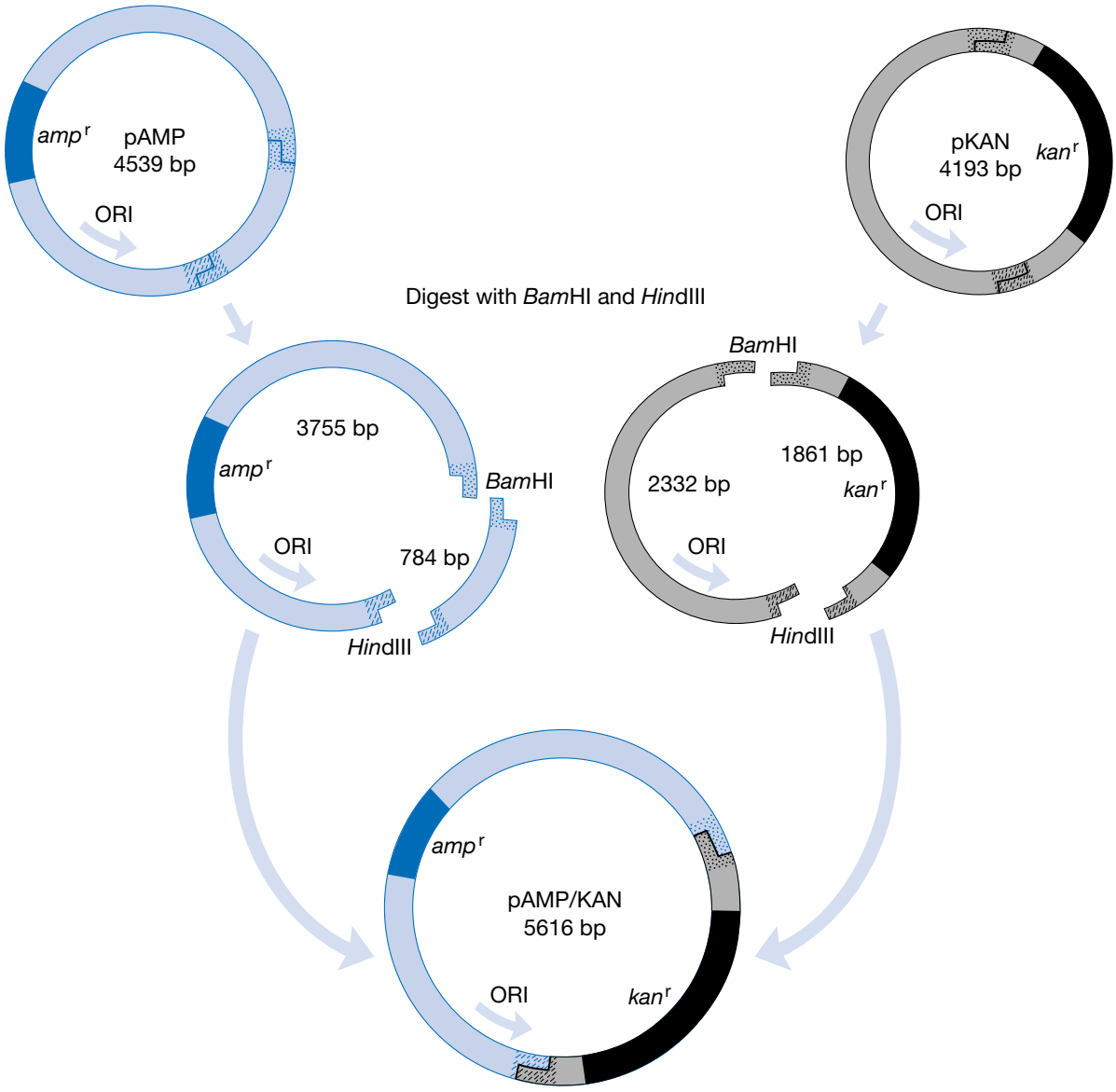
Recombination of Antibiotic Resistance Genes

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).

