

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. You will perform a gel analysis of your purified plasmid before and after cutting with restriction enzymes. You will perform a mock (control) digest of the plasmid, a single digest with two different restriction enzymes and a double digest. From this you should be able to determine both the purity of your Qiagen mini-prepared plasmid DNA but also the wgMDH insert in the pQE60 vector.

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. REQUIRED VIDEOS – Restriction Enzyme links on our web page prepared by New England BioLabs. The minimum videos required to watch are (Digestion with Restriction Enzymes, Standard Protocol for Restriction Enzyme Digests, and NEB Restriction Enzyme Double Digest Protocol videos). There are several others in this series that you should look at before doing the experiments. <https://www.neb.com/applications/cloning-and-synthetic-biology/dna-preparation/restriction-enzyme-digestion>

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 *EcoRI* 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.

STANDARD 10 µl RESTRICTION DIGEST (SINGLE) SET-UP

x µl DNA (total ~ 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.
- For a Double Digestion, use a second Enzyme and compensate with a smaller volume of water. Ensure the final volume remains at 10 µl.
- 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.

I. Restriction Digest of DNA AND Analysis by Agarose Gel Electrophoresis

Part I. Plan your experiment: Use the information at the end of this handout to predict the size of the plasmid and insert before and after each digestion. You will prepare an undigested (mock control), single for each restriction enzyme (*NcoI* and *BglII*) AND a double digest. Prepare a table of your digest with each addition and volume. Ensure that when all is finished the final volume of EACH reaction/control is 10 µl. From the NEB website – review which enzyme should be used and include your conclusions and source in your ELN.

1. Set Up Restriction Digests

- Tables and all indications should also be recorded in your ELN PRIOR to starting the experiment!
- 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.

2. After adding all reagents, close the lids and spin down the reagents in a microcentrifuge, briefly pulse the tube and spin the sample to the bottom of the tube.

3. 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

- 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.
- Using the appropriate tools (rubber dams or tape) carefully seal the gel tray.
- Rest the comb holder with TWO (2) that is TWO 8 well combs 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.
- 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).
- 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

- 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 = $0.02 \times 60 \text{ g} = 1.2 \text{ g}$ -- 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 - -

- 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.

SAFETY 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.

- SYBR Safe DNA: Add the dye to the cooled molten agar just prior to pouring the gel.

- 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.

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 Samples.

You will prepare samples from BOTH the plasmid preparation AND your restriction digest. Use all samples from the Qiagen mini-spin prep and only the purified plasmid from the alkali lysis method. Run all samples (Mock, single and double digest) for the RE experiment. This can get complicated. Plan ahead and talk with your laboratory partners to avoid confusion and starting ALL OVER!

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. For all samples (plasmid purification AND RE DIGEST). Add **2 μ l** of gel loading buffer/dye and mix with EACH reaction. For plasmid purification samples (lysis, the washes and elution) transfer 10 μ l of DNA sample to a clean and labeled tube. To THIS sample add gel loading buffer/dye.
3. Loading the gel. Ensure ALL information in table form is entered into the ELN PRIOR to loading the gel.
 - a. The top row should be used for your purification. Lane 1 will include 15 μ l of DNA ladder. Use your judgement which samples should be loaded from left to right. Consider starting with the most impure sample and in a sequential order ending with the eluted PURE Sample. For the alkaline lysis purification JUST USE THE FINAL plasmid sample.
 - b. The middle row will be for your RE digest. Molecular ladder should go first, then uncut (mock sample) followed by the single RE digests (there are two different singles... don't overload in same well) and the double digestion.
4. Load the full 12 μ l of each reaction/mixture + loading buffer 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.
6. 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.

