Rapid Colony Transformation of *E. coli* with Plasmid DNA

LABORATORY 5 DEMONSTRATES A RAPID METHOD to transform *E. coli* with a foreign gene. The bacterial cells are rendered "competent" to uptake plasmid DNA containing a gene for resistance to the antibiotic ampicillin. A bacteria that successfully takes up plasmid and expresses the gene for antibiotic resistance can be detected by its ability to grow in the presence of ampicillin.

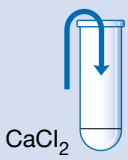
Samples of *E. coli* cells are scraped off a nutrient agar plate (LB agar) and suspended in two tubes containing a solution of calcium chloride. One of three plasmids (pAMP, pBLU, or pGREEN) is added to one cell suspension, and both tubes are incubated for 15 minutes at 0°C. Following a brief heat shock at 42°C, cooling, and addition of LB broth, samples of the cell suspensions are plated on two types of media: LB agar and LB agar plus ampicillin (LB/amp).

The plates are incubated for 15–20 hours at 37°C and then checked for bacterial growth. Only cells that have been transformed by taking up the plasmid DNA with the ampicillin resistance gene will grow on the LB/amp plate. Subsequent division of a single antibiotic-resistant cell produces a colony of resistant clones. Thus, each colony seen on an ampicillin plate represents a single transformation event. In addition, cells transformed with pBLU will have a blue color (on specially prepared plates), and cells transformed with pGREEN fluoresce under ultraviolet (UV) light.

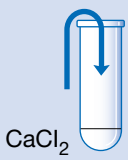
Kits based on this laboratory are available from the Carolina Biological Supply Company.

- Catalog no. 21-1142: Colony Transformation Kit
- Catalog no. 21-1082: Green Gene Colony Transformation Kit
- Catalog no. 21-1088: Glow-in-the-Dark Transformation Kit
- Catalog no. 21-1146: pBLU® Colony Transformation Kit

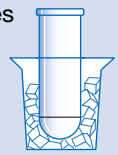
ADD + plasmid



– plasmid

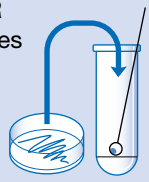


PLACE ON ICE both tubes



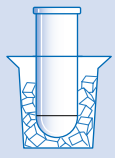
(3 min)

TRANSFER to both tubes



RESUSPEND cell mass in both tubes

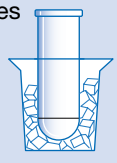
PLACE ON ICE both tubes



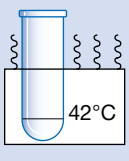
(3–5 min)

ADD + plasmid

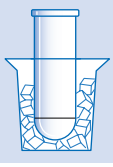
INCUBATE both tubes



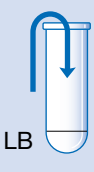
HEAT SHOCK both tubes 90 seconds



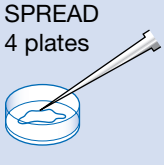
INCUBATE both tubes



ADD to each tube

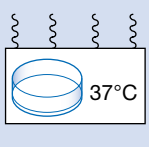


SPREAD 4 plates



(3–5 min)

INCUBATE plates 15–20 hours



PRELAB NOTES

Review Prelab Notes in Laboratories 1 and 2 regarding sterile technique and *E. coli* culture.

Colony transformation is a simplification of the classic transformation protocol used in Laboratory 10 which requires mid-log phase cells grown in liquid culture. This abbreviated protocol begins with *E. coli* colonies scraped from an agar plate. Since liquid culturing is not used, equipment for shaking incubation is not required. The procedure entails minimal preparation time and is virtually foolproof. However, what is gained in simplicity and time is lost in efficiency. This protocol, although fine for transforming intact plasmids, is not efficient enough to use when transforming ligated DNA.

Transformation Scheme

Most transformation protocols can be conceptualized as four major steps.