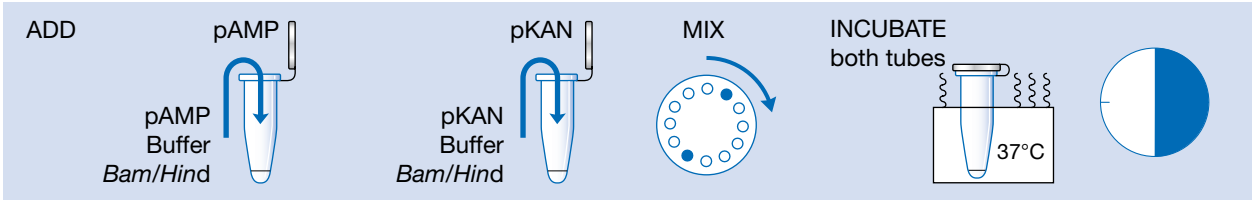


Formation of the "Simple Recombinant" pAMP/KAN

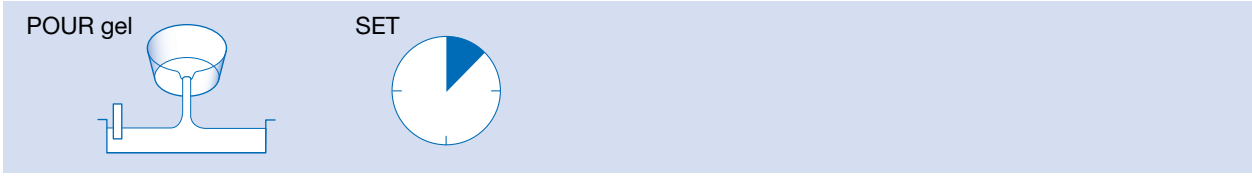
PART A

Restriction Digest of Plasmids pAMP and pKAN

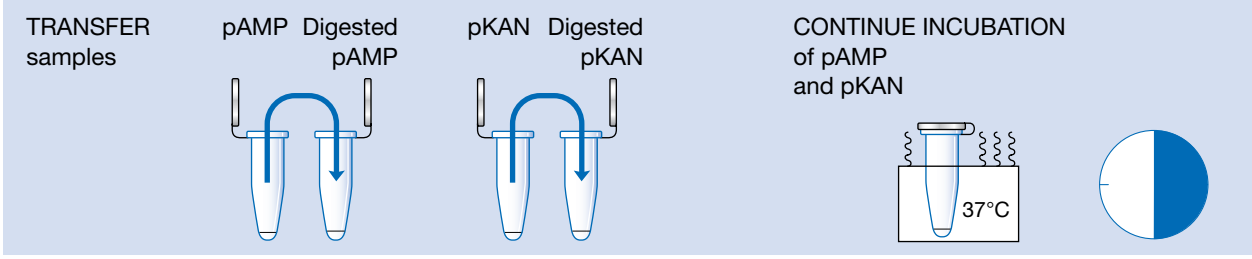
I. Prepare Restriction Digest

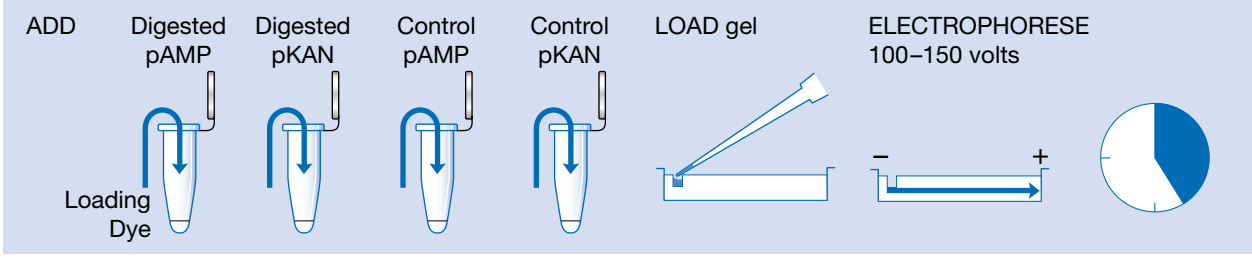


II. Cast 0.8% Agarose Gel

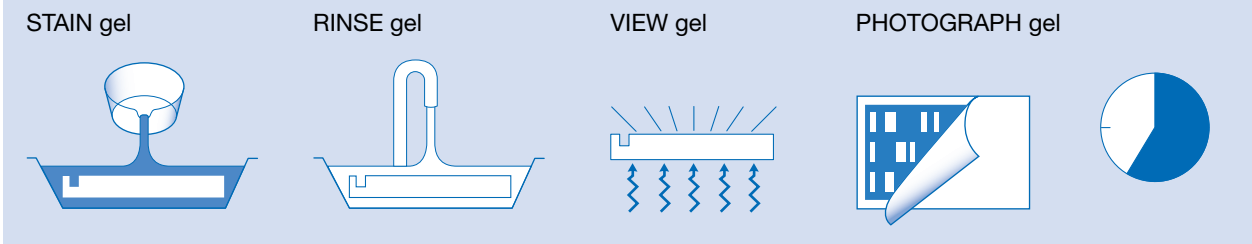


III. Load Gel and Separate by Electrophoresis





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

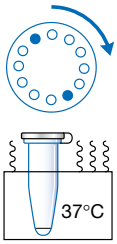
I. Set Up Restriction Digest

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

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

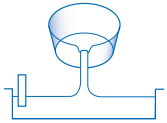
1. Use the matrix below as a checklist while adding reagents to each reaction. Read down each column, adding the same reagent to all appropriate tubes. *Use a fresh tip for each reagent.* Refer to detailed directions that follow.

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-

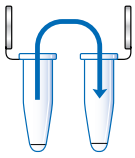
oo much buffer will chan-el the current over the top ather than through the el, increasing the time equired to separate DNA. BE buffer can be used sev-

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:

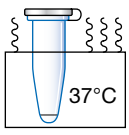
   Digested pAMP
   Digested pKAN



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.

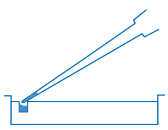


3. *Immediately* return the original Digested pAMP and Digested pKAN tubes to the water bath, and continue incubating at 37°C during electrophoresis.

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

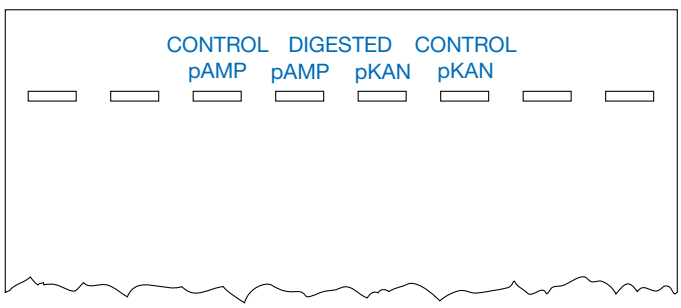
   Control pAMP
   Control pKAN

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.

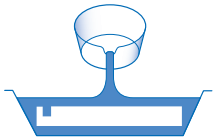
aining may be performed y an instructor in a con- olled area when students re not present.

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

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

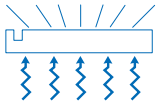
(10–15 minutes)

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.

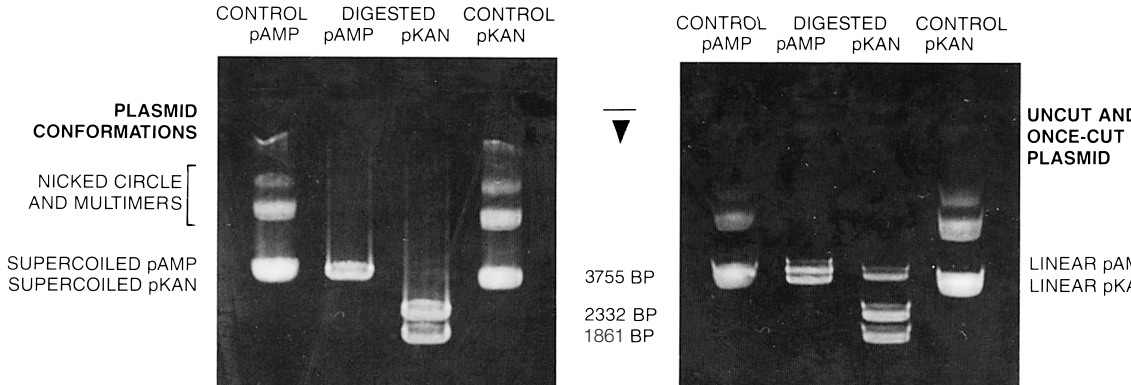


CAUTION
Ultraviolet light can damage eyes. Never look at unshielded UV light source with naked eyes. View only through a filter or safety glasses that absorb harmful wavelengths. For further information, see Appendix 4.

