Restriction endonucleases (REs) are part of bacterial defense systems. REs recognize and cleave specific sites in DNA molecules. REs are an indispensable tool in molecular biology for both analyzing and constructing DNA molecules. In this lab, you will prepare restriction digests to distinguish which of your plasmids carry the *S. cerevisiae* or *S. pombe* ORFs or the bacterial *lacZ* gene.

**Objectives**

At the end of this lab, students will be able to:

- describe the biological origins and functions of REs.
- use online tools to identify recognition sites for REs in a DNA molecule.
- devise a strategy to distinguish DNA molecules by selecting REs to use in DNA digests.
- interpret the patterns of restriction fragments separated on agarose gels.
Chapter 11

In the last experiment, your group isolated three different plasmids from transformed bacteria. One of the three plasmids, carries the *S. cerevisiae MET* gene that has been inactivated in your yeast strain. This *MET* gene was cloned into the pBG1805 plasmid (Gelperin *et al.*., 2005). A second plasmid carries the *S. pombe* homolog for the *MET* gene, cloned into the the pYES2.1 plasmid. The third plasmid is a negative control that contains the bacterial *lacZ* gene cloned into pYES2.1. In this lab, your team will design and carry out a strategy to distinguish between the plasmids using restriction endonucleases. In the next lab, you will separate the products of the restriction digests, or restriction fragments, by agarose gel electrophoresis, generating a restriction map.

**Restriction endonucleases**

**Bacterial restriction/modification systems protect against invaders**

The discovery of restriction enzymes, or restriction endonucleases (REs), was pivotal to the development of molecular cloning. REs occur naturally in bacteria, where they specifically recognize short stretches of nucleotides in DNA and catalyze double-strand breaks at or near the recognition site (also known as a restriction site). To date, thousands of REs with distinct specificities have been described. You might wonder why bacteria harbor these potentially destructive enzymes. REs are part of a bacterial defense system against foreign DNA, such as an infectious bacteriophage. The RE sites in the bacterium's own DNA are protected from cleavage because they have been modified by a methyltransferase that specifically modifies the RE sites. The combined activities of the endonuclease and methyltransferase are referred to as a restriction/modification system. Today, most commercially available REs are not purified from their natural sources. Instead, REs are usually isolated from bacteria that overexpress large quantities of REs from plasmids. These recombinant REs have often been engineered by molecular biologists to include amino acid changes that increase the catalytic activity or stability of the RE.

To understand how REs work, we will use EcoRI, one of the best-studied REs, as an example. Although the names of individual REs may sound a bit like baby talk, the nomenclature is actually very systematic and is based on its biological source. EcoRI is found naturally in the RY13 strain of *Escherichia coli*. Its name begins with the genus and species (Eco for *E. coli*), followed by a strain identifier (R for RY13), and ends with a Roman numeral that distinguishes the different REs found in the strain. Strain RY13 of *E. coli* contains multiple REs, but only EcoRI and EcoRV, are widely used in molecular biology.

**Restriction enzymes cleave specific sites in DNA**

Restriction enzymes like EcoRI are frequently called 6-cutters, because they recognize a 6-nucleotide sequence. Assuming a random distribution of A, C, G and Ts in DNA, probability predicts that a recognition site for a 6-cutter should occur about once for every 4000 bp (4<sup>6</sup>) in DNA. Of course, the distribution of nucleotides in DNA is not random, so the actual sizes of DNA fragments produced by EcoRI range from hundreds to many thousands of base pairs, but
the mean size is close to 4000 bp. DNA fragments of this length are useful in the lab, since they long enough to contain the coding sequence for proteins and are well-resolved on agarose gels.

EcoRI recognizes the sequence G A A T T C in double stranded DNA. This recognition sequence is a palindrome with a two-fold axis of symmetry, because reading from 5’ to 3’ on either strand of the helix gives the same sequence. The palindromic nature of the restriction site is more obvious in the figure below. The dot in the center of the restriction site denotes the axis of symmetry. EcoRI catalyzes the hydrolysis of the phosphodiester bonds between G and A on both DNA strands. The restriction fragments generated in the reaction have short single-stranded tails at the 5’-ends. These ends are often referred to as “sticky ends,” because of their ability to form hydrogen bonds with complementary DNA sequences.