1. *Preincubation:* Cells are suspended in a solution of cations and incubated at 0°C. The cations are thought to complex with exposed phosphates of lipids in the *E. coli* cell membrane. The low temperature freezes the cell membrane, stabilizing the distribution of charged phosphates.
2. *Incubation:* DNA is added, and the cell suspension is further incubated at 0°C. The cations are thought to neutralize negatively charged phosphates in the DNA and cell membrane. With these charges neutralized, the DNA molecule is free to pass through the cell membrane.
3. *Heat shock:* The cell/DNA suspension is briefly incubated at 42°C and then returned to 0°C. The rapid temperature change creates a thermal imbalance on either side of the *E. coli* membrane, which is thought to create a draft that sweeps plasmids into the cell.
4. *Recovery:* LB broth is added to the DNA/cell suspension and incubated at 37°C (ideally with shaking) prior to plating on selective media. Transformed cells recover from the treatment, amplify the transformed plasmid, and begin to express the antibiotic resistance protein.

The incubation and heat shock steps are critical. Since preincubation and recovery steps do not consistently improve the efficiency of colony transformation, they have been omitted from this protocol. If time permits, a preincubation of 5–15 minutes and/or a recovery of 5–30 minutes may be included.

Relative Inefficiency of Colony Transformation

The transformation efficiencies achieved with the colony protocol (5×10^3 to 5×10^4 colonies per microgram of plasmid) are 2–200 times less than those of the classic protocol (5×10^4 to 5×10^6 colonies per microgram). Colony transformation is perfectly suitable for transforming *E. coli* with purified intact plasmid DNA. However, it will give poor results with ligated DNA, which is composed of relaxed circular plasmid and linear plasmid DNA. These forms yield 5–100 times fewer transformants than an equivalent mass of intact supercoiled plasmid.

Maintenance of *E. coli* Strains for Colony Transformation

Prolonged reculturing (passaging) of *E. coli* can result in a loss of competence that makes the bacterium virtually impossible to transform using the colony method. There is some evidence that loss of transforming ability in MM294 may result from exposure of cells to temperatures below 4°C. Therefore, take care to store stab/slant cultures and streaked plates at room temperature. If there is a severe drop in number of transformants—from the expected 50–500 colonies per plate to essentially zero—discard the culture and obtain a fresh one.

Plasmids Used in This Experiment

Almost any plasmid containing a selectable antibiotic resistance marker can be substituted for pAMP for the purpose of demonstrating transformation of *E. coli* to an antibiotic-resistant phenotype. However, pAMP, pBLU, and pGREEN were constructed specifically as teaching molecules and offer advantages in other contexts:

1. All are derived from a pUC expression vector that replicates to a high number of copies per cell. Therefore, yields from plasmid preparations are significantly greater than those obtained with pBR322 and other less highly amplified plasmids.
2. pAMP was designed for use with another teaching plasmid, pKAN. Each produces unique and readily recognizable restriction fragments when separated on an agarose gel. Thus, recombinant molecules formed by ligating these fragments can be easily characterized.
3. pBLU carries ampicillin resistance and the full-length gene coding for β-galactosidase (*lacZ*). Other plasmids for expressing β-galactosidase contain only a small part of the *lacZ* gene and thus only make a small part of the protein. These vectors depend on using specific host cells that contain the remaining part of the *lacZ* gene. The pieces "complement" each other to make a complete protein. This is called α complementation.

   Note that wild-type *E. coli* does possess an endogenous *lacZ* gene. However, in the absence of lactose, this gene is suppressed. The *lacZ* gene in pBLU is expressed from the *lac* promoter but does not have the normal regulatory sequences (known as the operator), nor does it express the Lac repressor protein made from the *lacI* gene. This means that cells transformed with pBLU will constitutively express β-galactosidase, presumably at high levels. To detect the expression of β-galactosidase, prepare plates with X-gal (see Prelab Preparation).
4. pGREEN carries ampicillin resistance and the gene coding for GFP (green fluorescent protein). pGREEN contains an enhanced GFP mutant, which allows the expression of GFP to be visualized with ambient light alone. In addition, pGREEN does not use an inducible expression system. Therefore, no inducer (IPTG, arabinose, etc.) must be added to the media for the expression of recombinant GFP.

