Bio 6 – Restriction Enzyme Digestion Lab

Objectives

Upon completion of this laboratory you will understand how to: 1) set up and carry out a restriction enzyme digest of DNA, 2) carry out agarose gel electrophoresis of DNA, and 3) stain DNA in an agarose gel and determine the approximate length of DNA fragments present in the gel.

Introduction

A restriction endonuclease is an enzyme that catalyzes the hydrolysis or “cutting” of the DNA sugar-phosphate backbone at very specific sequences. Most people refer to restriction endonucleases as “restriction enzymes”, so from this point on the term restriction enzyme will be used. To understand what a restriction enzyme does, let’s consider three different restriction enzymes:

<table>
<thead>
<tr>
<th>restriction enzyme</th>
<th>restriction site</th>
<th>cut DNA product</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>5’…GAATTC…3’</td>
<td>5’…G AATTC…3’</td>
</tr>
<tr>
<td></td>
<td>3’…CTTAAG…5’</td>
<td>3’…CTTAA G…5’</td>
</tr>
<tr>
<td>SmaI</td>
<td>5’…CCCGGG…3’</td>
<td>5’…CCC GGG…3’</td>
</tr>
<tr>
<td></td>
<td>3’…GGGCCC…5’</td>
<td>3’…GGG CCC…5’</td>
</tr>
<tr>
<td>PstI</td>
<td>5’…CAGCTG…3’</td>
<td>5’…CAGCT G…3’</td>
</tr>
<tr>
<td></td>
<td>3’…GTCGAC…5’</td>
<td>3’…G TCGAC…5’</td>
</tr>
</tbody>
</table>

Each of the restriction enzymes shown above recognizes and cuts a very specific double-stranded DNA sequence referred to as a restriction site. There are literally hundreds of known restriction enzymes, each cutting a specific restriction site which is typically 4 to 8 base pairs. If you look carefully at each restriction site above you will notice that they are palindromic, i.e., the sequence on both strands is the same when read 5’ to 3’. This feature is characteristic of most restriction sites.

Another important feature of restriction enzymes is how they cut their corresponding restriction sites. Some enzymes such as SmaI cut the restriction site exactly in the middle on both strands producing cut DNA products with blunt ends. Most restriction enzymes cut their corresponding restriction sites in a staggered fashion leaving single-stranded overhangs. In the examples above, EcoRI produces ends with single-stranded 5’ overhangs and PstI produces ends with 3’ overhangs.

The majority of restriction enzymes cut 6 base pair palindromes as seen with the examples above, however not all restriction sites fit this pattern as shown in the examples on the next page:
<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Restriction Site</th>
<th>Cut DNA Product</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>AluI</em></td>
<td>5’…AGCT…3’</td>
<td>5’…AG CT…3’</td>
</tr>
<tr>
<td></td>
<td>3’…TCGA…5’</td>
<td>3’…TC GA…5’</td>
</tr>
<tr>
<td><em>NotI</em></td>
<td>5’…GCGGCCGC…3’</td>
<td>5’…GC GGCCGC…3’</td>
</tr>
<tr>
<td></td>
<td>3’…CGCCGG…5’</td>
<td>3’…CGCCGG CG…5’</td>
</tr>
<tr>
<td><em>HaeII</em></td>
<td>5’…RGCGCY…3’</td>
<td>5’…RGCGC Y…3’</td>
</tr>
<tr>
<td></td>
<td>3’…YCGCGR…5’</td>
<td>3’…Y RGCGC…5’</td>
</tr>
<tr>
<td><em>ScrFI</em></td>
<td>5’…CCNGG…3’</td>
<td>5’…CC NGG…3’</td>
</tr>
<tr>
<td></td>
<td>3’…GGNCC…5’</td>
<td>3’…GGN CC…5’</td>
</tr>
</tbody>
</table>

\[ \text{N} = \text{any base, R = purine, Y = pyrimidine} \]

Notice that *AluI*, *ScrFI* and *NotI* have 4, 5 and 8 base pair restriction sites, respectively. *HaeII* and *ScrFI* are also atypical in that they have restriction sites that accommodate more than one base at select positions. The first and sixth positions in the *HaeII* restriction site can be any purine (A or G) or pyrimidine (C or T), respectively, whereas the third position of the *ScrFI* restriction site can be any of the four bases (A, C, G or T).

