Purification and Identification of Plasmid DNA

GROWTH OF *E. COLI* ON AMPICILLIN PLATES demonstrates transformation to an antibiotic-resistant phenotype. In the basic version of Laboratory 5, the observed phenotype was due to uptake of plasmid pAMP, a DNA molecule that is well-characterized.

In experimental situations where numerous recombinant plasmids are generated by joining two or more DNA fragments, the antibiotic resistance marker only functions to indicate which cells have taken up a plasmid bearing the resistance gene. It does not indicate anything about the structure of the new plasmid. Therefore, it is standard procedure to isolate plasmid DNA from transformed cells and to identify the molecular genotype using DNA restriction analysis. In cases where the recombinant molecules are formed by combining well-characterized fragments, restriction analysis is sufficient to confirm the structure of a hybrid plasmid. In other cases, the nucleotide sequence of the insert must be determined. This protocol is divided into two parts: Plasmid Minipreparation of pAMP and Restriction Analysis of Purified pAMP.

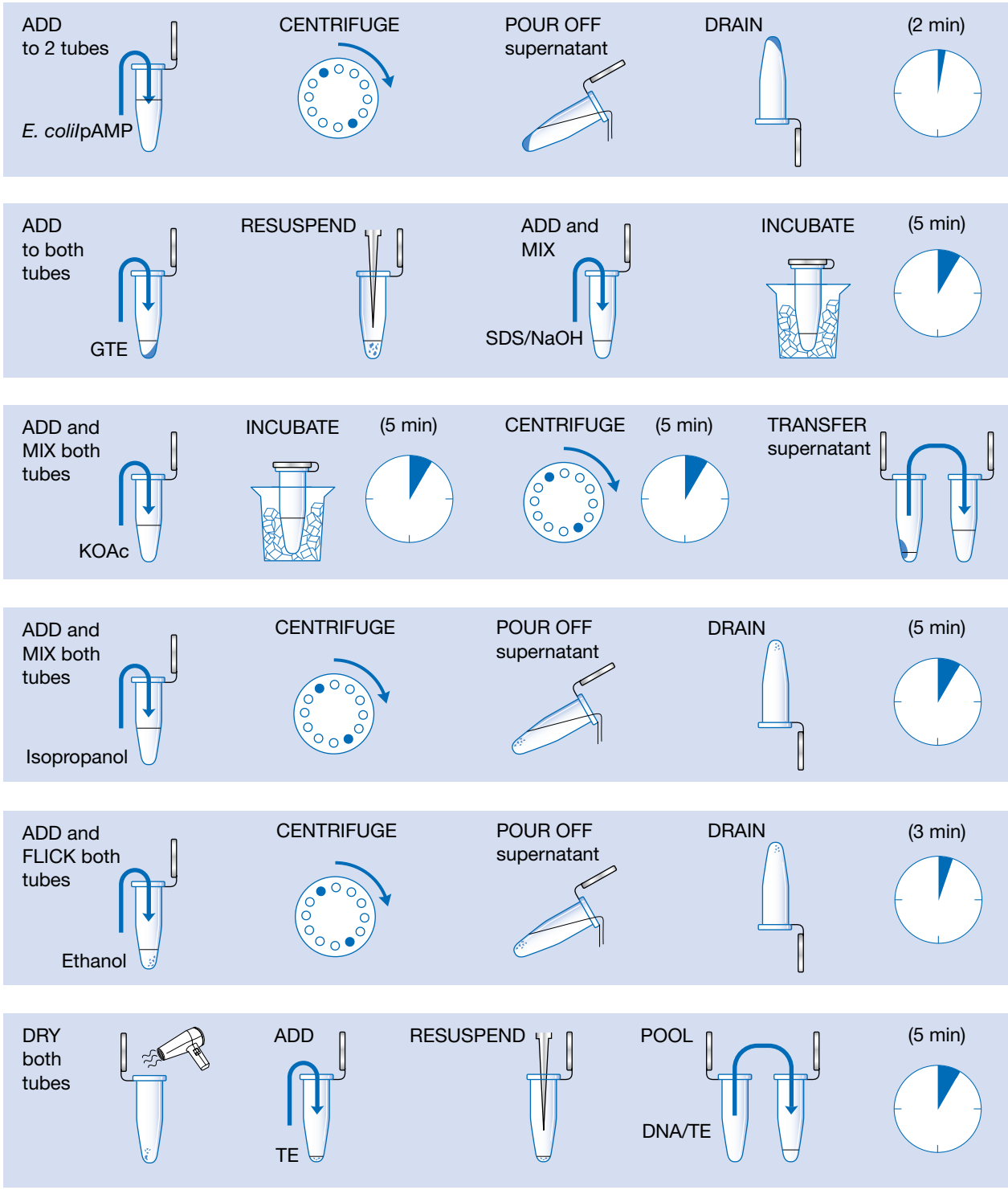
- Part A provides a small-scale protocol to purify from transformed *E. coli* enough plasmid DNA for restriction analysis. Cells taken from an ampicillin-resistant colony are grown to stationary phase in suspension culture. The cells from 1 ml of culture are harvested and lysed, and plasmid DNA is separated from the cellular proteins, lipids, and chromosomal DNA. This procedure yields 2–5 μg of relatively crude plasmid DNA, in contrast to large-scale preparations that yield 1 mg or more of pure plasmid DNA from a 1-liter culture.
- Part B provides a protocol using a sample of plasmid DNA isolated in Part A and a control sample of pAMP. These two samples are cut with the restriction enzymes *Bam*HI and *Hin*dIII and coelectrophoresed on an agarose gel, and the restriction patterns are stained and visualized. The purified DNA is shown to have a restriction "fingerprint" identical to that of pAMP. *Bam/Hin*d restriction fragments of the miniprep DNA comigrate with the 784-bp and 3755-bp *Bam/Hin*d fragments of pAMP. This provides genotypic proof that pAMP molecules were successively transformed into *E. coli* in Laboratory 5.