NOTE When viewing GFP expression, use a long-wavelength UV light source ("black light"). Do not use a short-wavelength light source. Use a Plexiglas shield or UV-blocking glasses when viewing with a *mid*-wavelength DNA transilluminator.

Antibiotic Selection

Ampicillin is the most practical antibiotic resistance marker for demonstration purposes, especially in the rapid transformation protocol described here. Ampicillin interferes with construction of the peptidoglycan layer and only kills dividing cells that are assembling new cell walls. It does not kill outright preexisting *E. coli* with intact cell walls. Thus, cells can be plated onto ampicillin-containing medium directly following heat shock, omitting the recovery step. Kanamycin selection, on the other hand, is less amenable to rapid transformation. A recovery step prior to plating is essential, because the antibiotic acts quickly to block protein synthesis and to kill *any* preexisting cells that are not actively expressing the resistance protein.

Test Tube Selection

The type of test tube used is a critical factor in achieving high-efficiency transformation and may also be important in the colony protocol. Therefore, we recommend using a presterilized 15-ml (17 x 100 mm) polypropylene culture tube. The critical heat-shock step has been optimized for the thermal properties of a 15-ml polypropylene tube. Tubes of a different material (such as polycarbonate) or thickness conduct heat differently. In addition, the small volume of cell suspension forms a thin layer across the bottom of a 15-ml tube, allowing heat to be quickly transferred to all cells. A smaller tube (such as 1.5-ml) increases the depth of the cell suspension through which heat must be conducted. Thus, *any* change in the tube specifications requires recalibrating the duration of the heat shock. The Becton Dickinson Falcon 2059 is the standard for transformation experiments.

Purified Water

Extraneous salts and minerals in the transformation buffer can also affect results. Use the most highly purified water available; pharmacy-grade distilled water is recommended. It might pay to obtain from a local research center or hospital several liters of water purified through a multistage ion-exchange system, such as Milli-Q.

Presterilized Supplies

Presterilized supplies can be used to good effect in transformations; 15-ml culture tubes and individually packaged 100–1000-µl micropipettor tips are handy. A 3-ml transfer pipette, marked in 250-µl gradations, can be substituted for a 100–1000-µl micropipettor with no loss of speed or accuracy.

Technically, everything used in this experiment should be sterilized. However, it is acceptable to use clean, but nonsterile, 1.5-ml tubes for aliquots of calcium chloride, LB broth, and plasmid DNA, *provided they will be used within 1 or 2 days*. Clean, nonsterile 1–10-µl micropipettor tips can be used for adding DNA to cells in Step 9. Plastic supplies, if not handled before use, are rarely contaminated. Antibiotic selection covers such minor lapses of sterile technique.

Plating Cell Suspensions

An alternative to using a traditional cell spreader is to use sterile glass spreading beads. Five to seven glass beads are placed on each agar plate after adding the

cell suspension. The beads are swirled around the plate until the cells have been evenly spread. No flame/ethanol is required for this method, thus lowering potential fire hazards. Use 3-mm silica beads. Beads can be used directly from the package or autoclaved prior to use.

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.

Hanahan D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166:** 557–580.

———. 1987. Techniques for transformation of *E. coli*. In *DNA cloning: A practical approach* (ed. D.M. Glover), vol. 1. IRL Press, Oxford.

