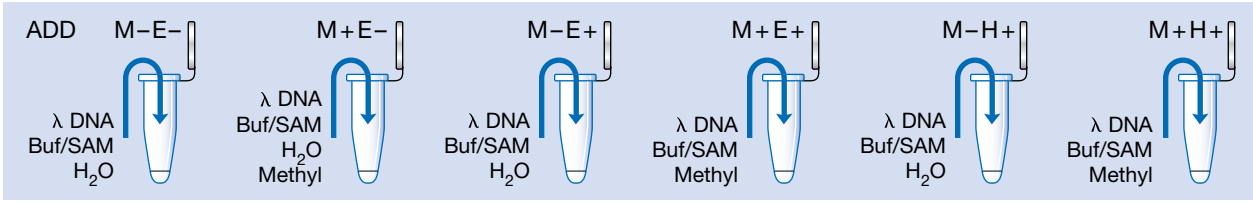
Effects of DNA Methylation on Restriction

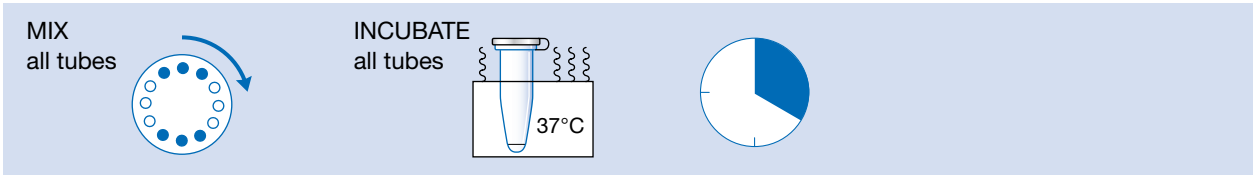
In Laboratory 4, the *Eco*RI methylation system is used to illustrate the sequence specificity of a modifying enzyme that protects DNA from restriction enzyme digestion. *Eco*RI methylase adds a methyl group to the second adenine residue in the *Eco*RI recognition site, thus preventing the endonuclease from binding and cutting the DNA. *S*-Adenosyl methionine (SAM), included in the methylation reaction, donates the methyl group that is attached to the DNA molecule by the methylase.

Three samples of bacteriophage λ DNA are incubated at 37°C with *Eco*RI methylase, one of which is subsequently incubated with *Eco*RI and another of which is incubated with *Hin*dIII. The third sample, a control, is incubated without a restriction enzyme. Three control samples of nonmethylated DNA are also incubated with *Eco*RI, *Hin*dIII, and no enzyme. All of the samples are separated by electrophoresis in an agarose gel and stained. Comparison of the band patterns reveals that the methylated DNA is protected from digestion by *Eco*RI. However, methylation at the *Eco*RI site has no effect on the activity of the restriction enzyme *Hin*dIII.

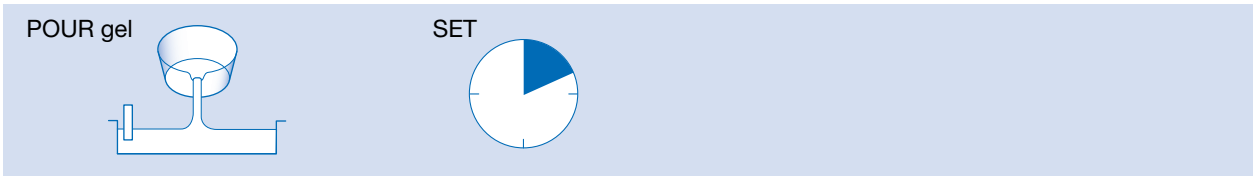
Equipment and materials for this laboratory are available from the Carolina Biological Supply Company (see Appendix 1).