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/

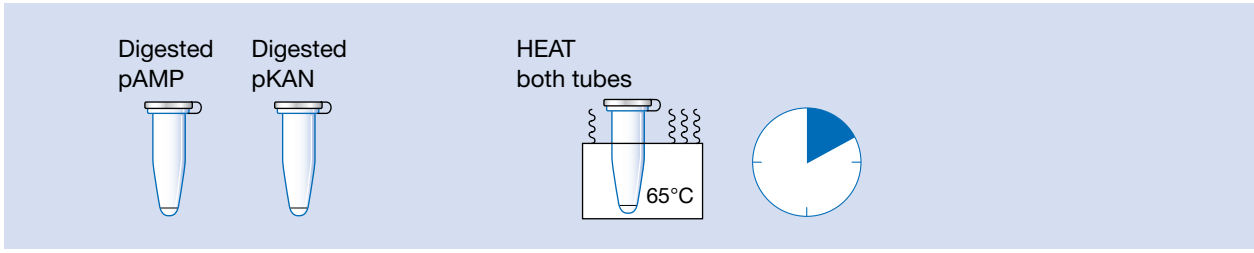
2. The Digested pKAN lane should show two distinct fragments: 1861 bp an 2332 bp.
3. Additional bands that comigrate with bands in the uncut Control pAMP an Control pKAN should be faint or absent, indicating that most or all of th pAMP and pKAN plasmid has been completely digested by both enzymes.
4. 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.

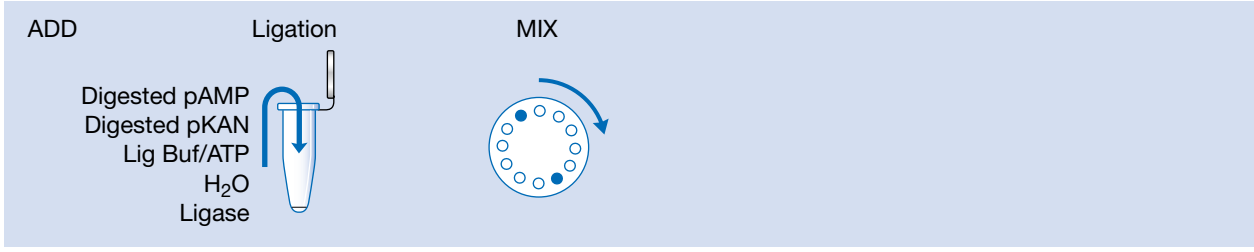
STOP Freeze *Bam*HI/*Hin*dIII reactions at –20°C until ready to continue. Thaw reac tions before proceeding to Part B, Ligation of pAMP and pKAN Restriction Fragments.

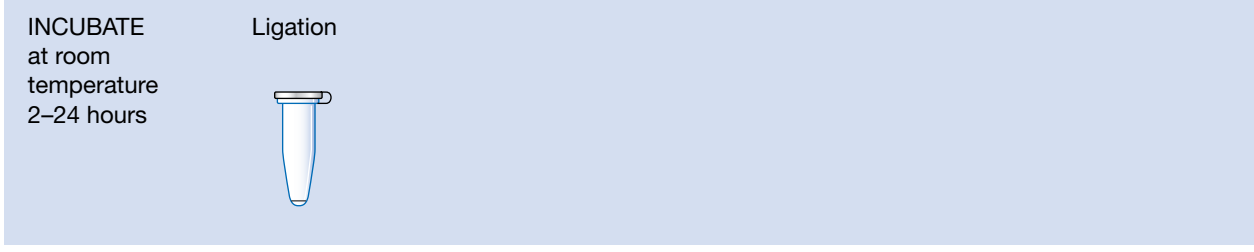


PART B

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

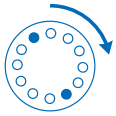
Digested pAMP (from part A)
Digested pKAN (from part A)
Distilled water
2x Ligation buffer/ATP
T4 DNA ligase

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.

Transformation of *E. coli* with Recombinant DNA

In Part A, Classic Procedure for Preparing Competent Cells, *E. coli* cells are rendered competent to uptake plasmid DNA using a method essentially unchanged since its publication in 1970 by Morton Mandel and Akiko Higa. The procedure begins with vigorous *E. coli* cells grown in suspension culture. Cells are harvested in mid-log phase by centrifugation and incubated at 0°C with two successive changes of calcium chloride solution.

This procedure is more involved than the rapid colony protocol introduced in Laboratory 5. However, the classical procedure typically achieves transformation efficiencies ranging from 5×10^4 to 5×10^6 colonies per microgram of plasmid—a 2–200-fold increase over the colony procedure. The enhanced efficiency is important when transforming ligated DNA (composed primarily of relaxed circular plasmids and linear DNA), which produces 5–100 times fewer transformants than plasmid DNA purified from *E. coli* (containing a high proportion of the supercoiled form).