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. The day before the laboratory, streak out several fresh "starter plates" of MM294 or other *E. coli* host strain. Follow the procedure in Laboratory 2A, Isolation of Single Colonies. Following initial overnight incubation at 37°C, use cells.
2. PLAN AHEAD. Be sure to have a streaked plate or stab/slant culture of viable *E. coli* cells from which to streak starter plates. Also, streak the *E. coli* strain on an LB/amp plate to ensure that an ampicillin-resistant strain has not been used by mistake.
3. 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). To eliminate autoclaving completely, store filtered solutions in presterilized 50-ml conical tubes.
4. Prepare for each experiment:

   two LB agar plates
   two LB + ampicillin plates (LB/amp)

   When transforming with pBLU, X-gal (5 bromo-4-chloro-3-indolyl-β-D-galactopyranoside) must be included in the LB/amp plate in order to detect the expression of the *lacZ* gene. Buy premade plates or make up a 2% solution of X-gal in dimethyl formamide▼. When pouring plates, cool media to 60°C and add ampicillin to 100 μg/ml and 2 ml of the X-gal stock solution per liter of media. These LB/amp/X-gal plates can also be used as regular LB-ampicillin plates.
5. Prepare aliquots for each experiment:

   1 ml of sterile 50 mM $CaCl_2$ in a 1.5-ml tube (store on ice)
   1 ml of sterile LB broth in a 1.5-ml tube
   12 μl of 0.005 μg/μl pAMP, pBLU, or pGREEN in a 1.5-ml tube (store on ice)

6. Adjust the water bath to 42°C. A constant-temperature water bath can be made by maintaining a trickle flow of 42°C tap water into a Styrofoam box. Monitor temperature with a thermometer. An aquarium heater can be used to maintain temperature.
7. Prewarm incubator to 37°C.
8. To retard evaporation, keep ethanol in a beaker covered with Parafilm, plastic wrap, or, if using a small beaker, the lid from a Petri dish. Retrieve and reuse ethanol exclusively for flaming.
9. If using spreading beads, carefully place five to seven beads into a sterile 1.5-ml tube. Tube can be used as a scooper. Prepare four tubes per experiment.

MATERIALS

CULTURES, MEDIA, AND REAGENTS

$CaCl_2$ (50 mM)
LB/amp plates (2) (or 2 LB/amp/X-gal▼ plates, if using pBLU)
LB broth
LB plates (2)
MM294 starter culture
Plasmid (0.005 µg/µl) (pAMP, pBLU, or pGREEN)

SUPPLIES AND EQUIPMENT

Beakers for crushed or cracked ice and for waste/used tips
Beaker of 95% ethanol▼ and cell spreader (or spreading beads)
"Bio-bag" or heavy-duty trash bag
Bleach (10%)▼ or disinfectant
Bunsen burner
Culture tubes (two 15-ml)
Incubator (37°C)
Inoculating loop
Micropipettor (100–1000-µl) + tips (or 3-ml transfer pipettes)
Micropipettor (0.5–10-µl) + tips
Permanent marker
Test tube rack
Water bath (37°C) (optional)
Water bath (42°C)

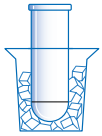
▼ See Appendix 4 for Cautions list.

METHODS

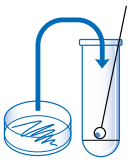
Prepare *E. coli* Colony Transformation (40 minutes)

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

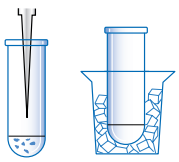
1. Use a permanent marker to label one sterile 15-ml tube +plasmid. Label another 15-ml tube –plasmid. Plasmid DNA will be added to the +plasmid tube; none will be added to –plasmid tube.



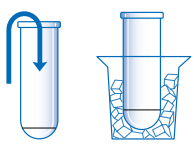
If there are no separate colonies on the starter plate, scrape up a small cell mass from a streak. Transformation efficiency decreases if too many cells are added to the calcium chloride.



Optimally, flame the mouth of the 15-ml tube after removing and before replacing cap. Cells become difficult to resuspend if allowed to clump together in $CaCl_2$ solution for several minutes. Resuspending cells in the +plasmid tube first allows the cells to preincubate for several minutes at 0°C while –plasmid tube is being prepared. If time permits, both tubes can be preincubated on ice for 5–15 minutes.



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



To save plates, different experimenters may omit either the +LB or the –LB plate.