Kits based on this laboratory are available from the Carolina Biological Supply Company.

- Catalog no. 21-1200 (with ethidium bromide stain)
- Catalog no. 21-1205 (with the *Carolina*Blu™ stain)

PART A

Plasmid Minipreparation of pAMP



PRELAB NOTES

Optimally, minipreps should be done on cells that have been recently manipulated for transformation. This completes a conceptual stream that firmly cements the genotype-phenotype relationship. Alternatively, use streaked plates of transformed *E. coli* to prepare overnight cultures.

Plasmid Selection

pAMP gives superior yields on minipreps compared to pBR322. A derivative of a pUC expression vector, pAMP is highly amplified—more than 100 copies are present per *E. coli* cell. If substituting a different plasmid for miniprep purposes, select a commercially available member of the pUC family, such as pUC18 or pUC19.

Centrifuge Requirements

A microfuge that generates approximately 12,000 times the force of gravity (12,000*g*) is needed for efficient and rapid purification of plasmid DNA. A slower-spinning clinical or preparatory centrifuge cannot be substituted.

Supplies

Sterile supplies are not required for this protocol. Standard 1-ml pipettes, transfer pipettes, and/or microcapillary pipettes can be used instead of micropipettors. Use good-quality, colorless 1.5-ml tubes, beginning with Step 11. The walls of poor-quality tubes, especially colored tubes, often contain tiny air bubbles that can be mistaken for ethanol droplets in Step 19. We have observed students drying DNA pellets for 15 minutes or more, trying to rid their tubes of these phantom droplets. (Typical drying time is actually several minutes.)

Fine Points of Technique

Be careful not to overmix reagents; excessive manipulation shears chromosomal DNA. The success of this protocol in large part depends on maintaining chromosomal DNA in large pieces that can be differentially separated from intact plasmid DNA. Mechanical shearing increases the amount of short-sequence chromosomal DNA, which is not removed in the purification of plasmid DNA. Make sure that the microfuge will be immediately available for Step 13. If sharing a microfuge, coordinate with other experimenters to begin Steps 12 and 13 together.

For Further Information

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

Birnboim H.C. and Doly J. 1979. A rapid alkaline extraction method for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7:** 1513–1523.

Ish-Horowicz D. and Burke J.F. 1981. Rapid and efficient cosmid cloning. *Nucleic Acids Res.* **9:** 2989–2998.

PRELAB PREPARATION

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

1. This protocol is designed to follow Laboratory 5. Ideally, students should pick colonies from their own transformed plates to begin this experiment. However, the colonies must grow overnight or for at least several hours, so unless the class meets on consecutive days, it may be necessary for the instructor to set up the cultures used in this laboratory. In any case, on the day before the laboratory, prepare an *E. coli* culture according to the protocol in Laboratory 2B, Overnight Suspension Culture. Inoculate the culture with a cell mass scraped from one colony selected from the +LB/amp plate from Laboratory 5, Rapid Colony Transformation of *E. coli* with Plasmid DNA. Maintain antibiotic selection with LB broth plus ampicillin. Alternatively, prepare the culture 2–3 days in advance and store at 4°C or incubate for 24–48 hours at 37°C without shaking. In either case, the cells will settle at the bottom of the culture tube. Shake the tube to resuspend cells before beginning procedure.
2. The SDS/sodium hydroxide solution should be fresh; prepare this solution within a few days of lab. Store solution at room temperature; a soapy precipitate may form at lower temperature. If a precipitate forms, warm the solution by placing the tube in a beaker of hot tap water, and shake gently to dissolve the precipitate.
3. Prepare aliquots for each experiment:

   250 µl of glucose/Tris/EDTA (GTE) solution (store on ice)
   500 µl of SDS/sodium hydroxide (SDS/NaOH) solution
   400 µl of potassium acetate/acetic acid (KOAc) solution (store on ice)
   1000 µl of isopropanol
   500 µl of 95% ethanol
   50 µl of Tris/EDTA (TE) solution
4. Review Part B, Restriction Analysis of Purified pAMP.

MATERIALS

CULTURES AND MEDIA

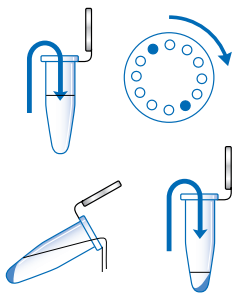
E. coli/pAMP overnight culture
Ethanol (95–100%)▼
Glucose/Tris▼/EDTA (GTE)
Isopropanol▼
Potassium acetate/acetic acid▼ (KOAc)
SDS/sodium hydroxide (SDS/NaOH)▼
Tris▼/EDTA (TE)

SUPPLIES AND EQUIPMENT

Beakers for crushed ice and for waste/used tips
Bleach▼(10%) or disinfectant
Clean paper towels
Hair dryer
Microfuge
Micropipettor (100–1000 µl and 0.5–10 µl) + tips
Permanent marker
Test tube rack
Tubes (1.5-ml)

▼ See Appendix 4 for Cautions list.

METHODS



The cell pellet will appear as a small off-white smear on the bottom-side of the tube. Although the cell pellets are readily seen, the DNA pellets in Step 14 are very difficult to observe. Make a habit of aligning the tube with the cap hinges facing outward in the microfuge rotor. Then, pellets should always be located at the tube bottom beneath the hinge.

