Laboratory 1: Digests and Gels: Analysis of DNA Using Restriction Mapping and Agarose Gel Electrophoresis

Background Reading in *DNA Science (2nd Edition)*:

2. *Restriction Endonucleases*: Chapter 4: pp. 109-113
4. *Laboratory Safety and Adherence to NIH Guidelines*: pp. 317-319

Objectives of Laboratory 1:

1. Review Laboratory Safety
2. Review micropipetting technique to transfer volumes accurately and reproducibly
3. Digest samples of methylated and non-methylated DNA
4. Pour an agarose gel
5. Analyze digests using agarose gel electrophoresis
6. Visualize DNA fragments in a gel with GelRed DNA stain
7. Investigate the significance of DNA methylation in human disease

Flow Chart for Laboratory 1:
GENERAL LABORATORY SAFETY

Professional standards of personal behavior are required in any laboratory:

Avoid distracting or startling other workers

Do not allow practical jokes or horseplay

Use laboratory equipment only for its designated purpose

Do not prepare, store (even temporarily), or consume food or beverages in the laboratory

Do not smoke in the laboratory.

Do not apply cosmetics when in the laboratory

Confine long hair and loose clothing in the laboratory. Wear shoes at all times. Open-toed shoes or sandals are not appropriate.

Under no circumstances should mouth suction be used to pipette chemicals or to start a siphon. Use a pipette bulb or a mechanical pipetting device to provide a vacuum.

Always wash your hands before leaving the lab.

Keep work areas clean and free from obstruction. Clean up spills immediately.

Notify your instructor of any allergies or health related concerns especially if you are pregnant.

**If you are unsure about something – ASK!!!!**

SAFETY PROCEDURES

CHEMICALS

A number of chemicals used in the laboratory are hazardous. All manufacturers of hazardous materials are required by law to supply the user with pertinent information on any hazards associated with their chemicals. The only particularly noteworthy hazardous chemical you will be working with is Ethidium Bromide which is a carcinogen. When we use this chemical your lab instructor will outline the procedures used when handling this substance.

These chemicals are not harmful if used properly: always wear gloves when using potentially hazardous chemicals and never mouth-pipet them. If you accidentally splash any of these chemicals on your skin, immediately rinse the area thoroughly with water and inform the instructor. Discard the waste in appropriate containers.

ULTRAVIOLET LIGHT

Exposure to ultraviolet light can cause acute eye irritation. Since the retina cannot detect UV light, you can have serious eye damage and not realize it until 30 min to 24 hours after exposure. Therefore, always wear appropriate eye protection when using UV lamps.

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ELECTRICITY
The voltages used for electrophoresis are sufficient to cause electrocution. Cover the buffer reservoirs during electrophoresis. Always turn off the power supply and unplug the leads before removing a gel.

GENERAL HOUSEKEEPING
All common areas should be kept free of clutter and all dirty lab supplies. Electrophoresis equipment should be dealt with appropriately. Since you have only a limited amount of space to call your own, it is to your advantage to keep your own area clean. Since you will use common facilities, all solutions and everything stored in an incubator, refrigerator, etc. must be labeled. In order to limit confusion, each person should use his/her initials or ID number for labeling plates and other samples.

CHEMICAL EXPOSURE
Eyes
If any substance is introduced into your eyes IMMEDIATELY get assistance and go to the eyewash station which is located at the rear sink of every laboratory. Holding your eyes open, flush for at least 15 minutes. Get medical attention as soon as possible. You will be instructed on how to use the emergency eyewash station in the introduction to your lab class. There are also instructions posted next to the station. If it is not covered in class, don't be afraid to ask.

Skin
If you get a substance on your hands or arms flush with cold water, over the sink, for 15 minutes. If there is a large amount of a substance spilled on you, IMMEDIATELY get assistance, remove any contaminated clothing, and use the emergency eyewash as a shower. Rinse yourself for at least 15 minutes. Get medical attention as soon as possible.

CUTS AND PUNCTURES
Handling glassware and pipets in the laboratory can be quite dangerous. Much of the laboratory glassware is delicate and easily broken, which contributes to cuts and punctures. Inspect glassware for chips and cracks which will weaken the glass. Contact your instructor who will discard of the damaged glass in the broken glass box. Pay close attention to your instructor's demonstration on how to handle these items. If you are washing glassware always use the brushes to clean the inside. Avoid putting your hands inside beakers, graduated cylinders and other glass items. In the event of an accident there is a first aid kits found in the prep room for your use. Control any bleeding and seek medical attention in case of an accident.

DISPOSAL OF SHARPS AND BROKEN GLASS
Sharps include razor blades, scalpels, and needles. Always remember to cut AWAY from your body. If you break any type of glassware, take care of yourself first, then CLEAN UP the broken glass to prevent others from cutting themselves. A broom and pan are located in the prep room and you should ask one of the teaching facility personnel to assist you. All broken glass is deposited in the broken glass box in the prep room. Sharps are disposed of in a separate box. This box is red plastic and labeled for sharps disposal.