I. Set Up Methylase Reaction

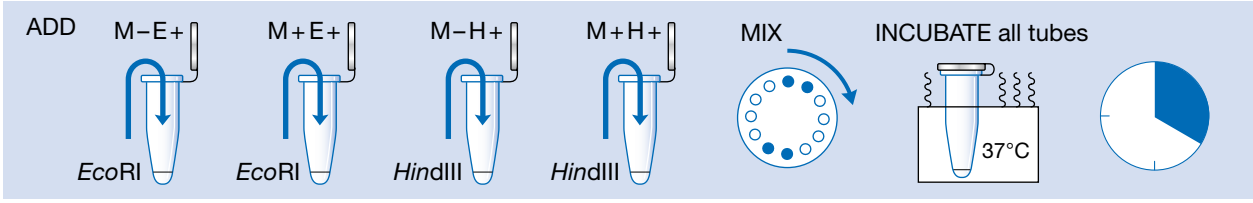




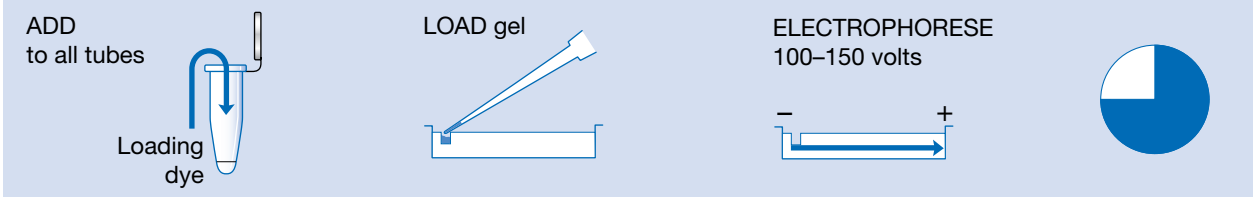
II. Cast 0.8% Agarose Gel



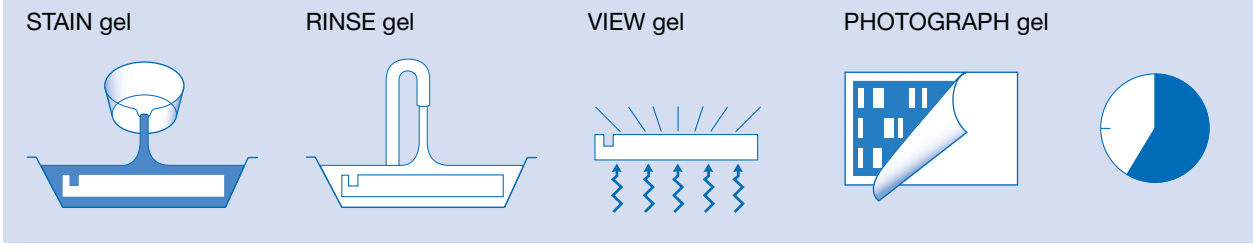
III. Set Up Restriction Reaction



IV. Load Gel and Separate by Electrophoresis



V. Stain Gel and View (Photograph)



PRELAB NOTES

Review Prelab Notes in Laboratory 3, DNA Restriction Analysis.

S-Adenosyl Methionine

S-Adenosyl methionine (SAM) is incorporated into a 2x restriction buffer, so that the same buffer is used for *both* methylation and restriction reactions. Because SAM is not very stable, mix the buffer/SAM solution just prior to the lab and discard after use. In addition, make sure to use a fresh stock of SAM not more than several months old.

To Avoid Confusion

This laboratory has two distinct steps involving two similar-sounding reagents. In the first step, DNA is preincubated with *Eco*RI methylase. In the second step, the methylated DNA is incubated with *Eco*RI restriction enzyme. To avoid mishaps, do not set out the endonuclease *Eco*RI restriction enzyme until *after* the methylation reactions are set up.

For Further Information

The protocol presented here is based on the following published methods:

Aaij C. and Borst P. 1972. The gel electrophoresis of DNA. *Biochim. Biophys. Acta* **269:** 192–200.

Greene P.H., Poonian M.S., Nussbaum A.L., Tobias L., Garfin D.E., Boyer H.W., and Goodman H.M. 1975. Restriction and modification of a self-complementary octanucleotide containing the *Eco*RI substrate. *J. Mol. Biol.* **99:** 237–261.

