Introduction

In the 40 - 50 years that molecular biology has existed, scientists have used restriction mapping to analyze the structure and sequence of DNA molecules. This powerful technique involves the use of restriction endonucleases (REs) that recognize specific DNA sequences, usually 4 to 8 bp in length, and cleave at these sequences. Nathans, Smith and Arber were awarded the Nobel Prize in 1979 for discovering restriction enzymes and having the insight and creativity to use these enzymes to map genes. More than 900 restriction enzymes, some sequence specific and some not, have been isolated from over 230 strains of bacteria since.

Restriction enzymes and the fragments produced by them have become powerful tools of molecular genetics. They are used to map DNA molecules physically, to analyze population polymorphisms, to rearrange DNA molecules, to prepare molecular probes, to create mutants, to analyze the modification status of the DNA, and other applications.

What is restriction digestion

Restriction Digestion is the process of cutting DNA molecules into smaller pieces with special enzymes called Restriction Endonucleases (sometimes just called Restriction Enzymes or RE). These special enzymes recognize specific sequences in the DNA molecule (for example GATATC) wherever that sequence occurs in the DNA. Restriction Enzymes are delicate and need to be treated carefully. Because enzymes are proteins and proteins denature as the temperature is increased, RE's are always stored in a freezer until they are used. In fact, all of the ingredients in a Restriction Digest are kept on ice until it is time for the reaction to begin. Restriction endonucleases are supplied in various concentrations with activities that are based upon cleavage rates of "standard" DNA samples. One unit of activity is typically defined as the amount of enzyme required to digest (or "restrict") one microgram of reference DNA in one hour at 37 °C. The endonuclease hydrolysis is a spontaneous reaction and does not, for example, require addition of ATP. Reaction buffers for restriction endonucleases usually contain a buffer component (typically 10 mM TRIS buffer around pH 8.0), magnesium salt (often 10 mM MgCl2), a reducing agent (usually 1mM dithiothreitol, or DTT), a protective carrier protein (typically 100 µg/ml bovine serum albumin, or BSA), and salt (sodium chloride). The biggest determinant of enzyme activity is typically the ionic concentration (NaCl content) of the buffer. Although there are hundreds of different restriction endonucleases, the majority of them can exhibit between 30-100% activity using a simple system of three
buffers, containing either low (20 mM), medium (100 mM) or high (250 mM) salt (NaCl) concentrations in the above described buffer.

When the restriction digestion has run for the appropriate amount of time, the reaction tube is put back on ice to prevent nonspecific degradation of your DNA. Once the Restriction Digest is completed, Agarose Gel Electrophoresis is performed to separate the digest fragments by size and you can visualize the fragments and perhaps purify them for further experiments.

There are three classes of restriction enzymes (type I, II and III). The most commonly used ones are type II enzymes. These recognize specific sequences that are 4, 5, and 6 nucleotides in length and display a twofold symmetry. Recognition sequences for many type II enzymes are the same on both strands. Such recognition sequences are said to be palindromic. Some cleave both strands exactly at the axis of symmetry, generating fragments of DNA that carry blunt or flushed ends (e.g. EcoRV); others cleave each strand at similar locations on opposite sides of the axis of symmetry, creating fragments of DNA that carry protruding single-stranded termini (also called “sticky” ends or overhangs).

**Restriction Enzyme Action of EcoRI**

What is gel electrophoresis?

Gel electrophoresis is a technique used for the separation of nucleic acids and proteins. Separation of large (macro) molecules depends upon two forces: charge and mass. When
a biological sample, such as proteins or DNA, is mixed in a buffer solution and applied to a gel, these two forces act together. The electrical current from one electrode repels the molecules while the other electrode simultaneously attracts the molecules. The frictional force of the gel material acts as a “molecular sieve,” separating the molecules by size. During electrophoresis, macromolecules are forced to move through the pores when the electrical current is applied. Their rate of migration through the electric field depends on the strength of the field, size and shape of the molecules, relative hydrophobicity of the samples, and on the ionic strength and temperature of the buffer in which the molecules are moving. After staining, the separated macromolecules in each lane can be seen in a series of bands spread from one end of the gel to the other. DNA is a negatively charged molecule, and is moved by electric current from the negative pole to the positive pole.

**Objectives**

To learn the basic principle of analyzing DNA fragments by restriction digestion and agarose gel electrophoresis. Several restriction enzymes (REs) will be used to digest the plasmid pCMV-GFP to build a restriction map of the plasmid.