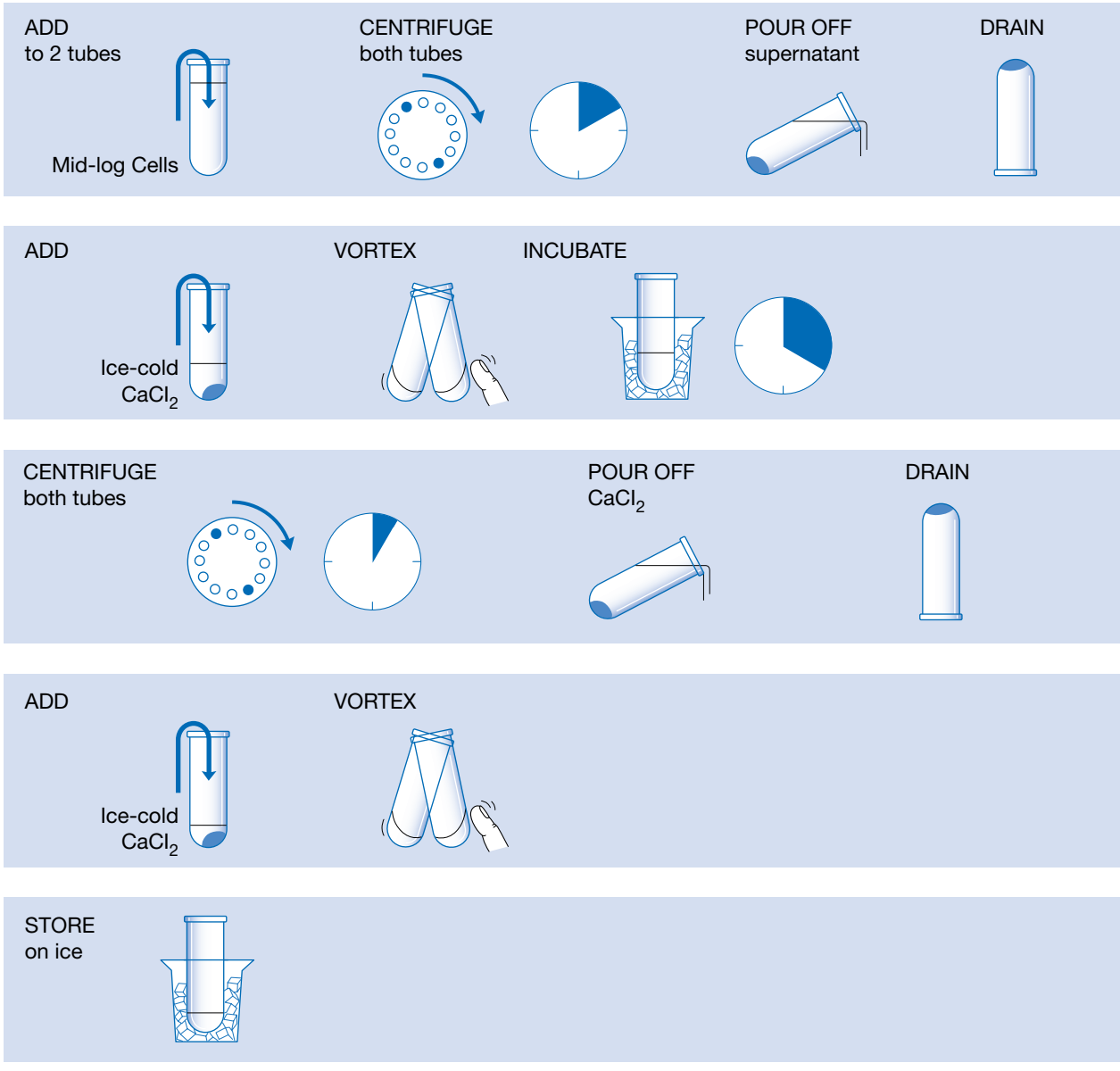
In Part B, Transformation of *E. coli* with Recombinant DNA, the competent *E. coli* cells are transformed with the ligation products from Laboratory 9, Recombination of Antibiotic Resistance Genes. Ligated plasmid DNA is added to one sample of competent cells, and purified pAMP and pKAN plasmids are added as controls to two other samples. The cell suspensions are incubated with the plasmid DNAs for 20 minutes at 0°C. Following a brief heat shock at 42°C, the cells recover in LB broth for 40–60 minutes at 37°C. Unlike ampicillin selection in Laboratory 5, Rapid Colony Transformation of *E. coli* with Plasmid DNA, the recovery step is essential for the kanamycin selection in this lab. Samples of transformed cells are plated onto three types of LB agar: with ampicillin (LB/amp), with kanamycin (LB/kan), and with both ampicillin and kanamycin (LB/amp+kan).

The ligation reaction produces many kinds of recombinant molecules composed of *Bam*HI/*Hin*dIII fragments, including the religated parental plasmids pAMP and pKAN. The object is to select for transformed cells with dual antibiotic resistance, which must contain a 3755-bp fragment from pAMP containing the ampicillin resistance gene (plus the origin of replication) and a 1861-bp fragment from pKAN containing the kanamycin resistance gene. Bacteria transformed with a single plasmid containing these sequences, or those doubly transformed with both pAMP and pKAN plasmids, form colonies on the LB/amp+kan plate.

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

PART A

Classic Procedure for Preparing Competent Cells



PRELAB NOTES

Review Prelab Notes in Laboratories 1, 2, and 5 regarding sterile techniques, *E. coli* culture, and transformation.

Seasoning Cells for Transformation

If possible, schedule experiments so that competent cells (Part A) are prepared one day prior to transformation with recombinant DNA (Part B). "Seasoning" cells for 12–24 hours at 0ºC (an ice bath inside the refrigerator) generally increases transformation efficiency five- to tenfold. This enhanced efficiency will help ensure successful cloning of the recombinant molecules produced in Laboratory 9.

For Further Information

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

Cohen S.N., Chang A.C., and Hsu L. 1972. Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci.* **69:** 2110–2114.

Dagert M. and Ehrlich S.D. 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene* **6:** 23.

Mandel M. and Higa A. 1970. Calcium-dependent bacteriophage DNA infection. *J. Mol. Biol.* **53:** 159–162.

PRELAB PREPARATION

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

1. On the day before this lab, begin an *E. coli* culture from a streaked plate of MM294, according to the protocol in Laboratory 2B, Overnight Suspension Culture.
2. PLAN AHEAD. Make sure that you have a streaked plate of viable *E. coli* cells from which to inoculate overnight. Also, streak your *E. coli* strain on LB/amp and LB/kan plates to ensure that a resistant strain has not been used by mistake.
3. Approximately 2–4 hours before the lab, begin an *E. coli* culture according to the protocol in Laboratory 2C, Mid-log Suspension Culture. Cells are optimal for transformation when the culture reaches an OD_{550} of 0.3–0.40. More simply, cells inoculated into room temperature LB will be ready to transform after 2 hours, 15 minutes. Hold cells in mid-log phase by storing the culture on ice for up to 2 hours prior to beginning calcium chloride treatment. *Each experiment requires 20 ml of mid-log suspension culture.*
4. Sterilize 50 mM calcium chloride ($CaCl_2$) solution and LB broth by autoclaving or filtering through a 0.45-μm or 0.22-μm filter (Nalgene or Corning). Filtered $CaCl_2$ can be stored in a filter collection container or transferred to sterile 50-ml conical tubes.

5. Prepare aliquots for each experiment:

   15 ml of 50 mM $CaCl_2$ in a sterile 50-ml tube (store on ice)
   two 10-ml cultures of mid-log *E. coli* cells in sterile 15-ml culture tubes (store on ice)
   12 µl of 0.005 µg/µl pAMP in a 1.5-ml tube (store on ice)
   12 µl of 0.005 µg/µl pKAN in a 1.5-ml tube (store on ice)

6. Review Part B, Transformation of *E. coli* with Recombinant DNA.

MATERIALS

CULTURE AND REAGENTS

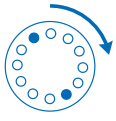
$CaCl_2$ (50 mM)
Mid-log MM294 cells
(two 10-ml cultures)

SUPPLIES AND EQUIPMENT

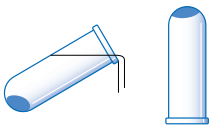
Beakers for crushed or cracked ice and for waste
Bleach (10%)▼ or disinfectant
Bunsen burner
Clean paper towels
Clinical centrifuge (2000–4000 rpm)
Micropipettor (100–1000 µl) + tips (or 1-ml pipette)
Pipettes (5-ml or 10-ml)
Spectrophotometer (optional)
Sterile pipette aid or bulb
Test tube rack

▼ See Appendix 4 for Cautions list.

METHODS



A tight pellet of cells should be easily seen at the bottom of the tube. If pellet does not appear to be consolidated, recentrifuge for an additional 5 minutes.



