INTRODUCTION

Biological macromolecules that have been isolated and purified from a heterogeneous mixture must be characterized to assess the quality and effectiveness of the purification scheme and to verify the identity of the purified material. A widely used approach for characterizing purified DNA is to perform restriction mapping. Restriction mapping is similar to solving a jigsaw puzzle. The “pieces of the puzzle” are the lengths of the restriction digests. When arranged properly, the experimentally determined digests match the theoretical fragment lengths so that the identity of the DNA can be confirmed. Significant deviation from the anticipated fragment lengths would dispute the DNA identity confirmation.

Digestion. Restriction endonucleases are enzymes that cut DNA at specific sites within the DNA sequence. While each restriction enzyme has a characteristic primary recognition sequence at which it preferentially cuts, most restriction enzymes are capable of cutting at secondary sites as well. Useful sources of information on restriction enzymes can be found on the web sites of commercial producers such as New England Biolabs and Invitrogen. Once DNA has been cleaved with various restriction enzymes, the sizes of the resulting fragments need to be determined. Typically fragment size determination is performed by agarose gel electrophoresis.

Electrophoresis. Nucleic acids can be separated on the basis of size from ~100 base pairs to ~200,000 base pairs on agarose gels. Samples are prepared by mixing them with a tracking dye that the biochemist uses to assess the progress of the electrophoresis. Samples are then loaded onto a gel that is submerged in a buffered solution. The gel is positioned between electrodes and a power supply is attached to the gel apparatus to supply the necessary voltage for the migration. Smaller fragments of DNA will migrate farther through the gel than larger ones. A molecular weight marker is generally run for each gel for subsequent estimation of DNA fragment size.

Agarose gels of nucleic acids are typically stained with Ethidium Bromide (EtBr). EtBr is a known mutagen and a potential carcinogen, therefore, care must be taken when handling it. EtBr is a fluorescent dye that intercalates into DNA between base pairs. The fluorescence of EtBr is enhanced when intercalated into DNA. EtBr binding to DNA is not sequence specific, however, the amount of fluorescence enhancement is proportional to the amount of DNA.

Mapping. A “restriction map” is a depiction of how fragments of DNA from a particular digest sum. If the map of actual data matches that of the theoretical pattern, then the sequence of the DNA is verified. There are many programs available for performing theoretical restriction mapping. We will use the program NEBcutter 2.0*. To use this tool, you must minimally paste the sequence of pYP** into the designated area, select “circular” as the type of DNA, and select all NEB enzymes for use in cutting. The result will give an exhaustive list of every restriction enzyme sold by NEB that cuts the sequence provided in any place.

**Sequence of pYP can be found as a link on the course home page.
GOAL

In the *E. coli* cells being used for this study, there are numerous types of DNA – genomic DNA, plasmid DNA, etc. During the previous laboratory period, a midi-prep purification of DNA (presumed to be pYP) should have resulted in 10-200 µg of purified plasmid DNA. The specific aim of today’s study is to characterize the material purified from the Midi-prep purification. You want to determine how pure was the resulting DNA solution and is the purified DNA actually pYP.

MATERIALS AND REAGENTS

Purified DNA presumably containing pYP plasmid (from previous lab)

Restriction Enzymes (each from New England Biolabs)
- *Ear I*
- *EcoRI*
- *Dra I*
- *Hae II*
- 10X Buffer containing:
  - 10 mM Tris-HCl, pH 7.9
  - 50 mM NaCl
  - 10 mM MgCl₂
  - 1 mM Dithiothreitol (DTT)
- Buffer for Hae II only is same as above spiked with bovine serum albumin (BSA)

Agarose Gel Electrophoresis
- High melting DNA agarose (note source)
- MW Marker: *pBR322* DNA - *BstN I* digest (New England Biolabs)
  - 1857 bp
  - 1058 bp
  - 929 bp
  - 383 bp
  - 121 bp (this band might be faint)
- Primo Horizontal Submarine Gel System (Thermo Electron)
- Tris-Acetate-EDTA (TAE) Buffer
- Sample Loading Dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 6X TAE
- Power supplies
- 10 mg/mL ethidium bromide (1mL)
- Fotophoresis UV Transilluminator (Fotodyne)
EXPERIMENTAL PROCEDURE

NOTE: You must wear gloves throughout this lab to protect your DNA.

Restriction Digestion

1. Generate a table to use to outline how you will conduct your restriction digests as modeled below. Be sure to check the actual concentrations of each restriction enzyme that you will use and adjust the volumes accordingly. Also, each student will have his/her own DNA concentration. Use approximately 1.5 µg of DNA in the restriction digest. For many restriction enzymes, 1 Unit of the enzyme will cleave 1 µg of DNA at 37 °C for 1 hour in a 50-µL reaction mix. The conditions below call for 20 Units of enzyme for every 1.5 µg of DNA in a 15-µL reaction mix.

