TECHNIQUES IN MOLECULAR BIOLOGY – RESTRICTION DIGEST AND AGAROSE GEL ELECTROPHORESIS

This lab will introduce you to DNA modification by restriction enzymes using the purified plasmids you prepared from your transformation. We will also perform analysis of the purified plasmid and the digestion using horizontal gel electrophoresis.

Be certain to read the information on restriction digests (textbook readings) and agarose gels (textbook and handouts) to understand the theory of each concept and learn the details needed to perform successful experiments.

Practical notes on Restriction Endonucleases (RE) and Their Use

Enzymatic Unit definition:

1 Unit = amount of enzyme necessary to digest 1 µg DNA in 1 hr (37°C, with appropriate buffer). Ensure you are using the right buffer, correct ratio of enzyme to substrate (DNA and the right conditions to achieve complete digest).

RE reaction buffers

Each restriction enzyme has a buffer in which the highest activity is achieved, usually as a 10X concentrate. Some enzymes have common buffers while others need to be used with a unique buffer for optimal activity. Several companies (New England BioLabs as an example) have common buffers formulated to work with several enzymes. There is a link on your website - explore several companies to get a feel for buffers and enzymes.

RE storage conditions

RE's are sensitive to loss of activity by repeated exposure to higher temperatures; stocks are kept at -20°C for long-term storage, and at ~ 0°C (on ice) for short times when in use. When digests are performed, activity of some enzymes is lost after an incubation of a few hours. Since RE's are expensive, stocks must be handled with care.

Enzymes should be stored in a *non-frost-free* freezer constantly at -20°C. (*Frost-free* freezers heat up above freezing periodically to limit ice accumulation.) It is also best to keep enzymes in an insulated container in the freezer that limits temperature change when the freezer is opened (this is particularly important if stored in a *frost-free* freezer).

Glycerol in RE digests

To prevent freezing at -20°C, RE stocks are in a solution containing 50% glycerol. Since RE activity can be inhibited or altered in the presence of >5% glycerol, no more than 10% of a final RE digest reaction mix should be stock RE. [A 1:10 dilution of RE takes 50% glycerol to 5% glycerol.]

Star (*) activity of RE's

Under incorrect buffer conditions, or in the presence of >5% glycerol, RE's can display altered DNA cleavage specificity, known as "star activity." Under such conditions, the enzyme may recognize, for example, a 4 base pair subset of its normal 6 bp recognition site, and therefore will cut the DNA at many more sites than expected. For example, the familiar enzyme *Eco*RI is notorious for its star activity in low ionic strength solutions.

Setting up RE digests

Keeping with the ratio of enzyme to substrate, many researchers use a rule of thumb that 10 units of RE is enough to overcome variability in DNA quality and purity. NEB suggests that 1 µg of purified DNA in a final volume of 50 µl (of course using the appropriate buffer) is enough to cut most, or all of the DNA in one hour at xx temperature.

Don't forget to avoid the * "star" activity the glycerol should be less than 5% final concentration (V/V). Most enzymes are supplied in a 50% glycerol solution and thus the volume of enzyme added should not exceed 10% of the total reaction volume.

It is vital to ensure your mixture is mixed and all of the components are "at the bottom of the tube". The reaction must be thoroughly mixed to achieve complete digestion. Pipette the final mixture "up and down" and flick the tube followed by a very brief microcentrifuge (just a touch of a spin) to bring the now homogeneous mixture to the bottom of the tube and avoid error.

x µl DNA (total 0.8 - 1.0 µg)
(8-x) µl water (molecular biology grade)
1 µl Restriction Enzyme buffer (10X concentrate)
≤1 µl Restriction Enzyme (5-20 U/µl)
10 µl total volume

Note that much more RE is used than necessary (given the standard Unit definition) – this is to ensure complete digestion in a short period of time.

The RE is always added last, and the reaction is mixed thoroughly and then the reaction is incubated in a water bath at the appropriate temperature for 1 hr. Highly purified DNAs can be incubated for extended periods of time (up to overnight) to ensure complete digestion, although some RE's may only be active for a few hours. Long incubations are not recommended for impure DNA preparations, as this may allow time for non-specific nucleases to degrade the DNA.

Ethidium Bromide [CAUTION]

Staining with ethidium bromide (EtBr) is a rapid, sensitive, and highly reliable method for visualizing DNA in gels. The stained gel is illuminated from below ('transillumination') with short- or medium-wavelength of UV light causing the EtBr,

bound to DNA, to fluoresce brightly. As a molecule that binds DNA, however, EtBr is a mutagen and likely carcinogen. EtBr should be handled with appropriate caution, and its use restricted to limited areas. Gloves and a lab coat should always be worn when using EtBr or handling items that might be contaminated with EtBr (such as gel staining containers).

