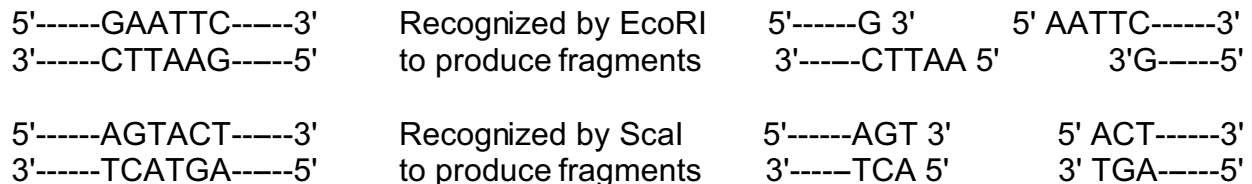# RESTRICTION ANALYSIS OF PLASMID pBR322

In this two week exercise, several restriction endonuclease digestions will be carried out on plasmid DNA, the resulting restriction fragments will be separated by electrophoresis on an agarose gel, and from the results you will build a "physical map" indicating where the restriction endonuclease cleavage sites lie relative to one another on the plasmid DNA molecule.

Introduction:

I. Restriction Endonucleases

Among the most important technical advances in molecular biology/genetics was the discovery and use of restriction endonucleases in the 1970's.  These enzymes cleave double stranded DNA at specific base pair recognition sequences.  Most restriction endonucleases recognize specific base sequences that are palindromic, that is, they have the same base sequence running antiparallel on the opposing strands, as shown below.

```
5'------GAATTC-----3'        Recognized by EcoRI      5'------G 3'         5' AATTC------3'
3'------CTTAAG-----5'        to produce fragments      3'-----CTTAA 5'          3'G-----5'


5'------AGTACT-----3'        Recognized by ScaI       5'------AGT 3'       5' ACT------3'
3'------TCATGA-----5'        to produce fragments      3'-----TCA 5'        3' TGA-----5'
```

Restriction enzymes are named for the organism from which they were isolated, *e.g.*, EcoRI comes from the bacterium *E. coli*.  As in the examples above, some restriction enzymes cleave DNA bluntly (like ScaI above), while others make staggered cuts (like EcoRI), leaving protruding ends of unpaired nucleotides.  These ends are often referred to as "sticky" or "cohesive" because they are complementary to the ends of all fragments produced by that restriction enzyme.  This allows for base pairing between any two DNA segments that have been cleaved by the same staggered cut restriction enzyme.

Different restriction enzymes recognize palindromes of different length, from four base pairs up to eight base pairs.  The frequency with which a certain recognition site occurs in a given genome depends on the length of the recognition sequence.  For example, an enzyme with a four base pair recognition sequence is expected to cut DNA more frequently than one with a six or eight base pair sequence.  The number of restriction fragments produced by a digestion depends therefore on the number of recognition sites.  It also depends on whether the starting DNA molecule is linear or circular, because a single cut in a circular DNA molecule yields one piece of full-length linear DNA whereas a single cut in a linear DNA molecule yields two fragments.

Restriction endonuclease methodology has been further developed to form recombinant DNA molecules and deletion mutants for cloning, mapping, and

determination of differences between individuals and species.

## II. Electrophoresis of DNA Fragments in an Agarose Gel

Electrophoresis is the process of separating particles in an electric field. Virtually all of the common forms of electrophoresis utilize a semisolid gel matrix in which the separation is conducted. The gel consists of an aqueous phase (buffer) and a solid phase composed of a natural or artificial polymer (agarose, polyacrylamide, starch, *etc.*). The solid phase has two roles: 1) it acts as a sieve to separate molecules according to a specific physical or chemical property and 2) it acts as a "trap" to keep separated molecules from diffusing at the conclusion of the electrophoretic treatment.

In an electric field E, a particle of charge q experiences a force of F = qE. That is, a negatively charged molecule will "feel" a force pushing it against the positive-to-negative electric field lines. So, any negatively charged molecule migrates toward the "anode" (+) end of the field, while positively charged molecules migrate toward the "cathode" (-) end. Since DNA is negatively charged, it migrates towards the anode. The rate at which a particular DNA fragment migrates through a gel is determined by its size. The larger the molecule, the more interaction it has with the gel matrix and the slower it moves. Smaller objects slip through the pores of the gel easily, while larger ones become trapped and move through more slowly. The distance traveled during a defined length of time is inversely proportional to the size of the fragment. The actual size of the pores in the gel influences the speed of migration, and thus this can be adjusted by varying the concentration of the matrix in the gel. Agarose, because it is safe and easy to handle, is the matrix of choice for gels for basic DNA analysis.