It should be no surprise that the number of times a DNA sample is cut by a given restriction enzyme depends on how often its restriction site appears in the sequence. This of course will vary from one DNA sample to another since every DNA sequence is unique. Nevertheless you can still calculate the probability of a given restriction site appearing in a DNA sample by random chance. This will allow you to estimate the average distance between each copy of a given restriction site, and thus the approximate number of times your DNA will be cut (assuming you know the total number of base pairs in your sample) as well as the average size the resulting DNA fragments. To illustrate this let’s look at the *EcoRI* restriction site:

\[
\text{EcoRI} \\
5’…GAATTC…3’ \\
3’…CTTAAG…5’
\]

Since there are four possible bases at any given position in a DNA sequence, there is a 1 in 4 chance that position will contain a particular base. For example the probability of any position in a DNA molecule containing the base G is \( \frac{1}{4} \). Since the *EcoRI* restriction site requires a G at position one, an A at position two, and so forth, the probability of any particular six base stretch of DNA being 5’-GAATTC-3’ is:

\[
\frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} = \frac{1}{4096}
\]

The nature of probability dictates that the probability of multiple events occurring together is the product of the probabilities of each individual event. This applies to flipping coins, rolling dice, or the identity of nucleotides in DNA. Thus the probability of any six base sequence in a DNA molecule being 5’-GAATTC-3’ is \( \left(\frac{1}{4}\right)^6 = \frac{1}{4096} \). What this means is that, simply due to random chance, the *EcoRI* restriction site should appear on average once every 4096 base pairs. Looking at this another way, *EcoRI* should cut DNA on average once every 4096 base pairs and thus the resulting DNA fragments should average ~4096 base pairs in length.
The calculation is slightly different when a restriction site contains positions with some flexibility as indicated by R, Y or N. The probability of a position containing any of the four possible bases is of course 4 out of 4 or 1, and the probability of a position containing a purine (A or G) or a pyrimidine (C or T) is 2 out of 4 or ½. With this in mind, let’s look at a couple more examples:

<table>
<thead>
<tr>
<th>restriction enzyme</th>
<th>restriction site</th>
<th>probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>HaeII</td>
<td>5'…RGCGCY…3'</td>
<td>½ x ⅛ x ⅛ x ⅛ x ⅛ = 1/1024</td>
</tr>
<tr>
<td></td>
<td>3'…YCGCGR…5'</td>
<td></td>
</tr>
<tr>
<td>ScrFI</td>
<td>5'…CCNGG…3'</td>
<td>⅛ x ⅛ x ⅛ x ⅛ = 1/256</td>
</tr>
<tr>
<td></td>
<td>3'…GGNCC…5'</td>
<td></td>
</tr>
</tbody>
</table>

DNA can also exist in a linear form or a circular form, something you will need to consider when cutting a DNA sample with a restriction enzyme. To see why this is important, let’s consider two hypothetical DNA molecules, one linear and one circular, each with a single EcoRI site:

As you can see, the DNA fragments resulting from restriction enzyme digestion of linear vs circular DNA will differ. With circular DNA you will get as many linear fragments as there are restriction sites in the molecule, whereas a linear molecule will yield one more fragment than the number of restriction sites. This is no different than cutting a piece of string with a pair of scissors. If a linear piece of string is cut 3 times you will end up with 4 pieces, whereas a circular piece of string cut 3 times will yield 3 pieces.

**Partial restriction enzyme digestion of DNA**

Any DNA sample used in a restriction enzyme digestion contains huge numbers identical molecules of DNA. For example, 1 µg of a 3000 bp plasmid contains over 300 billion (3 x 10^15) copies of the plasmid! So even if there is only one restriction site per plasmid for the enzyme being used, that’s over 300 billion sites that need to be cut. To cut them all and thus achieve “complete digestion” requires a sufficient amount enzyme and a sufficient amount of time. If complete digestion is not accomplished for any reason then you would have what is called “partial digestion”. This simply means that a significant proportion of the target restriction sites were not cut. You will be able to view the difference between a complete and partial digestion of a DNA sample at the end of this laboratory exercise.
Part 1: RESTRICTION ENZYME DIGESTION OF A DNA SAMPLE

Planning your reactions

Before you begin to put together a restriction enzyme digestion you need to have a plan. This is important to make efficient use of your time and materials. The materials you will use are expensive and you don’t want to make mistakes that force you to discard anything and start over. Before making your plan you need to consider each component of a restriction enzyme digest:

**DNA**
- What DNA sample do you want to cut and how much will you need per reaction?
- How many total reactions do you plan to carry out?