Prepare Competent Cells (40–50 minutes)

This entire experiment *must be performed under sterile conditions.* Review sterile techniques in Laboratory 1, Measurements, Micropipetting, and Sterile Techniques.

1. Place sterile tube of $CaCl_2$ solution on ice.
2. Obtain two 15-ml tubes each with 10 ml of mid-log cells, and label with your name.
3. *Securely close caps* and place both tubes of cells in a *balanced* configuration in the rotor of the clinical centrifuge. Centrifuge at 3000 rpm for 10 minutes to pellet cells on the bottom-side of the culture tube.
4. Sterilely pour off supernatant from each tube into the waste beaker for later disinfection. *Do not disturb the cell pellet.*

Plan out manipulations for Step 4. Organize lab bench, and work quickly.

Flaming is not necessary since contaminating bacteria do not grow well in $CaCl_2$.

If working as a team, one person handles the pipettes, and the other removes and replaces tube caps.

a. Remove cap from the culture tube, and briefly flame mouth. *Do not place cap on lab bench.*

b. Carefully pour off supernatant. Invert culture tube, and tap gently on the surface of a clean paper towel to drain thoroughly.

c. *Reflame mouth of culture tube, and replace cap.*

5. Use a 5- or 10-ml pipette to sterilely add 5 ml of ice-cold $CaCl_2$ solution to each culture tube:

a. Remove cap from $CaCl_2$ tube. *Do not place cap on lab bench.*

b. Withdraw 5 ml of $CaCl_2$ and replace cap.

c. Remove cap of the culture tube. *Do not place cap on lab bench.*

d. Expel $CaCl_2$ into culture tube and replace cap.

6. Immediately finger vortex to resuspend pelleted cells in each tube.

a. Close cap tightly.

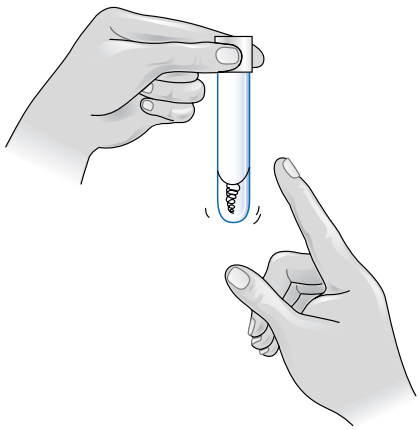
b. Hold upper part of tube securely with thumb and index finger.

The cell pellet becomes increasingly difficult to suspend the longer it sits in the $CaCl_2$ solution.

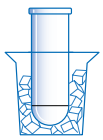
c. With the other hand, vigorously hit the bottom end of the tube with index finger or thumb to create a vortex that lifts the cell pellet off the bottom of the tube. Continue "finger vortexing" until all traces of the cell mass are completely resuspended. This may take a couple of minutes, depending on technique.

Double check both tubes for complete resuspension of cells, which is probably the most important variable in obtaining good results.

d. Hold the tube up to the light to check that the suspension is homogeneous. No visible clumps of cells should remain.



Finger Vortex (Steps 6 and 11)



$CaCl_2$ treatment alters adhering properties of E. coli *membranes. The cell pellet is much more dispersed after the second centrifugation.*

7. Return both tubes to ice, and incubate for 20 minutes.

8. Following incubation, respin the cells in a clinical centrifuge for 5 minutes at 2000–4000 rpm. This time the cell pellet will be more spread out on the bottom of the tube due to the $CaCl_2$ treatment.

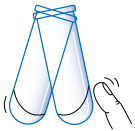
9. Sterilely pour off $CaCl_2$ from each tube into a waste beaker. *Do not disturb the cell pellet.*

a. Remove cap from $CaCl_2$ tube. *Do not place cap on lab bench.*

b. Withdraw 1000 µl (1 ml) of $CaCl_2$ and replace cap.

c. Remove cap of culture tube. *Do not place cap on lab bench.*

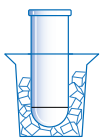
d. Expel $CaCl_2$ into culture tube and replace cap.



l pellet may appear more use than at beginning of cedure and will resuspend re easily. Double check h tubes for complete resus- sion of cells.

11. Close caps tightly, and immediately finger vortex to resuspend pelleted cells in each tube. Hold the tube up to the light to check that the suspension is homogeneous. No visible clumps of cells should remain.

STOP Store cells in a beaker of ice in the refrigerator (~0°C) until ready for use in Part B. "Seasoning" at 0°C for up to 24 hours increases competency of cells five- to tenfold.



12. If not proceeding immediately to Part B, take time for responsible cleanup:

a. Segregate for proper disposal culture plates and tubes, pipettes, and micropipettor tips that have come in contact with *E. coli*.

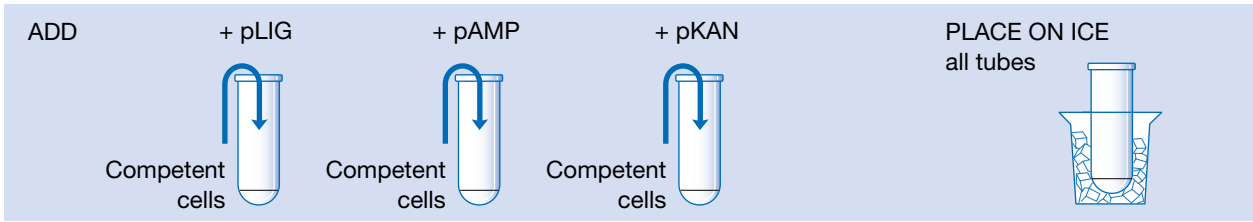
b. Disinfect mid-log culture, tips, and supernatant from Steps 4 and 9 with 10% bleach or disinfectant.

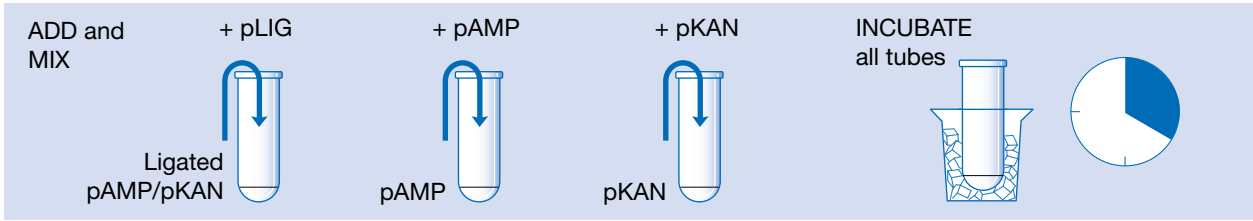
c. Wipe down lab bench with soapy water, 10% bleach solution, or disinfectant (such as Lysol).