To limit the generation of waste, the EtBr staining solution can be reused repeatedly until staining in gels begins to fade. Stained gels and the first rinse from destaining will be collected as hazardous waste.

SYBR Safe DNA Stain [Safe Alternative]

SYBR Safe is a cyan based, non-mutagenic dye (no observed in an acute oral toxicity study in rats) form of SYBR Green dye. The dye absorbs in the blue range, fluoresces only when complexes with DNA and then emits in the green (lmax 520 nm). The dye is purchased as a highly concentrated stock (often 10,000X). Add to cooling agarose gel before pouring into a casting stand.

Migration of DNA Molecules in Agarose Gel Electrophoresis

DNA molecules migrate through the gel matrix at a rate that is inversely proportional to the $\log_{10}$ of their molecular weight (for convenience, expressed in base pairs). Larger molecules move more slowly because of greater frictional drag and because they worm their way through the pores in the gel less efficiently than small molecules (p. 6.4, Sambrook *et al.*, 1989).

Note that the effective range of separation decreases as the voltage is increased. The best resolution of fragments of DNA >2 kb is obtained when the electric field is no greater than 5-8 V/cm.

DNA molecules migrate at different rates, depending on the concentration of agarose in the gel. Higher percentage gels are better for resolving small fragments; lower percentage gels are better for resolving large fragments.

Agarose concentration in gel (%, w/v)	Efficient range of separation of linear DNA molecules (kb)
0.3	5 - 60
0.5	1 - 20
0.7	0.8 - 10
0.9	0.5 - 7
1.2	0.4 - 6
1.5	0.2 - 3
2.0	0.1 - 2

[from p. 6.5, Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.]

Gel loading buffer (6X GLB)

The dyes **xylene cyanol FF** and **bromophenol blue** plus **30% glycerol** in water are present in the "6X GLB" provided. The glycerol makes the final solutions dense

so they sink to the bottom of the wells. The 'tracking dyes' are used to follow the progress of the electrophoresis. Under the conditions we use, BB migrates at about the same rate as a linear double-stranded **400 bp** DNA fragment whereas XC migrates at about the same rate as a **8000 bp** DNA fragment. See table below for the approximate migration of these dyes in other concentrations of agarose gels (using 1X TBE running buffer).

% agarose	xylene cyanol FF	bromophenol blue
0.5	20-40 kb	4,000 bp
0.8	8,000 bp	400 bp
1.0	4,000 bp	300 bp
1.3	1,800 bp	150 bp
1.5	1,200 bp	100 bp
2.0	700 bp	65 bp

[Source: New England Biolabs (http://circuit.neb.com/neb/products/nucleic/N3272.html)]

Restriction Digest of DNA, Analysis by Agarose Gel Electrophoresis

Reagents	**Supplies & Equipment**
0.2 µg/µl l DNA	Electrophoresis box with
Restriction Enzymes:	gel tray, rubber dams,
*example: Eco*RI, *Bam*HI, *Hin*dIII (TBA)	8 well comb, spacer
10x restriction buffers	gel box lid with leads
Water (molecular grade)	Power supply
10 or 6 X gel loading buffer (GLB)	37°C & 70°C water baths
Agarose (analytical grade)	
1x TBE buffer	
ethidium bromide stain/SYBR Safe Stain 10,000X	

Part I. Set Up Restriction Digests

1. Mark the tops of five 1.5 ml tubes indicating the reactions, such as:
1 = *Enzyme 1*, 2 = *Enzyme 2,* 3 = *Enzyme 1 & 2,* 1 = *no enzyme*

2. Use the matrix below as a checklist while adding reagents to each reaction.

As you add reagents, touch the tip to the side of the tube, at or very near the bottom of the tube. Except for the first pipetting into each tube, always use a fresh tip for each reagent and pipetting.

Always add the RE last.

Tube	I DNA*	10X buffer	Water	RE
1	4 µl	1 µl plasmid	4 µl	1 µl I
2	4 µl	1 µl plasmid	4 µl	1 µl
3	4 µl	1 µl plasmid	4 µl	1 µl
4	4 µl	1 µl plasmid	3 µl	1 µl *both enzymes*
0	4 µl	1 µl plasmid	**5** µl	**-**

* Using your purified plasmid DNA, adjust the DNA concentration to 0.2 µg/µl; therefore total mass = (0.2 µg/µl) x 4 µl = 0.8 µg. ***Show ALL math in lab notebook***.