**Restriction Enzyme**
- What restriction enzyme will you use to cut your DNA sample?
- How many units* of restriction enzyme will you need in a single reaction?

* By definition, one unit of a restriction enzyme is enough to completely cut 1 µg of DNA in 1 hour, though in practice this is not always the case.

**Reaction Buffer**
- What reaction buffer is best for the restriction enzyme you plan to use?
- Is it necessary to add bovine serum albumin (BSA) to the reaction?

**Ultrapure Water** (contaminants in impure water can interfere with the reaction)
- How much water will you need per reaction?

**Temperature**
- Every restriction enzyme works best at a particular temperature, so you need to determine the temperature at which you will incubate your reactions.

Once it is clear what components you will need per reaction and how many reactions you need to carry out, you can then make your plan. The most efficient way to put together your reactions is to pool together all components that are common to each reaction. This saves time and effort, is much more accurate, and minimizes mistakes. To illustrate this assume you want to cut 1 µg of 10 different DNA samples with 5 units of the restriction enzyme **BamHI**, and the available components are as follows:

- 10 different DNA samples (each at a concentration of 0.5 µg/µl)
- **BamHI** restriction enzyme (20 units/µl)
- reaction buffer supplied with the enzyme (10X concentration)
- bovine serum albumin or BSA (10X concentration)
- ultrapure water

The reaction buffer required for a given restriction enzyme and the need for BSA will be specified by the manufacturer of the enzyme. This information is available in the manufacturer’s catalog such as the one
found at your table, or online. For now let’s assume we have all necessary components and are ready to make our plan. First we need to determine the total volume of each reaction, and the volume needed of each component per reaction. Let’s plan on a total reaction volume of 30 µl which will dictate the volume we need of each component:

**Components needed for a single reaction**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA sample</td>
<td>2 µl (1 µg total)</td>
</tr>
<tr>
<td>BamHI</td>
<td>0.25 µl (5 units total)</td>
</tr>
<tr>
<td>10X buffer</td>
<td>3 µl (for a final concentration of 1X)</td>
</tr>
<tr>
<td>10X BSA</td>
<td>3 µl (for a final concentration of 1X)</td>
</tr>
<tr>
<td>water</td>
<td>21.75 µl (enough to make the total volume 30 µl)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>30 µl</td>
</tr>
</tbody>
</table>

Let’s review the volume added for each component to clarify how we arrived at these numbers. The original DNA sample is at a concentration of 0.5 µg/µl so we will need 2 µl to get 1 µg total (2 µl x 0.5 µg/µl = 1 µg). The BamHI enzyme is at a concentration of 20 units/µl so we will only need 0.25 µl to get 5 units total (0.25 µl x 20 units/µl = 5 units). The reaction buffer and BSA are both at a 10X concentration (10 times the working concentration), so we will need 1/10th of the total volume of each (1/10 x 30 µl = 3 µl) to make the final concentration 1X. Last and certainly not least, we need to add enough ultrapure water so that the total volume is 30 µl. This is extremely important. If you don’t add the correct amount of water, the final concentrations of all other components will be off which can adversely affect your reaction.

