A Time-Efficient & User-Friendly Method for Plasmid DNA Restriction Analysis

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A hands-on experience studying DNA in the laboratory enhances students' understanding of advances in biotechnology. However, standard laboratory experiments with DNA can be hazardous and require expensive equipment. We describe a simple, efficient, high-resolution, plasmid restriction enzyme digestion/agarose gel electrophoresis experiment in which a new DNA stain is substituted for ethidium bromide or methylene blue.

The discovery of restriction enzymes (or restriction endonucleases) in the 1970s was a key factor in the DNA revolution. Restriction enzymes are found in all microorganisms. They digest foreign nucleic acids at short sequences that are specific for each enzyme. A bacterial cell protects its own DNA from the effects of endogenous restriction enzymes by adding a methyl group to specific adenine or cytosine bases after replication. This modified DNA cannot be cleaved by the endogenous enzyme, but DNA from a different source will usually not have the protecting modification pattern and will be cleaved. Thus, restriction enzymes act as molecular scalpels (see Figure 1) that break a large molecule into many smaller pieces. In nature, they serve as a microbial "immune system," destroying most foreign DNA before it can become established.

A large number of restriction enzymes with different characteristic recognition sites have been identified: Some cleave up to 1000 base pairs (bp) from their specific recognition sites, but the ones that are most useful for recombinant work cleave within their recognition sites. Recognition sites for these restriction enzymes are usually 4–10 bp in length and are almost always palindromic (e.g. see Figure 1). Two types of cleavage can occur in the DNA, depending on the restriction enzyme used:

1. *Blunt ended*, which leaves no unpaired bases.
2. Staggered, leaving one or more unpaired bases.

Depending on the enzyme, the unpaired bases may be at the 3' or 5' end of the double stranded DNA.

DNA fragments of different sizes are usually separated by electrophoresis in agarose gels. Agarose creates a polymer matrix in which DNA fragments migrate when an electrical current is applied. Since DNA is negatively charged, it will migrate towards the positive pole, with small fragments moving faster than large fragments.

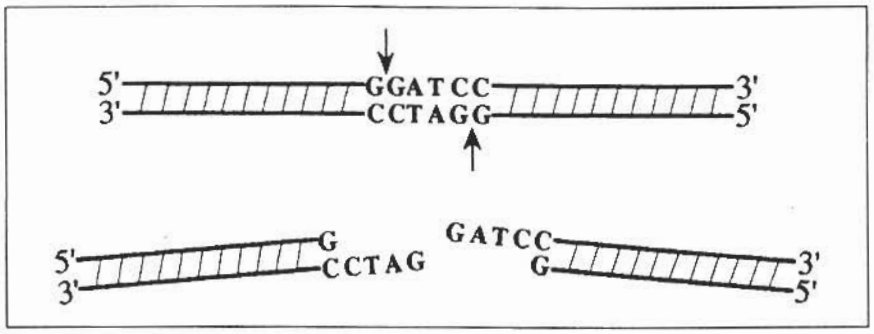

Figure 1. *Bam*HI restriction enzyme activity on target DNA. The six bp palindromic (inverted repeat) sequence required for *Bam*HI digestion is depicted at the top. The *Bam*HI-specific endonuclease recognizes this sequence and makes staggered cuts at the sites indicated by the arrowheads. When the DNA fragments separate, the four base single-stranded segments form cohesive or "sticky" ends (bottom). Note that because of the palindromy of the *Bam*HI recognition site, the single stranded segments are identical (5'-GATC-3').

*Eco*RI and *Hin*dIII also recognize six bp palindromic sequences and leave four base 5' single stranded sequences. However, *Eco*RI cleaves 5'-GAATTC-3' between G and A, while *Hin*dIII cleaves 5'-AAGCTT-3' between the two As.

