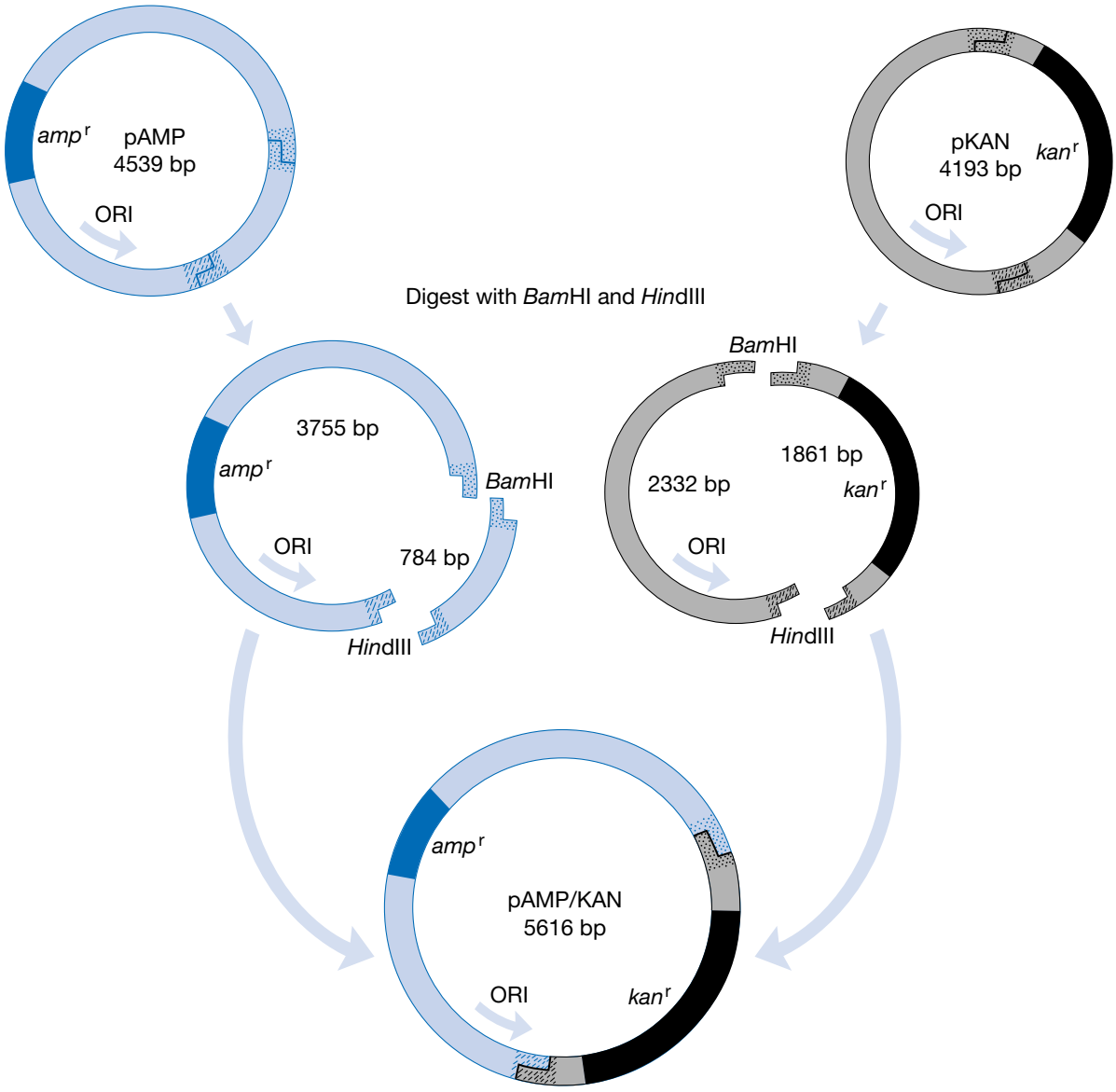
Recombination of Antibiotic Resistance Genes

LABORATORY 9 BEGINS AN EXPERIMENTAL STREAM designed to construct and analyze a recombinant DNA molecule. The starting reagents are the relaxed plasmids pAMP and pKAN, each of which carries a single antibiotic resistance gene: ampicillin in pAMP and kanamycin in pKAN. The goal is to construct a recombinant plasmid that contains both ampicillin and kanamycin resistance genes. This laboratory is divided into two parts: Restriction Digest of Plasmids pAMP and pKAN and Ligation of pAMP and pKAN Restriction Fragments.

