INSTRUCTOR GUIDE

Experiment 1004. Genotype to Phenotype

I. Chemicals and Materials Provided

DNA Manipulation

Plasmid A - pUC18	160 µl
Plasmid B - pUC18-Lambda	160 µl
Plasmid C - no plasmid	160 µl
DNA Markers	140 µl
Sample Buffer	0.5 ml
EcoR1 Endonuclease	250 units (25µl)
Endonuclease Buffer	0.6 ml of a 1.5 x solution

Bacterial Transformation and Growth

E. coli (Strain DH5)	0.5 ml
$CaCl_2$	5 ml
Nutrient Broth	10 ml
Nutrient Agar + Ampicillin	400 ml
Xgal	20 mg
Xgal Solvent	1 ml
Sterile Petri Dishes	20
Sterile Transfer Pipets	19
Sterile Microtubes	19
Inoculating Loops	25
Ampicillin	0.6 ml

The sample buffer contains glycerol, bromophenol blue, and electrophoresis buffer. The concentration of the plasmids is 350µg/ml and the concentration of the DNA markers is 150µg/ml. The markers are dissolved in sample buffer and are ready for electrophoresis.

II. Materials that are Needed but Not Provided

PROCELL Electrophoresis Unit and MB-170 Power Supply or equivalent Accessory Kit or equivalent
Water Bath
Agarose, Electrophoresis Buffer and Gel Stain from Electrophoresis Package 3/4. Instructions for preparing the buffer and stain are provided with Electrophoresis Package 3/4.

III. Laboratory Schedule

The procedure is written such that parts A and B of this exercise are performed during a single lab session. If the two parts are performed independently, the time the cells are incubated with DNA can be reduced to 15 minutes. (Step II-4)

IV. Pre and Post Lab Preparation

1. The X gal-Ampicillin-Agar plates must be prepared at least one day before the lab session.

2. Preparation of the competent cells must be initiated by the instructor shortly before or at the beginning of the laboratory session.

3. The plates should be placed in the refrigerator one day after the transformation.

V. Preparation of Solutions and Materials

EcoR1

The restriction enzyme EcoR1 is provided in a glycerol solution. Immediately before use, add 260 µl of restriction nuclease buffer to the tube containing the EcoR1. Mix the contents of the tube well to ensure that the enzyme comes in contact with the buffer. Place the tube in an upright position in the refrigerator.

Xgal-Ampicillin-Agar Plates

Twenty mg of Xgal, 1ml of Xgal solvent (Dimethyl Formamide), one bottle containing 400ml of nutrient agar plus ampicillin and 20 petri dishes are supplied. Due to the unstable nature of ampicillin, additional ampicillin is also provided and should be added to the nutrient agar plus ampicillin mixture to ensure that sufficient antibiotic is present in the agar plates. The plates must be prepared at least one day before the laboratory session. To prepare the plates:

A. Loosen the cap on the bottle.

B. Place the bottle in a beaker of boiling water over a burner until the agar has liquefied. This should take about 20-25 minutes.

C. Remove the bottle from the bath and let cool at room temperature for about 10 minutes.

D. Pour the entire 1ml of the Xgal solvent into the tube containing Xgal, cap the tube and carefully shake until the Xgal is dissolved.

Note: The Xgal solvent, dimethyl formamide, is toxic at this concentration. The solvent should be handled with caution in a well vented area (a fume hood, if available) and the instructor should wear gloves.

E. Pour the entire 0.6ml of the ampicillin solution and the 1ml of Xgal solution into the bottle containing the nutrient agar, replace the cap, and swirl the bottle to mix the contents. The ampicillin, Xgal, and Xgal solvent will be found in 1.5ml tubes along with the other frozen components of this Chemical Package

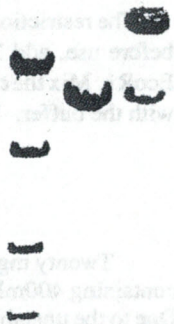
F. Uncover the petri dishes, one at a time, and pour a thin layer (10-20ml) of agar into the lower half of each dish. Immediately replace the covers. Let the agar harden for about one day at room temperature. If desired, the plates can be stored in the refrigerator in an inverted position for at least two weeks.

Sterile Microliter Pipets

The glass microliter pipets (but not the plungers) sold by Modern Biology, Inc., can be sterilized by baking in an oven for 20 minutes.