DNA is not visible without staining. The most economical and widely used research stain for DNA in agarose gels is the fluorescent dye ethidium bromide, despite the fact that this compound is highly mutagenic and carcinogenic. In addition, ethidium bromide-stained DNA has to be viewed and photographed under ultraviolet light, another known mutagen/carcinogen. Because of the hazards associated with misuse of ethidium bromide, some states prohibit its use in high schools. Methylene blue, a much less sensitive colored stain, is often used in the classroom because it is an intense stain that can be seen under normal lighting. However, methylene blue is also mutagenic, is messy to use, and requires a great deal of time for staining and destaining the gel outside of class. To avoid difficulties associated with use of ethidium bromide and methylene blue in the teaching laboratory, we have developed methods for using a new fluorescent DNA binding

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dye, SYBR®Green I nucleic acid gel stain (SG) (Singer et al. 1994). SG has several advantages over ethidium bromide and methylene blue for teaching purposes:

1. It is by far the least mutagenic.
2. It binds to DNA with high specificity.
3. SG-stained DNA can be seen without special equipment (although detection is most sensitive with UV illumination).
4. No time-consuming de-staining procedure is required.
5. Very low levels of DNA can be detected.
6. Exposure to SG is minimal because it can be incorporated in a small volume of loading buffer and used as an internal pre-stain, instead of as a post-stain.

We describe here an experiment in which plasmid DNA is digested with restriction enzymes that cleave the plasmid either once or twice. The DNA is then stained with SG, loaded on a gel, electrophoresed, and viewed under normal laboratory conditions during electrophoresis.

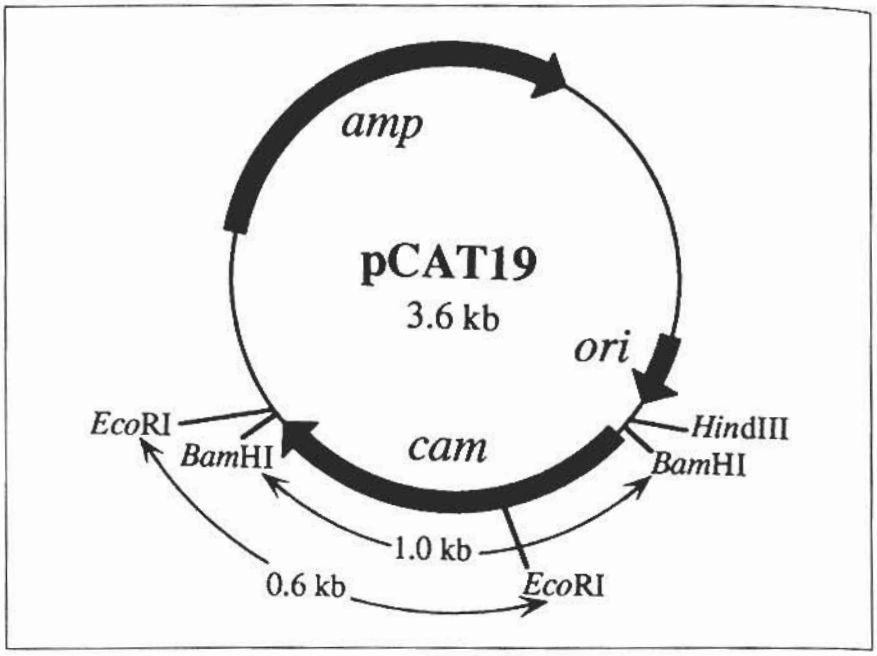

Figure 2. Diagram of plasmid pCAT19. Plasmid pCAT19 contains *amp* and *cam* antibiotic resistance genes. Presence of this plasmid is selected on nutrient medium containing either ampicillin or chloramphenicol. pCAT19 contains one *Hin*dIII site, two *Eco*RI sites, and two *Bam*HI sites. Digestion with each enzyme will yield a different set of fragments.

Restriction Digestion of Plasmid pCAT19

The pCAT19 plasmid (Fuqua 1992) used in this experiment is in a nonpathogenic strain of *E. coli* and can be obtained from the authors. pCAT19 is 3.6 kilobase pairs (kb) in size. It encodes resistance to the antibiotics chloramphenicol (*cam*) and ampicillin (*amp*), and was chosen for this experiment because digestion with relatively inexpensive restriction enzymes will cut it either once (*Hin*dIII), or twice (*Bam*HI or *Eco*RI) (see Figure 2). For plasmid isolation, the culture should be grown overnight in nutrient broth medium containing ampicillin (*amp*) with vigorous shaking or aeration at 37° C. Cells can be lysed and plasmid DNA isolated by a simple boiling method, previously described in this journal (Schmidt & Davis 1994). Alternatively, plasmid preparation kits can be purchased at reasonable cost from supply companies.