3. After adding all reagents, close the lids and spin down the reagents in a microcentrifuge, briefly pulse the

4. Put the tubes in a floating tube holder, place in a 37°C water bath. Incubate the reactions for a minimum of 40 min, up to 60 min or longer. (For this reaction, longer is better.)

Part II. Casting 0.8% Agarose gel

1. Preparing the gel tray

a. Make sure your gel tray, rubber dams and comb are clean. If not, rinse with deionized water. Remove any dried-on agarose with a moist Kimwipe.

b. Using the appropriate tools (rubber damns or tape) carefully seal the gel tray.

c. Rest the comb holder with 8 well comb down into the end slot of the gel tray. (There are two possible orientations of the comb. Place it in the configuration where the teeth of the comb are **further away** from the rubber dam. Face the comb into the gel tray to ensure enough space between the edge of the gel and the wells.

d. To ensure the proper spacing between the bottom of the tray and the comb, loosen the comb and place one of the white comb height spacers on the bottom of the tray and rest the comb on the thick part of the spacer. Then, holding the comb firmly in place, tighten the comb-holding screw (the comb can become skewed during this process if you don't hold on to it).

e. Place the gel box and tray away from the edge of the lab bench where it can be easily bumped. When the gel is poured, it should not be disturbed while it is setting.

2. Preparing the agarose for casting the gel

a. Calculate the mass of agarose to make 50 ml of a 0.8% agarose in TBE buffer.

The percentage gel is measured as a mass to mass ratio; in the case of water or buffer, we can use mass to volume, where 1 ml = 1 g. Thus a 1% agarose gel would contain 1 g of agarose per 100 ml of total gel volume.

Examples: 2% agarose gel with volume of gel 60 ml (approx mass = 60 g)
2% of 60 g = 0.02 x 60 g = 1.2 g -- Add 1.2 g agarose to 60 ml gel buffer.
The total volume of gel we will use today is 30 ml. So, for a 0.8% gel - -

b. Add the correct volume ml of 1X TBE gel running buffer to the flask and swirl. Calculate the weight of the combined flask, TBE and gel. Record in your notebook. Follow the general instructions from the Agarose Gel Handout to prepare the Gel.

SAFTEY NOTE: BE CAREFUL when microwaving, use short 1-2 min times to avoid over heating.

NOTE: The gel is molten when a small amount of boiling has occurred AND the gel is clear without any undissolved agarose remaining.

- IF adding SYBR Safe DNA, add the dye to the cooled molten agar just prior to poring the gel.

c. Once the gel is finished, determine the mass and make up the difference with WATER NOT TBE - mostly water will have evaporated or boiled off in the process leaving the Tris and other compounds behind. Bringing the mass back to the initial weight with TBE will result in a higher concentration of running buffer than necessary and errors with the gel.

3. Pour the gel into the sealed gel tray. Once the gel is set (it will become somewhat opaque), add a little 1X TBE to the top of the gel on the comb to lubricate it. Gently pull the comb straight out with no back and forth. Remove the rubber dams (starting at the center, not the ends) and place the gel tray in the gel box, with the wells on the – (negative/black) end. Immediately fill the gel box with 1X TBE to cover the gel completely so that the tops of the wells are under the fluid. (Never let a gel sit with dry wells since they will deform quickly.)

III. Load Gel and Electrophorese

1. Before loading, be sure your gel box is situated close enough to plug into one of the power supplies. You should not move the gel after it has been loaded. Once the DNA has been run into the gel for a few minutes, the apparatus can be moved if necessary.

2. Add **2 µl** of gel loading buffer/dye (labeled 6X GLB) and mix with the reaction.
3. Leave the outermost wells empty. Load in the following order (left to right):

Tube1 Enz1	*Tube 2 Enz2*	Tube 3 Enzy 1&2	Tube 4 Uncut	*Molecular Ladder*	Optional

4. Load the full 12 µl of each reaction + GLB into the well of the gel.

5. Run the gel at 125 V (constant voltage) until the leading dye has reached 2/3 – 3/4 the length of the gel.

Note when you are done that the 1X TBE running buffer is reusable. Return buffer in the gel box to the 1X TBE container. (Such as, while you are staining your gel in the next section.)

OPTIONAL - IV. Stain Gel with Ethidium Bromide and View. **WEAR GLOVES.**

1. Slide your gel from the gel tray to a small plastic container in the hood. Add enough ethidium bromide (EtBr) staining solution to cover the gel. Close the container and allow your gel to stain for 10-15 min in the hood. *Gentle* agitation of the gel can improve staining (and destaining) but is not essential. Use a lab rotator at a very slow speed.