VI. Experimental Results

I. Identification and description of the plasmids and the results of the electrophoretic studies from Part B.



Plasmid A-(pUC18) - The plasmid is 2686bp in length and contains a single EcoR1 site. Thus, digestion with EcoR1 converts the circular plasmid to a linear form of 2686bp. (See lane 2 on the adjacent gel)

Plasmid B-(recombinant plasmid) - The plasmid contains a 4878bp fragment from phage lambda inserted into the EcoR1 site of pUC18. Thus, digestion of the plasmid with EcoR1 gives rise to two bands of 4878 and 2686bp. (See lane 3)

Plasmid C - No plasmid (See lane 4)

DNA Markers - are 3621, 2040, 1120, and 784bp in length (See lane 1)

II. Phenotype of *E. coli* after transformation

Plasmid A yields blue E. coli colonies because it contains an active lac Z gene. Plasmid B yields white *E. coli* colonies because the lac Z gene was interrupted and inactivated by the lambda insert. Both plasmids promote growth of *E. coli* on ampicillin because both contain an ampicillin resistance gene. In contrast, transformation with "Plasmid C" (no plasmid) should not give rise to bacterial colonies

VII. Pooled Class Data

The sheet provided on the facing page can be distributed at the end of the exercise to provide students with pooled class data.

Student Initial	Plasmids Analyzed (A, B, C)	Colony Growth (yes or no)	Colony Color (Blue or White)	DNA Bands (Number)

Experiment 1004. Genotype to Phenotype

Background Information

A. Introducing a Plasmid into *E. coli*

Penicillin is one of the most important anti-infective agents used in clinical medicine because it is inexpensive, bactericidal and its toxicity for human cells is almost nonexistent. Penicillin interferes with the synthesis of the bacterial cell wall and will thus cause osmotic lysis of susceptible microbes. Penicillin is not a single compound but a group of compounds with related structures and activities. Many of these compounds are semi-synthetic in that part of each molecule is made by a mold to which the chemist adds another chemical group. Over 500 semi-synthetic penicillins have been made during the past 30 years. Ampicillin is a broad-spectrum semi-synthetic penicillin that will kill a number of gram-positive and gram-negative bacteria, including *Salmonella* and *Escherichia coli*. Occasionally, *E. coli* cells are found in nature that are resistant to the toxic effects of ampicillin. In today's laboratory, you will create such an ampicillin-resistant population of *E. coli*.

Plasmids are small circular DNA molecules that exist apart from the chromosomes in most bacterial species. Under normal circumstances, plasmids are not essential for survival of the host bacteria. However, many plasmids contain genes that enable bacteria to survive and to prosper in certain environments. For example, some plasmids carry one or more genes that confer resistance to antibiotics. A bacterial cell containing such a plasmid can live and multiply in the presence of the drug. Indeed, antibiotic-resistant *E. coli* isolated in many parts of the world contain plasmids that carry the genetic information for protein products that interfere with the action of many different antibiotics. In this laboratory, you will introduce a plasmid that contains an ampicillin resistance gene into *E. coli*.

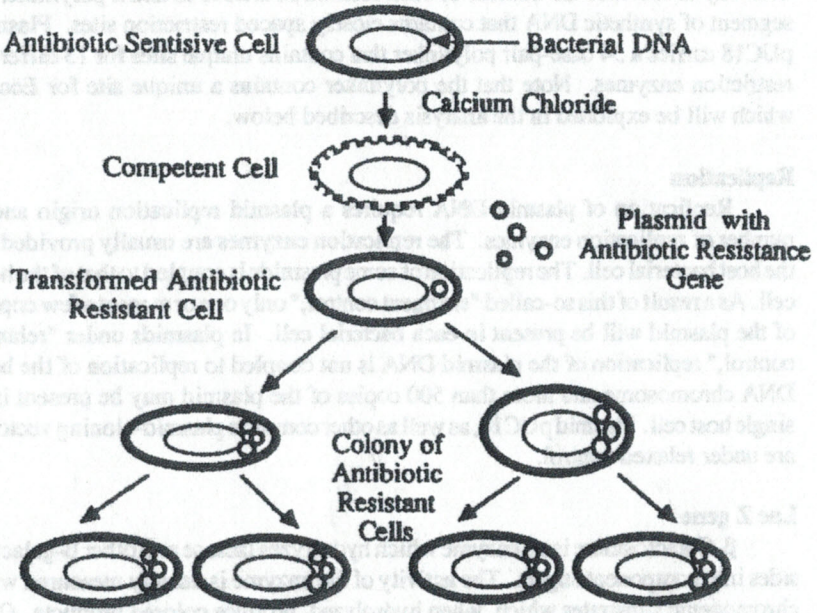
In the laboratory, plasmids can be introduced into living bacterial cells by a process known as transformation. A common procedure for transformation is shown in Figure 1 and entails:

1. The treatment of bacterial cells with calcium chloride in order to enhance the uptake of plasmid DNA. Such calcium chloride treated cells are said to be competent.