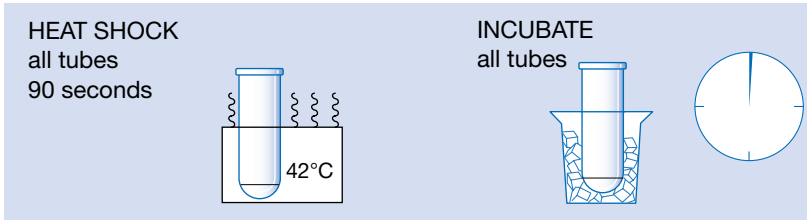
d. Wash hands before leaving lab.

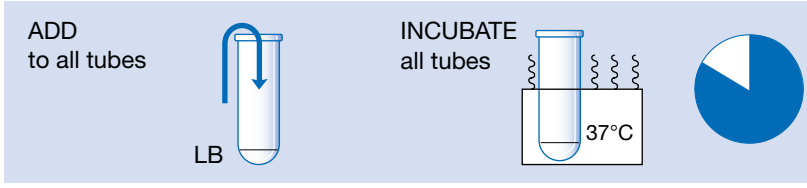
PART B

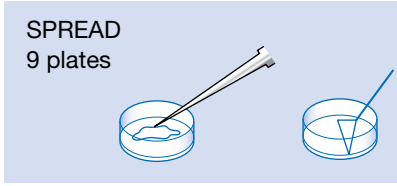
Transformation of *E. coli* with Recombinant DNA

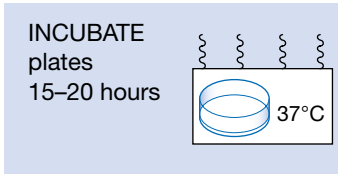












PRELAB NOTES

Review Prelab Notes in Laboratory 5, Rapid Colony Transformation of *E. coli* with Plasmid DNA.

Equipment Substitutions

A standard 1-ml pipette or transfer pipette can be substituted for a 100–1000-µl micropipettor.

Recovery Period

A 40–60-minute postincubation recovery at 37°C, with shaking, is essential prior to plating transformed cells on kanamycin, which acts quickly to kill any cell that is not actively expressing the resistance protein.

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

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

1. Prepare for each experiment:

   three LB+ampicillin plates (labeled LB/amp)
   three LB+kanamycin plates (labeled LB/kan)
   three LB+ampicillin+kanamycin plates (labeled LB/amp+kan)

   If only one control transformation, with *either* pAMP *or* pKAN, is done, then one less plate of each type is required.

2. Adjust water baths to 42°C and 37°C.
3. Prewarm incubator to 37°C.
4. To retard evaporation, keep beaker of ethanol covered with Parafilm, plastic wrap, or, if using a small beaker, the lid from a Petri dish. Retrieve and reuse ethanol exclusively for flaming.
5. If using spreading beads, carefully place five to seven beads into a sterile 1.5-ml tube. Tube can be used as scooper. Prepare nine tubes per experiment.

MATERIALS

CULTURE, MEDIA, AND REAGENTS

Competent *E. coli* cells (from Part A)
LB broth
LB/amp plates (3)
LB/kan plates (3)
LB/amp+kan plates (3)
Ligation tube (from Laboratory 9)
pAMP (0.005 µg/µl)
pKAN (0.005 µg/µl)

SUPPLIES AND EQUIPMENT

Beaker of 95% ethanol ▼
Beakers for crushed or cracked ice and for waste/used tips
"Bio-bag" or heavy-duty trash bag
Bleach (10%) ▼ or disinfectant
Bunsen burner
Cell spreader (or spreading beads)
Culture tubes (three 15-ml)
Incubator (37°C)
Micropipettors (0.5–10-µl and 100–1000-µl) + tips
Permanent marker
Shaking water bath (37°C)
Test tube rack
Water bath (42°C)

▼ See Appendix 4 for Cautions list.

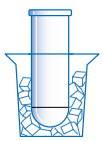
METHODS

Perform *E. coli* Transformation (70–90 minutes)

This entire experiment must be performed under sterile conditions. Review sterile techniques in Laboratory 1, Measurements, Micropipetting, and Sterile Techniques.

1. Use a permanent marker to label three *sterile* 15-ml culture tubes:

   +pLIG = ligated DNA
   +pAMP = pAMP control
   +pKAN = pKAN control



2. Use a 100–1000-µl micropipettor and *sterile tip* to add 200 µl of competent cells to each tube.
3. Place all three tubes on ice.

4. Use a 1–10-µl micropipettor to add 10 µl of ligated pAMP/KAN solution *directly into cell suspension* in tube labeled +pLIG.
5. Use a *fresh tip* to add 10 µl of 0.005 µg/µl pAMP solution *directly into cell suspension* in tube labeled +pAMP.
6. Use a *fresh tip* to add 10 µl of 0.005 µg/µl pKAN solution *directly into cell suspension* in tube labeled +pKAN.

save plates, experimenters ay omit either Set b or Set c.

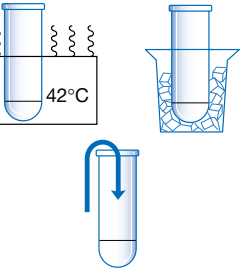
Mark L on one LB/amp, one LB/kan, and one LB/amp+kan plate.

Set b

Mark A on one LB/amp, one LB/kan, and one LB/amp+kan plate.

Set c

Mark K on one LB/amp, one LB/kan, and one LB/amp+kan plate.



10. Following a 20-minute incubation, heat-shock the cells in all three tubes. *It is critical that cells receive a sharp and distinct shock.*

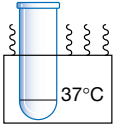
a. Carry the ice beaker to the water bath. Remove tubes from ice, and *immediately* immerse in a 42°C water bath for 90 seconds.

b. Immediately return all three tubes to ice for at least 1 additional minute.

11. Use a 100–1000-µl micropipettor with a sterile tip to add 800 µl of LB broth to each tube. Gently tap tubes with finger to mix.

a shaking water bath is ot available, warm cells for veral minutes in a 37°C ater bath, and then trans- r to a dry shaker inside a 7°C incubator. Alternatively, casionally swirl tubes by and in a nonshaking 37°C ater bath.

12. Allow cells to recover by incubating all three tubes at 37°C in a shaking water bath (with moderate agitation) for 40–60 minutes. If a shaking water bath is not available, incubate the tubes in a regular 37°C water bath. In this case, gently mix tubes periodically.