On Day 1, prior to class, the restriction enzymes to be used, *Eco*RI and *Hin*dIII, should be diluted to 2 units/μl (in 1X reaction buffer). Restriction enzyme concentration (units/μl) will be indicated on the tube. Students should use 4 units (2 μl) of enzyme per 10 μl reaction. In addition, each class set will require 50 μl of blue loading buffer mixed with 50 μl of undiluted SG (1:1). The mixture should be stored in a microfuge tube in a dark refrigerator. All materials must be kept on ice to avoid activation of the enzyme(s) and to maintain their stability.

When used in the classrooms, students should add the reagents to microfuge tubes, keeping them on ice. The mixture should then be transferred to a 37° C water bath and incubated for 30–60 minutes. The restriction enzyme is activated by the temperature shift and the reaction is terminated by the addition of the blue loading buffer/SG mixture.

Class Materials

- Chipped ice
- Ice bucket (A Styrofoam™ container can be used.)
- Restriction enzymes *Eco*RI and *Hin*dIII
- Reaction buffers for each enzyme
- Blue Loading Buffer:
  0.25 g Bromophenol blue
  40 g sucrose
  Fill to 100 ml with distilled water.
- SG
- Plasmid pCAT19 DNA
- Microfuge, or standard centrifuge with adapters for microfuge tubes
- 1.5-ml microfuge tubes
- Micropipettors and tips, or microcapillary tubes
- 37° C water bath

Class Procedure

1. Add, in order, to a microfuge tube on ice:

	undigested DNA	*Hin*dIII digestion	*Eco*RI digestion
1. Plasmid DNA	7 μl	7 μl	7 μl
2. 10X Reaction Buffer	0 μl	1 μl	1 μl
3. *Eco*RI restriction enzyme	0 μl	2 μl	0 μl
4. *Hin*dIII restriction enzyme	0 μl	0 μl	2 μl
5. Sterile distilled water	3 μl	0 μl	0 μl
Total	10 μl	10 μl	10 μl

2. Finger-flick the tube briefly to mix and either gently tap the tube or "pop-spin" (turn on-off) in the microfuge or centrifuge to collect the solution at the bottom of the tube.
3. Incubate the tube in a 37° C water bath for 30–60 minutes (floating racks can be made by punching holes in Styrofoam™ food trays).
4. Add 3 μl of loading buffer + SG to each reaction mixture to stop the restriction enzyme activity and to stain the DNA.
5. Finger-flick and tap the microfuge tube to collect the solution at the bottom of the tube.
6. Store the tubes in the refrigerator

or freezer until the next class period.

Electrophoresis of Restricted Plasmid DNA

The SG pre-stained DNA prepared during the preceding class period will be ready to be loaded in the agarose gel. As the electrophoresis proceeds, students will be able to observe the migration of the DNA bands through the gel.

Class Materials

- DC power supply
- Gel electrophoresis box, tray and comb (A commercial box should be used for safety and convenience.)
- Pan balance
- 50X TAE Buffer (can also be purchased from many supply houses):
  242 g Tris base
  57.1 ml glacial acetic acid
  100 ml 0.5M EDTA pH 8
  Bring up to 1 L with distilled water. TAE buffer should be diluted 1X concentration for experimentation.
- 0.5 M EDTA, pH 8:
  18.6 g disodium ethylene diamine tetraacetate (EDTA)
  80 ml distilled water
  2 g NaOH solid pellets
- Agarose
- Laboratory tape/masking tape
- Micropipettors and tips (or microcapillary tubes)
- A hand-held UV lamp, if available

Procedure

Agarose Gel Solution Preparation

The agarose gel should contain 0.8% weight/volume (0.8g agarose/100 ml buffer) and be about 5-mm thick. To make the agarose gel:

1. Weigh out agarose powder on a pan balance and add to the appropriate volume of 1X TAE buffer in an Erlenmeyer flask. Note: Do *NOT use water; it will cause im*proper distribution of the electric field during electrophoresis.
2. Microwave on HIGH for 2 minutes or heat the mixture in a pan of boiling water until all of the agarose granules are in solution (swirl the melted agarose to make sure that no granules can be seen).
3. Allow mixture to cool on a nonconducting surface until it is just warm to touch. Then pour into gel tray.

Casting the Gel

1. Seal the gel tray with laboratory or masking tape, unless a self-sealing tray is used.
2. Slowly pour the melted agarose into the gel tray. Avoid bubbles! If bubbles form, break them by touching with a pipet tip (or move them to the side of the tray).
3. Insert the well comb as soon as the gel is poured, before it starts to set. Allow the gel to stand for 10–15 minutes until hard. The gel will appear milky blue.
4. Remove the tape and insert the gel into the electrophoresis unit, with the wells closest to the negative electrode. (Gels can be made before class and stored wrapped securely in plastic wrap in the refrigerator for several weeks. The comb should be removed as described in 2, below).