2. The incubation of the competent cells with plasmid DNA and the uptake of the DNA into a small fraction of the cells. The cells that have taken up plasmid DNA are said to be transformed.

3. The selection of transformed cells by growth on an antibiotic-containing medium.

Figure 1. Transformation of Bacteria with Plasmid DNA



B. Plasmid pUC18

The plasmid that you will use in the analysis is called pUC18. A map of plasmid pUC18 is shown in Figure 2 and a discussion of some of the features of this plasmid is given below.

Antibiotic Resistance

Plasmid pUC18 contains an ampicillin-resistance gene that enables *E. coli* containing this plasmid to grow in the presence of the antibiotic. Thus, bacteria lacking this plasmid, or bacteria that lose the plasmid, generally will not grow in the presence of this antibiotic. The ampicillin-resistance gene of pUC18 codes for the enzyme beta-lactamase (penicillinase), which inactivates ampicillin and other penicillins.

Structure

Like all plasmids, pUC18 is a circular DNA molecule. Digestion of the plasmid with a restriction endonuclease that cleaves the DNA in only one site converts the circular form to a linear molecule.

Size

A small plasmid, pUC18 contains only 2686 base-pairs (molecular weight = 2×10^6). The small size of this plasmid makes it less susceptible to physical damage during handling. In addition, smaller plasmids generally replicate more efficiently in bacteria and produce larger numbers of plasmids per cell.

Restriction Sites

To be useful as a cloning vector, a plasmid should possess a single recognition site for one or more restriction enzymes into which foreign DNA can be inserted. One way to increase the number of such restriction sites is to add a polylinker , a segment of synthetic DNA that contains closely spaced restriction sites. Plasmid pUC18 carries a 54 base-pair polylinker that contains unique sites for 13 different restriction enzymes. Note that the polylinker contains a unique site for EcoR1 which will be exploited in the analysis described below.

Replication

Replication of plasmid DNA requires a plasmid replication origin and a number of replication enzymes. The replication enzymes are usually provided by the host bacterial cell. The replication of some plasmids is coupled to that of the host cell. As a result of this so-called "stringent control," only one or at most a few copies of the plasmid will be present in each bacterial cell. In plasmids under "relaxed control," replication of the plasmid DNA is not coupled to replication of the host DNA chromosome and more than 500 copies of the plasmid may be present in a single host cell. Plasmid pUC18, as well as other common plasmid-cloning vectors, are under relaxed control.

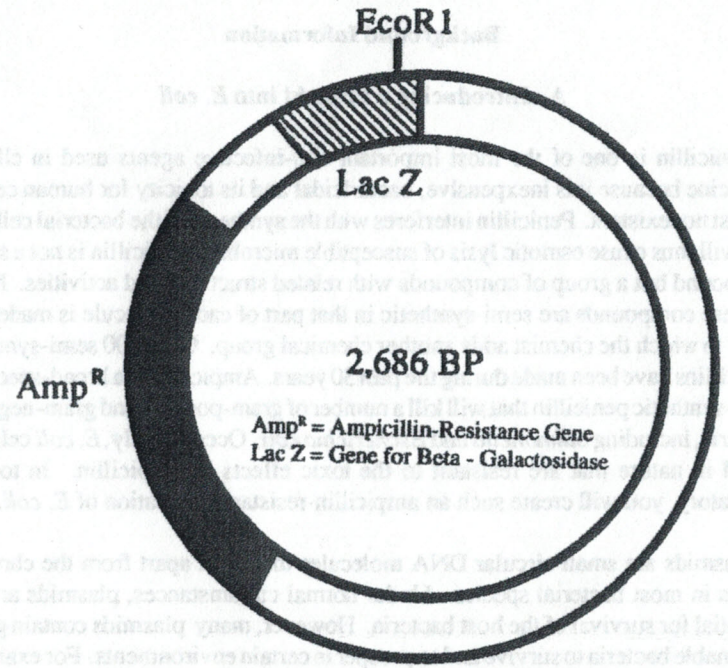
Lac Z gene

β-Galactosidase is an enzyme which hydrolyzes lactose and other β-galactosides into component sugars. The activity of the enzyme is usually measured with chromogenic substrates which, when hydrolyzed, produce colored products. One substrate is Xgal (5-bromo-4-chloro-3-indolyl-βD galactoside) which is converted to a blue product by β-galactosidase. Xgal is frequently used to detect β-galactosidase in cell extracts, bacterial colonies, and phage plaques. You will use this substrate to identify E. coli colonies containing β-galactosidase.