STOP Cells may be allowed to recover for up to 2 hours. A recovery period assures the growth of as many kanamycin-resistant recombinants as possible and can help compensate for a poor ligation or cells of low competence.

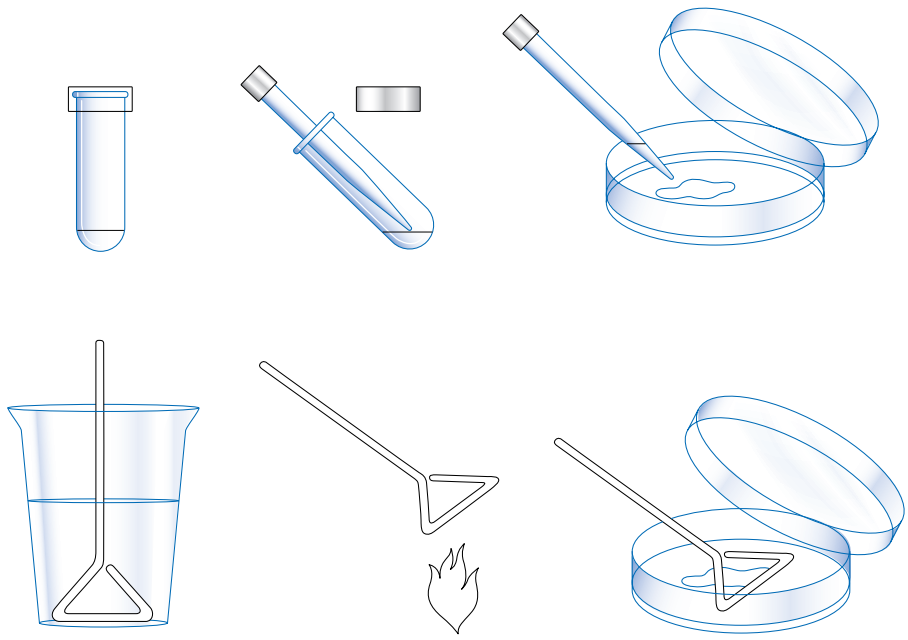
13. Use the matrix below as a checklist as +pLIG, +pAMP, and +pKAN cells are spread on each type of antibiotic plate in Steps 14–20:

	Ligated DNA L	pAMP control A	pKAN control K
LB/amp	100 µl	100 µl	100 µl
LB/kan	100 µl	100 µl	100 µl
LB/amp+kan	100 µl	100 µl	100 µl

The object is to evenly distribute and separate cells on agar so that each gives rise to a distinct colony of clones. It is essential not to overheat spreader in burner flame and to cool it before touching cell suspensions. A hot spreader will kill E. coli *cells on the plate.*

a. Dip spreader into the ethanol beaker and *briefly* pass it through Bunsen flame to ignite alcohol. Allow alcohol to burn off *away from* the Bunsen flame; spreading rod will become too hot if left in flame.

> **CAUTION**
> Be extremely careful not to ignite ethanol in the beaker. Do not panic if ethanol is accidentally ignited. Cover the beaker with a Petri lid or other cover to cut off oxygen and rapidly extinguish the fire.



Sterile Spreading Technique (Steps 14 and 15)

b. Lift the lid of the first L plate only enough to allow spreading. *Do not place lid on lab bench.*

c. Cool the spreader by gently rubbing it on surface of the agar *away* from the cell suspension or by touching it to the condensation on the plate lid.

d. Touch the spreader to the cell suspension, and gently drag it back and forth several times across the surface of agar. Rotate plate one-quarter turn, and repeat spreading motion. Try to spread the suspension evenly across the agar surface and be careful not to gouge the agar.

e. Replace plate lid. Return the cell spreader to ethanol *without flaming.*

f. Repeat Steps a through e in succession for the remaining two L plates.

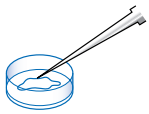
16. Use spreading beads to spread cells over the surface of each L plate in succession.

a. Lift the lid of first L plate enough to allow adding beads. *Do not place lid on lab bench.*

b. Carefully pour five to seven glass spreading beads from a 1.5-ml tube onto the agar surface.

c. Close plate lid and use a swirling motion to move glass beads around the entire surface of the plate. This evenly spreads the cell suspension on the agar suface. Continue swirling until the cell suspension is absorbed into the agar.

d. Repeat Steps a through c in succession for the remaining two L plates.



17. Use a *fresh sterile tip* to add 100 µl of cell suspension from tube labeled +pAMP onto three plates marked A.

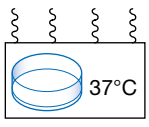
18. Repeat Step 15a–f or Step 16a–d to spread cells over the surface of each A plate in succession.

19. Use a *fresh sterile tip* to add 100 µl of cell suspension from tube labeled +pKAN onto three plates marked K.

20. Repeat Step 15a–f or Step 16a–d to sterilize cell spreader and spread cells over the surface of each K plate in succession.

21. If Step 15 was used, reflame spreader one last time before placing it on lab bench.

22. Let plates set for several minutes to allow suspension to become absorbed into agar. If Step 16 was used, invert plates and gently tap plate bottoms, so that the spreading beads fall into plate lids. Carefully pour beads from each lid into a storage container for reuse.



23. Stack plates and tape them in a bundle to keep the experiment together. Place plates upside down in a 37°C incubator, and incubate for 15–20 hours.

24. After initial incubation, store plates at 4°C to arrest *E. coli* growth and to slow the growth of any contaminating microbes.

Save L LB/amp and L LB/kan plates if planning to do Laboratory 11. Save L LB/amp+kan as a source of colonies to begin overnight suspension cultures if planning to do Laboratory 12.

25. Take time for responsible cleanup:

a. Segregate for proper disposal culture plates and tubes, pipettes, and micropipettor tips that have come in contact with *E. coli*.

b. Disinfect overnight cell suspensions, tubes, and tips with 10% bleach or disinfectant.

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

d. Wash hands before leaving lab.

RESULTS AND DISCUSSION

Observe colonies through the bottom of the culture plate, using a permanent marker to mark each colony as it is counted. If the experiment worked well, 5–50 colonies should be observed on the L LB/amp+kan experimental plate, 500–5000 colonies on the A LB/amp control plate, and 200–2000 colonies on the K LB/kan control plate. (If plates are very crowded, draw lines on the bottom of the plate to divide it into equal-sized sections. Count one sector estimated as being representative of the whole plate. After counting, multiply by the number of sectors.) Approximately ten times fewer colonies should be observed on the corresponding L LB/amp plate and L LB/kan plate. An extended recovery period would inflate these numbers. (Question 3 explains how to compute

transformation efficiency.) If plates have been overincubated or left at room temperature for several days, "satellite" colonies may be observed on the LB/amp plates. Satellite colonies are never observed on the LB/kan or LB/amp+kan plates.