- Part A provides a procedure whereby samples of both plasmids are digested in separate restriction reactions with *Bam*HI and *Hin*dIII. Following incubation at 37°C, samples of digested pAMP and pKAN are analyzed by agarose gel electrophoresis to confirm proper cutting. Each plasmid contains a single recognition site for each enzyme, yielding only two restriction fragments. Cleavage of pAMP yields fragments of 784 bp and 3755 bp, and cleavage of pKAN yields fragments of 1861 bp and 2332 bp.
- Part B provides a technique for ligation of pAMP and pKAN restriction fragments. The restriction digests of pAMP and pKAN are heated to destroy *Bam*HI and *Hin*dIII activity. A sample from each reaction is mixed with DNA ligase plus ATP and incubated at room temperature. Complementary *Bam*HI and *Hin*dIII "sticky ends" hydrogen-bond to align restriction fragments. Ligase catalyzes the formation of phosphodiester bonds that covalently link the DNA fragments to form stable recombinant DNA molecules.

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

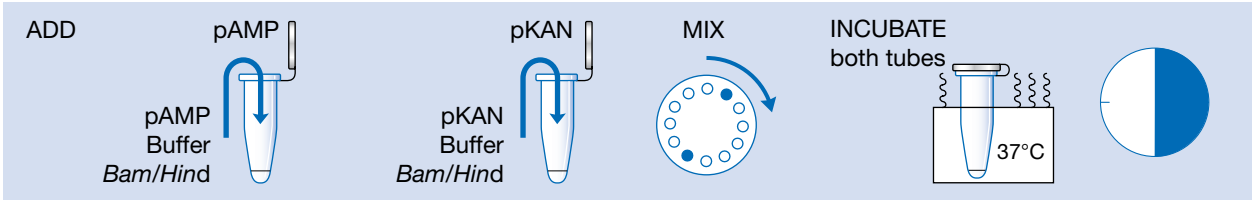


Formation of the "Simple Recombinant" pAMP/KAN

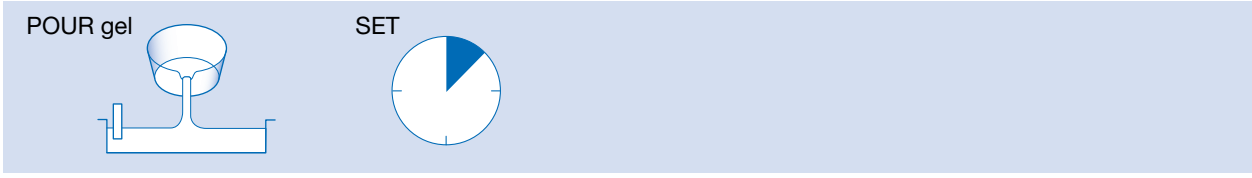
PART A

Restriction Digest of Plasmids pAMP and pKAN

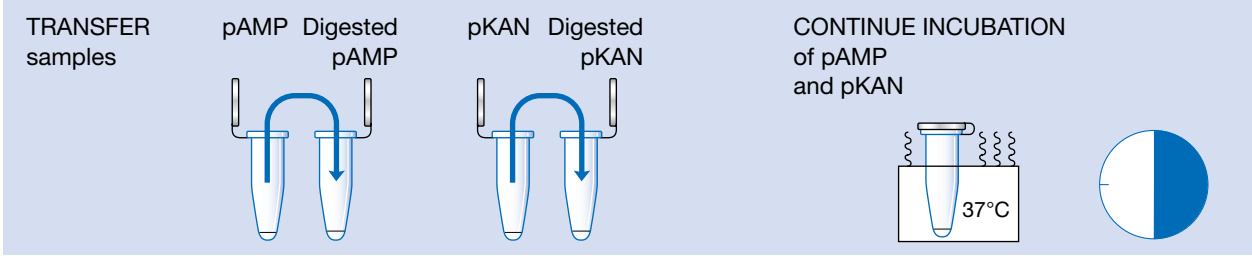
I. Prepare Restriction Digest

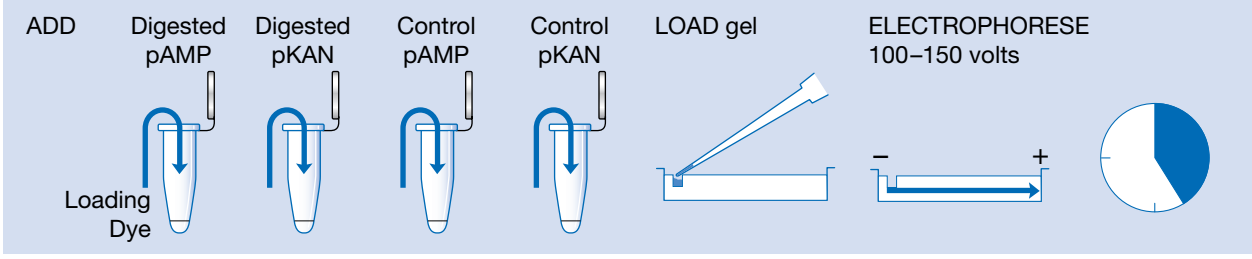


II. Cast 0.8% Agarose Gel

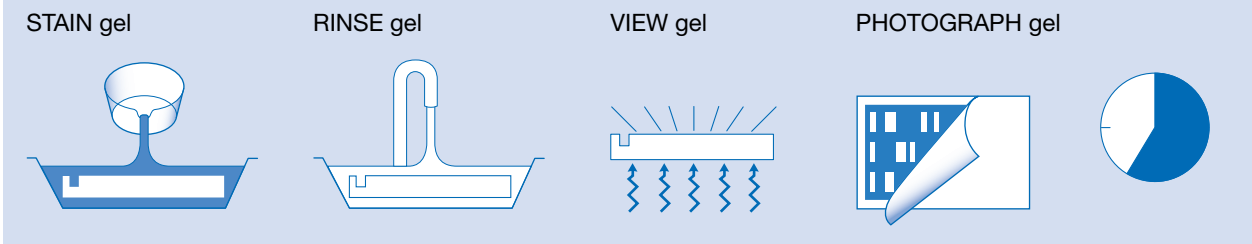


III. Load Gel and Separate by Electrophoresis





IV. Stain Gel and View (Photograph)



PRELAB NOTES

Review Prelab Notes in Laboratory 3, DNA Restriction Analysis.

Plasmid Substitution

The process of constructing and analyzing recombinant molecules is not trivial. However, good results can be expected if the directions are followed carefully. These protocols have been optimized for the teaching plasmids pAMP and pKAN, and the extensive analysis of results is based *entirely* on recombinant molecules derived from these parent molecules.

The Prudent Control

In Section III, samples of the restriction digests are analyzed by agarose gel electrophoresis, prior to ligation, to confirm complete cutting by the endonucleases. This prudent control is standard experimental procedure. If pressed for time, omit electrophoresis and ligate DNA directly following the restriction digest. However, be sure to pretest the activity of *Bam*HI and *Hin*dIII to determine the incubation time needed for complete digestion.

It is fairly impractical to use methylene blue staining for this step, which demands a rapid and sensitive assay to check for complete digestion of the plasmid DNAs. Methylene blue destaining requires *at least* 30 minutes, and it could fail to detect a small but possibly significant amount of uncut DNA. However, if using methylene blue staining for this lab, refer to the staining procedure in Step IVB of Laboratory 8 (Part B).