Accurate pipetting is essential to good plasmid yield. The volumes of reagents are precisely calibrated so that the sodium hydroxide added in Step 6 is neutralized by acetic acid in Step 8.

Prepare Duplicate Minipreps (50 minutes)

The instructions below are for making duplicate minipreps, which provide balance in the microfuge and insurance if a critical mistake is made.

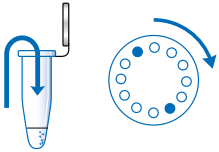
1. Shake culture tube to resuspend *E. coli* cells.
2. Label two 1.5-ml tubes with your initials. Use a micropipettor to transfer 1000 µl of *E. coli*/pAMP overnight suspension into each tube.
3. Close caps, and place the tubes in a *balanced* configuration in the microfuge rotor. Spin for 1 minute to pellet cells.
4. Pour off supernatant from both tubes into a waste beaker for later disinfection. Alternatively, use a micropipettor to remove supernatant. *Be careful not to disturb the cell pellets.* Invert the tubes, and tap gently on the surface of a clean paper towel to drain thoroughly.
5. Add 100 µl of ice-cold GTE solution to each tube. Resuspend the pellets by pipetting the solution in and out several times. Hold the tubes up to the light to check that the suspension is homogeneous and that no visible clumps of cells remain.
6. Add 200 µl of SDS/NaOH solution to each tube. Close caps, and mix solutions by rapidly inverting tubes five times.
7. Stand tubes on ice for 5 minutes. Suspension will become relatively clear.
8. Add 150 µl of *ice-cold* KOAc solution to each tube. Close caps, and mix solutions by rapidly inverting tubes five times. A white precipitate will immediately appear.

Step 11, the supernatant is ved and precipitate is dis- rded. The situation is rever- d in Steps 14 and 17, where e precipitate is saved and e supernatant is discarded.

o Step 12 quickly, and make re that the microfuge will e immediately available for ep 13.

e pellet may appear as a y smear or small particles the bottom-side of each be. Do not be concerned if llet is not visible; pellet size not a predictor of plasmid eld. A large pellet is com- sed primarily of RNA, and llular debris carried over om the original precipitate. smaller pellet often means cleaner preparation.

ucleic acid pellets are not sol- ble in ethanol and will not suspend during washing.



12. Add 400 µl of isopropanol to each tube of supernatant. Close caps, and mix vigorously by rapidly inverting tubes five times. *Stand at room temperature for only 2 minutes.* (Isopropanol preferentially precipitates nucleic acids rapidly; however, proteins remaining in solution also begin to precipitate with time.)

13. Place tubes in a *balanced* configuration in the microfuge rotor, and spin for 5 minutes to pellet the nucleic acids. Align tubes in rotor so that cap hinges point outward. The nucleic acid residue, visible or not, will collect on the tube side under the hinge during centrifugation.

14. Pour off supernatant from both tubes. *Be careful not to disturb nucleic acid pellets*. Alternatively, remove the supernatant with a 1000-ml micropipettor. Place tip away from the pellet. If you are concerned that the pellet has been drawn up in the tip, transfer the supernatant to another 1.5-ml tube, recentrifuge, and remove the supernatant again. Invert tubes, and tap gently on the surface of a clean paper towel to drain thoroughly.

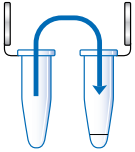
15. Add 200 µl of 100% ethanol to each tube, and close caps. Flick tubes several times to wash pellets.

STOP Store DNA in ethanol at –20°C until ready to continue.

16. Place tubes in a *balanced* configuration in microfuge rotor, and spin for 2–3 minutes.

17. Pour off supernatant from both tubes. *Be careful not to disturb nucleic acid pellets.* Alternatively, remove the supernatant with a 1000-ml micropipettor. Place tip away from the pellet. If you are concerned that the pellet has been drawn up in the tip, transfer the supernatant to another 1.5-ml tube, recentrifuge, and remove the supernatant again. Invert tubes, and tap gently on the surface of a clean paper towel to drain thoroughly.

18. Dry nucleic acid pellets by one of the following methods:



21. Pool DNA/TE solution into one tube.

STOP Freeze DNA/TE solution at –20°C until ready to continue. Thaw before using

22. Take time for responsible cleanup.

a. Segregate for proper disposal culture tubes and micropipettor tips tha have come in contact with *E. coli*.

b. Disinfect overnight culture, tips, and supernatant from Step 4 with 10% bleach or disinfectant.

c. Wipe down lab bench with soapy water, 10% bleach solution, or disin fectant (such as Lysol).

d. Wash hands before leaving lab.

RESULTS AND DISCUSSION

The minipreparation is a simple and efficient procedure for isolating plasmi DNA. Become familiar with the molecular and biochemical effects of eac reagent used in the protocol.

- *Glucose//Tris/EDTA:* The Tris buffers the cells at pH 7.9. EDTA binds divalen cations in the lipid bilayer, thus weakening the cell envelope.
- *SDS/sodium hydroxide:* This alkaline mixture lyses the bacterial cells. The dete gent SDS dissolves the lipid components of the cell membrane, as well as ce lular proteins. The sodium hydroxide denatures the chromosomal and pla mid DNA into single strands. The intact circles of plasmid DNA remain inte twined.
- *Potassium acetate/acetic acid:* The acetic acid returns the pH to neutral, allowin DNA strands to renature. The large, disrupted chromosomal strands cann rehybridize perfectly, but instead collapse into a partially hybridized tangle. A the same time, the potassium acetate precipitates the SDS (which is insoluab

a higher vapor point than does ethanol. The ethanol-isopropanol evaporates more rapidly in the drying step.