The Lac Z gene, which specifies β-galactosidase, is a member of the E. coli Lac operon. Plasmid pUC18 contains a portion of the E. coli Lac Z gene that codes for the first 146 amino acids of β-galactosidase. The remaining portion of the gene is encoded by appropriate strains of E. coli such as DH5 and JM 101. Thus, when these strains are transformed with pUC18, complementation occurs and the bacteria produce active β-galactosidase, which gives rise to blue colonies on nutrient agar containing Xgal.

The above complementation forms the basis of a powerful selection method that you will use to identify E. coli colonies containing pUC18 with foreign DNA. The polylinker is contained within the Lac Z gene of pUC18 although the insertion does not affect the ability of the β-galactosidase peptide to be complemented by the E. coli host. However, foreign DNA inserted into the polylinker destroys complementation and active β-galactosidase is not synthesized. Thus, in the later case, white colonies are produced on Xgal plates in contrast to the blue colonies produced by cells containing pUC18 without foreign DNA.

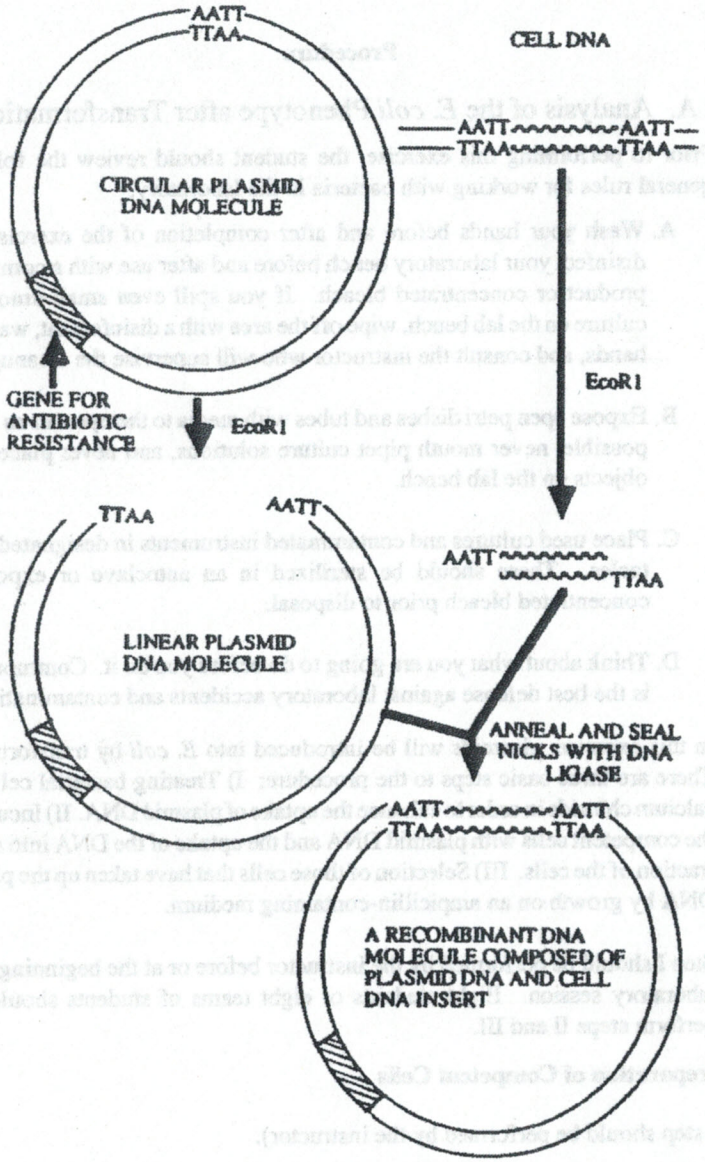
Figure 2. Partial Map of Plasmid pUC18.