EcoRI catalyzes the cleavage of a palindromic recognition site.

The recognition site for EcoRI has a two-fold axis of symmetry. Cleavage generates two fragments with 5’-sticky ends.

REs are sometimes referred to as molecular scissors because of their ability to generate restriction fragments that terminate with defined sequences. These “sticky ends” are important for recombinant DNA technology, because they enables researchers to construct designer DNA molecules. Any two DNA molecules with compatible sticky ends can be joined together by DNA ligases that serve as the “paste” by resealing broken phosphodiester bonds. We will not be generating recombinant molecules in this class, but it is important to understand their importance to modern biology. Consider the pBG1805 and pYES2.1 plasmids. From the plasmid maps in Chapter 10, you can see that these complex plasmids were constructed by stitching together DNA sequences from evolutionary distinct sources.

DNA molecules have unique restriction maps

The sequence of a DNA molecule determines the distribution of recognition sites for REs. Hundreds of REs with unique specificities have been described, so researchers can use the distribution of these recognition sites in a DNA molecule to construct a “map” of the sequence. In these experiments, DNA samples are digested with various REs to produce a restriction digest, a collection of smaller restriction fragments that have been cleaved at either end by the RE. The molecules in the digest are then separated by agarose gel electrophoresis (Chapter 8). From the sizes of the restriction fragments that are resolved on the gel, investigators are able to identify the original DNA molecule used in the restriction digest.
Careful planning is required for meaningful restriction maps. The first step in a mapping experiment is to identify the sizes of restriction fragments that will be generated from a target DNA molecule with different REs. A variety of software programs generate these restriction maps and provide tabular data with details about the lengths and positions of the restriction fragments in the DNA sequence. The list of enzymes that cut a particular sequence is always impressive, but only a few enzymes usually turn out to be practical for the purpose of the experiment. When choosing REs for a restriction map, there are many things to consider:

- How many restriction fragments will be generated?
- What are the predicted sizes of the restriction fragments?
- Will all the restriction fragments be clearly resolved on 1% agarose gels?
- Will the RE generate a distinctive set of fragments from each DNA sample?
- How expensive is the RE?

In this lab, you will use the NEB Cutter to identify REs sites in plasmid sequences. (NEB Cutter is provided by New England Biolabs, a commercial supplier of REs.) You will recall that plasmids are supercoiled circles. Digestion with a RE opens up a plasmid and relaxes its structure. (Without RE digestion, the apparent sizes of plasmids on agarose gels are unreliable.) The plasmids that we are using for our experiments are complex plasmids based on pYES2.1 (5886 bp) or pBG1805 (6573 bp). Search the results for REs that will generate clearly distinguishable restriction fragments from your plasmids. It is highly recommended that you select the same RE for all three digests! Since two plasmids are based on pYES2.1, it would not be surprising to observe some common restriction fragments in those digests, which could be a helpful diagnostic.

Handling restriction endonucleases in the laboratory

The REs that we are using in the lab are highly purified (and expensive!) proteins that have been purified from recombinant bacteria. Like all enzymes, each RE functions optimally under a defined set of reaction conditions, including temperature, pH, and the concentrations of metal ions and salts. The manufacturer of our REs has developed buffers that support high levels of activity for more than 200 REs. Each buffer contains 0.1 mg/mL bovine serum albumin (BSA), an abundant protein from cow serum, which helps to stabilize denaturation-prone REs and to prevent nonspecific absorption of REs to test tubes and tips.

Like all enzymes, REs are subject to spontaneous denaturation, so REs need to be handled with care. (By comparison, DNA is an exceptionally stable molecule.) The rate of protein denaturation increases with temperature and at air/water interfaces. Some simple precautions will minimize denaturation. Follow these simple rules when you prepare the restriction digests:

- Use the recommended buffer for a particular RE.
- Keep the reactions on ice until the incubation begins.
- Be careful not to introduce bubbles. Do not use the vortex mixer.
- Add the RE last, after the other components of the reaction mixture have been assembled.
**Exercise 1 - Plan the restriction digest**

Assign each person in your group a different plasmid to analyze.