- *Tris/EDTA:* Tris buffers the DNA solution. EDTA protects the DNA from degradation by DNases by binding divalent cations that are necessary cofactors for DNase activity. Buffering DNA is important, as low pH (<6) leads to the loss of purines (adenine and guanine) called depurination. The purines are actually cleaved from their sugars, creating an abasic site. Purine cleavage is a very common occurrence in cells (on the order of 10^5 times per cell per day) and is repaired by specific DNA repair systems. Of course, your DNA is in a tube and there is no DNA repair system present to repair it. Keep in mind that H_2O can have a pH as low as 5.

1. Consider the three major classes of biologically important molecules: proteins, lipids, and nucleic acids. Which steps of the miniprep procedure act on proteins? On lipids? On nucleic acids?
2. What aspect of the plasmid DNA structure allows it to renature efficiently in Step 8?
3. What other kinds of molecules, in addition to plasmid DNA, would you expect to be present in the final miniprep sample? How could you find out?

FOR FURTHER RESEARCH

Determine the approximate mass of plasmid DNA you isolated per milliliter of cells.

1. Set up 20-µl *Hin*dIII restriction reactions using 15 µl of your pAMP preparation and a known mass of λ DNA as a control.
2. Make 1:10, 1:50, and 1:100 dilutions of the digested pAMP and λ DNAs.
3. Separate by electrophoresis equal volumes of each dilution in an agarose gel, and stain with ethidium bromide.

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

4. Identify a lane of the λ digest where the 4361-bp fragment is *just* visible, and identify a lane of pAMP (4539 bp) that is of equal intensity. These bands should have a nearly equivalent mass of DNA.
5. Determine the mass of λ DNA in the selected fragment, using the formula below. Make sure to account for the dilution factor.

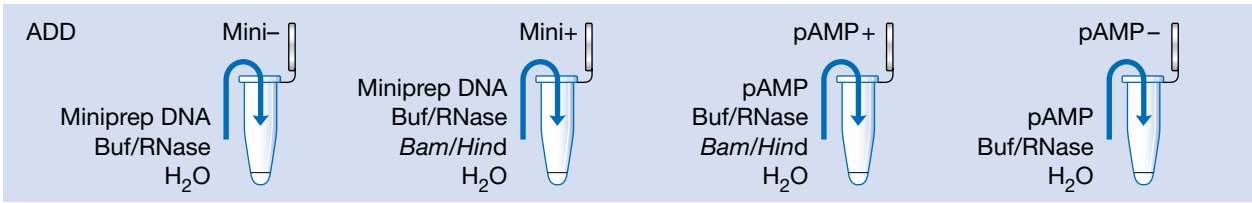
$$\frac{\text{fragment bp (conc. DNA) (vol. DNA)}}{\lambda \text{ bp}}$$

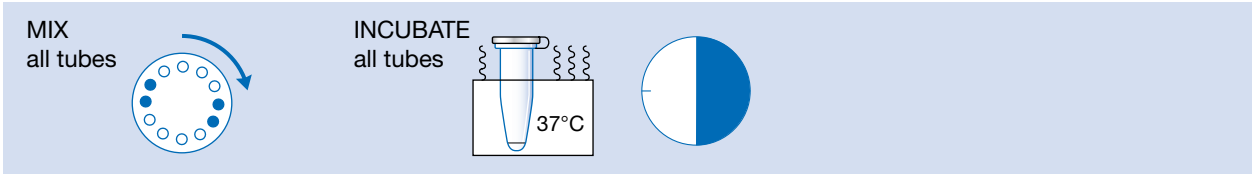
6. Multiply the mass from Step 5 by the dilution factor of the selected pAMP lane.