For more information you can visit the Princeton Web site for Laboratory safety at: http://www.princeton.edu/~ehs/labmanual/index.html
Discarding Laboratory Waste

All rules for disposal of waste in the biology lab are institutional and governed by the Environmental and Health Safety Committee (EHS).

What can go in the “regular trash”?  
Generally all paper and gloves that have not been used for work with biohazard material can be disposed of in the regular trash (grey trash cans in the lab)

Bench top red waste bins:  
These small bins can be used to discard all pipet tips and tubes used during lab. DO NOT put paper towels or gloves in these waste bins. These bins are emptied at the end of each lab into the biohazard waste. It is therefore appropriate to dispose of all tips and tubes that contain bacterial cells or DNA into these waste bins. DO NOT dispose of liquid waste in these containers.

Biohazard Waste (Large boxes in the back of the lab)  
These boxes should be used for all biohazard material which does not fit into the bench top bins. This includes gloves that were in contact with Ethidium Bromide and bacterial growth plates.

Water in the Laboratory

There are two main types of water available in the Schultz Teaching Lab:

Tap Water: The quality of tap water varies greatly. This water should not be used to make buffer solutions or to rinse out labware such as gel boxes. It is used mostly for rinsing glassware before washing and washing hands.

Deionized Water: This water is purified by a reverse osmosis procedure and then further deionized. This water is available through the white faucets located at each sink. This water should be used for washing out gel boxes and making buffers. Although purified, this water is not considered sterile.
What is in your molecular biology “toolbox”

1 – box of “yellow tips” – these are used with the P20 and P200
2 - box of “blue tips” – these are used with the P1000
   (all tips have been sterilized and should remain that way)
3 – timer (2): to clear the timer press both the min and sec button
   simultaneously
4 – black marker (2)
5 – P20 (this is used for pipetting 1 to 20 µl) (2)
6 – P200 (this is used for pipetting 20 to 200 µl) (2)
7 – P1000 (this is used for pipetting 200 to 1000 µl) (2)
8 – white microfuge tube rack (not pictured) (2)
9 – Loop (2) (not pictured)

Please remember that you will be using this equipment for the next two weeks. If something is
missing or not working correctly please let one of the lab staff know.

Each toolbox has a number. This number is your “lab pair” number. Each toolbox is assigned
specific micropipettes. It is your responsibility to ensure that the correct micropipettes are in your
box at the end of your lab section.
Please take a minute to look at the correct way to hold a micropipette. Your thumb should be on the top knob. When held correctly your fingers actually cover the number window.

Which Micropipette should I use?

- **P20**: 1 µl to 20 µl
- **P200**: 20 µl to 200 µl
- **P1000**: 200 µl to 1000 µl
Using Micropipettes

Almost all recombinant DNA work involves transferring volumes of liquid in the microliter (µl) range. A microliter is 1/1000th of a milliliter, which is 1/1000th of a liter. The success and reproducibility of your experiments in the lab will depend upon accurate measurement and delivery of these small volumes.

1. **Set the volume**
   All micropipettes have a volume control dial, and a window that shows the set volume. Determine whether the micropipette you are using shows tenths of microliters or whole microliters in the smallest place. This can be a little confusing because different sizes of pipettes can have different scales. Below is an example of volume settings for the three sizes of micropipettes you will be using.

   - **P-1000**
     - 640µl

   - **P-200**
     - 64µl

   - **P-20**
     - 6.4µl

2. **Put on a tip**
   Never use a micropipette without a tip! P-1000 micropipettes take a larger size pipette tip than the P-200 or P-20. Make sure the tip is snug; a loose tip can either fall off while you are transferring a sample, or it may not measure the correct volume.

3. **Draw up the sample**
   Using your thumb, press the plunger to the first stop, insert the tip into the liquid, and slowly release the plunger. Micropipettes have two stops as you depress the plunger; the second stop is used to get out any extra drops of liquid. The most common error when using micropipettes is missing the first stop and thus drawing too much liquid into the tip.

4. **Dispense the sample**
   Move the tip to the container you want to dispense the sample into and slowly press the plunger to the first stop. After the liquid has been released push the plunger to the second stop to expel the rest of the liquid from the tip.

5. **Dispose of the tip**
   Press the ejector button with your thumb to dispose of the tip into the red waste container located on your bench. Make sure to dispose of the old and load a new tip every time you pipette something!
The following diagrams are from the Gilson manual on pipetting:

1. **Preparation**
   - Hold the instrument in a nearly vertical position.
   - Depress the plunger smoothly to the first stop position.

2. **Aspiration**
   - Immerse the pipette tip in the liquid.
   - Allow the plunger to move up smoothly to the rest position.
   - Wait one second so that all the liquid has time to move up into the tip.

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3 Distribution
Place the pipette tip at an angle (10 to 45°) against the inside wall of the receiving vessel. Depress the plunger smoothly to the first stop position.

