

APh 162 – Molecular Biology

Day 2

Restriction Digests

The *LacZ* gene on our plasmid is flanked by restriction sites for the enzyme 'KpnI' on one side and 'HindIII' on the other, allowing us to cut the gene out and leave precisely controlled 'sticky-ends' on the plasmid DNA, onto which we will ligate our new reporter gene (or 'insert'), called -*Venus(YFP)*. The enzyme KpnI cuts at any site with the sequence...5-GGTAC'C-3... leaving the sticky-end GTAC, while the enzyme HindIII cuts at any site with the sequence...5-A'AGCTT-3... leaving the sticky-end AGCT. The fact that these sticky ends are distinct means we can discourage the plasmid from ligating on itself, and ensure that we insert the new reporter gene in the correct orientation. A myriad of available restriction enzymes ensures that we can control each piece of the plasmid independently. In today's lab session, we will digest pZE21-lacZ and the YFP (Venus) PCR amplicon from day 1.

Restriction enzyme concentrations are measured in the units of 'activity', where 1 unit of enzyme can digest 1 ug of DNA in 1 hour at 37C. To ensure that all of the DNA is cut by the enzyme, it is generally recommended to add enzyme in excess of the required amount.

Follow the protocol below, and mix the following reagents to begin this enzymatic reaction. Once the reaction is finished, we will examine the products on an agarose gel to determine their size and differences in topology. To properly gauge the size of DNA segments that appear on the agarose gel, we need to add a so-called 'DNA Ladder' that contains segments of known length, against which we will compare our plasmid digest. We will use two commercially available ladders, and we will create our own 'DNA Ladder' using the distinct enzyme EcoRI to digest λ -phage (viral) genomic DNA that has been pre-digested with HindIII.

Today you will perform the following restriction digests:

1. **KpnI/HindIII double digest of the purified, PCR-generated YFP (Venus) insert from Day 1.**
2. **KpnI single digest of vector pZE21-LacZ**
3. **HindIII single digest of vector pZE21-LacZ**
4. **KpnI/HindIII double digest of vector pZE21-lacZ**
5. **EcoRI single digest of pre-HindIII digested lambda phage DNA**

Following the digests, you will run some of the samples on an agarose gel for visualization, and save the others for the ligation on Day 3.

Double Digest of YFP (Venus) insert - Total Volume: 50ul

Reagent:	Amount:
YFP DNA (purified, from Day 1 PCR)	30 μl
NEB Buffer 2 (10X)(NEB-B7002S)	1X
BSA (10X)	1X
KpnI (10 units/ul)	2ul
HindIII (10 units/ul)	2ul
DDH₂O	Calc.

Double Digest of pZE21-lacZ vector - Total Volume: 30ul

Reagent:	Amount:
Plasmid (pZE21_LacZ)DNA	300ng
NEB Buffer 2 (10X)	1X
BSA (10X)	1X
KpnI (10 units/ul) *	1 ul
HindIII (10 units/ul) *	1 ul
DDH ₂ O	Calc.

***Omit either KpnI or HindIII in the corresponding single digests, or omit both enzymes in the negative control.**

λ - phage Digest - Total Volume: 30ul

Reagent:	Amount:
Digested λ DNA 0.5 ug/ul	1.5 ug
NEB EcoRI Buffer (10X)	1X
BSA (10X)	1X
EcoRI (10 units/ul)	2 ul
DDH ₂ O	Calc.

Notes:

NEB Buffer 2 is one of many potential buffers, chosen to optimize the performance of both KpnI and HindIII.

See "Enzyme Properties" at:

<http://www.neb.com/nebecomm/products/productR0104.asp>

<http://www.neb.com/nebecomm/products/productR0142.asp>

In many of the protocols to come, **Reagents** will be listed as 2X or 5X or 10X, etc, meaning that (unless otherwise directed) they should be added to relevant solutions such that the *final* concentration of the reagent is 1X.

Procedure:

1. Verify all calculated reagent volumes with your TA.
2. For the given concentration of input DNA, calculate the corresponding volume.
3. Mix this volume of DNA along with the specified amounts (see tables above) of buffer, restriction enzymes and water to bring the total volume to 25ul, in a small tube. When finished, ask your TAs to add the appropriate amounts of restriction enzyme
4. Mix thoroughly and spin in the micro-centrifuge for a few seconds (<6 sec) to sediment all the contents.
5. In addition to using commercial ladders, we will create our own ladder using lambda phage DNA.
 - a. **The λ -phage genome is 48,502bp, if the sequence was totally random, how many HindIII(5A'AGCTT3) and EcoRI(5G'AATTC3) sites would you expect? How many are there in reality?**
 - i. **Given the actual restriction sites for EcoRI and HindIII, calculate the expected segment lengths of the DNA ladder.**
 - ii. **See the attached restriction site map for the λ -phage genome.**

- Place all six of your tubes in a 37C incubator for 1 hour. During this incubation, we will prepare the agarose gel.