PART B

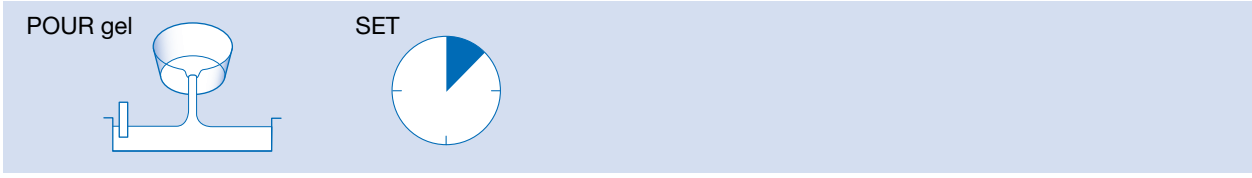
Restriction Analysis of Purified pAMP

I. Set Up Restriction Digest

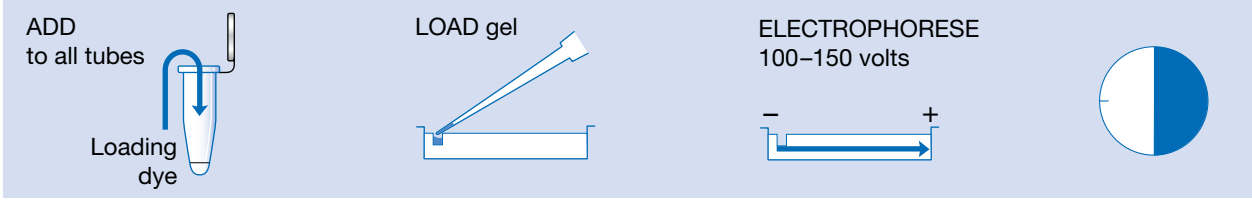




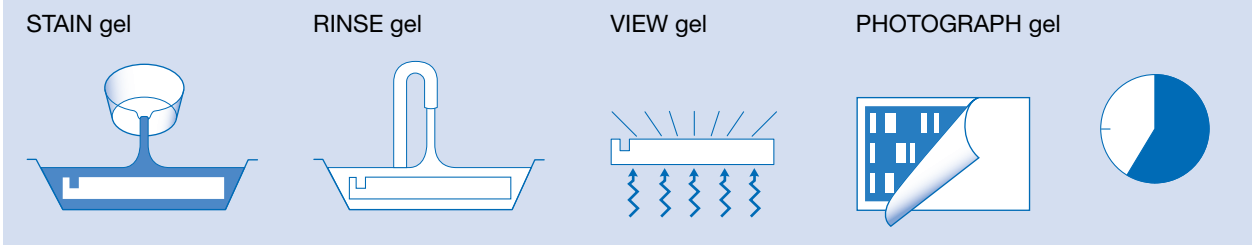
II. Cast 0.8% Agarose Gel



III. Load Gel and Separate by Electrophoresis



IV. Stain Gel and View (Photograph)



PRELAB NOTES

Review Prelab Notes in Laboratory 3, DNA Restriction Analysis.

Limiting DNase Activity

Unlike highly purified plasmid DNA available from commercial vendors, miniprep DNA is impure. A significant percentage of nucleic acid in the preparation is, in fact, RNA and fragmented chromosomal DNA. Typically, miniprep DNA is contaminated with nucleases (DNases) that cleave DNA into small fragments. Residual DNases will degrade plasmid DNA if minipreps are left for long periods of time at room temperature or even on ice. For this reason, store minipreps at –20°C, and thaw just prior to use.

The situation is further complicated during restriction digestion. DNases and restriction endonucleases both require divalent cations, such as Mg^{++}. Included in TE buffer at a low concentration of 1 mM, Na_2EDTA chelates (binds) divalent cations at a ratio of 2 cations/Na_2EDTA. Thus, a 1 mM solution can chelate about 2 mM of divalent cation. Although some divalent cations may remain free, we are limited to how much Na_2EDTA can be added because higher concentrations would chelate the Mg^{++} necessary for restriction enzyme activity. A balance is thus struck at an EDTA concentration that inhibits most of the contaminating DNases without significantly reducing the activity of the restriction enzymes.

Another balance must be struck. On the one hand, contaminants in the miniprep limit restriction enzyme activity—a 20-minute incubation is not usually sufficient for complete digestion. On the other hand, DNases are activated by Mg^{++} in the restriction buffer and will significantly degrade plasmid DNA if the restriction reaction is incubated too long. Experience has shown that a 30-minute incubation gives optimal results.

RNase

Miniprep DNA is contaminated by large amounts of ribosomal RNA and smaller amounts of messenger RNA and transfer RNA. If not removed from the preparation, this RNA will obscure the DNA bands in the agarose gel. Therefore, RNase is added to the restriction digest; during incubation, the RNase digests RNA into very small fragments (less than 100 nucleotides). These RNA fragments run well ahead of the DNA fragments of interest or are so small that they do not stain.

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.

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. Mix in 1:1 proportion: *Bam*HI + *Hin*dIII (6 µl per experiment). Keep on ice.
2. Prepare aliquots for each experiment:

   12 µl of 0.1 µg/µl pAMP (store on ice)
   12 µl of 5x restriction buffer/RNase (store on ice)
   6 µl of *Bam*HI/*Hin*dIII (store on ice)
   500 µl of distilled water
   500 µl of loading dye

   If another plasmid was substituted for pAMP in the transformation, use that plasmid as a control in the restriction digest.
3. Prepare 0.8% agarose solution (~40–50 ml per experiment). Keep agarose liquid in a hot-water bath (at ~60°C) throughout the experiment. Cover the solution 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), or other proprietary stain.
6. Adjust water bath to 37°C.

MATERIALS

REAGENTS

Agarose (0.8%)
*Bam*HI/*Hin*dIII (50:50 mix)
Distilled water
Ethidium bromide▼(1 µg/ml) (or 0.025% methylene blue▼)
Loading dye
Miniprep DNA
pAMP (0.1 µg/µl)
5x Restriction buffer/RNase
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)

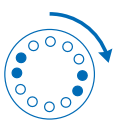
▼ See Appendix 4 for Cautions list.

Mini– = miniprep, no enzymes
Mini+ = miniprep + *Bam*HI/*Hin*dIII
pAMP+ = pAMP + *Bam*HI/*Hin*dIII
pAMP– = pAMP, no enzymes

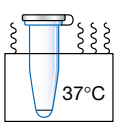
2. Use the matrix below as a checklist while adding reagents to each reaction. Read down each column, adding the same reagent to all appropriate tubes. *Use a fresh tip for each reagent.* Refer to detailed directions that follow.

Tube	Miniprep DNA	pAMP	Buffer/ RNase	*Bam*HI/ *Hin*dIII	H_2O
Mini–	5 µl	—	2 µl	—	3 µl
Mini+	5 µl	—	2 µl	2 µl	1 µl
pAMP+	—	5 µl	2 µl	2 µl	1 µl
pAMP–	—	5 µl	2 µl	—	3 µl

3. Collect reagents, and place in test tube rack on lab bench (*Bam*HI/*Hin*dIII on ice).
4. Add 5 µl of miniprep DNA to tubes labeled Mini– and Mini+.
5. Use a *fresh tip* to add 5 µl of pAMP to tubes labeled pAMP+ and pAMP–.
6. Use a *fresh tip* to add 2 µl of restriction buffer/RNase to a clean spot on each reaction tube.