If you plan to carry out a single restriction enzyme reaction then you are ready to proceed, however in this scenario we plan to carry out 10 reactions that differ only in the DNA sample being digested. As mentioned earlier, the best way to do this is to pool together all common components which are: the restriction enzyme BamHI, 10X buffer, 10X BSA, and ultrapure water. It is always a good idea to make enough of your “pool” to accommodate one extra reaction to ensure that you don’t come up short. So in this example we will make a pool to accommodate 11 reactions as shown below:

**Pooled components for 11 reactions**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PstI</td>
<td>2.75 µl</td>
</tr>
<tr>
<td>10X buffer</td>
<td>33 µl</td>
</tr>
<tr>
<td>10X BSA</td>
<td>33 µl</td>
</tr>
<tr>
<td>water</td>
<td>239.25 µl</td>
</tr>
<tr>
<td>TOTAL</td>
<td>308 µl (divided by 11 = 28 µl ea)</td>
</tr>
</tbody>
</table>

As you can see, each component common to all reactions was multiplied by 11. To ensure the amounts are correct, the total is divided by 11 resulting in 28 µl of the pool per reaction. This is exactly what we expect since the pool contains all components but the DNA which will account for 2 µl of each reaction (30 µl total – 2 µl DNA = 28 µl for everything else). *It is extremely important to verify your calculations in this way to avoid a mistake that will affect every reaction.*
Putting your reactions together

Now that you understand how to plan a set of restriction enzyme digests, you are ready to plan and carry out your own reactions. All of your reaction components except the water are very temperature sensitive, the enzyme in particular. Thus it is important to keep them as cool as possible which means keeping them on ice until needed. Before you begin, first get an ice bucket filled with crushed ice. You can then acquire all needed components, thaw them if necessary, and store them on ice.

Once you have made your plan and have all necessary components on ice, keep in mind that the order in which components are added to your pool is very important. You want to avoid exposing the enzyme to an environment drastically different from the reaction itself. For example, you don’t want to add the enzyme to the 10X buffer and 10X BSA and then add the water last. If you were to do it this way, the enzyme will be exposed to a much higher concentration of salts and other components than in the final reaction. As a rule of thumb it is always best to add the water first and the enzyme last. For this reason your pool should be put together in the order shown below:

1) ultrapure water
2) 10X buffer
3) 10X BSA (if necessary)
4) restriction enzyme

Now you are ready to carry out your restriction enzyme digest of DNA. The DNA you will be cutting is a small circular piece of DNA called a plasmid. Plasmids are commonly found in bacteria and contain additional genes not found in the bacterial chromosome. Plasmids are also very useful in the process of gene cloning as you will see later in the course. The plasmid DNA you will be cutting is 3342 base pairs (bp) in length, and the enzyme you will be cutting with is EcoRI.

Exercise 1 – EcoRI digestion of plasmid DNA

Each reaction should contain 1 µg of plasmid DNA (0.5 µg/µl) with 5 units of the restriction enzyme EcoRI (20 units/µl) in a total of 30 µl. Each student should prepare at least one reaction as described below:

1. Look up the restriction enzyme EcoRI in the New England Biolabs catalog at your desk and determine a) what 10X buffer should be used with this enzyme, b) if BSA is required, and c) at what temperature the digestion should carried out. Set the heat block at your table to the appropriate temperature.

2. Devise a plan (refer to previous two pages) to pool together all common ingredients so that there will be enough for 4 reactions plus 1 extra reaction.

3. Combine the components for your pool in a microcentrifuge tube in the order shown above, mixing gently by tapping the bottom of the tube after each addition. If necessary, do a quick spin at the end to bring all liquid to the bottom of the tube.

4. Label four microcentrifuge tubes “1, 2, 3 and 4” aliquot the calculated amount of pool into each tube.

5. Place each tube in the heat block at your table set to the appropriate temperature.

6. Remove tube 1 after 5 minutes and place on ice, remove tube 2 after 10 minutes and place on ice, remove tube 3 after 20 minutes and place on ice for 5 minutes, then place all 3 tubes in the freezer.

7. Leave tube 4 in your heat block for at least one hour (or overnight), then place in the freezer.
Part 2: AGAROSE GEL ELECTROPHORESIS

To visualize the products of your restriction digest you will need to carry out a very common laboratory technique called agarose gel electrophoresis. Agarose is a polysaccharide derived from red algae that is used to form a horizontal gel slab. When a DNA sample is loaded on an agarose gel and subjected to electric current, the DNA fragments in the sample move through the gel at a rate inversely proportional to DNA fragment length. Once sufficient separation has been accomplished the gel can be stained and the DNA fragment sizes visualized.