C. Plasmids as Gene Carrier Molecules

Plasmids are useful tools for the molecular biologist because they serve as gene-carrier molecules. A basic procedure of recombinant DNA technology consists of joining a gene of interest to plasmid DNA to form a hybrid, or recombinant molecule that is able to replicate in bacteria (Figure 3). In order to prepare a recombinant molecule, the plasmid and gene of interest are cut at precise positions by a restriction endonucleases and then the molecules are spliced together using an enzyme called DNA ligase. After the hybrid plasmid molecule has been prepared, it is introduced into E. coli cells by transformation. The hybrid plasmid replicates in the dividing bacterial cells to produce an enormous number of copies of the original gene. At the end of the growth period, the hybrid molecules are purified from the bacteria and the original gene of interest is recovered. This method has enabled scientists to obtain large quantities of more than 1000 specific genes including the genes for human interferon, insulin and growth hormone.

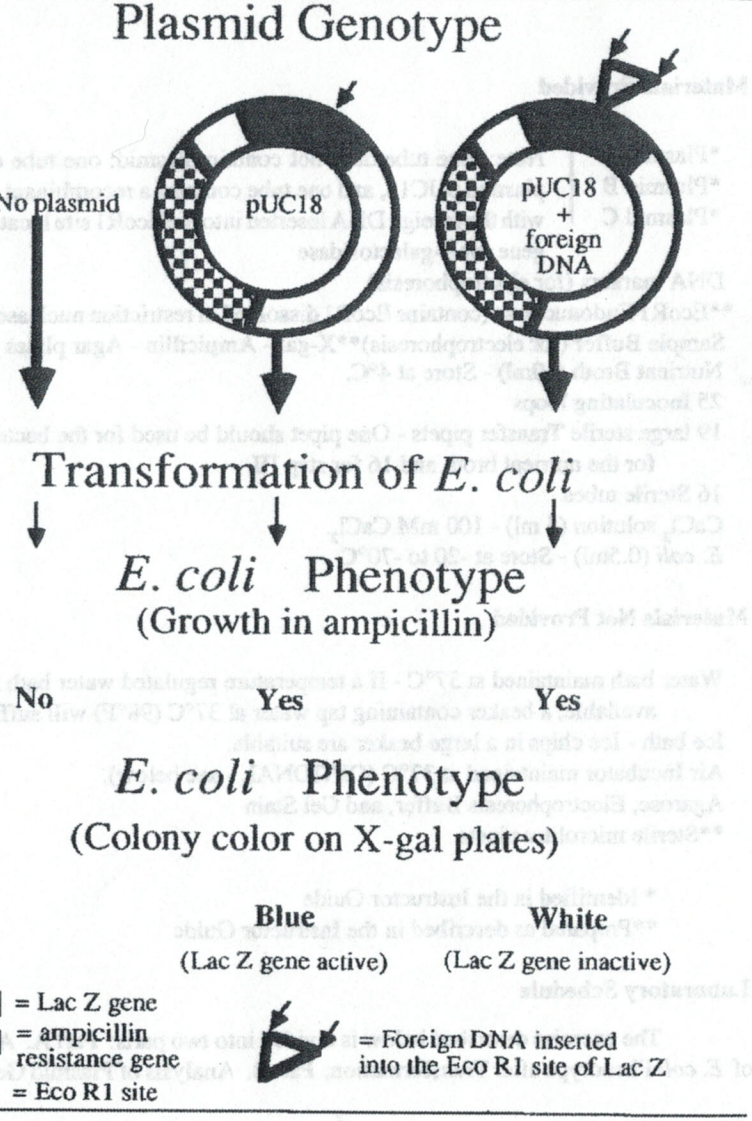
Figure 3. Preparation of a Recombinant DNA Molecule



D. Description of the Laboratory Exercise

An outline of this laboratory exercise is diagrammed in Figure 4. You will be given three tubes labeled Plasmid A, Plasmid B, and Plasmid C. One tube contains no plasmid, one tube contains plasmid pUC18 and one tube contains a recombinant plasmid. The recombinant plasmid carries a segment of foreign DNA from phage lambda inserted into the EcoR1 site of pUC18. This site is located in

Figure 4. Genotype and Phenotype



the lac Z gene which is the gene for β-galactosidase. The plasmid preparations will be characterized in two ways. Plasmid genotype will be studied by restriction nuclease digestion and electrophoresis. The phenotype of *E. coli* will also be examined after introduction of the plasmids into the bacteria by transformation. The cells will be plated onto nutrient agar containing ampicillin and Xgal. Analysis of bacterial colony growth and color will enable you to determine if the plasmid contains functionally active genes for ampicillin resistance and β-galactosidase. The results of the genotypic and phenotypic studies will permit you to determine the nature of the plasmids in tubes A, B, and C.