7. Use a *fresh tip* to add 2 µl of *Bam*HI/*Hin*dIII to tubes labeled Mini+ and pAMP+.
8. Use a *fresh tip* to add the proper volumes of distilled water to each tube.
9. Close tube tops. Pool and mix reagents by pulsing in a microfuge or by sharply tapping the tube bottom on the lab bench.



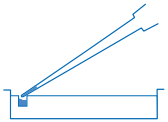
10. Place reaction tubes in a 37°C water bath, and incubate for 30 minutes only.

o not overincubate. During nger incubation, DNases in he miniprep may degrade lasmid DNA.

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

buffer remaining in electrophoresis box from a previous experiment, rock chamber back and forth to remix ions that have accumulated at either end.

Buffer solution helps to lubricate comb. Some gel boxes are designed such that the comb must be removed prior to inserting casting tray into box. In this case, flood casting tray and gel surface with running buffer before removing comb. Combs removed from a dry gel can cause tearing of wells.



A piece of dark construction paper beneath the gel box will make the wells more visible.

6. Gently remove comb, taking care not to rip wells.
7. Make certain that sample wells left by the comb are completely submerge If "dimples" appear around the wells, slowly add buffer until they disappea

STOP Cover the electrophoresis tank and save the gel until ready to continue. Ge will remain in good condition for at least several days if it is completely sub merged in buffer.

III. Load Gel and Separate by Electrophoresis (30–50 minute

1. Add loading dye to each reaction. Either
   a. Add 1 µl of loading dye to each reaction tube. Close tube tops, and m by tapping the tube bottom on the lab bench, pipetting in and out, pulsing in a microfuge. Make sure that the tubes are placed in a *balanc* configuration in the rotor.

      or

   b. Place four individual droplets of loading dye (1 µl each) on a small squa of Parafilm or wax paper. Withdraw contents from reaction tube, and m with a loading dye droplet by pipetting in and out. Immediately load dy mixture according to Step 2. Repeat successively, *with a clean tip*, for eac reaction.
2. Use a micropipettor to load 10 µl of each reaction tube into a separate we in the gel, as shown on the following page. Use a *fresh tip* for each reaction
   a. Before loading sample, make sure that there are no bubbles in the well If bubbles exist, remove them with a micropipettor tip.
   b. Use two hands to steady the micropipettor over the well.
   c. If there is air in the end of the tip, carefully depress the plunger to push th sample to the end of the tip. (If an air bubble forms a "cap" over the we DNA/loading dye will flow into the buffer around the edges of the well.

lternatively, set power pply on lower voltage, nd run gel for several ours. When running two ls from the same power pply, current is double at for a single gel at the me voltage.

he Bam*HI*/Hin*dIII digest elds two bands containing nall fragments of 784 bp nd 3755 bp, which are sily resolved during a ort electrophoresis run. he 784-bp fragment runs rectly behind the purplish and of bromophenol blue quivalent to ~300 bp), hereas the 3755-bp frag- ent runs in front of the qua band of xylene cyanol quivalent to ~9000 bp).*

aining may be performed an instructor in a con- olled area when students e not present.

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 20–40 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 the bromophenol blue band runs off the end of the gel.
6. Turn off power supply, disconnect leads from the inputs, and remove the top of the electrophoresis box.
7. Carefully remove the casting tray from the electrophoresis box, and slide the gel into a disposable weigh boat or other shallow tray. Label staining tray with your name.

STOP Cover the electrophoresis tank and save the gel until ready to continue. Gel can be stored in a zip-lock plastic bag and refrigerated overnight for viewing/photographing the next day. However, over longer periods of time, the DNA will diffuse through the gel and the bands will become indistinct or disappear entirely.

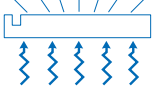
8. Stain and view gel using one of the methods described in Sections IVA and IVB.

IVA. Stain Gel with Ethidium Bromide and View (Photograph)

(10–15 minutes)

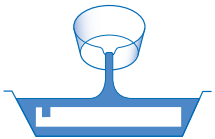
CAUTION

Review Responsible Handling of Ethidium Bromide in Laboratory 3. Wear latex



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.



IVB. Stain Gel with Methylene Blue and View (Photograph)

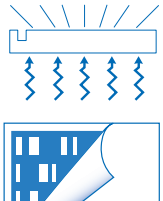
(30+ minute

1. Wear latex gloves during staining and cleanup.
2. Flood the gel with 0.025% methylene blue, and allow to stain for 20–3 minutes.
3. Following staining, use a funnel to decant as much methylene blue solutio as possible from the staining tray back into the storage container.

Destaining time is decreased by rinsing the gel in warm water, with agitation.

4. Rinse the gel in running tap water. Let the gel soak for several minutes i several changes of fresh water. DNA bands will become increasingly distin as gel destains.

STOP For best results, continue to destain overnight in a *small volume* of water. (Ge may destain too much if left overnight in large volume of water.) Cover staining tray to retard evaporation.