Table 1. Example of a Theoretical Restriction Digestion Outline*

<table>
<thead>
<tr>
<th>Reaction Mix Contents</th>
<th>Ear I 10,000 Units/mL</th>
<th>Hae II 20,000 Units/mL</th>
<th>EcoR I + Dra I each @ 20,000 Units/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vol of Purified DNA (150 µg/mL)</td>
<td>10 µL</td>
<td>10 µL</td>
<td>10 µL</td>
</tr>
<tr>
<td>Vol of Buffer</td>
<td>1.5 µL of NEBuffer #1</td>
<td>1.7 µL of NEBuffer #4 supplemented with BSA</td>
<td>1.5 µL of NEBuffer #4</td>
</tr>
<tr>
<td>Vol of dI H2O</td>
<td>1.5 µL</td>
<td>2.3 µL</td>
<td>1.5 µL</td>
</tr>
<tr>
<td>Vol of Restriction Enzyme</td>
<td>2 µL</td>
<td>1 µL</td>
<td>1 µL of EcoR I</td>
</tr>
<tr>
<td>Total Reaction Volume</td>
<td>15 µL</td>
<td>15 µL</td>
<td>15 µL</td>
</tr>
</tbody>
</table>

*When you generate your own table, insert the appropriate concentrations and volumes everywhere. The data here serve as an example only.

2. Prepare each of the components of the reaction mix as required. The purified DNA should be thawed completely and stored on ice until further use. NEBuffers are commercially available. Each should be fully thawed and stored on ice until further use. Prepare the NEBuffer #4 supplemented with BSA by adding 5 µL of 100X BSA to 45 µL of NEBuffer #4. This volume can be shared with others in the class. All restriction enzymes should be thawed fully and centrifuged before aliquoting to ensure that every drop is available for sampling. Keep all restriction enzymes on ice!

3. Label microcentrifuge tubes with your initials and the restriction enzyme. Combine the reagents according to your restriction digestion outline. Be sure to add the restriction enzyme last since that starts the reaction. Keep all samples on ice while working with them.

4. “Flick” each sample tube to gently mix the reactants, then quickly centrifuge all liquid to the bottom of the tube. See your TA for guidance on the specific centrifugation conditions and record them in your lab notebook.

5. Place the samples into the 37°C incubator and record the start of your incubation time in your notebook. Allow the sample to remain at 37°C for 1 hour.
6. Meanwhile, put the remainder of your pYP plasmid back into the -20 °C freezer and then pour a 1% agarose gel.

**Agarose Gel Electrophoresis and Visualization**

1. Place 0.35 gram of high melting DNA agarose in a small Erlenmeyer flask with a stir bar in it. Add 35 ml of 1X TAE buffer to generate a 1% agarose solution.

2. Cover the flask with aluminum foil to trap evaporated water. Heat the mixture on a stirring hot plate until the agarose dissolves. Do not let it boil!

3. Assemble the gel casting tray and comb. The comb should not touch the bottom of the tray. The TA will demonstrate apparatus assembly, if necessary.

4. When the agarose solution has cooled to about 50°C (i.e. warm but not scalding), pour the agarose solution carefully into the mold, ensuring that no bubbles get into the gel. (NOTE: Do not pour the agarose solution into the casting tray if the temperature is greater than 50°C, as the casting tray will crack or warp. Rinse the flask immediately after filling the tray.)

5. Allow the gel to cool. It should solidify and become slightly opaque within 20 to 30 minutes.

6. Carefully remove the comb by lifting it gently at one end, tilting the comb as it comes out. Pulling the comb straight up creates a vacuum in the wells, which tends to lift the whole gel out of the tray.

7. Lift the gel and plastic running tray from the casting tray and remove any agarose adhering to the bottom of the tray. Place the gel on the center platform of the running unit.

8. Add 1X TAE running buffer to the reservoirs of the gel apparatus. Submerge the gel in running buffer so that the buffer level is ~0.5 cm above the gel.

9. Prepare the DNA samples for loading onto the gel by adding 2 µL of 6X loading buffer to 10 µL of each restriction digest. Mix well with pipetting.

10. Prepare a sample of the DNA MW marker by combining 1 µL of marker, 4 µL of 1X TAE buffer, and 1 µL of 6X loading buffer into a tube. Mix with pipetting.

11. Record each sample name and the corresponding lane number in your notebook.

12. Carefully load 10 µL of each restriction digest into the gel with a 20 µL pipettor, which is easier to control than a 200 µL pipettor. Place the pipet tip containing the sample into the well. Be careful not to puncture the well. Slowly expel the sample. Do not use the "blow out" feature of the pipettor, which will expel an air bubble and blow the sample out of the well. Do not stir the contents of the well.