Saving DNA

Restriction reactions and controls in this experiment use a relatively large amount of plasmid DNA, which is the most expensive reagent used in the course. *To minimize expense, the protocol directs that the lab be prepared by setting up aliquots of exactly the required volumes of pAMP and pKAN into 1.5-ml tubes. Then the reagents for restriction digestion are added directly to these aliquots of DNA.*

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).
2. Prepare aliquots for each experiment:

   5.5 µl of 0.20 µg/µl pAMP (store on ice)
   5.5 µl of 0.20 µg/µl pKAN (store on ice)
   5 µl of 0.10 µg/µl pAMP (store on ice)
   5 µl of 0.10 µg/µl pKAN (store on ice)
   20 µl of 2x restriction buffer (store on ice)
   6.0 µl of *Bam*HI/*Hin*dIII
   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 (at ~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 staining solution (50 ml per experiment).
6. Adjust water bath to 37°C.
7. Review Part B, Ligation of pAMP and pKAN Restriction Fragments.

MATERIALS

REAGENTS

For digest:
- pAMP (0.20 µg/µl)
- pKAN (0.20 µg/µl)

For control:
- pAMP (0.1 µg/µl)
- pKAN (0.1 µg/µl)

Agarose (0.8%)
*Bam*HI/*Hin*dIII
Distilled water
Ethidium bromide▼ (1 µg/µl) (or 0.025% methylene blue▼)
Loading dye
2x Restriction buffer
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

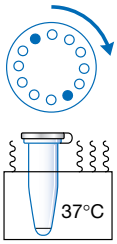
I. Set Up Restriction Digest

(40–60 minutes, including incubation through Section III)

Refer to Laboratory 3, DNA Restriction Analysis, for more detailed instructions.

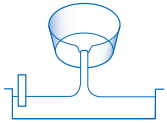
1. Use the matrix below as a checklist while adding reagents to each reaction. Read down each column, adding the same reagent to all appropriate tubes. *Use a fresh tip for each reagent.* Refer to detailed directions that follow.

to these tubes.



4. Use a *fresh tip* to add 2 µl of *Bam*HI/*Hin*dIII to each tube.
5. Close tube tops. Pool and mix reagents by pulsing in a microfuge or by sharply tapping the tube bottom on the lab bench.
6. Place the reaction tubes in a 37°C water bath, and incubate for a minimum of 30 minutes. Reactions can be incubated for a longer period of time.

STOP After a full 30-minute incubation (or longer), freeze reactions at –20°C until ready to continue. Thaw reactions before proceeding to Section III, Step 1.



II. Cast 0.8% Agarose Gel (15 minutes)

1. Seal the ends of the gel-casting tray with tape, and insert a well-forming comb. Place the gel-casting tray out of the way on the lab bench so that the agarose poured in the next step can set undisturbed.
2. Carefully pour enough agarose solution into the casting tray to fill to a depth of about 5 mm. Gel should cover only about one-third the height of comb teeth. Use a pipette tip to move large bubbles or solid debris to the sides or end of the tray while gel is still liquid.
3. Gel will become cloudy as it solidifies (~10 minutes). *Be careful not to move or jar the casting tray while the agarose is solidifying.* Touch the corner of the agarose *away* from the comb to test whether the gel has solidified.
4. When the agarose has set, unseal the ends of the casting tray. Place the tray on the platform of the gel box, so that the comb is at negative black electrode (cathode).
5. Fill box with TBE buffer, to a level that just covers the surface of the gel.
6. Gently remove the comb, taking care not to rip the wells.
7. Make sure that sample wells left by the comb are completely submerged. If "dimples" appear around the wells, slowly add buffer until they disap-

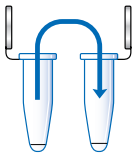
oo much buffer will chan-el the current over the top ather than through the el, increasing the time equired to separate DNA. BE buffer can be used sev-

III. Load Gel and Separate by Electrophoresis (20–30 minutes)

Only a fraction of the *Bam*HI/*Hin*dIII digests of pAMP and pKAN are separated by electrophoresis to check whether plasmids are completely cut. These restriction samples are separated by electrophoresis along with uncut pAMP and pKAN as controls.