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

1. A background "smear" of degraded and partially digested chromosomal DNA, plasmid DNA, and RNA is typically seen running much of the length of the miniprep lanes. The smear is composed of faint bands of virtually every nucleotide length that grade together. A heavy background smear, along with high-molecular-weight DNA at the top of the undigested lane, indicates that the miniprep is contaminated with large amounts of chromosomal DNA.
2. Frequently, undissolved material and high-molecular-weight DNA are seen "trapped" at the front edge of the well. These anomalies are not seen in commercial preparations, where plasmid DNA is separated from degraded nucleic acids by ultracentrifugation in a cesium chloride gradient.
3. A "cloud" of low-molecular-weight RNA is often seen in both the cut and uncut miniprep lanes at a position corresponding to 200 bp or less. Again, variously sized molecules are represented, which are the remnants of larger molecules that have been partially digested by the RNase. However, the majority of RNA is usually digested into fragments that are too small to intercalate the ethidium bromide dye or that migrate off the end of the gel.
4. Only two bands (784 bp and 3755 bp) are expected to be seen in the cut miniprep lane. However, it is common to see one or more faint bands higher up on the gels that comigrate with the uncut plasmid forms described below. Incomplete digestion is usually due to contaminants in the preparation that inhibit restriction enzyme activity or may occur when the miniprep solution contains a very high concentration of plasmid DNA. The plasmid might also be cut at only one site, creating a linear plasmid that will also migrate slower than the 3755-bp band. This is called a partial digest.

It is especially confusing to see several bands in a lane known to contain only uncut plasmid DNA. This occurs because the migration of plasmid DNA in an agarose gel depends on its molecular conformation, as well as its molecular weight (base-pair size). Plasmid DNA exists in one of three major conformations:

- *Form I, supercoiled:* Although a plasmid is usually pictured as an open circle, within the *E. coli* cell (in vivo), the DNA strand is wound around histone-like proteins to make a compact structure. Adding these coils to the coiled DNA helix produces a *super*coiled molecule. The extraction procedure strips proteins from plasmid, causing the molecule to coil about itself. Supercoiling is best demonstrated with a piece of string. Double the string and hold an end in each hand without slack. Now twist the string in one direction. At first, the coils form easily and spread evenly along the length of the string. However, as you add more twists, the string begins to bunch and form knots. If you relax the tension on the string, the string become tangled. This is the equivalent of removing the protein from supercoiled plasmid DNA. Under most gel conditions, the supercoiled plasmid DNA is the fastest-moving form. Its compact molecular shape allows it to move most easily through the agarose matrix. Therefore, the fastest-moving band of uncut plasmid is assumed to be supercoiled.
- *Form II, relaxed or nicked circle:* During DNA replication, the enzyme topoisomerase I introduces a nick into one strand of the DNA helix and rotates the strand to release the torsional strain that holds the molecule in a supercoil.

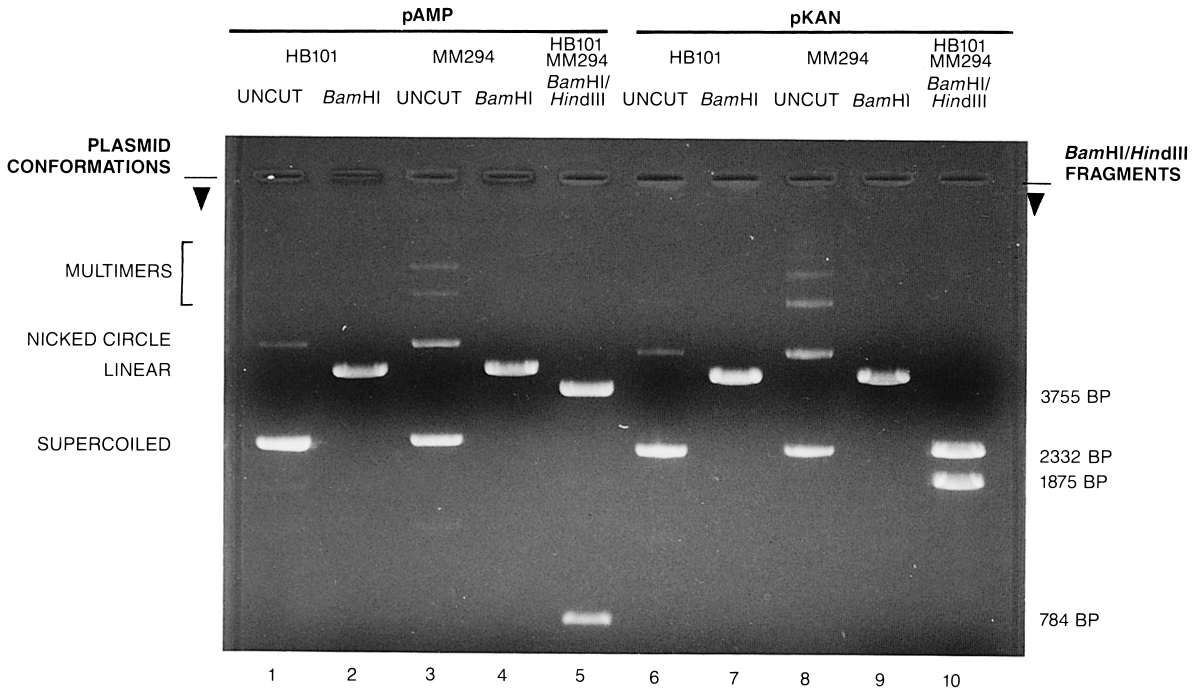
The relaxed section of the DNA uncoils, allowing access to the replicating enzymes. Introducing nicks into supercoiled plasmid DNA produces the open circular structure with which we are familiar. Physical shearing and enzymatic cleavage during plasmid isolation introduce nicks in the supercoiled plasmid DNA. Thus, the percentage of supercoiled plasmid DNA is an indicator of the care with which the DNA is extracted from the *E. coli* cell. The relaxed circle is the slowest-migrating form of plasmid DNA; its "floppy" molecular shape impedes movement through the agarose matrix.

