Replica Plating to Identify Mixed *E. coli* Populations

Ligation of *Bam*HI/*Hin*dIII fragments of pAMP and pKAN in Laboratory 9 created a number of recombinant plasmids containing either an ampicillin resistance gene (amp^r), a kanamycin resistance gene (kan^r), or both genes together (amp^r/kan^r). Samples of this ligated DNA (L) were then transformed into competent *E. coli* cells, which were plated onto LB/amp, LB/kan, and LB/amp+kan plates in Laboratory 10. The results clearly indicate that colonies growing on the L LB/amp+kan plate (having an amp^r/kan^r phenotype) contain both resistance genes (the amp^r/kan^r genotype).

It is not possible, however, to be certain of the amp^r/kan^r genotypes of bacteria growing on the L LB/amp or L LB/kan plates. Although a colony growing on the L LB/amp plate possesses an amp^r gene, it is not possible to say whether it also possesses a kan^r gene. Conversely, although a colony growing on the L LB/kan must possess a kan^r gene, it is not possible to know whether it also possesses an amp^r gene. A conclusion can be made about the presence of an antibiotic resistance gene only when the organism has been challenged with that antibiotic.

In this laboratory, replica plating provides a rapid means to distinguish between single- and dual-resistant colonies growing on the L LB/amp and L LB/kan plates. Cells from 12 colonies on the L LB/amp plate and from 12 colonies on the L LB/kan plate are transferred onto one fresh LB/amp plate and one fresh LB/kan plate to which numbered grids have been attached. An L colony is scraped with a sterile toothpick (or inoculating loop), and a sample of cells is streaked successively into the same numbered squares of the fresh LB/amp and LB/kan plates. Following overnight incubation at 37°C, colonies that grow in the same squares of both the LB/amp and LB/kan plates have the amp^r/kan^r genotype.

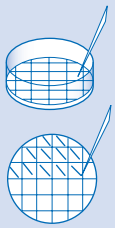
Equipment and materials for this laboratory are available from the Carolina Biological Supply Company (see Appendix 1).

INOCULATE same number square on both plates from each colony

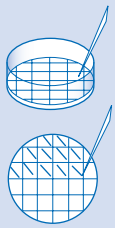
squares 1–12

L LB/amp Culture Plate

LB/amp



LB/kan

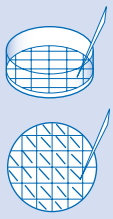


INOCULATE same number square on both plates from each colony

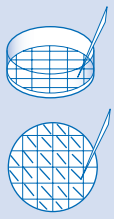
squares 13–24

L LB/kan Culture Plate

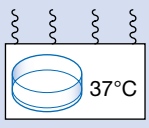
LB/amp



LB/kan



INCUBATE 15–20 hours



PRELAB NOTES

Review Prelab Notes in Laboratory 2A, Isolation of Individual Colonies.

Replica plating provides a rapid means to screen L LB/amp and L LB/kan plates for dual-resistant colonies that potentially contain pAMP/KAN recombinant plasmids. If colonies were not obtained on the L LB/amp+kan plate, replica plating provides another chance to identify dual-resistant colonies from which to isolate recombinant plasmids for Laboratory 12, Purification and Identification of Recombinant DNA.

For Further Information

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

Lederberg J. and Lederberg E.M. 1952. Replica plating and indirect selection of bacterial mutants. *J. Bacteriol.* **63:** 399.

PRELAB PREPARATION

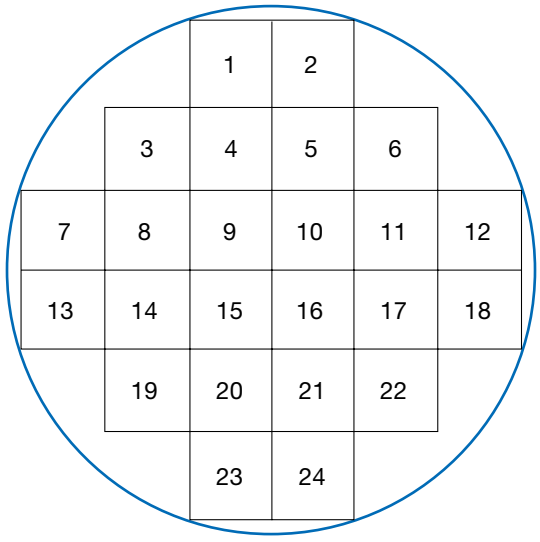
Before performing this Prelab Preparation, please refer to the cautions indicated on the Laboratory Materials list.