**Outline**

1. Restriction enzyme digest of plasmid DNA with
   (a) EcoR I
   (b) EcoR V
   (c) Mlu I
2. Load digested DNA samples in 1% agarose gel
3. Gel electrophoresis
4. Photograph
5. Determine restriction map of plasmid

**Protocol**

**(A) Digestion of your plasmid DNA pCMV-GFP:**
The plasmid pCMV-GFP consists of the cDNA encoding the green fluorescence protein (GFP) cloned into the mammalian expression vector pcDNA3.1(+). To make a restriction map of pCMV-GFP, it is necessary to determine the number and sizes of the fragments that result from digestion of this plasmid DNA with several REs. This is accomplished by first digesting your pCMV-GFP DNA with different REs and then using agarose gel electrophoresis to separate the resulting fragments on the basis of size. You should **keep the components of your digests on ice (especially your REs)**, while you are working.

1. Fill an icebox with ice. Collect the tubes with the solutions and enzymes from the bench at the front of the lab and place them in the icebox.
2. Label seven 0.5 ml Eppendorf tubes 1, 2, 3, 4, 5, 6 and 7.
3. Add the components to the tubes according to Table 1. Always use a fresh tip for dispensing each component into individual tubes to avoid contamination. Tube 1
contains your uncut DNA sample.

Note:
The order in which you add components to a digest is important. Usually, you add water to the tube first, the buffer second, followed by bovine serum albumin (BSA), then the DNA, and the RE last. Thus, all other components will be present in the tube and the required conditions for digestion will have been established before the RE is added. Adding the RE to the tube before adding water and buffer could result in inactivation of the RE. As you will notice from Table I, a specific buffer should be used for each RE. For double digestions, the buffer that permits maximum activities of both enzymes is used.

<table>
<thead>
<tr>
<th>Tube#</th>
<th>H₂O</th>
<th>10X Buffer 3</th>
<th>BSA (100 ug/ml)</th>
<th>pCMV-GFP DNA</th>
<th>MluI</th>
<th>EcoRI</th>
<th>EcoR5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16 µl</td>
<td>2 µl</td>
<td>--</td>
<td>2 µl</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>15 µl</td>
<td>2 µl</td>
<td>--</td>
<td>2 µl</td>
<td>1 µl</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>15 µl</td>
<td>2 µl</td>
<td>--</td>
<td>2 µl</td>
<td>--</td>
<td>1 µl</td>
<td>--</td>
</tr>
<tr>
<td>4</td>
<td>13 µl</td>
<td>2 µl</td>
<td>2 µl</td>
<td>2 µl</td>
<td>--</td>
<td>--</td>
<td>1 µl</td>
</tr>
<tr>
<td>5</td>
<td>14 µl</td>
<td>2 µl</td>
<td>--</td>
<td>2 µl</td>
<td>1 µl</td>
<td>1 µl</td>
<td>--</td>
</tr>
<tr>
<td>6</td>
<td>12 µl</td>
<td>2 µl</td>
<td>2 µl</td>
<td>2 µl</td>
<td>1 µl</td>
<td>--</td>
<td>1 µl</td>
</tr>
<tr>
<td>7</td>
<td>12 µl</td>
<td>2 µl</td>
<td>2 µl</td>
<td>2 µl</td>
<td>--</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

4. Flick the tube with your finger to mix well and spin for 5 sec in the microfuge at maximum speed to bring all components to the bottom of the tube.

5. Begin incubating the digest at 37°C in the water-bath for 45 min.

6. Inactivate the restriction enzyme by incubating the reaction tubes at 65°C for 10 min.

(B) Agarose gel for electrophoresis:
Digested DNA are often separated on a porous matrix like an agarose gel and this separation is dependent on several parameters such as (i) the molecular size of the DNA (ii) the agarose concentration (iii) the conformation of the DNA (iv) amount of voltage applied and (v) the composition of the running buffer. In general DNA from 100-50 kb in length can be separated on agarose gels of various concentrations.

Note:
Making the gel requires about 30 min. The members of the lab staff have prepared 1% agarose for your use.

Caution:
The agarose gel contains 0.5 mg/ml ethidium bromide for visualization of digested
DNA (see Section C) and should be handled with care. Ethidium bromide is a powerful mutagen and is moderate toxic. Gloves must be worn at all times.

**C** Preparation of DNA digests and loading into gel.
1. Add 5 µl of 5X loading dye directly to each digest. Flick the tubes gently to mix and centrifuge briefly for 5 sec as before. You are provided with a clear tube that contains a sample of the One Kilobase Ladder DNA standard, which already contains loading dye. Label this tube #8.

2. Place the agarose gel in the electrophoresis chamber. Fill the chamber with 400 ml of running buffer (0.5X TBE). Make sure the gel is completely submerged with a depth of at least 3 mm.

3. Load 20 µl of each digest/sample into Lanes 1 - 7 in the order shown in the first column of the Table on the previous page using the P20 pipette provided. Load 10 µl of the One Kilobase Ladder into Lane 8. If you mix up the order of the samples, record the actual order in your notes.