- *Form III, linear:* Linear DNA is produced when a restriction enzyme cuts the plasmid at a single recognition site or when damage results in strand nicks directly opposite each other on the DNA helix. Under most gel conditions, linear plasmid DNA migrates at a rate intermediate between supercoiled and relaxed circle. The presence of linear DNA in a plasmid preparation is a sign of contamination with nucleases or of sloppy lab procedure (overmixing or mismeasuring SDS/NaOH and KOAc).

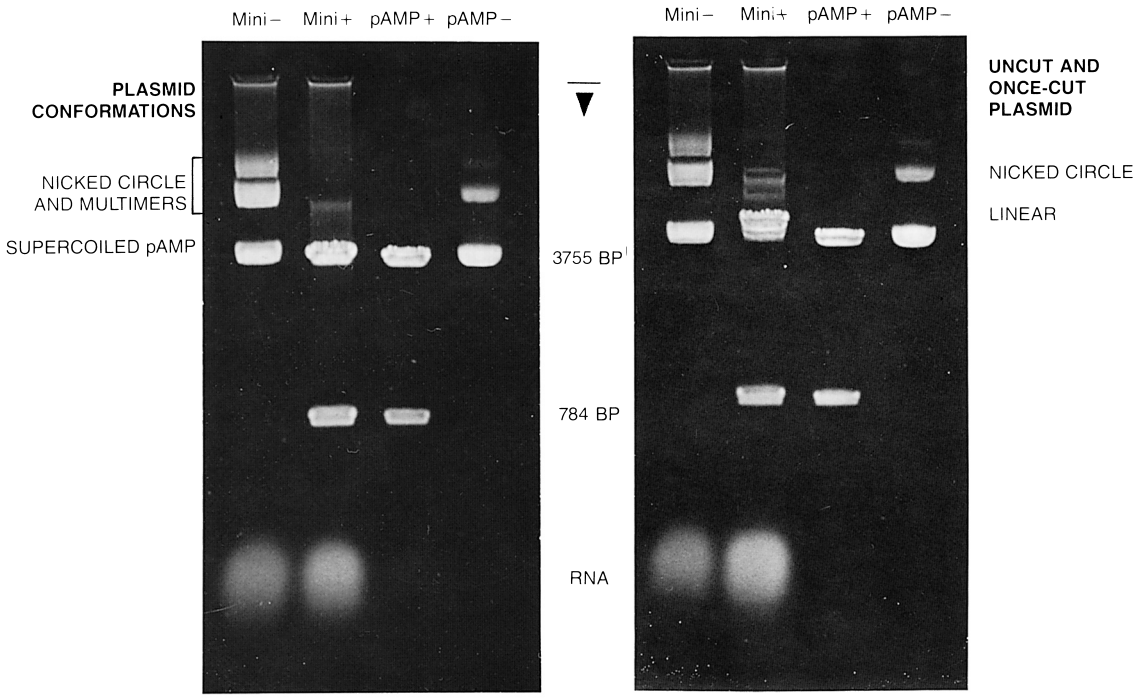
MM294 and other strains of *E. coli*, termed *recA*$^+$, have an enzyme system that recombines plasmids to form large concatemers of two or more plasmid units. A general mechanism for shuffling DNA strands, homologous recombination, occurs when identical regions of nucleotides are reciprocally exchanged between two DNA molecules. Homologous recombination occurs frequently between plasmids, which exist as multiple identical copies within the cell.

The RecA protein binds to single-stranded regions of nicked plasmids, promoting crossover and rejoining of homologous sequences. This results in multimeric ("super") plasmids that appear as a series of slow-migrating bands near the top of the gel. Since the concatemers form head-to-tail, they produce restriction fragments identical to those of a monomer (single plasmid) when cut with restriction enzymes. To confuse matters further, multimers can exist in any of the three forms mentioned above. Supercoiled multimers may appear further down on the gel than relaxed or linear plasmids with fewer nucleotides.

1. Examine the photograph of your stained gel (or view on a light box or overhead projector). Compare your gel with the ideal gel. Label the size of fragments in each lane of your gel.
2. Compare the two gel lanes containing miniprep DNA with the two lanes containing control pAMP. Explain possible reasons for variations.
3. A plasmid preparation of pAMP is composed entirely of dimeric molecules (pAMP/pAMP). The two molecules are joined *head-to-head* at a "hot spot" for recombination located 655 bp from the *Hin*dIII site near the origin of replication.
   a. Draw a map of the dimeric plasmid described above.
   b. Draw a map of the dimeric pAMP that actually forms by head-to-tail recombination at the site described above.
   c. Now draw the gel-banding patterns that would result from double digestion of each of these plasmids with *Bam*HI and *Hin*dIII, and label the base-pair size of fragments in each band.
4. Explain why EDTA is an important component of TE buffer in which the miniprep DNA is dissolved.



Component of Plasmid DNA Isolated from a *recA*⁻ Strain (HB101) and a *rec*A⁺ Strain (MM294)



Ideal Gel

Partial Digest

FOR FURTHER RESEARCH

1. Isolate and characterize an unknown plasmid. Make overnight cultures of *E. coli* strains containing any of several commercially available plasmids (such as pAMP, pKAN, pUC19, and pBR322). Digest miniprep and control samples of each plasmid with *Bam*HI/*Hin*dIII, and separate by electrophoresis in an agarose gel.
2. Transform pAMP and/or other plasmids into a *recA*$^+$ strain (MM294) and a *recA*$^-$ strain (HB101). Do minipreps from overnight cultures of each strain, and incubate samples of each with no enzyme, *Hin*dIII, and *Bam*HI+*Hin*dIII. Separate the samples by electrophoresis as far as possible in an agarose gel. Compare the banding patterns of the two strains, especially in the uncut lanes.
3. Research the potential use of homologous recombination in targeted gene therapy.