13. Once all the samples are loaded place the cover on the gel apparatus. Connect the leads so that the red (positive) lead is at the end of the gel to which the DNA will migrate and the black (negative) lead is at the end of the gel containing the wells.

14. Turn on the power supply and set the voltage to 120 V. *Caution: Do not remove the lid from the gel apparatus without disconnecting the power leads. Electrical shock may result.*
(15) When the blue tracking dye (which runs in these gels along with a DNA fragment of about 200-400 bp) has migrated about 50% of the distance to the end of the gel (this usually takes less than 60 minutes), turn off the power supply and disconnect the power leads.

(16) While the gel is running, add 100 mL of dI water to your plastic staining box. Add 20 μL of 10 mg/mL ethidium bromide to the water. Caution: Ethidium bromide is a mutagen and a potential carcinogen. Ethidium bromide solutions must be handled with disposable latex gloves. The gel must be handled with gloves from this step on!

(17) Once gel electrophoresis is complete, transfer the gel to your plastic staining box to stain the gel with ethidium bromide. Allow the gel to stain for at least 15 minutes on the rocker.

(18) Visualize the DNA with UV light using the Fotophoresis system. (NOTE: UV light damages skin and eyes, wear eye protection at all times!) Place the gel on the light box.

(19) Photograph the gel under the UV light, as instructed by a TA. Allow the Polaroid about 45 seconds to develop before opening it. Note that there is a caustic reagent in the border of the film. Use caution when opening your photograph. The film border can be disposed of in the trash.

(20) If you have a clear picture, dispose of the gel in a designated plastic EtBr waste bag.

(21) Carefully pour the ethidium bromide solution into the appropriate waste container. Leave an ethidium bromide staining tea bag in the liquid waste stirring at room temperature overnight. This will extract the ethidium from the waste solution so that the liquid waste can simply be disposed of down the drain.

(22) Rinse the light box and tray with dI water and dry it with paper towels. Dispose of the paper towels in the EtBr waste bags.
LABORATORY REPORT GUIDELINES

Copying figures from the literature for your lab report constitutes plagiarism. All materials in the published literature are copyrighted. Do not copy any figures from the literature. Generate your own figures!

Introduction (15 points)

- Give some background on DNA plasmids in general and any other relevant topic (7 points)
- Explicitly state the goal of this study (8 points)

Experimental Procedures (15 points)

- Materials (2 points)
- Silica Adsorption Chromatography (4 points)
- Determination of DNA Concentration (2 points)
- Restriction Digestion of pYP including agarose gel electrophoresis (7 points)
  - Do not insert your restriction digestion table in your lab report since that would offer too much detail.
  - Succinctly describe in sentences how the restriction digestion was performed.
  - Avoid giving every detail of the agarose gel electrophoresis.
    - Report the concentration of the gel,
    - Report the electrophoretic voltage and duration time for running the gel
    - Report the method of visual detection of bands in the gel.
    - Also include a statement on how you determined theoretical fragment lengths for comparison with your experimental data.

Results (30 points)
Remember to describe your data in words! Be very thorough here.

- DNA Yield (5 points) – explicitly state how much DNA was obtained
- DNA Purity (10 points) – explicitly describe in words the agarose gel by lane. Include an annotated figure of your agarose gel. Give some qualitative measure of purity. If possible, deduce a quantitative measure of purity and describe how you quantify purity.
- Restriction Mapping (15 points) – describe in words how your actual fragment sizes compare to your theoretical fragment sizes. Also describe any other aspects of your data that are noteworthy.
  - Include a figure of a calibration curve from your gel showing the migration distance vs. log (# bp) in MW marker
  - Table of restriction fragment sizes compared to theoretical sizes.
Discussion (30 points)

☐ State explicitly whether or not your data supports that you purified pYP DNA. (5 points)

☐ Describe the implications of your results using a figure of a restriction map made from your data. You may also wish to indicate in that figure similarities or discrepancies from the theoretical version of your restriction map to strengthen your point. (15 points)

☐ How does your recovery compare to what is expected for purification of a 100-mL culture using a Promega Wizard® Plus Midiprep kit? (5 points)

☐ Any additional discussion. (5 points)

References (10 points)

☐ Use the American Chemical Society guidelines for formatting references in the journal Biochemistry. The “Instructions to Authors” link gives the formatting style online.

☐ Refer to references in the order in which they are discussed in the text. Use numerals in parentheses or superscripts to cite references within the text.

☐ Include a minimum of 3 sound references for this lab report.