2. Pour the EtBr stain back into the stock container, holding your gel with a plastic spatula. Add deionized water to cover the gel and allow the gel to destain for 5-10 min in the hood.

3. Pour the wash into the EtBr waste container in the hood.

4. Take your gel to photodocumentation system in the equipment room next door and get a good image. (Using the system will be demonstrated. There are also detailed instructions there.) Print out a copy (mainly as a backup), and save a copy of the image (exported to TIFF) to the computer, then to your own flashdrive or email to yourself. Once you have a good image, dispose of your gel in the EtBr waste container in the hood.

By convention, gels are displayed with the wells at the top and are read left to right. The region extending down from a well in a vertical column is called a **lane**. (If lanes are numbered, the leftmost lane will be '1'.)

During lab, we will discuss the lab write-up and how to solve your unknown (and you will receive a detailed written set of instructions for this task).

NOTEBOOK: Data Analysis necessary for writing the notebook discussion section.
Plot a molecular weight standard line of the standards vs the Rf of each band.

Estimating the size of DNA molecules from the gel photograph
Need: gel photo (or enlargement), mm ruler, semi-log graphing paper

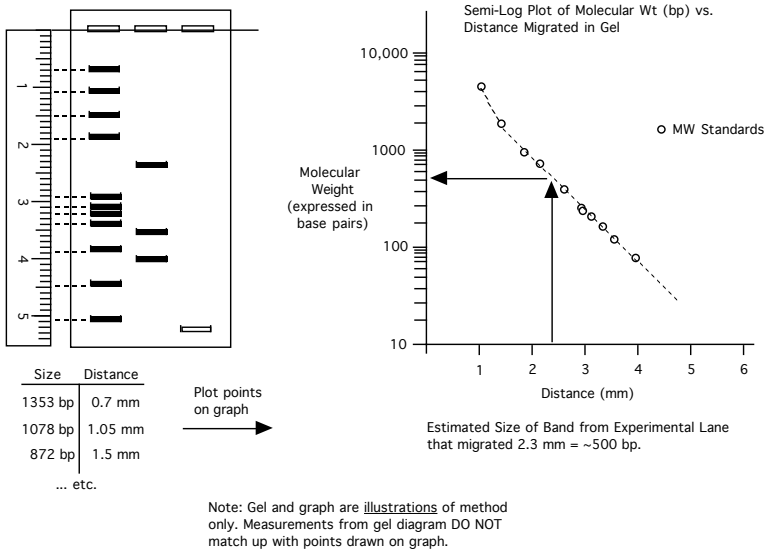
1) Because the standard gel photo printout is small, you should enlarge your saved digital image and print out one that is roughly an 8.5" x 11" sheet size (full page). Although it is possible to make a good graph without enlarging your gel, your resolution is likely to suffer. Be sure that you can still see all the bands in your printout, especially the smallest, faintest bands. You can adjust the brightness/contrast of the image as needed to bring out bands.

Note that to save ink on your printer, you can also invert the image to a white background with black bands using an appropriate image software (or doing it at the beginning on the 'Gel-Doc').

2) Measure the distance from the origin (the well) of the bands of your known intact and plasmid digests and plot their molecular weights on the log scale vs. distance migrated on the linear scale. (See the figure below.) Plot on semi-log graph paper rather than making a computer-generated plot. For this plot label the bottom line on the y-axis as 100 bp, the next '1' as 1000 bp, then 10,000 bp. The top line will be 100,000 or 10^5 bp. This is because it is much easier to estimate the size of unknown bands using the graph paper with its densely-packed series of lines in both axes. Be sure to be consistent in where you choose to measure the bands - it is probably best to use the leading edge of the band. Then, use a ruler to draw lines between the points, or draw a "best-fit" line among the points. You should get a straight line through most of the range of molecular weights, although the line will curve upward in the higher molecular weight range where bands are compressed into a smaller region of the gel. See the examples provided below.

3) Measure the distance migrated by bands in each lane. Use your molecular weight standard curve to estimate sizes of the fragments. Go back to your gel photograph and make sure the sizes make sense. The best-fit line of scattered points can sometimes lead to inaccuracies - compare the distance migrated by fragments of known size in adjacent lanes. For example, if an unknown fragment has migrated farther than a fragment of known size in another lane, the unknown fragment *must* be smaller. If an unknown fragment is exactly parallel to a known fragment, then simply assign it the approximate size of the known

Figure: How to plot your standards, and estimate your unknown fragment sizes



[This cartoon is more relevant to the 'old days' when everyone looked at gels using an exposed UV transilluminator. One had to wear eye protection (or full face shield as in the cartoon above), and had to take care not to get a 'sunburn' from looking at one's gel for too long or too close.]