The process of agarose gel electrophoresis involves 3 general steps which we will address in order:

1) Preparing the gel
2) Running the gel
3) Staining the gel

Preparing an agarose gel

An agarose gel is a combination of agarose powder and an electrolyte solution called 1X TAE. “1X” refers to “one times the working concentration” (a stock solution of 50X TAE is diluted to make 1X TAE). “TAE” refers to tris-acetate (pH buffer system, electrolytes) plus EDTA (prevents DNA degradation). To prepare an agarose gel you need to know two things: 1) the percentage of agarose required in the gel and 2) the total volume of the gel. You are actually making a percent solution (grams/100 ml) in which the percent agarose is simply the weight of agarose powder in grams divided by the total volume in ml, multiplied by 100. The calculation to determine how much agarose powder you will need is simple. Once you know the volume you want, the amount of agarose is simply the desired agarose concentration (% agarose) multiplied by the desired total volume:

\[
0.8\% \times 50\text{ ml} = 0.4\text{ g}
\]

Notice that we ignore the volume of the agarose itself, which means that the actual percentage of agarose is slightly lower. The difference, however, is insignificant and the convention in laboratories is to assume that the total volume of your gel is determined by the electrolyte solution alone.

Once the agarose powder and TAE have been measured, they are combined and boiled in a microwave oven after which the agarose solution is cooled to 50°C in a water bath. This is important since the agarose solution must be warm enough to remain liquid but not too hot to pour into a gel casting tray (if the gel mixture is too hot it can warp the casting tray). A comb suspended over the casting tray will result in the formation of cavities in the gel called wells as the gel cools. The solidified agarose gel slab will be placed in an electrophoresis chamber and completely submerged in 1X TAE running buffer. DNA samples can then be loaded into the wells and subjected to electric current, i.e., electrophoresis.
**Exercise 2A – Prepare an agarose gel**

The DNA sample you are currently subjecting to restriction enzyme digestion will be run on a 1% agarose gel. The gel casting apparatus you will be using requires a total volume of 60 ml. Calculate the amount of agarose you will need and follow the instructions below as you prepare your gel:

1. Weigh the necessary amount of agarose powder and add it to a 250 ml flask.
2. Add 60 ml of 1X TAE to the agarose in the flask.
3. Place a folded paper towel on the scale and weigh the flask. Record this weight and zero the scale.
4. Heat the mixture uncovered for 1 minute 30 seconds in a microwave. This will boil the mixture long enough to completely dissolve the agarose.
5. Using the rubber “hot hand” protector, remove the flask from the microwave and swirl to ensure the agarose is completely dissolved. If not, then boil in the microwave for an additional 30 seconds.
6. Place the flask on the scale and, using a water bottle, add deionized water until the weight is ~3 ml above the original weight of the flask/agarose mixture which you recorded in step 3. Swirl to mix.
7. Place the flask in a 50°C water bath for ~15 minutes to allow the gel mixture to reach 50°C. The gel mixture is cool enough when the base of the flask is comfortably warm to the touch.
8. While the gel mixture is cooling, set up the gel casting tray:
   - ensure the rubber seals are firmly in the grooves along each end of the casting tray
   - insert the tray into the electrophoresis chamber so that the ends of the tray are pressed snugly against the sides
   - insert the comb (use side with thicker teeth) into the notches at one end of the casting tray
9. Pour the 50°C gel mixture into the casting tray and allow the gel to cool for ~15 or 20 minutes.
10. Remove the casting tray containing the gel and turn it 90 degrees so that it rests parallel in the chamber with the wells on the left and the plugs facing away from you.
11. Add just enough 1X TAE to completely submerge the gel.
12. Carefully remove the comb from the gel. The gel is now ready for your DNA samples.

**Loading and running an agarose gel**

“Loading a gel” refers to adding DNA samples to the wells of an agarose gel. To ensure that the DNA samples remain in the wells prior to electrophoresis, a gel loading dye solution is added to each sample. Gel loading dye contains two key components:

- **glycerol** or **ficoll** – makes the solution denser than water
- **bromophenol blue** – a negatively charged dye

The glycerol (or ficoll) ensures that the DNA sample will sink to the bottom of the well and stay there when loaded. If not for the glycerol, the DNA sample would rapidly diffuse out of the well and be lost. The bromophenol blue dye, like DNA, is negatively charged and will migrate through the gel in the same direction as DNA. The migration of this dye thus allows one to monitor the progress of electrophoresis.
The actual loading of DNA samples into a well requires careful technique as shown below to ensure that the sample ends up in the well with minimal loss and without damaging the surrounding gel.