Helling R.B., Goodman H.M., and Boyer H.W. 1974. Analysis of R-*Eco*RI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. *J. Virol.* **14:** 1235–1244.

Sharp P.A., Sugden B., and Sambrook J. 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose–ethidium bromide electrophoresis. *Biochemistry* **12:** 3055–3063.

PRELAB PREPARATION

Before performing this Prelab Preparation, please refer to the cautions indicated on the Laboratory Materials list.

1. Prepare 2x restriction buffer plus SAM within 1–2 days before the experiment.
2. Prepare aliquots for each experiment:

   30 µl of 0.1 µg/µl λ DNA (store on ice)
   40 µl of 2x restriction buffer/SAM (store on ice)
   5 µl of *Eco*RI methylase (store on ice)
   3 µl each of *Eco*RI and *Hin*dIII (store on ice)
   500 µl of distilled water
   500 µl of loading dye

3. Prepare 0.8% agarose solution (40–50 ml per experiment). Keep agarose liquid in a hot-water bath (~60°C) throughout lab. Cover with aluminum foil to retard evaporation.
4. Prepare 1x Tris/Borate/EDTA (TBE) buffer for electrophoresis (400–500 ml per experiment).
5. Prepare ethidium bromide or methylene-blue-staining solution (100 ml per experiment).
6. Adjust water bath to 37°C.

MATERIALS

REAGENTS

Agarose (0.8%)
Distilled water
Ethidium bromide▼(1 μg/ml) (or 0.025% methylene blue▼)
λ DNA (0.1 μg/μl)
Loading dye
2x Restriction buffer/SAM▼
Enzymes:
*Eco*RI
*Hin*dIII
*Eco*RI methylase
1x Tris▼/Borate/EDTA (TBE) buffer

SUPPLIES AND EQUIPMENT

Aluminum foil
Beakers for agarose and for waste/used tips
Camera and film (optional)
Electrophoresis box
Latex gloves
Masking tape
Microfuge (optional)
Micropipettor (0.5–10-μl) + tips
Parafilm or wax paper (optional)
Permanent marker
Plastic wrap (optional)
Power supply
Test tube rack
Transilluminator (optional)▼
Tubes (1.5-ml)
Water baths (37°C and 60°C)

▼ See Appendix 4 for Cautions list.

METHODS

I. Set Up Methylase Reaction (30 minutes, including incubation)

1. Use a permanent marker to label six 1.5-ml tubes, in which methylation and restriction reactions will be performed:

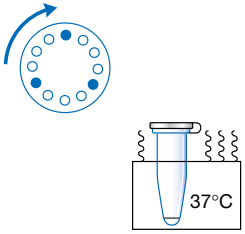
M– E– = no methylase, no *Eco*RI
M+ E– = methylase, no *Eco*RI
M– E+ = no methylase, *Eco*RI
M+ E+ = methylase, *Eco*RI
M– H+ = no methylase, *Hin*dIII
M+ H+ = methylase, *Hin*dIII

M+E+	4 µl	5 µl	—	1 µl
M–H+	4 µl	5 µl	1 µl	—
M+H+	4 µl	5 µl	—	1 µl

It is not necessary to change tips when adding the same reagent. The same tip may be used for all tubes, provided the tip has not touched the solution already in the tubes.

To avoid confusing methylase with reagents in Part III, discard after completing Step 7.

3. Collect and place reagents in the test tube rack on the lab bench.
4. Add 4 µl of DNA to each reaction tube. Touch the micropipettor tip to th side of the reaction tube, as near to the bottom as possible, to create capi lary action to pull the solution out of the tip.
5. Use a *fresh tip* to add 5 µl of restriction buffer/SAM to a clean spot on eac reaction tube.
6. Use a *fresh tip* to add specified volume of distilled water to appropriate tube
7. Use a *fresh tip* to add 1 µl of *Eco*RI methylase to Tubes M+E–, M+E+, an M+H+.
8. Close tube tops. Pool and mix reagents by pulsing in a microfuge or sharpl tapping the tube bottom on the lab bench.
9. Place the reaction tubes in a 37°C water bath, and incubate for a minimu of 20 minutes. Reactions can be incubated for longer periods of time.