4 Purge
Wait one second, then depress the plunger to the second stop position. This removes any remaining sample from the tip. Remove the pipette tip from the vessel.

5 Home
Allow the plunger to move up to the rest position. Dispose of the tip in a waste container.
A quick review of metric prefixes

The basic units used in molecular biology include meters, grams, moles and liters. The following table lists the metric prefixes that are used with these units.

<table>
<thead>
<tr>
<th>Metric Prefix</th>
<th>Abbreviation</th>
<th>Power of 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>giga-</td>
<td>G</td>
<td>$10^9$</td>
</tr>
<tr>
<td>mega-</td>
<td>M</td>
<td>$10^6$</td>
</tr>
<tr>
<td>kilo-</td>
<td>k</td>
<td>$10^3$</td>
</tr>
<tr>
<td>milli-</td>
<td>m</td>
<td>$10^{-3}$</td>
</tr>
<tr>
<td>micro-</td>
<td>µ</td>
<td>$10^{-6}$</td>
</tr>
<tr>
<td>nano-</td>
<td>n</td>
<td>$10^{-9}$</td>
</tr>
<tr>
<td>pico-</td>
<td>p</td>
<td>$10^{-12}$</td>
</tr>
<tr>
<td>femto-</td>
<td>f</td>
<td>$10^{-15}$</td>
</tr>
<tr>
<td>ato-</td>
<td>a</td>
<td>$10^{-18}$</td>
</tr>
</tbody>
</table>

The prefix is usually chosen so that the value can be written as a number greater than one but less than 1000.

In the lab manual, we may sometimes use 0.8 ml and other times 800 µl, but both are the same amount!
Today’s laboratory will focus on making sure your micropipetting skills are correct. As mentioned before, the success of your laboratory experiments depends on your ability to pipette accurately!

**Materials**

- P20, P200, and P1000 micropipettes
- Disposable pipette tips
- Red, Blue and Yellow food coloring diluted 1:10 in H2O
- Clear test tubes
- Lab markers

**Each workshop participant** will do the following exercise individually. If you remember the following steps each time you pipette you should have no problems:

1. **Check the volume setting**
2. **Firmly secure the proper size tip onto the end of the micropipette**
3. **Push to the first stop **before you draw up the sample**
4. **Push to the second stop to expel the sample**
5. **Eject the tip into the waste container after you have finished**

1. **Find 2 clear plastic test tubes in the test tube rack at your bench.**
2. **Label one clear tube with your initials and the number 1.**
3. **Use the P1000 micropipette to transfer 1.0 ml (this is 1000 µl) of water into the tube.**

   You must use the large size (blue) tips on this micropipette. Depress the plunger to the first stop and hold it in this position. Dip the tip into the water just below the surface and SLOWLY release the plunger. This will slowly draw the water into the tip. Only take the tip out of the water after you have fully released the plunger. If you look at the water in the tip there should no air bubbles.
   To expel the sample into tube 1 SLOWLY depress the plunger to the first stop, and then to the second stop to push out the last bit of liquid.
4. Use the **P20 micropipette** to transfer 2.0 µl of Blue dye into tube 1.
   You need to use the smaller size (yellow) tips on this micropipette.
   When adding small volumes it is a good practice to add the sample to the side wall of the tube
   and then mix the contents.

5. Use the **P200 micropipette** to transfer 25 µl of Yellow dye into tube 1.
   You need to use the smaller size (yellow) tips on this micropipette. Use the same technique as
   above.

6. Cap the tube tightly, mix, by finger flicking the tube, and place it in rack 1 on the front
desk.

Now that you are a pro – here is another practice exercise with fewer helpful hints attached.

1. Label the other clear tube with your initials and the number 2.

2. Pipet 1.0 ml of water into tube 2.

3. Pipet 16.0 µl of yellow dye into tube 2.

4. Pipet 3.0 µl of red dye into tube 2.

5. Cap the tube tightly, mix, and place it in rack 2 on the front desk.
**Introduction – Restriction Enzymes**

**Restriction endonucleases** are enzymes that cut double-stranded DNA in a sequence-specific manner. This ability to cut DNA is both predictable and reproducible and has made these enzymes among the most powerful tools in molecular biology. Restriction endonuclease cleavage of DNA is one of the most basic procedures in molecular biology.

There are hundreds of different restriction endonucleases (also called restriction enzymes) that have been discovered and are available to scientists today. Each is a protein isolated from a specific bacterium and each recognizes a specific DNA sequence usually 4 to 8 nucleotides in length. After recognizing this sequence, the enzyme cuts the backbone (the covalent bonds!) of the double stranded DNA, which cleaves the DNA into two pieces. The specific sequence that a restriction enzyme recognizes is called the **restriction site**. Some restriction enzymes cut the backbone of the two strands of DNA symmetrically; this leaves DNA fragments with **blunt ends**. Other restriction enzymes cleave the two DNA strands at different positions (or asymmetrically) within the restriction site and this leaves DNA fragments with small single stranded overhangs called **sticky ends**. These sticky ends are very important in recombinant DNA techniques.