Materials Provided

*Plasmid A
*Plasmid B
*Plasmid C

Note: one tube does not contain plasmid: one tube contains plasmid pUC18, and one tube contains a recombinant plasmid with the foreign DNA inserted into the EcoR1 site located in the gene for β-galactosidase

DNA markers (for electrophoresis)
**EcoR1 Endonuclease (contains EcoR1 dissolved in restriction nuclease buffer)
Sample Buffer (for electrophoresis)**X-gal - Ampicillin - Agar plates (16)
Nutrient Broth (10ml) - Store at 4°C.
25 Inoculating loops
19 large sterile Transfer pipets - One pipet should be used for the bacteria, one for the nutrient broth and 16 for step III.
16 Sterile tubes
$CaCl_2$ solution (5 ml) - 100 mM $CaCl_2$.
E. coli (0.5ml) - Store at -20 to -70°C

Materials Not Provided

Water bath maintained at 37°C - If a temperature regulated water bath is not available, a beaker containing tap water at 37°C (98°F) will suffice.
Ice bath - Ice chips in a large beaker are suitable.
Air Incubator maintained at 37°C (OPTIONAL - see below).
Agarose, Electrophoresis Buffer, and Gel Stain
**Sterile microliter pipets

* Identified in the Instructor Guide
**Prepared as described in the Instructor Guide

Laboratory Schedule

The exercise described below is divided into two parts: Part A. Analysis of *E. coli* Phenotype after Transformation; Part B. Analysis of Plasmid Genotype

by Electrophoresis. The procedure is written such that both parts can be performed during a single 3-hour laboratory session. Alternatively, parts A and B can be performed independently during two 2-hour laboratory periods.

Procedure

Part A. Analysis of the *E. coli* Phenotype after Transformation

1. Prior to performing this exercise, the student should review the following general rules for working with bacteria in the laboratory.

   A. Wash your hands before and after completion of the exercise, and disinfect your laboratory bench before and after use with a commercial product or concentrated bleach. If you spill even small amounts of culture on the lab bench, wipe off the area with a disinfectant, wash your hands, and consult the instructor who will supervise the cleanup.

   B. Expose open petri dishes and tubes with media to the open air as little as possible, never mouth pipet culture solutions, and never place sterile objects on the lab bench.

   C. Place used cultures and contaminated instruments in designated receptacles. These should be sterilized in an autoclave or exposed to concentrated bleach prior to disposal.

   D. Think about what you are going to do before you do it. Common sense is the best defense against laboratory accidents and contamination.

2. In this exercise, plasmids will be introduced into *E. coli* by transformation. There are three basic steps to the procedure: I) Treating bacterial cells with calcium chloride in order to enhance the uptake of plasmid DNA. II) Incubating the competent cells with plasmid DNA and the uptake of the DNA into a small fraction of the cells. III) Selection of those cells that have taken up the plasmid DNA by growth on an ampicillin-containing medium.

   Step I should be performed by the instructor before or at the beginning of the laboratory session. Eight students or eight teams of students should then perform steps II and III.

I. Preparation of Competent Cells

(This step should be performed by the instructor).

1. Place the vial of $CaCl_2$ and the tube of *E. coli* in the ice bath.

2. Using a sterile pipet, transfer about 1/2 ml of the $CaCl_2$ solution to the tube containing the bacteria.

3. Using the same pipet, transfer the contents of this tube back into the larger vial that contains most of the $CaCl_2$ solution.

4. Tap the vial with the tip of your index finger to mix the solution.

5. Incubate the cells for about 20 minutes on ice. The cells are then called competent because they can take up DNA from the medium. If desired, the cells can be stored in the $CaCl_2$ solution for up to 12 hours on ice before use.

II. Uptake of DNA by competent cells

1. Choosing plasmid samples.

The analysis described below was designed for 8 groups of students. There are three tubes labeled Plasmid (A, B, and C). Each group will analyze two plasmids as indicated below.