To Electrophorese the DNA

1. Fill the electrophoresis unit with 1X TAE buffer so that there is about 1 mm of buffer over the gel.
2. Gently remove the comb from the gel while it is submerged in the buffer solution. Be careful to avoid damaging the wells.
3. Slowly load each sample (8 µl) into a well. Support your hand as you load the samples, being careful not to damage the bottom or edges of wells. The sample will displace the buffer. Be sure to record the location of each sample.
4. Connect the electrodes to the unit and apply 100 volts of current.
5. Darken the room and start viewing the DNA bands as they migrate out of the well and separate based on molecular weight. A typical run in a 10-cm long gel will take about one hour.

Notes for the Instructor

Gel Tray

Because maximum contrast enhances the viewing of the SG-stained DNA, unless the gel box has a black background, black contact paper can be applied to the surface of the gel tray (it stays attached for many uses), or black velvet or felt can be placed under the gel box.

Antibiotics

Either ampicillin or chloramphenicol can be used in the growth medium to select for the pCAT19 plasmid, but ampicillin is more readily available and easier to use.

Ampicillin stock solution is prepared by adding 0.25 g of ampicillin to 10 ml of sterile distilled water. The solution should be mixed well and stored in the refrigerator. Four ml of the ampicillin stock solution should be added per liter to sterile broth after it is cool and to nutrient agar just before pouring plates. Medium containing ampicillin is stable for up to six months if it is kept refrigerated.

Chloramphenicol stock solution is prepared by adding 0.1 g of chloramphenicol to 10 ml of 95% ethanol. The solution should be mixed well and stored in the freezer. Two ml of the chloramphenicol stock solution should be added per liter of broth or agar. Medium containing chloramphenicol is stable for a period of several months if it is kept refrigerated

Plasmid Preparation

Plasmids can be prepared as described by Schmidt & Davis (1994) or a commercial kit can be used.

Restriction Enzymes

Restriction enzymes become inactivated if they are not kept frozen or on ice. They should not be allowed to warm up on the benchtop or in someone's hands. Restriction enzymes are generally stored in glycerol, which makes the solution viscous.

SG

SG is sensitive to heat and light. Therefore, it should be kept frozen in the dark in the original package. Under these conditions, it is stable for a year or longer. SG should be thawed completely before being dispensed.

At the concentrations recommended here, SG retards DNA migration (Berg et al. 1996). If a hand-held UV lamp is available, the level of SG used can be reduced to one part SG to nine parts of blue loading buffer. Be sure to use eye protection if using UV illumination.

Electrophoresis Band Patterns

When isolated from cells, plasmid DNA is circular and supercoiled, twisted on itself like an over-twisted phone cord. The quick plasmid preparation method suggested here does some damage to the DNA, introducing single-stranded nicks into some molecules. Therefore, when undigested plasmid DNA is electrophoresed, two bands are usually seen: the faster supercoiled band and the slower nicked band. Supercoiled DNA migrates faster than nicked DNA because it is more compact and can move through the agarose matrix more rapidly.

DNA that is linearized by digestion at a single site will migrate at a rate that is intermediate between the supercoiled and nicked bands, while smaller

fragments will migrate at faster rates, depending on fragment size.

If more bands than expected are found, they are due to partial digestion, which occurs when there is too little active restriction enzyme to digest all of the sites in the incubation time used (due to partially inactivated enzyme and/or an excess of DNA). For example, partial digestion by *Hin*dIII (single site) could give two extra bands (supercoiled and nicked circular DNA), while partial digestion by *Eco*RI or *Bam*HI (two sites each) could give three extra bands (supercoiled and nicked circular DNA, and singly cut linear DNA). Poor digestion could be due to many causes; among the most likely are inactive enzymes due to improper storage or use of the wrong buffer in the reaction mixture. Note that a reaction buffer from one manufacturer should not be used with the enzyme from another because each manufacturer optimizes the buffer salt concentrations in relation to the salt concentrations in the enzyme solution.

pCAT19

This experiment involves pCAT19 because this plasmid can be digested with inexpensive restriction enzymes that cut either once or twice (Figure 2).