To digest a sample of DNA with a restriction enzyme, you simply need to incubate the enzyme with the DNA in the appropriate reaction conditions. Today in lab you will be digesting genomic DNA isolated from bacteriophage lambda (λ). The genome of bacteriophage lambda is 48,502 bp in length. It is a linear genome.

Restriction enzyme activity is measured in units (U). One unit is defined as the amount of enzyme needed to digest to completion 1µg of λ DNA in 1 hour at 37°C. The unit concentration of different restriction enzymes varies, but typical commercially available enzymes have activities in the range of 5-50 U/µl. Most researchers add excess enzyme to restriction digests to overcome impurities in DNA or to shorten incubation times.

**Introduction – DNA methylation in bacteria**

There are two different types of DNA methylation that occur in bacteria. The first type of methylation is a component of the restriction modification system that can be thought of as a very primitive immune system in the bacterial cell. The purpose of this system is to help the cell discriminate between its own DNA and foreign DNA. The restriction modification system is made up of two enzymes, a restriction enzyme (described above) and a methyltransferase enzyme that recognizes the same sequence as the restriction enzyme and adds a methyl group to a base in that sequence of DNA. The restriction enzyme can only cut unmethylated DNA. Due to the presence of the methyltransferase, the chromosomal DNA is methylated and thus protected from the restriction
enzyme. Any foreign DNA, for example from a bacteriophage, is not methylated and would be cleaved by the restriction enzyme. Each restriction enzyme has a specific methyltransferase that recognizes the same DNA sequence.

The second type of DNA methylation that occurs in bacteria is used to regulate the timing of DNA replication and also functions in the DNA mismatch repair system. This DNA methylase (called Dam methylase) recognizes the sequence GATC and adds a methyl group to the adenine in the sequence. This occurs at all GATC sequences in the bacterial genome.

In this experiment we are providing both methylated and unmethylated lambda DNA for analysis by restriction enzymes. The methylated lambda DNA has been treated with the enzyme Dam methylase. The result of this is that all GATC sequences will have a methyl group added to the A nucleotide. The lab illustrates how the addition of a methyl group to an adenine in the GATC sequence can protect this sequence from cleavage by a restriction enzyme. The restriction enzymes you will use are HindIII and BclI. Students can look up the recognition sites for these enzymes prior to performing the digest to predict experimental outcomes, or students can analyze results of the digests and investigate the sequence differences after the experiment.

**Introduction: DNA methylation in Eukaryotes**

Methylation of eukaryotic DNA is slightly different than the methylation of bacterial DNA. Bacteria methylate their chromosomal DNA to identify it as ‘self’ and to protect it from digestion by restriction enzymes. They also methylate their DNA as an aid in the timing of DNA replication. In contrast, eukaryotes methylate DNA as a way to modify gene expression.

DNA methylation in eukaryotes occurs at the dinucleotide sequence ‘CG’. This is sometimes written ‘CpG’ where the p is the phosphate bond between the two nucleotides. The methylation pattern of eukaryotic DNA is maintained during DNA replication, in that the newly synthesized strand of DNA gets methylated to match the parent strand. Methylation of C residues in eukaryotic DNA is a way to silence or turn off the expression of a gene. Amazingly, about 70-80% of all CG sequences in the mammalian genome are methylated.

**Materials**

- λ DNA (methylated and unmethylated)
- 10X restriction enzyme buffer
- Restriction enzymes (Hind III and BclI)
- dH₂O
- 1.5 ml microfuge tubes
- 37 °C water bath or heating block
**Procedure: lambda DNA digests**

Each lab pair will digest both methylated and unmethylated samples of lambda DNA with two restriction enzymes, Bcl I and Hind III.

1. Lab pair person 1 should digest both lambda DNA samples with Bcl I.

2. Lab pair person 2 should digest both lambda DNA samples with Hind III.

3. For each digest you need to digest 0.1 µg of λ DNA (either methylated or unmethylated) in a total reaction volume of 20 µl.

   Do not throw out your left over λ DNA because you will also load the uncut DNA on the gel.