Student Group Number	Plasmids that will be analyzed
1, 2 -	Plasmids A and B
3, 4 -	Plasmid A and B
5, 6 -	Plasmid A and C
7, 8 -	Plasmid B and C

2. Obtain two sterile tubes (1.5ml) and label the tubes with the letters that correspond to the plasmids that you will analyze (A and B, A and C, or B and C)

3. Using a sterile micropipet, add 10µl of the corresponding plasmid DNAs to the two tubes and place them in an ice bath. Use a fresh sterile micropipet for each addition.

4. Gently tap the vial of competent cells with the tip of your index finger to ensure that the cells are in suspension. Then, using a sterile transfer pipet, add 5 drops (5 drops ≈ 100 µl) of the competent cells to each of the two tubes. Tap each of these tubes with the tip of your index finger to mix these solutions and store both tubes on ice for 30 - 90 minutes. During this time, proceed to Part B of this exercise: Analysis of Plasmid Genotype. Return to step 5 (below) during the electrophoretic run.

5. Transfer the tubes to a water bath, preheated to 37°C, for 5 minutes. This heat shock facilitates the uptake of plasmid DNA.

6. Add about 0.7ml of nutrient broth to each tube and incubate at 37°C for 30 minutes. The nutrient broth should be dispensed with a sterile transfer pipet. This incubation period allows the bacteria time to recover from the $CaCl_2$ treatment and to begin to express the ampicillin-resistance gene on the plasmid.

III. Selection of cells that have taken up the plasmid by growth on an ampicillin-containing medium.

1. Obtain two X gal- ampicillin-nutrient agar plates from your instructor. Label the plates A, B, or C according to the plasmids that were used for the transformation.

2. Using a sterile pipet, remove 0.25ml of the mixed bacterial suspension from the first tube, remove the lid from the corresponding plate and dispense the bacteria onto the agar. Use an inoculating loop to spread the bacteria evenly onto the agar surface.

3. Repeat step 2 using the bacterial suspension from the remaining tube.

4. Replace the lids on the plates and leave the plates at room temperature until the liquid has been absorbed (about 10-15 minutes).

5. Invert the plates and incubate at 37°C (preferred) for about 24 hours or at room temperature for 2-3 days.

6. Plates should then be placed in the refrigerator until the next laboratory session.

7. Count the number and color of visible colonies on each of your plates and record these values below.

Plasmid	Number of Colonies	Color of Colonies
A		
B		
C		

Part B. Analysis of Plasmid Genotype

In this section, you will characterize the DNA in tubes labeled Plasmid A, B, and C.

1. Number two small (0.5ml) tubes according to the plasmid letters (A, B, or C) that you used for transformation.

2. Place 10µl of the EcoR1-buffer solution into each tube.

3. Add 5µl of the corresponding plasmids to the tubes. Gently tap the tubes with the tip of your index finger to mix the solutions. Incubate the tubes for 60 minutes at 37°C.

4. During this incubation, prepare 1.2% agarose gels as described in the first part of this manual.

5. At the end of the 60 minute incubation period, add 5µl of electrophoresis sample buffer to each tube.

6. Load 15µl of the following samples into the sample wells.

Sample Well	Sample	
1		
2	Tube A, B, or C	Group 1
3	Tube A, B, or C	
4	DNA Markers*	
5	Tube A, B, or C	Group 2
6	Tube A, B, or C	
7	DNA Markers*	
8		

*The sizes of these DNA markers are: 784, 1120, 2040, and 3621 base pairs.

7. Electrophorese until the bromophenol blue in the samples has migrated to within 1mm of the positive electrode end of the gel.

8. During the electrophoretic run, return to Part A of this exercise (Step II - 5) and complete the bacterial transformation.

9. At the end of the electrophoretic run, remove the gels from the unit and stain them as described in the first part of this manual.

Data Analysis: Determining the Size of the Plasmid DNA Fragments

1. Place your gel over a light source and measure the distances of all DNA bands (in mm) from the sample origin. Record these values below.

lanes	mm migrated
2	_____
3	_____
4	_____
5	_____
6	_____
7	_____

2. On the semilog paper provided on the following page, plot the distance migrated by the DNA bands in the DNA marker sample. The length of these DNA molecules are: 784, 1120, 2040, and 3621 base pairs.