2. Use a 100–1000-µl micropipettor and sterile tip (or sterile transfer pipette) to add 250 µl of $CaCl_2$ solution to each tube.
3. Place both tubes on ice.
4. Use a sterile inoculating loop to transfer one or two large (3-mm) colonies from the starter plate to the +plasmid tube:
   a. Sterilize the loop in a Bunsen burner flame until it glows red hot. Then pass the lower one half of the shaft through the flame.
   b. Stab the loop several times at the edge of the agar plate to cool.
   c. Pick a couple of large colonies and scrape up a visible cells mass, but be careful not to transfer any agar. (Impurities in the agar can inhibit transformation.)
   d. Immerse the loop tip in the $CaCl_2$ solution and *vigorously* tap it against the wall of the tube to dislodge the cell mass. Hold the tube up to the light to observe the cell mass drop off into the $CaCl_2$ solution. Make sure that the cell mass is not left on the loop or on the side of the tube.
   e. Reflame the loop before placing it on the lab bench.
5. Immediately resuspend the cells in the +plasmid tube by repeatedly pipetting in and out, using a 100–1000-µl micropipettor with a sterile tip (or sterile transfer pipette).

> **CAUTION**
> Keep nose and mouth away from the tip end when pipetting suspension culture to avoid inhaling any aerosol that might be created.

   a. Pipette carefully to avoid making bubbles in suspension or splashing suspension far up the sides of the tube.
   b. Hold the tube up to the light to check that the suspension is homogeneous. No visible clumps of cells should remain.
6. Return the +plasmid tube to ice.
7. Transfer a second mass of cells to the –plasmid tube as described in Steps 4 and 5 above.
8. Return the –plasmid tube to ice. Both tubes should be on ice.
9. Use a 1–10-µl micropipettor to add 10 µl of 0.005 µg/µl plasmid solution *directly into the cell suspension* in the +plasmid tube. Tap tube with a finger to mix. Avoid making bubbles in the suspension or splashing the suspension up the sides of tube.
10. Return the +plasmid tube to ice. Incubate both tubes on ice for an additional 15 minutes.
11. While the cells are incubating on ice, use a permanent marker to label two LB plates and two LB/amp plates with your name and the date. Remember, if transforming with pBLU, to use LB/amp/X-gal plates in place of regular LB/amp plates.

    Label one LB/amp plate +. This is the experimental plate.
    Label the other LB/amp plate –. This is the negative control.
    Label one LB plate +. This is a positive control.
    Label one LB plate –. This is a positive control.

cells to recover for 5–30 minutes at 37°C. Gentle shaking is also helpful.

formation. If necessary, store the +plasmid and –plasmid tubes on ice in the refrigerator (0°C) for up to 24 hours, until there is time to plate cells. Do not put cell suspensions in the freezer.

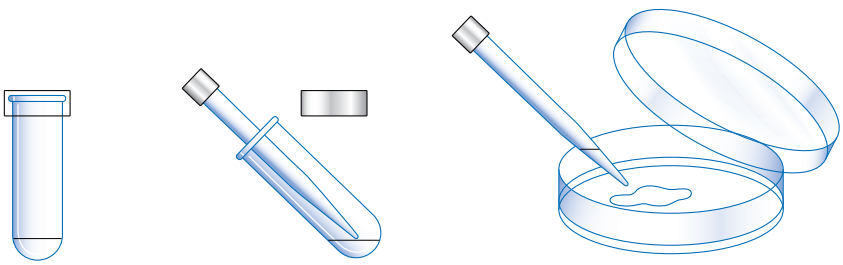
13. Place +plasmid and –plasmid tubes in the test tube rack at room temperatur
14. Use a 100–1000-µl micropipettor and sterile tip (or sterile transfer pipette) t add 250 µl of LB broth to each tube. Gently tap tubes with finger to mix.
15. Use the matrix below as a checklist as +plasmid and –plasmid cells are sprea on each type of plate:

	Transformed cells +plasmid	Nontransformed cells –plasmid
LB/amp	100 µl	100 µl
LB	100 µl	100 µl

If too much liquid is absorbed by agar, cells will not be evenly distributed.