1. Record your observation of each plate in matrix below. If cell growth is too dense to count individual colonies, record "lawn." Were the results as expected? Explain possible reasons for variations from expected results.

	Ligated DNA L	pAMP control A	pKAN control K
LB/amp			
LB/kan			
LB/amp+kan			

2. Compare and contrast the growth on each of the following pairs of plates. What does each pair of results tell you about transformation and/or antibiotic selection?

   L LB/amp and A LB/amp
   L LB/kan and A LB/kan
   A LB/amp and K LB/kan
   L LB/amp and L LB/kan
   L LB/amp and L LB/amp+kan
   L LB/kan and L LB/amp+kan

3. Calculate transformation efficiencies of A LB/amp and K LB/kan positive controls. Remember that transformation efficiency is expressed as the number of antibiotic resistant colonies per microgram of intact plasmid DNA. The object is to determine the mass of pAMP or pKAN that was spread on each plate and was therefore responsible for the transformants observed.

   a. Determine the total mass (in micrograms) of pAMP used in Step 5 and of pKAN used in Step 6: concentration x volume = mass.

   b. Determine the fraction of cell suspension spread onto the A LB/amp plate (Step 17) and K LB/kan plate (Step 19): volume suspension spread/*total* volume suspension (Steps 2 and 11) = fraction spread.

   c. Determine the mass of plasmid pAMP and pKAN in the cell suspension spread onto the A LB/amp plate and K LB/kan plate: total mass plasmid (*a*) x fraction spread (*b*) = mass plasmid spread.

   d. Determine the number of colonies per microgram of pAMP and pKAN. Express answer in scientific notation: colonies observed/mass plasmid spread (*c*) = transformation efficiency.

4. Calculate transformation efficiencies of the L LB/amp, L LB/kan, and L LB/amp+kan plates.

   a. Calculate the mass of pAMP and pKAN used in the restriction reactions of Laboratory 9 (see matrix in Part A, Step 1). Then calculate the concentration of plasmid in each restriction reaction.

   b. Calculate the mass of pAMP and pKAN used in the ligation reaction (Laboratory 9, Part B, Step 3). Then calculate the *total* concentration of plasmid in the ligation mixture.

c. Use this concentration in calculations following Steps a–d of Question 3 above.

5. Compare the transformation efficiencies that you calculated for the A LB/amp plate in this laboratory and the +pAMP plate in Laboratory 5. By what factor is the classical procedure more or less efficient than colony transformation? What differences in the protocols contribute to the increase in efficiency?

6. Compare the transformation efficiencies that you calculated for control pAMP and pKAN *versus* the ligated pAMP and pKAN. How can you account for the differences in efficiency? Take into account the formal definition of transformation efficiency.

FOR FURTHER RESEARCH

Interpretable experimental results can only be achieved when the classic transformation protocol can be repeated with reproducible results. Only attempt the experiments below when you are able to routinely achieve 500–2000 colonies on the A LB/amp plate.

1. Design and execute an experiment to compare the transformation efficiencies of linear versus circular plasmid DNAs. Keep molecular weight constant.

2. Design and execute a series of experiments to test the relative importance of each of the four major steps of most transformation protocols: (1) preincubation, (2) incubation, (3) heat shock, and (4) recovery. Which steps are absolutely necessary?

3. Design and execute a series of experiments to compare the transforming effectiveness of $CaCl_2$ *versus* salts of other monovalent (+), divalent (++), and trivalent (+++) cations.

   a. Make up 50 mM solutions of each salt.

   b. Check the pH of each solution, and buffer to pH 7 when necessary.

   c. Is $CaCl_2$ unique in its ability to facilitate transformation?

   d. Is there any consistent difference in the transforming ability of monovalent *versus* divalent *versus* trivalent cations?

4. Test the effect of pH differences on transformation. First, prepare a series of 50 mM $CaCl_2$/20 mM Tris transformation solutions at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. Next grow a 100-ml culture of *E. coli* to mid-log phase (OD_{550} 0.4–0.6). Remove nine 10-ml aliquots and prepare competent cells with the above series of transformation solutions. Then, transform each set of competent cells using a plasmid DNA, such as pAMP. For consistency, select from only one dilution tube of plasmid DNA for all of your transformations. Follow identical protocols for each transformation so that pH is the only variable. Plate transformations on selective media and incubate overnight. The next day, count and determine which pH transformation solution has the highest efficiency.

5. Carry out a similar experiment to determine the effect of adding dithiothreitol (DTT) to the transformation at various concentrations. Use 50 mM

$CaCl_2$/20 mM Tris transformation solution at pH 7 (or whichever pH you have determined works best). Try DTT at 0 mM, 0.5 mM, 1 mM, 2 mM, 5 mM, and 10 mM.

6. Design a series of experiments to determine saturating conditions for transformation reactions.
   a. Transform *E. coli* using the following DNA concentrations:
      0.00001 μg/μl
      0.00005 μg/μl
      0.0001 μg/μl
      0.0005 μg/μl
      0.001 μg/μl
      0.005 μg/μl
      0.01 μg/μl
      0.05 μg/μl
      0.1 μg/μl
   b. Plot a graph of DNA mass *versus* colonies per plate.
   c. Plot a graph of DNA mass *versus* transformation efficiency.
   d. At what mass does the reaction appear to become saturated?
   e. Repeat the experiment with concentrations clustered on either side of the presumed saturation point to produce a fine saturation curve.
7. Repeat Experiment 6 above, but transform with a 1:1 mixture of pAMP and pKAN at each concentration. Plate transformants on LB/amp, LB/kan, and LB/amp+kan plates. *Be sure to include a 40–60-minute recovery, with shaking.*
   a. Calculate the percentage of double transformations at each mass.

$$\frac{\text{colonies amp+kan plate}}{\text{colonies amp plate + colonies kan plate}}$$

   b. Plot a graph of DNA mass *versus* colonies per plate.
   c. Plot a graph of DNA mass *versus* percentage of double transformations. Under saturating conditions, what percentage of bacteria are doubly transformed?
8. Plot a recovery curve for *E. coli* transformed with pKAN. Allow cells to recover for 0–120 minutes at 20-minute intervals.
   a. Plot a graph of recovery time *versus* colonies per plate.
   b. At what time point is antibiotic expression maximized?
   c. Can you discern a point at which the cells began to replicate?
9. Attempt to isolate pAMP/KAN recombinants using the colony transformation protocol in Laboratory 5. What trick would increase the likelihood of retrieving ampicillin/kanamycin-resistant colonies?