3. Estimate the length of the bands in the plasmid samples and record these values below.

	Fragment(s) length (in base-pairs)
Plasmid A	_____
Plasmid B	_____
Plasmid C	_____

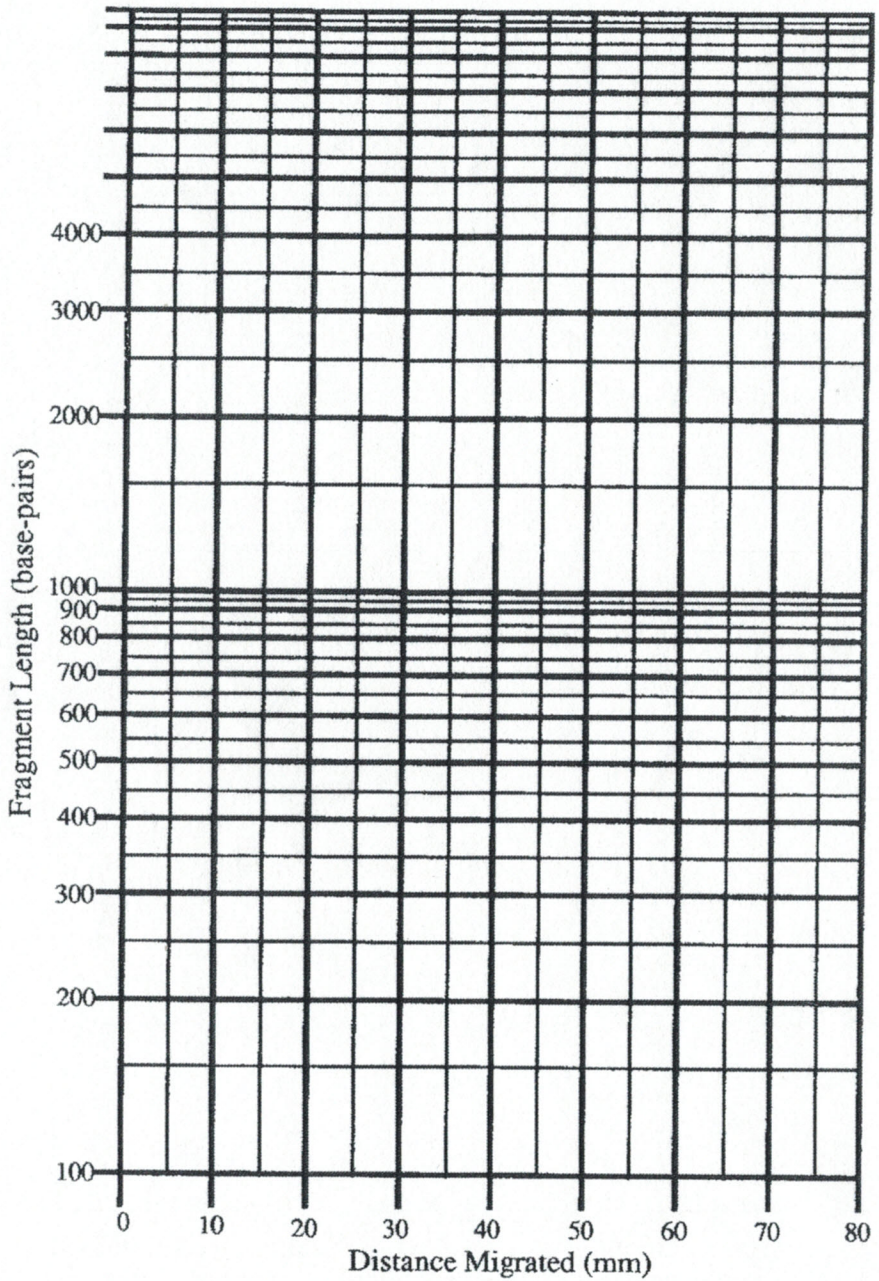
Data Analysis: Identification of the Plasmids

1. Record your observations in the Table below on the transformed *E. coli* phenotype and genotype of plasmids A, B, and C.

Plasmid	Colony Growth (yes or no)	Colony Color (blue or white)	DNA bands (number)
A	_____	_____	_____
B	_____	_____	_____
C	_____	_____	_____

2. From these results, identify the nature of the two plasmids that you characterized in this analysis and provide a detailed rational for your identification.

3. You may wish to compare your identities to those of your classmates by recording your data on a class data sheet that may be distributed by your instructor.


Fragment Length (base-pairs)
4000
3000
2000
1000
900
800
700
600
500
400
300
200
100
0
10
20
30
40
50
60
70
80
Distance Migrated (mm)