1. Use a permanent marker to label two clean 1.5-ml tubes:

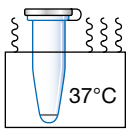
   Digested pAMP
   Digested pKAN



2. Remove original tubes labeled Digested pAMP and Digested pKAN from the 37°C water bath.

   Transfer a 5-µl sample of plasmid from the original Digested pAMP tube into the clean Digested pAMP tube.

   Transfer a 5-µl sample of plasmid from the original Digested pKAN tube into the clean Digested pKAN tube.

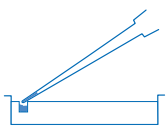


3. *Immediately* return the original Digested pAMP and Digested pKAN tubes to the water bath, and continue incubating at 37°C during electrophoresis.

4. Collect 1.5-ml tubes containing 5 µl each of purified plasmid at 0.1 µg/µl; label tubes:

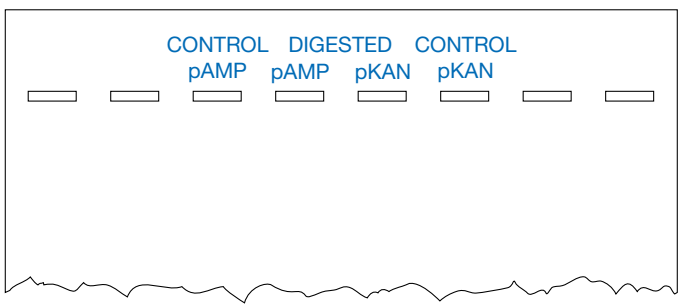
   Control pAMP
   Control pKAN

5. Add 1 µl of loading dye to each tube of Digested and Control pAMP and pKAN. Close tube tops, and mix by tapping the tube bottom on the lab bench, pipetting in and out, or pulsing in microfuge.



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

6. Load entire contents of each sample tube into a separate well in the gel, as shown in diagram below. *Use a fresh tip for each sample. Expel any air in the tip before loading, and be careful not to punch the tip of the micropipettor through the bottom of the gel.*



The 784-bp Bam*HI/*Hin*dIII fragment of pAMP migrates just behind the bromophenol blue marker. Stop electrophoresis before the bromophenol blue band runs off the end of the gel or this fragment may be lost.*

7. Separate by electrophoresis at 100–150 volts for 15–30 minutes. Adequate separation will have occurred when the bromophenol blue band has moved 2–4 cm from the wells.

8. Turn off power supply, disconnect leads from the inputs, and remove top of electrophoresis box.

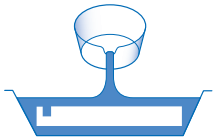
aining may be performed y an instructor in a con- olled area when students re not present.

10. Stain and view gel as described in Section IV.

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

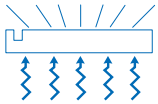
(10–15 minutes)

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.



thidium bromide solution ay be reused to stain 15 or ore gels. Dispose of spent aining solution as explained Laboratory 3.

1. Flood the 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 storage container.
3. Rinse the gel and tray under running tap water.
4. If desired, the gel can be destained in tap water or distilled water for 5 minutes or more to remove background ethidium bromide.
5. View under UV transilluminator or other UV source.

