with little difficulty, whereas a string of four students would require more time and have difficulty working their way through the maze.

Making DNA Visible

DNA is colorless so DNA fragments in the gel cannot be seen during electrophoresis. A loading dye containing two blue dyes is added to the DNA solution. The loading dye does not stain that DNA itself but makes it easier to load the gels and monitor the progress of the DNA

electrophoresis. The dye fronts migrate toward the positive end of the gel, just like the DNA fragments. The dye has Xylene Cyanol FF (~4000 bp), Bromophenol blue (~300 bp) and Orange G (50 bp).

To visualize your DNA, in the gel, you will use ethidium bromide (EXTREMELY CARCINOGENIC). This molecule (see below) intercalates between the planar DNA bases. When UV light is shined on it, it fluoresce orange/pink. You will take a picture of your gel while its exposed to UV light and paste it in your notebook.



The overall goal of this experiment is to digest pGLO™ DNA with various restriction enzymes and determine the lengths of the fragments.

Supplies/Equipment Provided

Bio-Rad Mini-Sub Cell GT cell (12 x 26 x 6.5 cm)

Bio-Rad Mini-Sub Cell GT tray (7 x 10 cm)

Bio-Rad Gel Caster

Bio-Rad PowerPac™ Basic Power Supply

Bio-Rad Ultrarocker™ Rocking Platform

Promega *EcoR* I (System Lot # 196028) 12 U/μL

New England Biolabs *Nde* I () 20 U/μL

NEBuffer 4 10X (200 mM Tris-OAc, 500 mM KOAc, 100 mM MgOAc, 10 mM DTT pH 7.9)

NEBuffer *EcoRI* 10X

(1 M Tris-HCl, 500 mM NaCl, 100 mM MgCl₂, 0.25% Triton X-100, pH 7.5)

Bio-Rad 5X loading dye

New England Biolabs λ DNA/*Bst*EII digest MW marker (System Lot # N3014S) 500 μg/mL

Bio-Rad 1X FastBlast dye

1X TBE buffer (90 mM Tris-Borate, 1 mM EDTA, pH 8.3)

Ethidium bromide (**WEAR GLOVES AT ALL TIMES!!!**)

DDI H₂O

Procedure

- I. Digest DNA
- II. Cast a 1% agarose gel
- III. Load and run the gel
- IV. Develop the gel and analyze the data.

I. Restriction Digestion

1. Obtain micro test tubes that contain each enzyme stock solution, pGLO™, and restriction buffer. Keep all the stock solutions on ice.

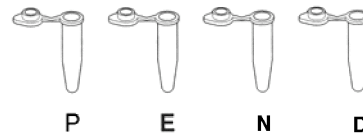
2. Obtain one eppie tube for each reaction and label them as follows:

P (pGLO™ DNA)

E (*Eco*RI pGLO™ digest)

N (*Nde*I pGLO™ digest)

D (*Eco*RI and *Nde*I pGLO™ digest)



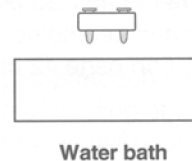
3. Using a fresh tip for each sample, pipet the reagents into each tube according to the table below (all volumes in uL). Each reaction requires a specific buffer known as NEBuffer 4 (4) or NEBuffer *Eco*RI (E). Please be pay attention to which buffer goes with which reaction.

	P	E	N	D
DDI H ₂ O	12.5	8.5	8.5	6.5
Buffer 4/E	0	(E) 2.0	(4) 2.0	(E) 2.0
DNA	7.5	7.5	7.5	7.5
<i>Eco</i> RI	-----	2.0	----	2.0
<i>Nde</i> I	-----	-----	2.0	2.0

Total 20.0 20.0 20.0 20.0

4. Mix them up and down by pipeting gently up and down with your pipetman set to 10 μ L.

5. Place the tubes in the floating rack and incubate for 1 hr at 37°C.



6. After incubation, put the samples on ice.

II. Casting an Agarose Gel

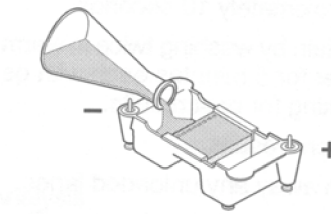
1. Level the gel caster.
2. Insert gel casting tray into gel caster. Make sure the cam lever is pointing towards the gel tray. Test the casting for leaks by pouring DDI water into the tray.
3. Mix 0.48 g of agarose and 40 mL of 1X TBE (90 mM Tris-Borate, 1 mM EDTA, pH 8.3) buffer in a 125 mL Erlenmeyer flask.
4. Microwave the agarose for 0.5-2 minutes (until all the agarose dissolves), stopping the microwave every 30-45 seconds to swirl the flask. Make sure it doesn't boil. Let cool to $\sim 60^\circ\text{C}$ (when you can hold the flask with your gloved hand). Overly warm agarose will warp the gel tray.
5. Add 4 μL of ethidium bromide to your agarose mixture. **(WEAR GLOVES)**
6. Pour the dissolved agarose into the gel tray.
7. Insert comb immediately at the top of the gel.
8. Let the gel solidify. This may take 20-40 minutes. Carefully remove the comb.
9. To disengage the tray from the gel caster, rotate the cam lever. You may need to use a flathead spatula to break the seal between the gel and the rubber of the gel caster.