The keys to successfully loading an agarose gel are listed below:

- position the electrophoresis chamber so that you can comfortably load your samples without moving the chamber when you are finished (“sloshing” can result in the loss of your samples)
- get in a comfortable position (sitting is recommended unless it is easier to stand)
- steady your arms by leaning your elbows on the bench top
- use your opposite hand to steady the micropipettor as shown in the picture
- position the micropipettor tip just above, but not inside, the well to avoid puncturing the gel
- load the sample slowly
- remove the tip from the liquid before releasing the plunger

If you follow these suggestions, you will be sure have minimal sample loss when your load your gel.

Running a gel is fairly straightforward. The electrophoresis chamber is connected to a power supply (without disturbing the gel now loaded with DNA samples) such that the cathode is opposite the DNA samples. The power supply is set to between 90 and 100 volts, and the power is turned on. The gel should be allowed to run until the bromophenol blue dye is approximately halfway across the gel.
As electrophoresis progresses, the DNA fragments migrate toward the cathode (due to the negative charge of DNA molecules) at a rate inversely proportional to the length of the DNA fragment. In other words, shorter DNA fragments will migrate faster and longer DNA fragments will migrate more slowly. The net result is a separation of DNA fragments based on length which is revealed upon staining.

While the relative length of DNA fragments is easy to see upon staining a gel, the actual length of a DNA fragment cannot be determined without running a sample of DNA fragments with known lengths on the same gel. Such a sample is called a DNA ladder. An example of a stained gel containing a lane with a DNA ladder (*) is shown below:

![Stained gel with DNA ladder](image)

Relative to the known sizes of the ladder (k = kilobase pair), the length of the DNA fragments in each lane can be estimated. For example, the fragments in lane 2 are ~3000 base pairs (bp) and ~400 bp.

**Exercise 2B – Load and run an agarose gel**

1. Consult your labmates to plan what samples will be loaded into what lane on your gel. Be sure to include one lane for the DNA ladder, ideally in the middle of the gel, and one lane for uncut plasmid DNA, both of which will provided by your instructor.

2. Add 3 µl of gel loading dye to each restriction digest. Mix by tapping and then quick spin your sample to bring all liquid to the bottom of the tube.

3. Make sure your gel is oriented so that the negatively charged DNA samples will run towards the positively charged anode, then load 10 µl of the DNA ladder and 10 µl of the uncut plasmid DNA.

4. Load all 33 µl of each restriction digest into the appropriate lanes.

5. Connect the electrophoresis chamber to the power supply, set the toggle switches to “low” and “volts”, and turn the knob to “min”. Turn on the power and adjust the knob until the readout is between 90 and 100 volts (NOTE: if the gel is set up correctly a large amount of bubbles should rise from the anode)

6. Run the gel until the bromophenol blue dye has moved a little more than halfway across the gel (about 90 minutes), then turn off the power.
Staining an agarose gel

Visualizing DNA fragments as bands on a gel requires the addition of a dye that is attracted to DNA. Two common dyes used for this purpose are **ethidium bromide** and **methylene blue**. Ethidium bromide is a more sensitive stain used routinely in research labs despite its classification as a mutagen (chemical that can cause mutations in DNA). Ethidium bromide is perfectly safe as long as it is handled properly. Methylene blue is a less sensitive dye that takes more time to stain commonly used in educational labs. To ensure that all bands on your gel are clearly visible and to save time, you will stain your gels with ethidium bromide.

Exercise 2C – Stain your agarose gel

1. Put on a pair of gloves and keep them on throughout the staining procedure.
2. Remove the casting tray with the gel from the electrophoresis chamber and carefully slide the gel off the tray into the plastic container at your table.
3. Drip a few drops of running buffer (1X TAE) on top of the gel to moisten it.
4. Peel the plastic film off an InstaStain ethidium bromide card and place it face down on top of the region of the gel you want to stain.
5. Place the gel casting tray on top of the staining card and a beaker on top of the casting tray. Leave for 5 minutes. (**NOTE:** the extra weight will ensure good contact between the staining card and the gel)
6. Remove the staining card from the gel and discard as biohazard waste.
7. Photograph your gel as indicated by your instructor and store a copy in your notebook.