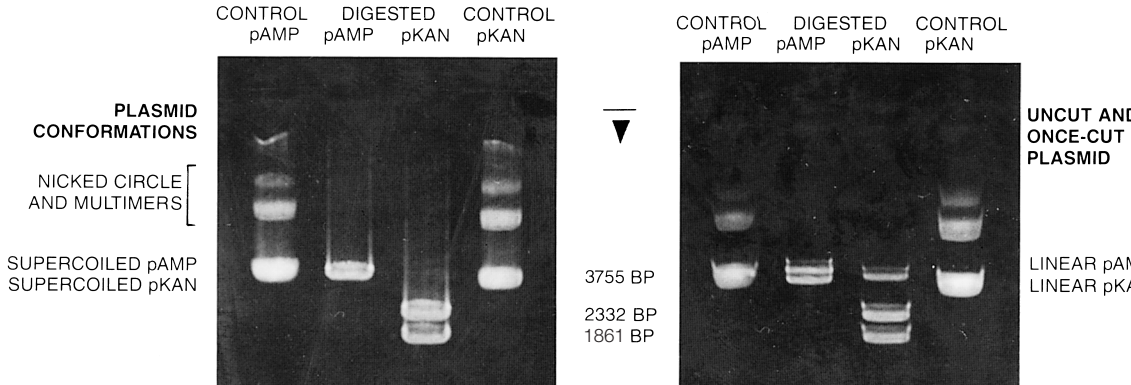


CAUTION
Ultraviolet light can damage eyes. Never look at unshielded UV light source with naked eyes. View only through a filter or safety glasses that absorb harmful wavelengths. For further information, see Appendix 4.

6. Photograph with a Polaroid or digital camera.
7. If both digests look complete, or nearly so (see Results and Discussion), continue on to Part B, Ligation of pAMP and pKAN Restriction Fragments. The reaction will have gone to completion with the additional incubation during electrophoresis.
8. If either or both digests look very incomplete, add another 1 µl of *Bam*HI/

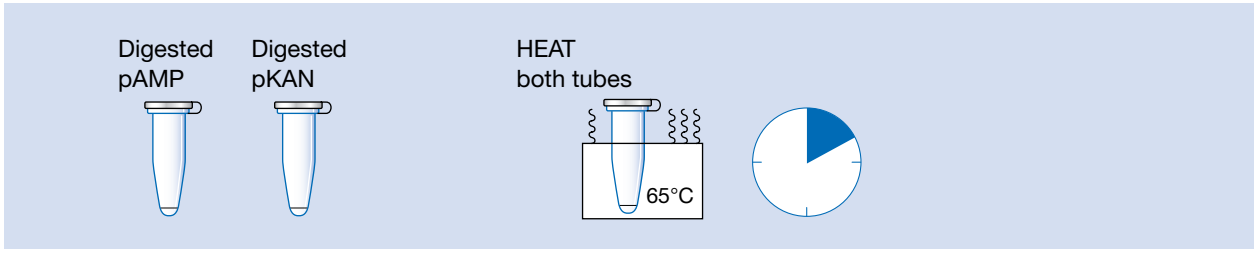
2. The Digested pKAN lane should show two distinct fragments: 1861 bp an 2332 bp.
3. Additional bands that comigrate with bands in the uncut Control pAMP an Control pKAN should be faint or absent, indicating that most or all of th pAMP and pKAN plasmid has been completely digested by both enzymes.
4. If both digests look complete, or nearly so, continue on to Part B, Ligation pAMP and pKAN Restriction Fragments. The reaction will have gone to com pletion with the additional incubation during electrophoresis.
5. If either or both digests look very incomplete, add another 1 µl *Bam*HI/*Hin*dIII solution and incubate for an additional 20 minutes befo continuing to Part B, Ligation of pAMP and pKAN Restriction Fragments.

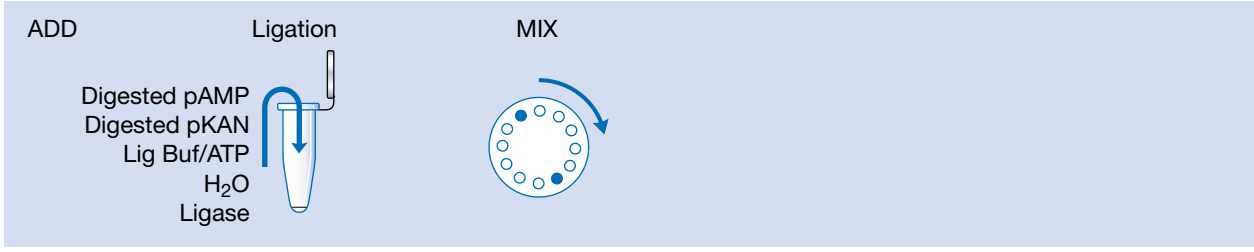
STOP Freeze *Bam*HI/*Hin*dIII reactions at –20°C until ready to continue. Thaw reac tions before proceeding to Part B, Ligation of pAMP and pKAN Restriction Fragments.