DNA Agarose Gel Electrophoresis

The above enzymatic reactions and accompanying controls have produced a range of DNA lengths and the no-enzyme (uncut DNA) control even has a distinct polymer topology. It is immensely useful to be able to measure the lengths produced during DNA manipulations. The primary method employed throughout molecular biology is to subject DNA in an ultra-viscous agarose gel to an electric field, wherein we utilize the $\sim -5.88e/nm$ charge on DNA to 'pull' it through the gel. In a given period of time the DNA migrates through the gel roughly proportional to the inverse of its mass.

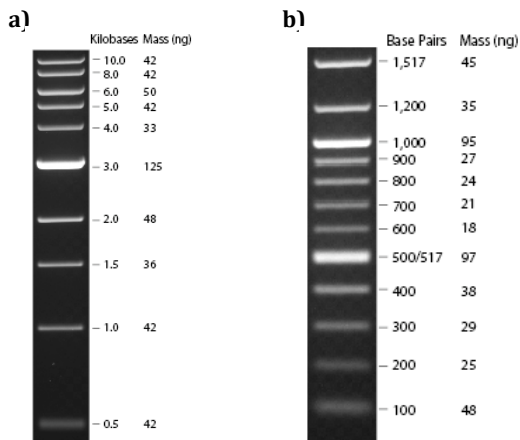
Required Materials

1X TAE Buffer	~500 ml
1X TAE- 1% agarose gel	~ 35 ml
100bp DNA Ladder (NEB-N0467S)	2ul
1000bp DNA Ladder (NEB-N3232)	2 ul
6X gel loading dye	1X

Note: TAE (Tris-acetate EDTA) buffer

Additional materials:

gel box gel power supply
gel tray gel comb



DNA Ladders

a) Example of 1 kb DNA ladder on a 0.8% agarose gel, stained with EtBr.

b) Example of the 1000bp DNA ladder on a 1.3% agarose gel, stained with EtBr.

You will run the following samples on the gel:

- 100 bp DNA ladder
- Non-purified, non-digested PCR product from day 1
- Lambda phage double digest
- pZE21-LacZ – KpnI single digest
- pZE21-LacZ – KpnI single digest
- pZE21-LacZ – KpnI/HindIII double digest
- pZE21-LacZ – no enzyme control digest
- 1 kb DNA ladder

Procedure:

1. Using DI water, clean the gel box, gel comb and gel tray.
2. In short bursts of ~10s, heat the 1X TAE- 1% agarose gel in the microwave until it is *completely* liquefied.
3. Secure the gel comb in the gel tray and orient so that the red rubber seals form a liquid-tight dish. Pour ~35ml of the liquid agarose into the gel tray. Be careful not to introduce any bubbles.
4. Allow the gel to solidify over the next 30 minutes, without disturbing it.
5. Once fully solidified, carefully remove the gel comb by pulling it straight up, taking care not to damage the wells. Carefully remove the gel tray (with the gel) and rotate it 90 degrees so that the wells are closer to the negative terminal.
6. Gently pour 1X TAE buffer until the reservoirs on both sides of the gel are filled and a thin layer (~1mm) of buffer is covering the gel. The wells / holes left by the gel comb should now be filled with 1X TAE buffer.
7. Set the gel power supply to 110 VDC and 65 minutes, but do not start it yet.
8. To 30 μ l of the appropriate products from the previous sections, add 6ul of 6X loading dye (now total volume 36ul) and mix gently with the pipette tip. The loading dye permits visualization of the lanes as the gel runs. Additionally, the added glycerol will sediment the DNA to the bottom of the lane.
 - a. Be sure to record in which lane you put which sample! The ladders (see step 9b, should be loaded in the far left and far right lanes.
9. With a steady hand, place 30ul(or as much as you can fit without overflowing the well) of each digest sample in each lane – remember to record the lane number of each sample.
 - a. Mix 1 ul of the 100 bp DNA ladder with 1 ul of 6X loading dye, add 4 ul of DDH₂O, and load this onto the gel. Repeat this for the 1kb ladder.
 - b. Later you will use the ladder lanes as a calibration for the total amount of DNA in each lane. What are some sources of error in this calibration?
10. Secure the gel box lid and start the power supply—you should immediately see bubbles form around the electrodes (as a result of electrolysis). Make sure the gel is running in a flat, stable orientation.
11. Within a few minutes you should see the loading dye moving in the gel.

Visualizing DNA in the Gel

1. The gel power supply will automatically shut-off after the 65 minute cycle ends. Your TAs will image the gels for you and email you the results.
2. Recall that the gel contains the dangerous chemical EtBr. This compound uniformly intercalates in the DNA and becomes fluorescent upon binding. Hence, the EtBr fluorescence intensity is proportional to the amount of DNA present in each band.
 - a. **Knowing the concentration of DNA in either the 100bp or 1kb ladder is 500 ng/ μ l, you can integrate the fluorescence in each band to determine the initial concentration of each piece.**
 - b. **Likewise, using MatLab and the ladder of your choosing, calibrate the distances in your gel, and verify that you produced the correct DNA lengths.**
 - i. **How are migration distance and DNA length/mass related?**
 - ii. **Are you missing any bands? If so, where might they be?**
 - iii. **Did the uncut plasmid control and each of the single digest controls migrate the same distance? If so, why? If not, why not?**