You will first need to assemble the complete sequences of your overexpression plasmids by combining the plasmid and MET gene sequences. Recall that the *S. cerevisiae* genes have been cloned into the pBG1805 vector and that the *S. pombe* genes and *LacZ* have been cloned into the pYES2.1 vector. You will then generate a restriction map that can be used to predict restriction fragments generated in an RE digestion. To generate the map, we will use one of several online tools that is available at the website of New England Biolabs, a commercial supplier of REs.

Use the table below to calculate the length of your plasmid as you complete the first few steps of this exercise.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Vector size (bp)</th>
<th>Inserted CDS (bp)</th>
<th>Final length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBG1805-MET</td>
<td>6573</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pYES2.1-</td>
<td>5886</td>
<td>3078</td>
<td>8964</td>
</tr>
<tr>
<td>pYES2.1-LacZ</td>
<td>5886</td>
<td>3078</td>
<td>8964</td>
</tr>
</tbody>
</table>

1. **Locate the coding sequence of your gene.**
   - Open the record for your *MET* gene or its homolog at SGD or Pombase, respectively.
   - Find the number of amino acids in your protein. Multiply this number by 3 to find the number of nucleotides in the CDS. Add this number to the table above.
   - Find the CDS sequence for your gene. You will paste this sequence to the end of the plasmid sequence in step 2.
   - The pYES2.1- *lacZ* sequence is posted on Canvas, so steps 1 and 2 are already done for this plasmid. Note that plasmid *lacZ* gene has been modified from naturally-occurring *lacZ* genes.

2. **Assemble the complete nucleotide sequence of your plasmid.**
   - Open the Word document containing the pBG1805 or pYES2.1 vector sequence posted on Canvas. (Alternatively, you can find the sequence record for pBG1805 in the NCBI Nucleotide database. The accession number is JN560956.) The plasmid sequences are numbered so that the *GAL1* promoter is at the 3’-end of the DNA sequence.
   - Copy the CDS from SGD or Pombase and paste it at the end of the plasmid sequence.
   - Delete the last three nucleotides of the CDS, which comprise the gene’s stop codon. The overexpression plasmids are designed to encode fusion proteins with C-terminal extensions. (Note: the stop codon in the *LacZ* sequence has been removed by the manufacturer.)
3. **Prepare a restriction map of the complete plasmid sequence.**
   - Paste the sequence from step 2 into the search box in the NEBCutter tool:
     
     tools.neb.com/NEBcutter2/
   - Check the box to indicate that the plasmid is CIRCULAR, rather than linear.
   - You might also want to give your plasmid a name. The NEB site will store your queries for 24 hours, which can be very convenient. Click submit.
   - The search tool will return results for a bewildering number of REs. The vast majority of the RE sites are not useful, because the fragments are too large or too small, the enzyme is not available in the lab, or the endonuclease is sensitive to DNA methylation (which can be unpredictable).

4. **Perform custom digests with enzymes that look promising.**
   - Click the custom digest link. This brings up a chart of REs that cut the plasmid, their recognition sites, the number of recognition sites, and the amount of enzyme activity in each of four buffers.
   - Analyze the fragments that would be produced by the four REs below by checking the box and clicking the green Submit button.
     
     AccI  HincII  ScaI  XbaI
     
     (Note: There may be some changes to this list of available REs before the lab.)

5. **Prepare a table summarizing the restriction maps for your three plasmids.**
   - Complete the table below, indicating the sizes of the restriction fragments generated with each RE.
   - Include the total length of the plasmid in the table. The sum of the restriction fragment lengths should sum up to this number.