The object is to evenly distribute and separate cells on agar so that each gives rise to a distinct colony 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.*

16. Use a micropipettor with a sterile tip (or transfer pipette) to add 100 µl of ce suspension from the –plasmid tube onto the –LB plate, and another 100 µ onto the –LB/amp plate. *Do not allow the suspensions to sit on the plates too lon before proceeding to Step 17.* Spread cells using one of the methods described i Steps 17 and 18.



17. Sterilize cell spreader, and spread cells over the surface of each –plate in succession.

    a. Dip the spreader into the ethanol beaker and *briefly* pass it through a 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 the ethanol in the beaker. Do not panic if the ethanol is accidentally ignited. Cover the beaker with a Petri lid or other cover to cut off oxygen and rapidly extinguish fire.

    b. Lift the lid of one –plate just enough to allow spreading; *do not place lid on lab bench*.

    c. Cool spreader by gently rubbing it on the surface of the agar *away* from the cell suspension or by touching it to 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 the agar. Rotate plate one-quarter turn, and repeat spreading motion. Try to spread the suspension evenly across agar surface. *Be careful* not to gouge the agar.

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

18. Use spreading beads to spread cells over the surface of each –plate in succession.

    a. Lift the lid of one –plate enough to allow adding beads; *do not place the lid on the lab bench.*

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

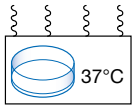
    c. Close plate lids and use a swirling motion to move glass beads around the entire surface of the plate. This evenly spreads the cell suspension on the agar surface. Continue swirling until the cell suspension is absorbed into the agar.

19. Use a micropipettor with a sterile tip (or transfer pipette) to add 100 µl of cell suspension from +plasmid tube onto +LB plate and to add another 100 µl of cell suspension onto +LB/amp plate. *Do not allow the suspensions to sit on the plate too long before proceeding to Step 20.*

20. Repeat Step 17a–e or Step 18a–c to spread cell suspension on +LB and +LB/amp plates.

21. If Step 17 was used, reflame the spreader one last time before placing it on the lab bench.

22. Allow the plates to set for several minutes so that the suspension absorbs into the agar. If Step 18 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 storage container for reuse.



23. Stack plates and tape into a bundle to keep the experiment together. Place the 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.

25. If planning to do Laboratory 8, Purification and Identification of Plasmid DNA, save the +LB/amp plate as source of a colony to begin an overnight suspension culture.
26. Take time for responsible cleanup.
    a. Segregate for proper disposal culture plates and tubes, pipettes, and micropipettor tips that have come into contact with *E. coli*.
    b. Disinfect 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

Count the number of individual colonies on the +LB/amp plate. Observe colonies through the bottom of the culture plate, and use a permanent marker to mark each colony as it is counted. If the transformation worked well, between 50 and 500 colonies should be observed on the +LB/amp plate; 100 colonies is equal to a transformation efficiency of 10^4 colonies per microgram of plasmid DNA. (Question 3 explains how to compute transformation efficiency.)

If plates have been overincubated or left at room temperature for several days, tiny "satellite" colonies may be observed that radiate from the edges of large, well-established colonies. Nonresistant satellite colonies grow in an "antibiotic shadow" where ampicillin has been broken down by the large resistant colony. Do not include satellite colonies in the count of transformants. Also examine the colonies carefully to detect any possible contamination. Contaminating organisms will usually look different in color, shape, or size of colony. Over time, you will improve at distinguishing *E. coli* colonies from other organisms. A "lawn" should be observed on positive controls, where the bacteria cover nearly the entire agar surface and individual colonies cannot be discerned.