III. Agarose Gel Electrophoresis

1. Add 5 μL of 5X loading buffer into each tube. Mix the contents by pipeting slowly up and down with your pipetman.
2. Obtain the DNA marker (M) from the front of the room. Be sure to mix well before taking your sample. Take only 1 μL more than you will use as they are expensive.

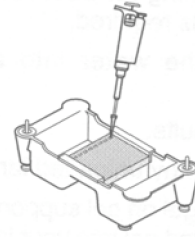
M = 2-log DNA ladder (10.0 μL)

3. Fill the electrophoresis chamber and cover the gel with 1X TBE buffer (about 275 mL of buffer).
4. Check that the walls of the agarose gels are near the black (-) electrode and the bottom edge of the gel is near the red (+) electrode.

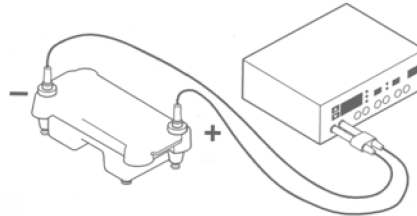


5. Load 10 μL of each sample into separate wells in the gel chamber in the following order:

<u>Lane</u>	<u>Sample</u>
1	M marker
2	P, uncut pGLO DNA
3	E, <i>EcoRI</i> digest
4	N, <i>NdeI</i> digest
5	D, double digest



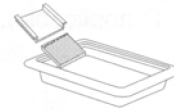
6. Carefully place the lid on the electrophoresis chamber. Connect the electrical leads into the power supply, red to red and black to black.
7. Turn on the power and run the gel at 120 V for 1 hour or until the dye is a little over half way down the gel.



The dye has Xylene Cyanol FF (~4000 bp), Bromophenol blue (~300 bp) and Orange G (50 bp). pGLO is at 5,371 bp.

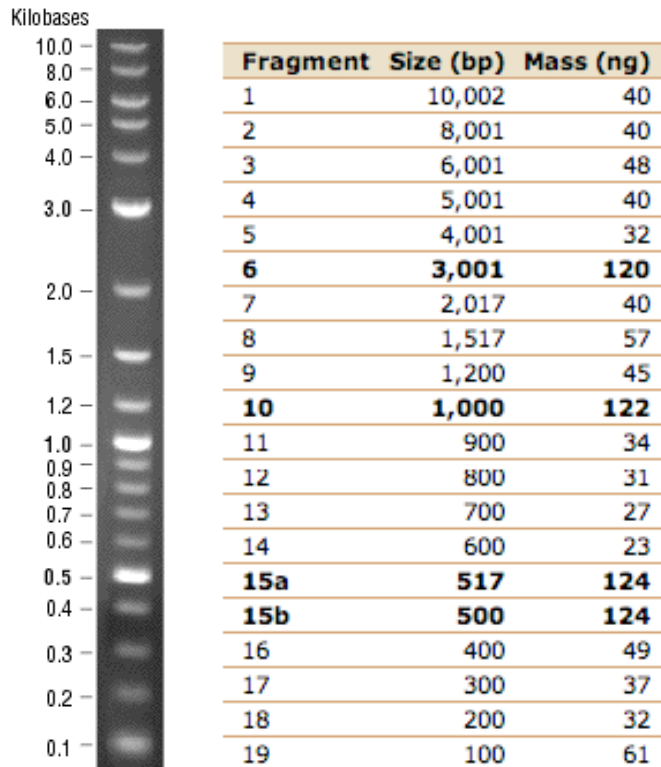
IV. Visualization of DNA Fragments

1. When the electrophoresis run is complete, turn off the power and remove the top of the chamber. Carefully remove the gel and tray from the gel box. Be careful – the gel is very slippery. Slide the gel into a tray.
2. Ask the professor or a TA to help you take a picture of the gel in room MG 2070.
3. Place the gel in the waste bag (in the hood).



IV cont'd. Results

- 1) Measure the migration distances (mm) for each band from the bottom of each well.
- 2) Make a standard curve (log bp vs. migration distance) using the marker. The MW marker is from New England Biolabs (Quik-Load 2-log DNA ladder). Note that your units for DNA is bp not kbp.



- 3) Use the standard curve to determine the lengths of the DNA fragments.
- 4) Compare your digested pGLO DNA lengths to the known pGLO restriction map. Look at the New England Biolabs website (www.neb.com). Click on NEBcutter at the bottom of the screen. Open the pGLO sequence found at <http://www2.truman.edu/~mnagan/pgloseq.doc>. Copy and paste the sequence into NEBcutter. Make sure the sequence doesn't have linebreaks in it. The sequence is circular. Select all NEB enzymes and click submit. You will get the restriction map for pGLO. Then click on Custom Digest under Main Options. Choose *EcoRI*, *NdeI*. It will display the restriction map with just these enzymes. You can also get a list of fragments.

Reference

Sanger, F., Coulson, A.R., Hong, G.F., Hill, D.F. and Petersen, G.B. *J. Mol. Biol.* **1982**, *162*, 729-773.