## III. Visualization of DNA

After electrophoresis is completed, the DNA must be stained in order to visualize the separated restriction fragments. The most common method of direct staining is the incubation of the gel in a weak solution of ethidium bromide (EtBr). This compound fluoresces brightly when irradiated with ultraviolet light. EtBr has a very high affinity for DNA, to which it rapidly and strongly binds (this property also renders EtBr a potent mutagen, so it must be handled with extreme caution). EtBr has almost no affinity for agarose. Thus, EtBr concentrates in the DNA molecules within the gel. When a stained gel is viewed under UV light, the DNA restriction fragments are revealed as bright bands in the gel.

Protocol:

During the next two weeks each lab group will conduct a set of restriction digests of a plasmid, electrophorese the digested DNA and appropriate molecular weight marker standards through an agarose gel, and visualize the restriction fragments in the gel via EtBr staining. The results of the procedure will be used to generate a restriction map of the plasmid DNA.

**FIRST WEEK**

Restriction Endonuclease Digestion of Plasmid DNA

1. **IMPORTANT PRECAUTION: WEAR GLOVES** during all procedures.  Unless directed otherwise, keep all tubes on ice at all times.  Pure DNA is fairly stable, but restriction enzymes are highly labile and will degrade if left at room temperature.  As always, goggles must also be worn.

2.  Obtain and clearly label six microcentrifuge tubes with your group number and a letter, A though F.  Place these tubes into the ice bucket.

3.  Examine the pipetting device (Pipetman, P-20) and the accompanying pipette tips. Listen to the TA as she/he demonstrates its use.  Practice pipetting water several times before proceeding to the next section.  Before you start pipetting make sure you can see and tell the difference between 2 and 20 microliters of liquid in the pipette tip.  2µls of liquid just fills the very end of the pipette tip while 20 µls fills about one-third of the tip.

4.  Locate the ice bucket containing the following items:

       Plasmid  pBR322  (uncut)
       Restriction Enzymes:  BamHI,  NdeI,  PstI
       10X Buffer ("Multicore")
       Sterile (DNase free) water
       6X gel loading dye

5.  Pipette the indicated amount of each of the solutions into the appropriate microcentrifuge tube.  Be sure to change pipette tips after each pipetting to prevent contamination of the DNA, enzymes, or buffer solutions.

Add to tube (microliters):

|  | **A** | **B** | **C** | **D** | **E** | **F** |
|---|---|---|---|---|---|---|
| **dH$_2$O** | 16 | 14 | 14 | 12 | 12 | 12 |
| **DNA** | 2 | 2 | 2 | 2 | 2 | 2 |
| **10X Buffer** | 2 | 2 | 2 | 2 | 2 | 2 |
| **BamHI** |  | 2 |  | 2 | 2 |  |
| **NdeI** |  |  | 2 | 2 |  | 2 |
| **PstI** |  |  |  |  | 2 | 2 |

When adding the enzymes, mix by pipetting up and down gently once or twice. Do not create bubbles. The volume in each tube adds up to 20 microliters. Some tubes contain single digests and some double digests. Spin all tubes briefly in the microcentrifuge to mix the samples and concentrate the samples at the bottom.

6. Float the completed digestion mixes in the 37°C water bath. Be certain that the tubes are labeled with your group number. The tubes will be incubated for 18 hours. At the end of the incubation, the tubes will be placed at -20°C. The digests will remain frozen until next week's laboratory at which time the electrophoretic analysis will be conducted.

7. While the tubes are incubating, pour a mock gel and practice loading mock samples. DO NOT YIELD TO THE TEMPTATION TO SKIP THIS PART OF THE LAB. Gel pouring and loading practice is very important to the success of the experiment. There will be only one opportunity to load the samples next week. So practice now!


#### Practice Gel Making and Sample Loading

1. Prepare a gel tray for pouring a sample gel. The gel boxes and trays should be clean and dry. Tape the ends of the gel trays securely to prevent leakage of the molten agarose. Gel boxes should be placed on a level surface.

2. Set up the slot-forming comb as instructed by the TA. The comb will form the wells in the gel into which you will load your DNA samples.

3. Obtain a flask containing molten (50-55°C) 0.8% agarose in TBE buffer (40 mM Tris-borate,1 mM EDTA, pH 8.0). Carefully pour the agarose into the prepared gel tray. Do not disturb the gel until it has cooled and solidified.

4. Once the gel has set and is cool, carefully remove the comb and tape, following the TA's instructions. Then place the gel into the electrophoresis chamber. Pour on enough water to fill the chamber above the level of the gel. (TBE buffer will be used when an actual gel is being run). Pour the water (buffer) directly but gently over the slots of the gel to make sure they are clean and have no bubbles in them.