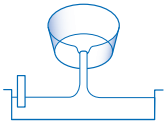


After several hours, methylase loses activity and the reaction stops.

STOP Following incubation, freeze reactions at –20°C until ready to continue. Thaw reactions before continuing to Section III, Step 1.

II. Cast 0.8% Agarose Gel

(5 minute



Gel is cast directly in the box in some electrophoresis apparatuses.

Too much buffer will chan-

1. Seal the ends of the gel-casting tray with tape, and insert a well-formin comb. Place gel-casting tray out of the way on the lab bench, so that agaros poured in next step can set undisturbed.
2. Carefully pour enough agarose solution into the casting tray to fill to a dept of about 5 mm. Gel should cover only about one-third the height of com

moved from a dry gel can ar the wells.

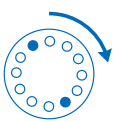
III. Set Up Restriction Reaction

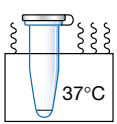
(30 minutes, including incubation)

1. Remove methylation reactions from the water bath or thaw the tube stored in the freezer.
2. Use the matrix below as a checklist while adding reagents to each reaction. Read down each column, adding the same reagent to all appropriate tubes. *Use a fresh tip for each reagent.* Refer to detailed instructions that follow.

Tube	*Eco*RI	*Hin*dIII
M–E–	—	—
M+E–	—	—
M– E+	1 µl	—
M+E+	1 µl	—
M–H+	—	1 µl
M+H+	—	1 µl

3. Collect *Eco*RI and *Hin*dIII, and place them on ice on the lab bench.
4. Add 1 µl of *Eco*RI to Tubes M–E+ and M+E+.
5. Use a *fresh tip* to add 1 µl of *Hin*dIII to tubes labeled M–H+ and M+H+.
6. Close tube tops. Pool and mix reagents by pulsing in a microfuge or sharply tapping the tube bottom on the lab bench.
7. Place the reaction tubes in a 37°C water bath, and incubate restriction reactions for a minimum of 20 minutes. Reactions can be incubated for longer periods of time.

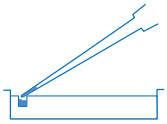




fter several hours, enzymes se activity and the reaction pps.

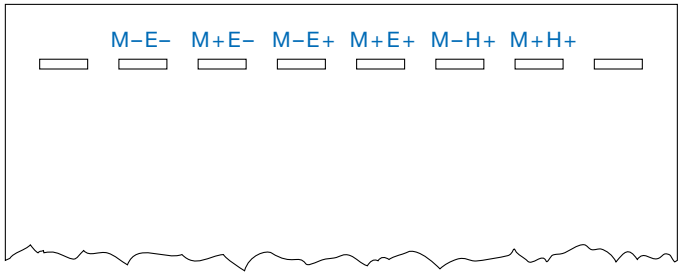
STOP Following incubation, freeze reactions at –20°C until ready to continue. Thaw reactions before continuing to Section IV, Step 1.

b. Place six individual droplets of loading dye (1 μl each) on a small square of Parafilm or wax paper. Withdraw contents from the reaction tube, and mix with a loading dye droplet by pipetting in and out. Immediately load dye mixture according to Step 2. Repeat successively, *with a clean tip*, for each reaction.



A piece of dark construction paper beneath the gel box will give the wells more visibility.