1. Prepare for each experiment:

   one LB + ampicillin plate (LB/amp)
   one LB + kanamycin plate (LB/kan)

2. Sterilize 30 toothpicks per experiment. Put toothpicks in 50-ml beaker, cover with aluminum foil, and autoclave for 15 minutes at 121°C. (Although much less rapid, a flamed and cooled inoculating loop can be used to transfer colonies.)

3. Make two copies of the replica-plating grid (below) per experiment.



4. Prewarm incubator to 37°C.

MATERIALS

CULTURES AND MEDIA

L LB/amp plate w/colonies (from Laboratory 10)
L LB/kan plate w/colonies (from Laboratory 10)
LB/amp plate (1)
LB/kan plate (1)

SUPPLIES AND EQUIPMENT

Beaker for waste
"Biobag" or heavy-duty trash bag
Bleach▼(10%) or disinfectant
Incubator (37°C)
Permanent marker
Replica-plating grids (2)
Sterile toothpicks (or inoculating loop + Bunsen burner)

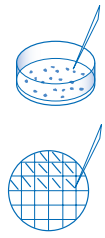
▼ See Appendix 4 for Cautions list.

METHODS

Prepare Replica Plates (10 minutes)

A 24-square grid may be drawn on the bottom of the plate with a permanent marker.

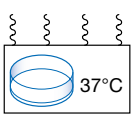
Lift plate lids only enough to select colony and streak. Do not place lids on the lab bench.



1. Attach a replica-plating grid to the *bottom* of an LB/amp plate and to the *bottom* of an LB/kan plate. Use a permanent marker to label each plate with your name and the date.
2. Replica plate a sample of cells from one colony on the L LB/amp plate onto the fresh LB/amp and LB/kan plates.
   a. Use a sterile toothpick (or inoculating loop) to scrape up a cell mass from a well-defined colony on the L LB/amp plate.
   b. Immediately drag the *same* toothpick (or loop) gently across the agar surface to make a short diagonal (/) streak *within Square 1* of the LB/amp plate.
   c. Immediately use the *same* toothpick (or loop) to make a diagonal (/) streak *within Square 1* of the LB/kan plate.
   d. Discard the toothpick in a waste beaker (or reflame and cool inoculating loop).
3. Repeat Step 2a–d *using fresh toothpicks* (or *flamed* and *cooled* inoculating loop) to streak cells from 11 *different* L LB/amp colonies onto Squares 2–12 of both LB/amp and LB/kan plates.

If you have fewer than 12 colonies on either plate, obtain a plate from another experimenter.

4. Repeat Step 2a–d *with fresh toothpicks* (or a *flamed* and *cooled* inoculating loop) to streak cells from 12 *different* L LB/kan colonies onto Squares 13–24 of both LB/amp and LB/kan plates.
5. Place plates upside down in a 37°C incubator, and incubate for 15–20 hours.



6. After initial incubation, store plates at 4°C to arrest *E. coli* growth and to slow the growth of any contaminating microbes.
7. Take time for responsible cleanup:

Save replica plates as a source of cells from which to isolate plasmid DNA in Laboratory 12 if your L LB/amp+kan plate has fewer than two colonies.

a. Segregate for proper disposal bacterial cultures *and* used toothpicks.

b. Disinfect plates and toothpicks 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

In general, the results of replica plating indicate the success of the ligation in Laboratory 9 and parallel the results observed on the L LB/amp+kan plate from Laboratory 10. Thus, if there were a large number of colonies on the LB/amp+kan plate, it is likely that there will be a high percentage of dual-resistant colonies that grow on both the LB/amp and LB/kan replica plates. Experience indicates that 30–70% of transformants selected with only ampicillin *or* kanamycin actually have dual resistance. Roughly equal numbers of dual-resistant colonies are identified from the L LB/amp and L LB/kan plates.

1. Observe the LB/amp and LB/kan plates. Use the matrix below to record as + the squares in which new bacterial growth has expanded the width of the initial streak. Record as – the squares in which no new growth has expanded the initial streak. Remember that nonresistant cells may survive, separated from the antibiotic on top of a heavy initial streak; however, no *new growth* will be observed.