5. Each person in the group should obtain a clean microcentrifuge tube and use the P20 pipetman to mix 20 microliters of water and 4 microliters of a 6X gel loading dye (30% glycerol with xylene cyanole and bromophenol blue added). DO NOT CONTAMINATE THE VIAL OF LOADING DYE. It will be used again next week. Mix thoroughly with the pipette tip, then set the pipetman to 20 microliters. Pipette as much of the diluted dye mix as you can and try to load it into one of the wells in the gel. This procedure takes patience, concentration, and a steady hand. Repeat the procedure as needed until each person in the group becomes proficient. Pour another gel if you need more slots. The most skilled gel loader should be assigned the task of loading the actual samples next week.

6.  Once the gels are loaded, the TA will demonstrate the proper method of attaching the chamber to the power supply.  Listen very carefully.  Improper attachment will result in no electrophoresis (bad) or backward electrophoresis (very bad) or worse (don't even think about it!).

7.  Clean up by discarding the gels and washing the gel apparatus with water.  Inspect the area for stray pipette tips and return the dye vials to the TA.


## SECOND WEEK

1.  Prepare a gel and set up the electrophoresis chamber, as described in the previous section.

2.  Obtain your digests and molecular weight standard tubes from the TA and let them thaw.

3.  Add 4 microliters of loading dye to tubes A through F.  Mix each thoroughly and spin the tubes briefly in the microcentrifuge to mix and get all the samples down into the bottom of the tube.  Follow the TA's instructions for the standard tubes.  The standards contain a mixture of DNA fragments of known length*.  By comparing the migration of the standard DNA fragments with the migration of the DNA fragments in your restriction digests, you can determine the size in base pairs of each DNA fragment in the gel.

4.  Remove the comb, place the gel into the chamber and cover with TBE buffer.

5.  Load the samples into the gel slots in the following order: λHindIII standard markers, your samples A, B, C, D, E, F, and pGEM standard markers.  This should yield the best side-by-side comparison of the fragment patterns from the DNA digests.  Be sure to load 10 microliters of each of the molecular weight standards and 20 microliters of samples A through F.  If the comb you used has more than 8 wells, use the innermost wells for your samples.

6.  Place the cover on the electrophoresis chamber, attach the electrode leads, and turn on the power supply.  Adjust the voltage to 100 volts and allow the electrophoresis to proceed for approximately one hour, or until the leading dye marker is about 1 cm from the far end of the gel.  Do not run the dye marker completely off the end of the gel or you will lose some of the smaller DNA fragments.

7.  At the end of  the run, turn the voltage to zero, turn the power supply off, and detach the leads from the power supply.  Use a razor blade to cut the upper left corner of the gel to indicate the position of your first sample, and use a pipette tip to punch a hole in the edge to identify the gel of your group.  Remove the gel from the electrophoresis chamber.

8.  Working with the instructor, place the gel into the EtBr solution (1 µg/ml EtBr) for

staining.  After 10 minutes in the EtBr, the gel should be transferred to a water rinse for 10 minutes to remove excess EtBr from the gel.

9.  View the destained gel under UV light for visualization of the DNA, and photograph the gel using Polaroid film.  Be sure to follow the TA's instruction regarding contact with the EtBR stained gel.  Use gloves, goggles and long sleeves to prevent UV burns to skin and eyes.

10.  Examine the photograph for the presence of bands.  Observe their positions and the differences between single and double digest patterns.  Determine the size (in base pairs) of the DNA in each band.

11.  From the DNA fragment sizes, figure out the "restriction map" of plasmid pBR322 and include in your lab report an image that represents the map on the circular form of plasmid pBR322.

The analysis you have done here is similar to problem 11.16 in Hartl and Jones.  You should construct your restriction map in the same general manner as the solution to problem 11.16, shown on page 579 in the text.

**\*Standard restriction fragments for pGEM and  λHIND III (in base pairs)**

       Standard digests are prepared commercially by digesting plasmid DNA with enzymes that give fragments of known molecular weight.  The pGEM markers are prepared by combining three separate digests in equimolar amounts.  The restriction enzymes used are Hinf I, Ras I and Sin I.  The substrate in this case is called pGEM-3 DNA.  The other set of markers used in this experiment are derived from a digestion of λ DNA by the restriction enzyme, HindIII.   The known molecular weights are listed below.  In the present protocol we would not expect to be able to see fragments under 200bp in size.

| pGEM Markers | λHind III Markers |
|:---:|:---:|
| 2,645 | 23,130 |
| 1,605 | 9,416 |
| 1,198 | 6,557 |
| 676 | 4,361 |
| 517 | 2,322 |
| 460 | 2,027 |
| 396 | 564 |
| 350 | 125 |
| 222 | |
| 179 | |
| 126 | |
| 75 | |
| 65 | |
| 51 | |
| 36 | |