2. Use a micropipettor to load 10 μl of each reaction tube into a separate well in the gel, as shown in diagram below. *Use a fresh tip for each reaction.*
   a. Use two hands to steady the micropipettor over the well.
   b. Before loading sample, make sure that there are no bubbles in the wells. If bubbles exist, remove them with a micropipettor tip.
   c. If there is air in the end of the tip, carefully depress the plunger to push the sample to the end of the tip. (If an air bubble forms a "cap" over the well, DNA/loading dye will flow into buffer around edges of well.)
   d. Dip the micropipettor tip through the surface of the buffer, center it over the well, and gently depress the micropipettor plunger to slowly expel the sample. Sucrose in the loading dye weighs down the sample, causing it to sink to the bottom of the well. *Be careful not to punch the tip of the micropipettor through the bottom the gel.*



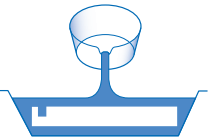
Alternately, set power supply on lower voltage, and run the gel for several hours. When running two gels from the same power supply, the current is double that for a single gel at the same voltage.

3. Close the top of the electrophoresis box, and connect the electrical leads to a power supply, anode to anode (red-red) and cathode to cathode (black-black). Make sure that both electrodes are connected to the same channel of power supply.
4. Turn power supply on, and set to 100–150 volts. The ammeter should register approximately 50–100 milliamperes. If current is not detected, check connections and try again.
5. Separate by electrophoresis for 40–60 minutes. Good separation will have occurred when the bromophenol blue band has moved 4–7 cm from the wells. If time allows, carry out electrophoresis until the bromophenol blue band nears the end of the gel. *Stop* electrophoresis before bromophenol blue band runs off end of gel.
6. Turn off power supply, disconnect leads from the inputs, and remove top of electrophoresis box.
7. Carefully remove casting tray from electrophoresis box, and slide the gel into a disposable weigh boat or other shallow tray. Label staining tray with your name.

Staining may be performed by an instructor in a controlled area when students are not present.

8. Stain and view gel using one of the methods described in Sections VA and VB.

CAUTION
Review Responsible Handling of Ethidium Bromide in Laboratory 3. Wear latex gloves when staining, viewing, and photographing gels and during cleanup. Confine all staining to a restricted sink area. For further information, see Appendix 4.

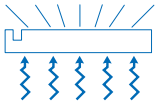


aining time increases arkedly for thicker gels.

hidium bromide solution ay be reused to stain 15 or ore gels. When staining ne increases markedly, spose of ethidium bromide lution as explained in aboratory 3.

1. Flood gel with ethidium bromide solution (1 µg/ml), and allow to stain for 5–10 minutes.
2. Following staining, use a funnel to decant as much ethidium bromide solution as possible from the staining tray back into the storage container.
3. Rinse the gel and tray under running tap water.
4. If desired, gels can be destained in tap water or distilled water for 5 minutes or more to remove background ethidium bromide.

STOP Staining intensifies dramatically if rinsed gels set overnight at room temperature. Stack staining trays, and cover top gel with plastic wrap to prevent desiccation.



5. View under UV transilluminator or other UV source.

CAUTION
Ultraviolet light can damage eyes. Never look at unshielded UV light source with naked eyes. View only through a filter or safety glasses that absorb harmful wavelengths. For further information, see Appendix 4.

6. Photograph with a Polaroid or digital camera.
7. Take time for responsible cleanup.
   a. Wipe down camera, transilluminator, and staining area.
   b. Wash hands before leaving lab.

5. View gel over light box; cover surface with plastic wrap to prevent staining
6. Photograph with a Polaroid or digital camera.