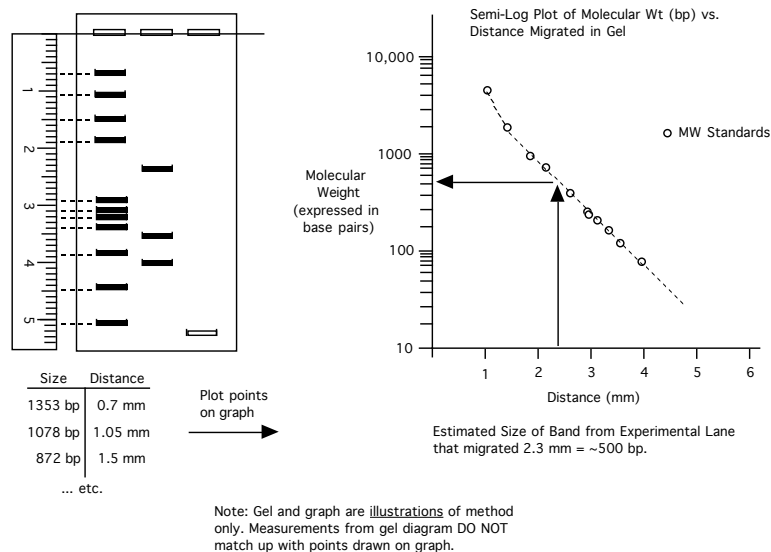
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By convention, gels are displayed with the wells at the top and are read left to right. The region extending down from a well in a vertical column is called a **lane**. (If lanes are numbered, the leftmost lane will be '1'.)

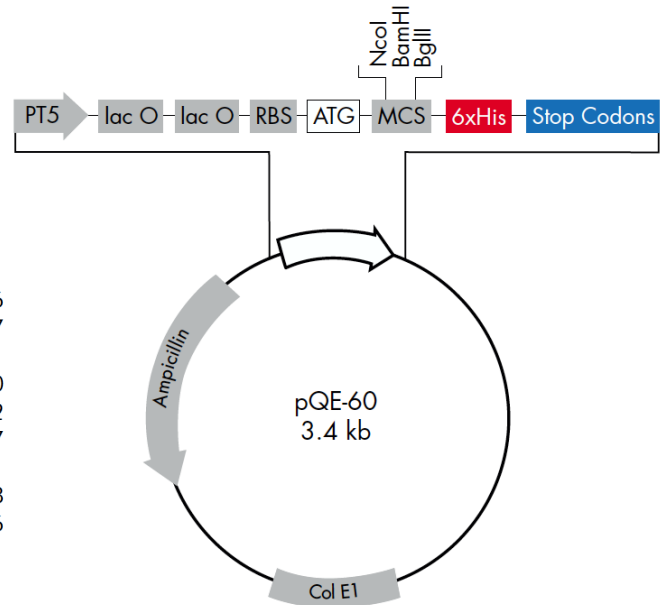
During lab, we will discuss the lab write-up and how to solve your unknown (and you will receive a detailed written set of instructions for this task).

7. Measure the distance migrated by bands in each lane. Either use a graphical interface following the instruction on the BioRad Imager (MAKE SURE YOU APPLY THE RIGHT Molecular Mass to the Standard), or 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



Watermelon Glyoxysomal Malate Dehydrogenase (wgMDH)

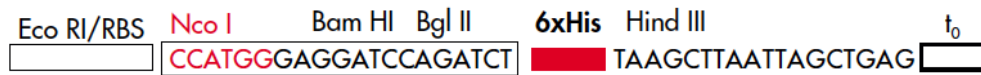


pQE-60 Vector

Positions of elements in bases

Vector size (bp)	3431
Start of numbering at XhoI (CTCGAG)	1–6
T5 promoter/lac operator element	7–87
T5 transcription start	61
6xHis-tag coding sequence	133–150
Multiple cloning site	113–132
Lambda t_0 transcriptional termination region	173–267
<i>rrnB</i> T1 transcriptional termination region	1033–1131
ColE1 origin of replication	1608
β -lactamase coding sequence	3226–2366

pQE-60



GI: 60593492. Cut into pQE-60 with *NcoI* and *BglII*
Biochimicaet BiophysicaActa 1274(1996)48-58

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1 rakggapgfk vailgaaggi gqplamlmkm nplvsvlhly dvvnapgvta dishmdtgav
61 vrgflgqqql eaaltgmdli ivpagvprkp gmtrddlfki nagivktlce giakccprai
121 vnlisnpvns tvpiaaevfk kagtydpkrl lgvtmldvvr antfvaevlg ldprdvdpv
181 vgghagvtil pllsqvkpps sftqeeisyl tdriqnggte vveakagags atlsmayaav
241 kfadaclrgl rgdagvieca fvssqvtelp ffaskvrlgr ngieevyslg plneyerigl
301 ekakkelags iekgvsfirs hhhhhh
    
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