The experiment could be adapted to use with another plasmid, such as pUC19, but it should be noted that most small plasmids which are used for cloning DNA contain only single restriction sites for the inexpensive, easy-to-use enzymes, and that these sites are clustered.

The pCAT19 plasmid is also amenable to use in more advanced teaching laboratories. Analysis of a *Bam*HI/*Eco*RI or *Eco*RI/*Hin*dIII double digestion can teach students how to build restriction maps. Double digestion with *Bam*HI and *Hin*dIII would yield only two fragments because the *Bam*HI and *Hin*dIII sites at 5 o'clock on the map (Figure 2) are separated by only a few bases. Double digestion with *Eco*RI and either *Bam*HI or *Hin*dIII will yield three fragments, permitting the second *Eco*RI site to be mapped to a site within the *cam* gene.

Because the *cam* gene is bracketed by *Bam*HI sites (Figure 2), pCAT19 can also be used to clone the chloramphenicol gene into another plasmid.

Sources of Supplies & Equipment

Electrophoresis equipment, agarose, plasmid preparation kits, restriction enzymes, and ampicillin can be purchased from Ward's Biology (800-962-2660). These items can also be obtained from other supply companies such as Carolina Biological Supply Co. (800-334-5551), Edvotek (800-338-6835), Sigma Chemical Company (800-325-3010), Life Technologies (800-828-6686), New England Biolabs (800-632-5227) or Stratagene (800-424-5444). Free catalogs are available on request.

SG can be obtained from the manufacturer, Molecular Probes, Inc. (800-438-2209), or from FMC Corporation (800-341-1574).

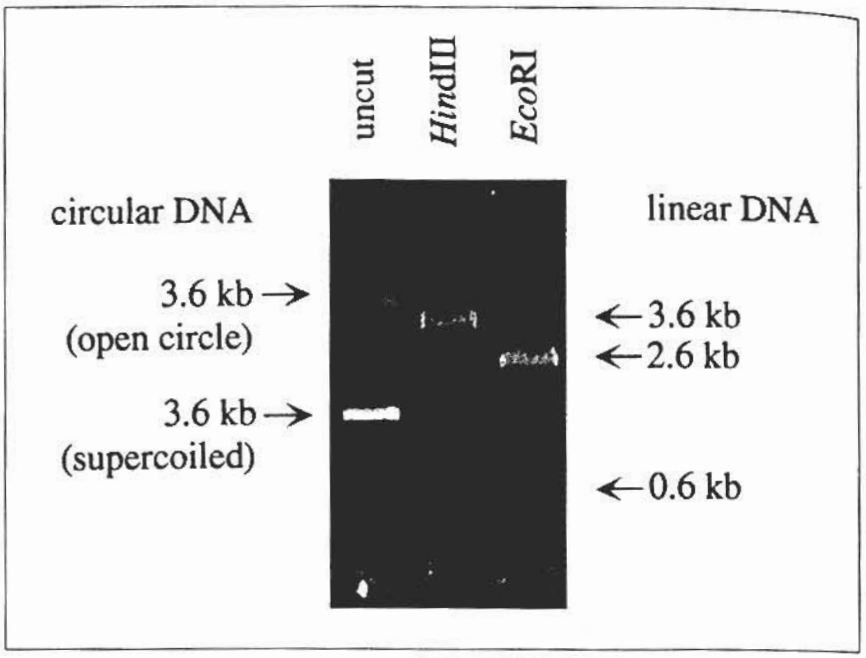

Fig. 3. Photograph of pCAT19 DNA bands after agarose gel electrophoresis. Lane 1 contains undigested DNA, Lane 2 contains *Hin*dIII-digested DNA, and Lane 3 contains *Eco*RI-digested DNA.

Conclusions

This experiment permits the students to view undigested and restriction enzyme-digested DNA during the course of DNA migration in the gel. pCAT19 is good for illustrating the use of restriction enzymes for digesting DNA because it is a small plasmid that can be cut by inexpensive restriction enzymes to yield one or two fragments. In addition, pCAT19 is present in many copies in each bacterial cell, making it easy to isolate plasmid DNA. The prime benefits of using the SG staining system described here, rather than ethidium bromide or methylene blue, are time-efficiency and safety. No post-staining or de-staining steps are required, and potential hazards to the student are greatly reduced. Using SG, the students obtain immediate results from their efforts and learn a great deal about common procedures in biotechnology.

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