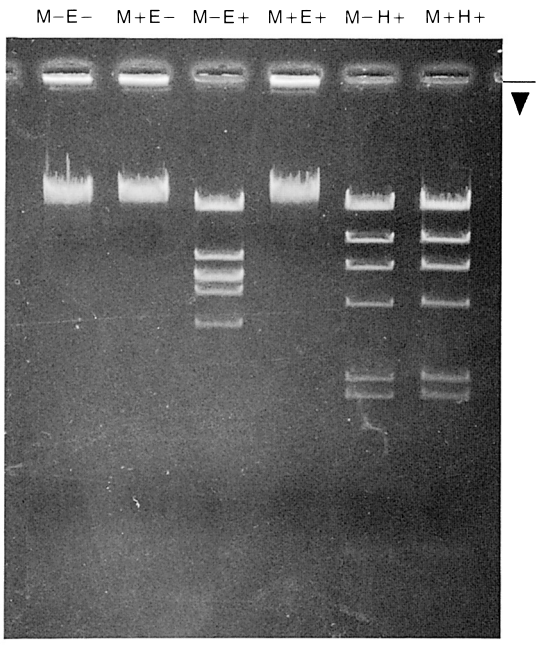
RESULTS AND DISCUSSION

Each type II restriction enzyme has a corresponding methylase that recognize the same nucleotide sequence. However, rather than cutting the DNA at th point, the methylase adds a methyl group within the recognition sequence. Th "modification" blocks the restriction endonuclease from recognizing and bindin to the restriction site. Within the bacterium, methylation protects the host DN from cleavage by its own restriction enzyme. Unmethylated foreign DNA is n protected from cleavage.

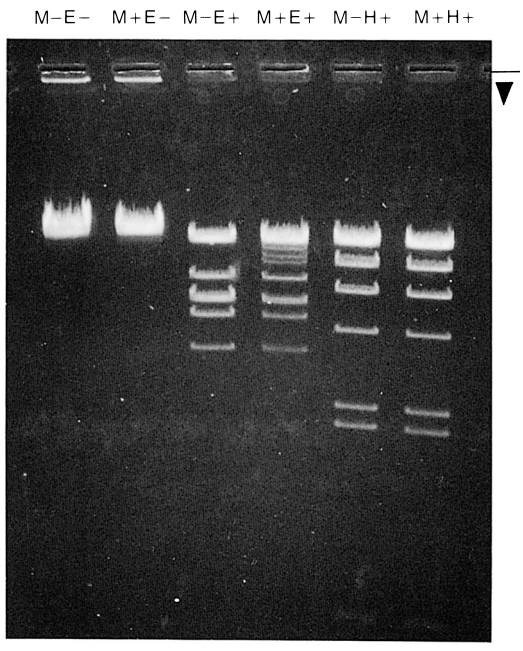
The methylation reaction requires *S*-adenosyl methionine (SAM), the un versal methyl-donating molecule in both prokaryotes and eukaryotes. As i name implies, SAM is composed of the nucleoside adenosine and the amino ac methionine. The donation of a methyl from the methionine portion of the mo ecule converts it into *S*-adenosyl homoserine.

One common occurrence in this laboratory is partial methylation, whe methyl groups are added to only a fraction of the *Eco*RI sites within the λ DN molecules. Cleavage at the unprotected sites produces a partial digest, yieldin restriction fragments of varying lengths. These fragments are evidenced a lower-molecular-weight bands in an agarose gel. The intensity of the bands inversely proportional to the level of DNA methylation.

1. Examine the photograph of your stained gel (or view on a light box or ove head projector). Compare your gel to the ideal gel shown on the next pag How can you account for differences in separation and band intensity?
2. What does the M+H+ control tell you about *Eco*RI methylation?
3. What does the M+E− control tell you about methylation?



Ideal Gel



Incomplete Methylation
Faint bands in Lane M+E+ DNA partially cut by *Eco*RI

the pAMP "backbone" also contains an *Eco*RI site, which is not the intended cloning site for the *Eco*RI fragment in Step 2.

a. Draw a diagram of this cloning experiment.

b. Explain how *Eco*RI methylase could be used to solve this experimental problem.

6. Which nucleotide(s) is (are) methylated by *Eco*RI methylase? Draw the structure of the newly methylated base.

FOR FURTHER RESEARCH

1. Design and execute a series of experiments to study the kinetics of a methylation reaction.

   a. Determine the approximate percentage of sites protected at various time points.

   b. Repeat the experiments with several methylase dilutions and several DNA dilutions.

   c. In each case, at what time point does protection appear to be complete?

2. Design and execute experiments to use *Eco*RI methylase to map the locations of *Eco*RI restriction sites in the λ genome.

3. Research the use of methylases in constructing a genomic library.

4. Research the role of DNA methylation in gene regulation in higher organisms.

5. Research the role of methylation in controlling the movement of transposable elements in maize (corn).