4. Determine the amounts needed for each component of the digestion before adding anything to your tube! Please use the tables below to help you plan your digest components. (Check with one of the lab staff if you are unsure about your calculations)

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>_______ µl</td>
<td>0.1 µg unmethylated λ DNA (stock is 0.025 µg/µl)</td>
</tr>
<tr>
<td>_______ µl</td>
<td>10x restriction enzyme buffer (this is 10 times concentrated, the final digest concentration should be 1x)</td>
</tr>
<tr>
<td>_______ µl</td>
<td>dH₂O</td>
</tr>
<tr>
<td>1.0 µl</td>
<td>restriction enzyme Bcl I or Hind III</td>
</tr>
<tr>
<td>20 µl</td>
<td>Final Volume</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>_______ µl</td>
<td>0.1 µg methylated λ DNA (stock is 0.025 µg/µl)</td>
</tr>
<tr>
<td>_______ µl</td>
<td>10x restriction enzyme buffer (this is 10 times concentrated, the final digest concentration should be 1x)</td>
</tr>
<tr>
<td>_______ µl</td>
<td>dH₂O</td>
</tr>
<tr>
<td>1.0 µl</td>
<td>restriction enzyme Bcl I or Hind III</td>
</tr>
<tr>
<td>20 µl</td>
<td>Final Volume</td>
</tr>
</tbody>
</table>
5. Label two clean 1.5 ml microfuge tube with your initials, the enzyme name, and M or U to designate methylated (M) or unmethylated (U). Please remember there are two enzymes to test, one lab partner will set up both lambda DNA digests with the first enzyme, and the other lab partner will set up the two lambda DNA sample digests with the second enzyme.

6. Add the components of your digestion in the following order:
   DNA,
   water
   10x Buffer
   restriction enzyme (enzymes are stored in stratacoolers at the front of the lab)
   **When adding the buffer and enzyme to your digest dispense the small volume onto the side wall of the tube. This gives you visual confirmation that you have added the component. You can cap the tube and “wrist flick” the tube to bring the contents to the bottom.

7. Flick the bottom of the microfuge tube with your finger to mix the contents. An extremely important, yet often overlooked, element of a successful restriction digest is mixing. The reaction must be thoroughly mixed to achieve complete digestion.

8. Pulse-spin your tube in the tabletop centrifuge to collect all liquid components at the bottom of the tube.
   YOU MUST ALWAYS BALANCE THE TUBES IN THE MICROCENTRIFUGE!
   Do not forget to put on the lid of the rotor.

9. Check the final volume of your digests by comparing them both to the reference tube (20 µl) taped to the heating block.

10. If the volume looks correct incubate your digests for 15 minutes in a heating block at 37°C. If the volume appears different, check with one of the lab staff before starting the incubation.
**Introduction**

Agarose gel electrophoresis is a relatively simple and very efficient method for separating DNA fragments in the size range of 0.5 – 20 kilobases. Agarose is a polysaccharide derived from red algae. The agarose dissolves in solution when heated and hardens to a gel as it cools, similar to pectin in jelly. When it gels it forms a porous lattice, the more agarose you use, the tighter the lattice will be. Most gels that are used in the lab for separating DNA are between 0.5% and 4% agarose. Today each group will pour a 1% agarose gel.

Electrophoresis is the transport of charged molecules through a solvent by an electrical field. A molecule that carries a net charge will migrate when placed in an electric field. The mobility of the molecule will depend on the strength of the electric field, the net charge of the molecule and the size and shape of the molecule. Agarose serves as a matrix that the DNA will migrate through.

To separate the DNA fragments resulting from a restriction enzyme digestion you simply put the mixture of DNA fragments into a gel and apply an electrical current. DNA is negatively charged at neutral pH, due to the phosphate backbone, and it will move toward the positive (red) electrode. The DNA has to slip through the lattice of agarose molecules as it moves. Smaller DNA can fit through the holes easier than the larger fragments. The result is that a mixture of DNA fragments can be separated according to size. Small pieces of DNA migrate farther from the original starting position than do larger fragments of DNA. While both size and charge can affect the distance that a molecule of DNA moves in the gel, the charge to mass ratio for different sizes of DNA fragments is the same, so it becomes just the size of DNA that determines how far a DNA fragment will travel through the gel.

**Materials**

1.0% agarose solution (in 1X TAE)
Transfer pipet
Gel electrophoresis apparatus
Tris-Acetate EDTA (TAE) Running buffer (1X)
GelRed DNA stain
Procedure

Each lab pair needs to pour only one 1% agarose gel. Please wear safety goggles when pouring the gels.

1. Insert the black “dams” into the slots next to the gel tray. Do not place the white comb into place until after pouring the gel.

2. One member of the lab pair at the front of each bench should obtain:
   - a flask of melted 1% agarose from the 55°C water bath. Leave the red or green “donut” next to the water bath, it is only a weight.
   - a plastic transfer pipet from the beaker next to the water bath
   - a tube of GelRed DNA stain from the foil covered tube rack

3. Transfer 10 µl of GelRed from the tube to the flask of melted agarose (100 ml). Swirl gently to mix.

4. Using the transfer pipet, seal the inside edges of the gel tray next to the dams with a thin stripe of agarose.
   After sealing with agarose pass the flask of agarose to the lab pair next to you on your side of the bench.

5. When the flask of agarose makes it back to your group and when the sealing stripe of agarose is hard (you can touch it to test it), pour the melted agarose up to the sides of the gel bed.
6. Insert the comb into the agarose at the end with the black electrode with the flat surface facing the black electrode.
   If there are any bubbles on the gel surface, you can drag them to the side with a yellow tip.