4. When all the samples are loaded, close the lid on the gel box, taking care not to jostle the box. Make sure that the electrical leads are attached in the correct orientation and turn the power on and adjust the voltage to a maximum of 110 V. If the leads have been attached correctly, bubbles will be generated at the anode and cathode due to electrophoresis and within a few minutes the blue dye will have migrated out of the gels.

5. Let the gel run for 30 min or until you see that the blue dye-front has migrated three-quarters the length of the gel. Turn off the electric current and remove the leads. Carefully remove the gel from the electrophoresis tank and place it in the plastic tray provided.

Note:
DNA migrates from the negatively charged electrode (cathode; black-coloured leads) towards positively charged electrode (anode; red-coloured leads). Ethidium bromide migrates towards the cathode (opposite direction to DNA).

**D** Visualisation of digested DNA samples by ultraviolet light.
Ethidium bromide is a fluorescent dye that intercalates between the stacked bases of DNA. It can be used to detect both single- and double stranded nucleic acids i.e. both DNA and RNA although its affinity for the latter is lower. Ultraviolet radiation at 254 nm is absorbed by the DNA and transmitted to the dye, radiation at 302 nm and 366 nm is absorbed by the bound dye itself. The energy is re-emitted at 590 nm in the red-orange region of the visible spectrum, hence allowing visualization by eye.

1. Carry the gel in the holding tray to the photographic area. Lift the gel carefully out of
the tray, tip it slightly so that the excess solution will drain off, and transfer it to the surface of the light box.

2. Replace the protective cover over the surface of the light box and switch on the uv light to observe the digested DNA. Place the camera over the gel and take one photograph.

3. Remove your gel from the light box and wipe the surface of the box thoroughly with hand-towels.

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure make sure the uv light source is shielded by the cover on the illuminator and wear protective goggles or a full safety mask for blocking.

4. **Discard your gel** in your waste-bag and rinse the tray with water in the sink. Discard all tubes and tips in the waste-bag provided.

5. Use your electrophoresis data to make a restriction map of pCMV-GFP and answer the questions below.

**(E) Restriction Mapping Questions and Analysis**

1. The migration of DNA molecules in agarose gels is roughly proportional to the inverse of the log of their molecular weights (sizes). Refer to the Appendix I for the sizes of the DNA fragments in the One Kilobase Ladder standard and plot their sizes (Y-axis) against the distances migrated (mm, X-axis) on the semi-log paper provided. Draw a curve or line to connect all points.

   Use this standard curve to estimate the sizes of all the fragments generated by digestion of pCMV-GFP. The sum of the sizes of all fragments in a given digest should equal the size of the pCMV-GFP plasmid. Use this data to make a restriction map of the plasmid, remembering that pCMV-GFP is a circular DNA.
Appendix I.
Photograph of 1 kb DNA ladder

0.5 µg of 1 kb DNA Ladder visualized by ethidium bromide staining on a 0.8% TAE agarose gel.
**Materials**

**Equipment:**

1. PCR thermal cycler at 37°C and 65°C (5)
2. Vortex mixer
3. Microfuges
4. Gilson/Eppendorf pipette (P10, P20 and P200)
5. DNA gel apparatus (mini gel tank) 25 sets
6. Powerpak (120V) 25 sets
7. Microwave oven
8. Gene Wizard gel documentation system with thermal paper
9. latex gloves (small/medium; powdered and non-powdered)
10. ruler
11. masking tape
12. Autoclaved yellow pipette tips
13. 500 ml of 0.5X Tris-borate EDTA (TBE) buffer
14. Icebox

**Consumables/reagents required per 2 pairs of students:**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>No. of tubes</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restriction Buffer</td>
<td>10 x</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>dd H2O</td>
<td></td>
<td>60</td>
<td>1000</td>
</tr>
<tr>
<td>BSA</td>
<td>100 ug/ml</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>pCMV-GFP DNA</td>
<td>0.5 ug/ul</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>MluI</td>
<td></td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>EcoRI</td>
<td></td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>EcoRV</td>
<td></td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>6X loading dye</td>
<td></td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>1000 bp DNA ladder</td>
<td>0.5 ug/ul</td>
<td>60</td>
<td>0.5 ul+19.5 ul+4 ul dye</td>
</tr>
</tbody>
</table>

Parafilm

0.5 ml PCR tubes

Ethidium bromide

Rack for 0.5 ml Eppendorf tubes

Plastic bag for waste

20 tubes each two groups

one tube for all students

50
2. Report

1. List three techniques you have learnt from this practical.

2. Paste your gel photo on the left and answer the following questions:
   a. What is the size of the plasmid DNA?
   b. What is the size of each fragment after restriction digestion?
   c. Draw a restriction map of this plasmid showing the positions of the restriction enzymes.
   d. Why do you need to add loading dye into the digestion reactions before gel electrophoresis?
   e. Discuss the reasons if the experiment did not work well.