Estimating DNA fragment sizes

Once your agarose gel is stained and bands of DNA are visible on the gel, you can estimate the approximate length of various DNA fragments by comparison with the DNA ladder. The DNA ladder you have used is called “**1 kb Ladder**” since most of the DNA fragments in the ladder have lengths that are multiples of 1000 bp (aka, 1 kilobase pair or 1 kb). The specific lengths of each DNA fragment in the 1 kb ladder are shown on the right.

DNA fragment length can be estimated by plotting the distance each band travelled on semilog graph paper, however this is tedious and really no more accurate than doing a visual estimate as shown on the previous page.
Before you examine your gel you need to be aware that a circular piece of DNA (i.e., uncut plasmid) can exist in two different forms as illustrated below: **relaxed** and **supercoiled**.

During agarose gel electrophoresis, circular DNA in its relaxed form migrates at a different rate than supercoiled circular DNA. For this reason uncut plasmid DNA run on a gel usually results in two bands – a lower, faster migrating band of supercoiled DNA and higher, slower moving band of relaxed circular DNA. A linearized plasmid of the same size will migrate on the gel at a rate different from either circular form. Thus the same DNA molecule can migrate at 3 different rates and therefore end up in 3 different positions on a gel depending on whether it is linear, supercoiled or a relaxed circle. Since circular DNA runs at a different rate on a gel than linear DNA, a DNA ladder (which consists entirely of linear DNA fragments) can only be used to estimate the size of linear DNA fragments. Keep this in mind as you analyze your stained gel.

**Exercise 2D – Estimate the length of each DNA fragment in your restriction digests**

1. Compare each of your restriction digests with the uncut plasmid DNA on your gel and identify any bands that match and thus are uncut supercoiled or relaxed circular DNA. The lengths of DNA molecules in these bands cannot be estimated in relation to the DNA ladder, so don’t bother to do so.

2. The remaining bands on your gel should contain linear DNA fragments. Compare each of the remaining bands for each restriction digest to the 1 kb ladder and estimate the length of each fragment to the nearest 50 or 100 base pairs.

3. Examine the bands in the lane containing the DNA sample digested for the longest period of time (at least one hour) and:
   - determine how many EcoRI sites are in the plasmid
   - estimate the distance in base pairs (bp) between each EcoRI site
1. What is a restriction enzyme? Give an example of one and it restriction sequence.

2. Indicate how often the following restriction sites should appear (on average) in a DNA molecule.

   - **AlwNI**
     - 5’ - CAGNNNCTG - 3’
     - 3’ - GTCNNNGAC - 5’

   - **AvaI**
     - 5’ - CYCGRG - 3’
     - 3’ - GRGCYC - 5’

3. What do you notice about each sequence above that is characteristic of most restriction sites?

4. Describe what is meant by the terms “sticky ends” and “blunt ends” with regard to linear DNA.

5. Why should you include a DNA ladder every time you run DNA samples on an agarose gel?

6. What is the relationship between DNA fragment length and the distance it will run in a gel?

7. A plasmid and a linear DNA molecule are each cut 7 times by a restriction enzyme. How many DNA fragments will be produced in each case?
8. You want to cut 1 µg of a plasmid with 4 units of the restriction enzyme *HindIII* for two hours at each of five different temperatures: 10°C, 20°C, 30°C, 40°C & 50°C. The plasmid stock is 250 ng/µl, the enzyme is 10 units/µl, the reaction buffer is 5X, and the total volume of each reaction is 20 µl. Using the New England Biolabs catalog, indicate your plan for setting up these reactions below.

9. What is a *partial* restriction enzyme digestion and how would you recognize partial digestion of a DNA sample on a stained agarose gel?

10. Describe how the rate of migration and thus the position on a gel of a DNA fragment will differ if the fragment is linear, supercoiled or a relaxed circle.