7. The gel should take about 15 minutes to harden at room temperature. When it is hard, it will appear opaque white.

8. If there is agarose remaining in the flask you should place the flask back into the water bath.

9. When the gel is solid, gently slide the black dams upward to remove them before running your gel.

10. Obtain a bottle of TAE running buffer (located at the rear sink), shake it and pour it into the electrophoresis chamber. The gel must be completely covered.
    There will be more than enough TAE running buffer in your bottle to cover the gel.

11. Carefully remove the comb from the gel.
    Do this slowly so that you do not rip the wells. One partner should put on gloves and hold down the gel on either side of the comb while the other partner pulls up slowly at a slight angle. The lab instructor will demonstrate the correct way to remove a comb.

12. You can practice loading technique by loading 3 µl of sample buffer into the wells of a mock gel (small section of the gel containing the wells). The mock gels are located in a petri dish and are floating in buffer.
    Once you are comfortable loading wells you can proceed to the next step. If you are having trouble loading the sample buffer into the wells please ask a lab assistant for help.

13. Check that the wells of your gel are not broken or leaking by adding 3 µl of loading dye to each well.
    Adding the loading dye to the wells will not affect your DNA samples or the running of the gel.

14. If you find that one or two of the wells in your gel are broken, you can still use the gel as you will only need 6 lanes for this experiment. If you have more than three wells that are broken please ask one of the lab staff for a replacement gel.
Preparation and loading the lambda DNA samples

**Introduction**

Sample loading dye is added to DNA samples before loading them on a gel for two main reasons. The loading dye contains either sucrose, ficoll 400 or glycerol which when mixed with the DNA sample allows it to sink into the wells rather than float out. The loading dye also contains a tracking dye which allows one to monitor the progress of an electrophoretic separation. The dye you will use today contains bromophenol blue. This tracking dye co-migrates during gel electrophoresis with DNA molecules around 0.5 kb.

**Materials**

- 10x sample loading dye (1.5 ml microfuge tube with purple dot on the lid)
- 1 Kb ladder (clear tube/green screw cap; this already has loading dye added) (0.25 µg/20 µl)
- dH₂O
- Your DNA samples
- Microfuge tube

**Procedure**

1. Prepare your lambda digest samples for loading on the 1% agarose gel. Each lab pair will need one clean 1.5 ml microfuge tube to prepare a sample of uncut DNA.

2. You should follow the chart below for how much dye needs to be added to each tube and the amount of each sample that should be loaded into each well.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume</th>
<th>10x loading dye</th>
<th>Volume to load</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ DNA (uncut)</td>
<td>4 µl DNA + 16 µl dH₂O</td>
<td>3 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>λ unmethylated DNA Bcl I digest</td>
<td>20 µl</td>
<td>3 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>λ methylated DNA Bcl I digest</td>
<td>20 µl</td>
<td>3 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>λ unmethylated DNA Hind III digest</td>
<td>20 µl</td>
<td>3 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>λ methylated DNA Hind III digest</td>
<td>20 µl</td>
<td>3 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>1 kb ladder</td>
<td>All components already added</td>
<td>20 µl</td>
<td></td>
</tr>
</tbody>
</table>
3. **Use the P20 to load the gel and remember to use a new pipette tip for each sample.**
   When you load the sample into a well you should NOT push the micropipette plunger to the second stop. This will produce an air bubble that could dislodge the sample.
   Air bubbles at the end of the pipette tip can cause the sample to dislodge the sample. If you cannot take up the sample without having a residual air bubble, lower the volume by 1 or 2 µl and try again.

4. **Load your gel according to the following order:**
   - Lane 1: 1 Kb ladder
   - Lane 2: Lambda uncut
   - Lane 3: Partner 1 Bcl I digest (U)
   - Lane 4: Partner 1 Bcl I digest (M)
   - Lane 5: Partner 2 Hind III digest (U)
   - Lane 6: Partner 2 Hind III digest (M)
   - Lane 7: Blank
   - Lane 8: Blank

5. **When all samples have been loaded, put down the cover of the electrophoresis tank and connect the electrodes to both the tank and the power supply.** Connect “red to red” and “black to black”
   The black electrode is negative and the red is positive.
   Remember: DNA “RUNS RED”

6. **Set the power supply to 100 volts.**
   The voltage adjusts in increments when you turn the knob to the right. You may not be able to set 100 V exactly – just get as close as you can. Be sure to check that the power supply is set to Volts and not milliamps.

7. **The gel should run until the blue dye is in between the second and third red line on the gel bed.**

8. **When the gel is finished, turn off the power supply and disconnect the electrodes.**
1. **Wearing gloves, one person should remove the gel by gently grasping the gel bed underneath it.**

2. **Transfer only the gel to the surface of the UV light box.**

3. **Close the cover and turn on the UV light.** The safety magnet ensures that the UV light only turns on when the cover is closed.

