Day 5: April 12, 2005

Restriction enzyme digests, agarose gel electrophoresis and restriction mapping

Today you will do restriction enzyme digests of your recombinant DNA and visualize them by agarose gel electrophoresis.

You will do restriction digests on one of the minipreps from the white colonies and one of the minipreps from the blue colonies.

You will digest your plasmid DNA with the restriction endonucleases EcoRI, ApaI, SacI, and XhoI *. Restriction enzymes have optimal buffers and temperatures for digestion. Information about restriction sites can always be found in the product literature or catalogs. All 4 of these restriction enzymes will digest at 37˚ C, but you will have to use specific buffers for each. If you are setting up many restriction digests with the same enzyme you can make a master mix. Practice sterile molecular biology techniques when pipetting and do not contaminate the enzyme stocks.

5µL miniprep DNA
2 µL restriction enzyme buffer
1 µL restriction enzyme
12 µL sterile water

Total volume 20 µL

Incubate at 37˚ C for 1 hour

* Note: restriction enzymes are heat labile and stocks should be kept in the freezer (-20˚ C) except for a brief period on ice just before you are ready to add them to your digest. Restriction enzymes are stored in glycerol so they will not be frozen, and they do not need to be thawed. Put the pipette tip just into the enzyme and pipette slowly. You want to avoid getting excess enzyme on the outside of the pipette tip and adding too much enzyme to your digest.

During incubation you should prepare your agarose gel for electrophoresis.
**Agarose gel electrophoresis:**

Electrophoresis can be used to separate nucleic acids by size. Since DNA is negatively charged it will move towards the positive electrode (run to red). Gels for electrophoresis can be made of polyacrylamide or of agarose. Agarose (a highly refined form of agar) is most commonly used. The percentage gel (w/v) will vary depending on the size of the molecules that you are trying to resolve (See table below). Today we will pour a 1% agarose gel.

**Table 2.1 (from Short Protocols in Molecular Biology): Agarose concentrations for separating DNA fragments of various sizes**

<table>
<thead>
<tr>
<th>Agarose (%)</th>
<th>Range of resolution of linear DNA fragments (kb)</th>
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</thead>
<tbody>
<tr>
<td>0.5</td>
<td>30 to 1</td>
</tr>
<tr>
<td>0.7</td>
<td>12 to 0.8</td>
</tr>
<tr>
<td>1.0</td>
<td>10 to 0.5</td>
</tr>
<tr>
<td>1.2</td>
<td>7 to 0.4</td>
</tr>
<tr>
<td>1.5</td>
<td>3 to 0.2</td>
</tr>
</tbody>
</table>

**Pouring an agarose gel:**

1. Tape the ends of a gel mold or place in casting chamber. Choose appropriate combs (you will need 12 wells- 8 digested samples, 2 undigested minipreps, and 2 DNA ladders/ molecular weight markers) and put in place.
2. Make a 1% (1 g in 100 ml) agarose gel (TA will provide you with the size of the gel and buffer volume for individual electrophoresis apparatus) in 1X Tris-acetate-EDTA (TAE). The lab stock of TAE provided is usually 50X.
3. Heat in microwave on high until all agarose is melted. Watch to make sure it does not boil over (large size flask will minimize this risk). Use a flask with a foam plug.
4. Allow it to cool (~ 60°C; cool to touch but not enough that agarose is polymerizing). You may run it under cool water or let sit and cool.
5. Some labs add ethidium bromide (EtBr) to the gel (final concentration 0.2 to 0.5 µg/ml) when casting. We will stain the gel after it has run. **EtBr is a mutagen.** **Always wear gloves.** Assume all gel boxes, combs, etc are contaminated and do not touch them without gloves.
6. Pour the gel into the mold. Let it solidify (will turn cloudy/ white).
7. Pull off tape and carefully remove combs.
8. Running buffer is 1X TAE. Pour into gel box so that it just covers the gel (to fill line).
9. When current is applied, DNA, which is negatively charged in solution, will migrate toward the anode (+). Remember run to red. If you have your gel in the box in the wrong direction, the DNA will run off the top of the gel. When moving through the agarose, small fragments of DNA will move faster (farther) than large fragments.
Agarose Gel electrophoresis of restriction digested miniprep DNA:

1. Take your entire restriction enzyme digest (20 µl) and add 4 µL of 6X loading dye. The loading dye has glycerol, which helps the sample fall to the bottom of the well and a dye that helps you visualize the migration of the DNA.
   - Bromophenol blue migrates at the same rate as DS DNA 300 bp in size.
   - Xylene cyanol migrates at the same rate as DS DNA 4000 bp in size.

2. Load your samples in the wells. Pipette slowly so that DNA falls down into the wells.

3. Also load 5 µl of your undigested miniprep (for each of the minipreps that you digested). Typically uncut plasmids will appear to migrate more rapidly than the same plasmid when linearized.

4. Load 10 µl of the Fisher 100 bp PCR ladder and load 10 µl of the Takara 1.0 kb ladder.

5. Run the gel at ~90 volts. A general rule of thumb is to have your electric field between 1 and 5 V/cm. This cm value is the distance between the two electrodes rather than the distance of the gel.

6. Stop the gel when the smallest dye band has migrated approximately ¾ of the length of the gel.

7. DNA fragments are visualized by staining with EtBr. EtBr is a fluorescent dye that intercalates between bases of DNA and RNA. We will soak the gel in a solution of 1X TAE containing 0.5 µg/ml final concentration EtBr (stock is 10mg/mL) for 15 min.

8. Gels will be visualized using a UV transilluminator and photographed using a gel documentation system (Syngene). Everyone in the group will need a picture of the gel for their notebook.

Calculating the molecular weight of a DNA fragment separated by agarose gel electrophoresis and restriction enzyme mapping:

The log of a DNA molecules molecular weight is proportional to the distance that the molecule has migrated. To determine the size of unknown DNA fragments you must create a standard curve using fragments of known size from the molecular weight markers (DNA ladders). Using your gel photograph, measure the distance that your unknowns and standards have migrated. Make a plot of the log MW versus relative mobility for your MW standards (distance they have traveled from the well). Use this to determine the size of your restriction digest fragments and make a restriction map of your recombinant plasmid. On the course website you can find the sequence of cDNA#2 and cDNA#4. Using this sequence, map the restriction sites for EcoRI, ApaI, SacI, and XhoI within the cDNA. This will allow you to confirm the restriction map generated above and will also allow you to determine the orientation of the insert within the plasmid. You can use any restriction enzyme mapping program available on the web (see website suggestions in additional resources list on the class website). Make sure you cite in your lab write up what website you used.
A nice introduction to restriction mapping can be found at:
http://opbs.okstate.edu/~melcher/MG/MGW1/MG11231.html

http://arbl.cvmbs.colostate.edu/hbooks/genetics/biotech/enzymes/maps.html

**Determining the identity of your insert:**

Determining the sequence of an unknown piece of DNA can allow you to determine its putative identity and function. I have provided you with the sequence for unknown cDNAs #2 and #4.

**For your lab writeup:** determine the putative identity of your cDNA clone. Use the BLAST search program to search the nucleotide database at GenBank (http://www.ncbi.nih.gov/Genbank/). Report the top 5 hits from this search.

**Questions to be addressed in your lab writeup:**

1). What is the difference between shrimp alkaline phosphatase (SAP) and calf intestinal alkaline phosphatase (CIAP)?

2). What are the controls for the ligation experiment?

3). What is the difference between selection and screening. What is the selection and screening in pGEM-7Zf(+).

4). What type of overhang is created by digestion with EcoRI? Apal? SacI? And XhoI?

5). What are the different sized DNA bands you see when you run the uncut plasmid DNA on an agarose gel?