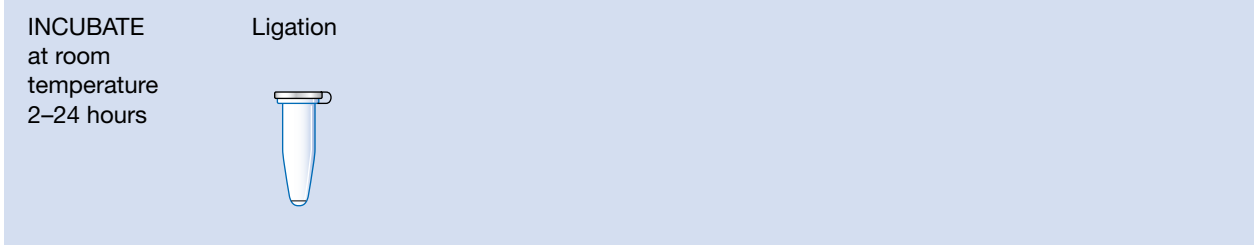


PART B

Ligation of pAMP and pKAN Restriction Fragments







PRELAB NOTES

DNA Ligase

Use only T4 DNA ligase. *E. coli* DNA ligase requires different reaction conditions and cannot be substituted in this experiment. *Cohesive-end units* are used to calibrate ligase activity: One unit of enzyme ligates 50% of *Hin*dIII fragments of λ DNA (6 μg in 20 μl) in 30 minutes at 16°C. This unit is used by New England Biolabs (NEB) and Carolina Biological Supply Company (CBS).

Researchers typically incubate ligation reactions overnight at room temperature. *For brief ligations, down to a minimum of 1 hour, it is essential to choose a high-concentration T4 DNA ligase with at least 100–500 cohesive-end units/μl.*

For Further Information

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

Cohen S.N., Chang A.C.Y., Boyer H.W., and Helling R.B. 1973. Construction of biologically functional bacteria plasmids in vitro. *Proc. Natl. Acad. Sci.* **70:** 3240–3244.

PRELAB PREPARATION

1. Obtain fresh 2x ligation buffer/ATP solution. ATP is somewhat unstable in solution, so do not use very old buffer/ATP and take care to keep frozen when not in use.
2. T4 DNA ligase is critical to the experiment and rather expensive. Make one aliquot of ligase sufficient for all experiments, and hold on ice during the laboratory. We suggest that the instructor dispense ligase directly into each experimenter's reaction tube.
3. Prewarm water bath to 65°C.
4. Dispose of 2x restriction buffer from Part A, Restriction Digest of Plasmids pAMP and pKAN, to avoid mistaking it for 2x ligation buffer/ATP.

MATERIALS

REAGENTS

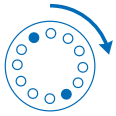
Digested pAMP (from part A)
Digested pKAN (from part A)
Distilled water
2x Ligation buffer/ATP
T4 DNA ligase

SUPPLIES AND EQUIPMENT

Beaker for waste/used tips
Microfuge (optional)
Micropipettor (0.5–10-μl) + tips
Test tube rack
Tube (1.5-ml)
Water bath (65°C)

res protein, thus inactivat-
g the restriction enzymes.

igase may be dispensed by
ur instructor.



r brief ligations of 2–4
urs, it is essential to use a
gh-concentration T4 DNA
ase with at least 100–500
hesive-end units/µl.

3. Use the matrix below as a checklist while adding reagents to the LIG tube. *Use a fresh tip for each reagent.* Refer to detailed directions that follow.