4. **Photograph your gel according to the following steps:**

   a. Lift the safety cover up and fit the hood of the camera over the gel ensuring that the magnet in the hood lines up with the magnet in the UV light box.

   b. Turn on the light and squeeze the trigger of the camera.

   c. Turn off the UV light.

   d. Pull your picture from the camera by first pulling the thin protruding white paper tab.

   e. Grasp the Polaroid picture that is now sticking out of the camera and slowly pull it out of the cassette.

   f. Wait 30 sec and peel your picture (the gray rectangle) from the backing

   g. **Record the camera settings in your notes** for use with your classes. Start with f stop of 4.5 and shutter speed of 2 which is 1/2 sec.

5. **If you are satisfied with your photo dispose of the gel in the ‘gel disposal bin’ located on the bendh with the UV light boxes.**

6. **Rinse out and dry all components of the gel box with deionized water (white faucet at each sink).**
Understanding the DNA ladder

1 kb DNA Ladder

Description:
A number of proprietary plasmids are digested to completion with appropriate restriction enzymes to yield 10 bands suitable for use as molecular weight standards for agarose gel electrophoresis. The digested DNA includes fragments ranging from 0.5-10.0 kilobases (kb). The 3.0 kb fragment has increased intensity to serve as a reference band. The approximate mass of DNA in each of the bands is provided (assuming a 0.5 μg load) for approximating the mass of DNA in comparably intense samples of similar size.

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

Effective Size Range: 500 bp to 10 kb
Data Analysis and Discussion Questions for Lab 1

1. As you have learned shorter DNA fragments run farther in agarose gels than larger DNA fragments. The rate at which a fragment of DNA travels through a gel is related to the log of the fragment’s length in base pairs. Make a standard curve by measuring the migration distance in mm from the well for each band in the 1 kb ladder and make a list of these distances. Use the sheet of three cycle semi-log graph paper from the front table and plot the size of each fragment on the Y-axis, and the distance migrated on the X-axis. Draw a line to connect the points, either freehand or with a straight edge.

2. Is your standard curve a straight line? If not, where does it deviate from linearity? Why do you think this happens?

3. Are all the DNA fragments in the Figure of the 1 kb ladder on p. 23 visible in your gel? If all the fragments are not visible, briefly explain why this might be.

4. Measure the distance that each fragment traveled in one of your digest lanes. Use the standard curve to determine the size in kb of each of these DNA fragments. You can use NEBcutter to determine if the sizes of the fragments determined by gel electrophoresis are close to the actual fragment size. Are your estimates of fragment size close to the expected sizes?

5. Must you always run standard(s) every time you run a gel? Explain briefly.

6. Is the number of cleavage sites for HindIII in the lambda genome approximately what you would expect? Explain your answer briefly.

7. How does the methylation of DNA affect its digestion by restriction enzymes? Is this the same for all enzymes tested?

8. Assuming that there are 3.1 x 10^9 base pairs of DNA in the human genome, how many times would you expect EcoRI, which has a 6 bp recognition site, and HaeIII, which has a 4 bp cutting site to cut the human genome (assume that there is equal distribution of all the nucleotides throughout the genome)?

8. If your photograph of your gel using the recommended settings (f = 4.5 and shutter 2 sec) proved to be too light, describe briefly how you would adjust the camera setting(s) to remedy the problem. Answer the same question assuming that your photograph was too dark.
How to use the NEBcutter program

On the New England Biolabs website (www.neb.com) you will find a link to the NEBcutter program under the heading NEB Tools. NEBcutter will produce a comprehensive report of all the restriction enzymes that will cleave a DNA sequence. It can produce restriction enzyme maps, and offers a variety of displays of custom digests in which you chose the restriction enzymes. The opening webpage is shown below.

How to input DNA sequences for analysis:

- There are a few options that you can use to input the sequence you wish to analyze with NEBcutter.
  1 – use the GenBank accession number to retrieve the DNA sequence
  2 – paste the DNA sequence directly into the box
  3 – choose one of the standard plasmid, viral or phage sequences from the pull down menus.
- Remember to specify if the sequence is linear or circular
- You can give the sequence analysis a name if you will return to work on the sequence within 2 days (from the same computer).
Understanding the results page:
A linear or circular map of the DNA sequence will appear in the results page.

- On a linear map the grey box arrows that appear above the map show all the open reading frames identified in the sequence along with the restriction enzymes that could be used to isolate that specific sequence.
- The enzymes shown under the linear map are the enzymes that will cut the DNA sequence just one time. The color of the enzyme name indicates if it is available from NEB, another company or is not commercially available.

Other Features and Options
- If you would like to see a list of all enzymes that cut the DNA sequence listed in order of cut site click on “All sites” in the List menu. This list does not tell you how many times each enzyme cuts the DNA sequence.
- “ORF” summary will show in a table format all the identified open reading frames, what proteins they code for and which enzyme sites are closest to their 5’ and 3’ ends
- When you put the mouse over the name of a restriction enzyme you will see the restriction enzyme recognition sequence and the exact position of the cut.