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Length (bp)</th>
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<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>(Fragment lengths)</th>
<th>(Fragment lengths)</th>
<th>(Fragment lengths)</th>
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6. **Choose a RE that distinguishes your three plasmids.**
   The team should use the data table above to select the RE that best allows you to distinguish the three plasmids. We will be analyzing the restriction fragments on 1% agarose gels, which do a good job of resolving fragments ranging in size from ~500 bp to ~5000bp. Refer to the figure in Chapter 8, which shows the distribution of molecular size markers on 1% agarose gels. Choose an RE that will produce restriction digests with a nice range of fragment sizes.
Exercise 2 - Set up the restriction digests

The concentrations of RE and plasmid DNA need to be matched in a restriction digest. Manufacturers assay the activity of each batch of RE and express the activity in units of enzyme activity per µL. A unit of activity (U) is assessed in a standardized assay for measuring RE concentrations. Restriction digests are usually set up to contain at least 2-5 U per µg plasmid DNA. The Zyppy\textsuperscript{TM} kits typically yields plasmid concentrations ranging from 10 to 30 ng/µL. (You will be able to estimate your plasmid DNA concentrations when you run the agarose gels in the next lab.) In this lab, we are using 7 µL of plasmid miniprep DNA in each reaction and 1 U of RE. This should be more than enough RE to ensure complete digestion of the plasmid DNA.

In your lab notebook, note which RE(s) you have decided to use.

- Prepare a separate tube for each of your RE digests.
- Combine the following components in each tube in order listed:
  - 7.0 µL plasmid
  - 1.0 µL 10X CutSmart\textsuperscript{TM} buffer or 10X Buffer 3.1 (for HincII digests only)
  - 2.0 µL (1.0 U) restriction enzyme

The total reaction volume should be 10 µL.

- Ensure that the components of each reaction are well-mixed by centrifuging them for a few seconds in the microcentrifuge.
- Incubate the samples at 37 °C for at least 2 hr.
- Store the reactions in the freezer.

Exercise 3 - Analyze the restriction digests on agarose gels

This exercise will be performed in the next laboratory session.

1. Plan your gel. Each group of students will prepare one agarose gel with 8 sample lanes.
   - Each student will run one lane with undigested plasmid and a second lane with plasmid that has been digested with RE. It is important to run a lane with undigested plasmid to determine if the REs have effectively cleaved the plasmids. Keep in mind that undigested plasmids will electrophorese with anomalous sizes because of their supercoiled structures.
   - One lane should be reserved for molecular weight standards.
   - Record in your notebook how you plan to load your gel.

2. Prepare a 1\% agarose gel in TAE buffer as described in Chapter 8.
3. **Prepare the samples for electrophoresis.** Use the entire restriction digest on the gel. For undigested plasmid samples, set up tubes containing 7 µL plasmid and 3 µL deionized water.

   IMPORTANT: Return any unused plasmid to the freezer. You will use the plasmids for yeast transformation in a later lab.

   **Combine:**
   - 10 µL restriction digest or undigested plasmid
   - 5 µL deionized water
   - 3 µL 6X sample buffer

4. **Load and run the agarose gel as described in Chapter 8.**

5. **Estimate the DNA concentrations in your plasmid preparations.** After the gel has been run and stained, attempt to estimate the DNA concentrations in your three plasmid preparations, using the size standards as a reference. Each of the markers, with the exception of the 3 kb marker, contains ~40 ng. The 3 kb band contains 125 ng. Use these values to visually estimate the amount of DNA in your lane. Correct for the volume of sample in the lane to calculate the concentration of DNA in each plasmid preparation. (Although imprecise, this value will be useful for calculating your transformation efficiency in the next lab.)

**Test yourself**

The pBG1805 plasmid was derived from an earlier yeast shuttle vector, pRS426. The pRS426 plasmid is 3419 bp long and has restriction sites recognized by AvaII at positions 1514, 1736 and 2683 in its sequence. Draw a map of pRS426 below, showing the positions of the AvaII restriction sites. (Be sure to show the position of nucleotide 1 in the sequence.)

Next to the drawing, list the sizes of the restriction fragments that would be generated by digesting pRS426 with AvaII.