Tube	Digested pAMP	Digested pKAN	2× Ligation Buffer/ATP	Water	Ligase
LIG	3 µl	3 µl	10 µl	3 µl	1 µl

4. Collect reagents (except ligase), and place them in test tube rack on lab bench.
5. Add 3 µl of Digested pAMP.
6. Use a *fresh tip* to add 3 µl of Digested pKAN.
7. Use a *fresh tip* to add 10 µl of 2× ligation buffer/ATP.
8. Use a *fresh tip* to add 3 µl of distilled water.
9. Use a *fresh tip* to add 1 µl of DNA ligase. Carefully check that the droplet of ligase is on the *inside* wall of the tube.
10. Close tube top. Pool and mix reagents by pulsing in a microfuge or by sharply tapping the tube bottom on the lab bench.
11. Incubate the reaction for 2–24 hours at *room temperature*.
12. If time permits, ligation may be confirmed by electrophoresing 5 µl of the ligation reaction, along with *Bam*HI/*Hin*dIII digests of pAMP and pKAN. None of the parent *Bam*HI/*Hin*dIII fragments should be observed in the lane of ligated DNA, which should show multiple bands of high-molecular-weight DNA high up on the gel.

STOP Freeze the reaction at –20°C until ready to continue. Thaw the reaction before proceeding to Laboratory 10.

ESULTS AND DISCUSSION

Ligation of the four *Bam*HI/*Hin*dIII restriction fragments of pAMP and pKAN (refer to diagram on page 444) produces many types of hybrid molecules, including plasmids composed of more than two fragments. However, only those

1. Make a scale drawing of the simple recombinant molecule pAMP/pKAN described above. Include fragment sizes, locations of *Bam*HI and *Hin*dIII restriction sites, location of origin(s), and location of antibiotic resistance gene(s).
2. Make scale drawings of other two-fragment recombinant plasmids with the following properties.
   a. Three kinds of plasmids having two origins.
   b. Three kinds of plasmids having no origin.

   Whenever possible ,include fragment sizes, locations of *Bam*HI and *Hin*dIII restriction sites, location of origin(s), and location of antibiotic resistance gene(s).
3. Ligation of the 784-bp fragment, 3755-bp fragment, 1861-bp fragment, and 2332-bp fragment produces a "double plasmid" pAMP/pKAN (or superplasmid). Make a scale drawing of the double plasmid pAMP/pKAN.
4. Make scale drawings of several recombinant plasmids composed of any three of the four *Bam*HI/*Hin*dIII fragments of pAMP and pKAN. Include fragment sizes, locations of *Bam*HI and *Hin*dIII restriction sites, location of origin(s), and location of antibiotic resistance gene(s). What rule governs the construction of plasmids from three kinds of restriction fragments?
5. What kind of antibiotic selection would identify *E. coli* cells that have been transformed with each of the plasmids drawn in Questions 1–4?
6. Explain what is meant by "sticky ends." Why are they so useful in creating recombinant DNA molecules?
7. Why is ATP essential for the ligation reaction?

FOR FURTHER RESEARCH

Clone a DNA fragment using either pUC18 or pBLU as a vector. These vectors contain part or all of a gene coding for β-galactosidase (see discussion in Laboratory 5). The β-galactosidase enzyme acts on the synthetic substrate X-gal to produce a blue product, so cells containing one of these vectors will grow blue on plates containing X-gal. However, the pUC18 and pBLU vectors contain unique restriction sites within the β-galactosidase gene. Cloning a DNA fragment into these sites will disrupt the gene so that it does not make functional β-galactosidase. Cells containing only the disrupted gene will grow white on plates containing X-gal. Thus, pUC18 and pBLU allow you to distinguish the colonies containing plasmids with cloned DNA (white) from the colonies containing plasmids without cloned DNA (blue). Obtain a commercial source of bacteriophage λ DNA or *E. coli* DNA. Digest the DNA with *Bam*HI and *Hin*dIII. Ligate digested DNA to pUC18 or pBLU DNA that is also digested with *Bam*HI and *Hin*dIII. Plate onto X-gal plates and isolate several white colonies. Grow up each colony in a few milliliters of LB and make a miniprep. Carry out a *Bam*HI and *Hin*dIII restriction digest on the miniprep DNA and separate your DNA fragments on an agarose gel to visualize your cloned DNA fragment.