Colony source	Replica plates		*Colony source*	Replica plates	
L LB/amp	LB/amp	LB/kan	*L LB/kan*	LB/amp	LB/kan
1			13		
2			14		
3			15		
4			16		
5			17		
6			18		
7			19		
8			20		
9			21		
10			22		
11			23		
12			24		

2. On the basis of your observations:

   a. Calculate the percentage of dual-resistant colonies taken from the L LB/amp plate (Squares 1–12).

   b. Calculate the percentage of dual-resistant colonies taken from the L LB/kan plate (Squares 13–24).

   c. Give an explanation for the similarity or difference in the percentages of dual-resistance colonies taken from the two source plates.

3. Draw restriction maps for different plasmid molecules that could be responsible for the dual resistance phenotype.

FOR FURTHER RESEARCH

1. We have discussed the phenomenon of satellite colonies on LB/amp plates—small nonresistant colonies that grow in a halo around large resistant colonies. To prove this, replica plate satellite colonies from an LB/amp plate onto fresh LB and LB/amp plates.
2. The Amp^r protein, β-lactamase, is not actively secreted into the medium, but it is believed to "leak" through the cell wall of *E. coli*. Satellite colonies do not form on kanamycin plates because the antibiotic kills all nonresistant cells outright. The following experiment tests whether resistance protein escapes from ampicillin- and kanamycin-resistant cells.
   a. Grow separate overnight cultures of an *amp*^{r} colony from the A LB/amp plate and a *kan*^{r} colony from the K LB/kan plate (from Laboratory 10, or use other *amp*^{r} and *kan*^{r} strains). Inoculate 5 ml of *plain* LB broth, according to the protocol in Laboratory 2B, Overnight Suspension Culture.
   b. Pass each overnight culture through a 0.22-μm or 0.45-μm filter, and collect the filtrate in a clean, sterile 15-ml tube. Filtering removes all *E. coli* cells.
   c. Use a permanent marker to mark one LB/amp plate and one LB/kan plate. Draw a line on the plate bottom to divide each plate into two equal parts; mark one half +.
   d. Sterilely spread 100 μl of the A filtrate onto the + *half only* of the LB/amp plate. Sterilely spread 100 μl of the K filtrate onto the + *half only* of the LB/kan plate. Allow filtrates to soak into plates for 10–15 minutes.
   e. Sterilely streak wild-type (nontransformed) *E. coli* cells on each filtrate-treated plate, taking care to streak back and forth across the dividing line.
   f. Incubate plates for 15–20 hours at 37°C. Compare growth on the treated sides *versus* untreated sides of each plate.
3. The Amp^r protein is believed to leak primarily from stationary-stage cells. The following experiment tests the hypothesis that leakage of β-lactamase is growth-phase-dependent.
   a. Grow an overnight culture of an ampicillin-resistant colony from the A LB/amp plate in Laboratory 10 (or other *amp*^{r} strain). Inoculate 1 ml of *plain* LB broth, according to the protocol in Laboratory 2B, Overnight Suspension Culture.
   b. Use an overnight culture to inoculate 100 ml of fresh LB broth, and grow according to the protocol in Laboratory 2C, Mid-log Suspension Culture.
   c. Sterilely withdraw 10-ml aliquots from the culture after 1, 2, and 4 hours, holding aliquots on ice.
   d. Take the OD_{550} of each aliquot.

NOTE The objective is to test resistance protein "leakage" as a function of culture age, *not as a function of cell number.* Because cell number increases over time, it must be equalized by diluting the 2-hour and 4-hour samples with sterile LB to the *E. coli* concentration of the 1-hour sample. Since the OD_{550} values are proportional to the cell number, they can be used to compute the dilution factor.

e. Pass each of the three samples through a 0.22-μm or 0.45-μm filter to remove the bacteria.

f. Prepare a 10-fold and 100-fold dilution for each filtrate, using sterile LB broth.

g. Use a permanent marker to draw a line dividing each of six LB/amp plates into equal parts.

h. Sterilely spread 100 μl of undiluted filtrate from the 1-hour sample over half of the first plate. Label the plate with time point and dilution factor. Allow filtrate to soak into the plate for 10–15 minutes.

i. Spread 100 μl of undiluted 2-hour and 4-hour filtrates over separate halves of the second plate, as described above.

j. Repeat spreading procedure for the 10-fold and 100-fold dilutions, as described in Steps h and i.

k. After filtrates have soaked into the plates, sterilely streak wild-type (non-transformed) *E. coli* cells on each half of each plate.

l. Incubate the plates for 15–20 hours at 37°C. Compare growth for each time point across each dilution.