Performing a Custom Digest:
- Click on “Custom Digest” in the Main Options menu. This allows you to select specific enzymes and perform a virtual digest of the DNA sequence.
- You can view the calculated fragment sizes by clicking on “fragments” in the results page or you can view how the DNA fragments will run on a gel by clicking on “View gel”
**Lab 1 Prep Room Notes:**

**Pipetting practice**
- Dilute food coloring (Mc Cormick) 1:10 (1ml dye & 9ml water) before use

**Preparation of Agarose Gels**
- TAE buffer (50X, 10X or 1X) can be stored at room temperature. Be sure to mix well before use as sometimes the salts can precipitate.
- 1% agarose gel is 1g agarose mixed into 100ml 1X TAE.
- To melt agarose bring mixture to a boil in either a microwave or in a boiling water bath. Watch carefully in microwave as agarose easily boils over.
- Melted agarose mixture can be held in a 55 °C waterbath.
- Agarose gels can be poured ahead of time and stored at 4°C in the gel apparatus with 1X TAE covering the gel.
- Agarose gels can be stored for longer times (with comb removed) by carefully removing the gel from the apparatus and wrapping it in plastic wrap. Store wrapped gels at 4°C for up to six months. To keep gels from drying out we use a plastic pipette to squirt a little 1XTAE onto the gels before wrapping.

**Lambda RE digest**
- If you will be preparing your own Lambda DNA it needs to be solubilized and diluted before use.
  - i. NEB cat # N3013 (Lambda DNA, N6-methyladenine free, 500ug/ml) or N3011 (Lambda DNA, 500ug/ml). The tube may contain some viscous bits of DNA, so careful inverting and pipetting may be necessary. The solubilization may take some time and effort (30 min to an hour, with occasional inverting and pipetting). With stubborn solid particles brief incubations at 37°C will help.
  - ii. Once the lambda is solubilized it can be diluted with TE 1:1, which will help with the solubility for future use. If the original stock concentration was 500 ug/ml, the 1:1 dilution will now give a concentration of 250 ug/ml = 0.25 ug/ul. This slightly diluted lambda can be stored frozen and should not require extra effort to make it soluble.
- TE (10 mM Tris, pH 8, 1 mM EDTA) Not much TE is needed for this lab so a new bottle could be subdivided (to minimize going in and out of the same bottle) and the TE will keep for years at room temperature.
- Restriction enzymes should be kept in the freezer until right before use, to preserve enzyme activity. Best to store in a stratacooler @-20°C if your freezer cycles through temperatures. Whenever thawing, aliquoting or combining components it is important to make sure the solutions are well mixed. With microfuge tubes it is best to ‘flick’ or tap the tube several times, then, if there is a microfuge available, do a quick spin to bring the droplets down. Alternatively, a hard flick could bring most of the liquid to the bottom of the tube.
• For our protocol the digest time is 15 min to ensure digestion. However, manufacturer’s instructions state 5-15 min, so if time is an issue the shorter time could be tried.
• Digests can be stored in the freezer if the gel will be run at a later time.

Running Agarose Gels
• Quick Load 1Kb Ladder; NEB cat #N0468L (50µg/ml); Our working concentration for gels stained with GelRed is 0.0125µg/µl (mix 5 µl of stock ladder, 13 µl TE, and 2 µl 10X loading dye). If you pre-aliquot the ladder for students be sure to flick and quickly spin the contents before use.
• GelRed; Biotium cat #41003. Store at room temperature in dark (cover w/foil). 10 µl GelRed/100ml melted agarose. Store any unused agarose w/gel red at 4°C in dark.
• 10X Loading dye; Purchase from vendor or make ahead of time and store; 45g Ficoll PM400, 0.75g Bromophenol Blue, up to 300ml with npwater in a 500ml sterile bottle. Stir well w/stir bar on hot plate w/no heat (This may take all day). Store at room temperature or 4°C. Mix well before aliquoting. Make sure ficoll sediment in tube is vortexed well before use.

Kit instructions:
Make up 1X TAE buffer from the provided 50X concentrate by diluting with water 1:50.

1% Agarose Gels:
- Mix 3g of agarose with 300 ml of 1X TAE buffer in a heat proof container at least twice the size
- Heat in microwave or in a water bath to melt the agarose
- Swirl to mix while still hot
- Hold at 55°C

Using GelRed in the gel to stain DNA:
- Wearing gloves and safety glasses add 30µl of GelRed to the entire flask of agarose if you will use all agarose for gels. Swirl to mix.
- If you only need to pour a few gels, add 2.5 µl of GelRed to the bottom of a 50ml orange cap tube. Gently pour 25 ml of melted 1% agarose into the tube. Cap and swirl gently to mix. Pour gel as usual.
- Gels containing GelRed can be made ahead of time and kept in the refrigeratoir wrapped in both plastic and foil.