If pBLU was used for transformation, you will observe blue colonies on the +LB/amp/X-gal plate because of the expression of β-galactosidase. The X-gal in the plates mimics the normal substrate for β-galactosidase, the disaccharide lactose. β-galactosidase cleaves the X-gal, removing the compound 5-bromo-4-chloro-3-indolyl from galactopyranoside, which is blue. Thus, the presence of a blue colony indicates the presence of β-galactosidase activity.

If pGREEN was used for transformation, you will observe green colonies under long-wavelength UV light (black light). Green colonies indicate the presence of GFP (green fluorescent protein).

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.

	Transformed cells +plasmid	Nontransformed cells –plasmid
LB/amp	experiment	negative control
LB	positive control	positive control

2. Compare and contrast the growth on each of the following pairs of plates. What does each pair of results tell you about the experiment?
   a. +LB and –LB
   b. –LB/amp and –LB
   c. +LB/amp and –LB/amp
   d. +LB/amp and +LB
3. Transformation efficiency is expressed as the number of antibiotic-resistant colonies per microgram of plasmid DNA. The object is to determine the mass of plasmid that was spread on the experimental plate and was therefore responsible for the transformants observed.
   a. Determine total mass (in micrograms) of plasmid used in Step 9.

      concentration x volume = mass

   b. Determine the fraction of the cell suspension spread onto +LB/amp plate (Step 19): volume suspension spread/*total* volume suspension (Steps 2 and 14) = fraction spread.
   c. Determine the mass of plasmid in the cell suspension spread onto +LB/amp plate: total mass plasmid (*a*) x fraction spread (*b*) = mass plasmid spread.
   d. Determine number of colonies per microgram of plasmid. Express answer in scientific notation: colonies observed/mass plasmid spread (*c*) = transformation efficiency.
4. What factors might influence transformation efficiency?
5. Your Favorite Gene (*YFG*) is cloned into pAMP, and 0.2 μg of pAMP/YFG is used to transform *E. coli* according to the protocol described in this laboratory. Using the information below, calculate the number of molecules of pAMP/YFG that are present in a culture 200 minutes after transformation.
   a. You achieve a transformation efficiency equal to 10^6 colonies per microgram of intact pAMP/YFG.
   b. pAMP/YFG grows at an average copy number of 100 molecules per transformed cell.
   c. Following heat shock (Step 12), the entire 250 μl of cell suspension is used to inoculate 25 ml of fresh LB broth. The culture is incubated, with shaking, at 37°C. Transformed cells enter log phase 60 minutes after inoculation and then begin to replicate an average of once every 20 minutes.
6. The transformation protocol above is used with 10 μl of intact plasmid DNA at different concentrations. The following numbers of colonies are obtained when 100 μl of transformed cells are plated on selective medium:

0.00001 μg/μl	4 colonies
0.00005 μg/μl	12 colonies
0.0001 μg/μl	32 colonies
0.0005 μg/μl	125 colonies
0.001 μg/μl	442 colonies
0.005 μg/μl	542 colonies
0.01 μg/μl	507 colonies
0.05 μg/μl	475 colonies
0.1 μg/μl	516 colonies

a. Calculate transformation efficiencies at each concentration.
b. Plot a graph of DNA mass *versus* colonies.
c. Plot a graph of DNA mass *versus* transformation efficiency.
d. What is the relationship between mass of DNA transformed and transformation efficiency?
e. At what point does the transformation reaction appear to be saturated?
f. What is the true transformation efficiency?

7. For cells transformed with pBLU or pGREEN, what color would you expect nontransformed satellite colonies to be?

FOR FURTHER RESEARCH

Interpretable experimental results can only be achieved when the colony transformation can be repeated with reproducible results. Attempt experiments below only when you are able to routinely achieve 100–500 colonies on the +LB/amp plate.

1. Design and execute an experiment to compare 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* the salts of other monovalent (+), divalent (++), and trivalent (+++) cations.
   a. Make up 50 mM solutions of each salt.
   b. Check pH of each solution, and buffer to approximately 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. Carry out a series of experiments to determine the saturating conditions for transformation reactions.
   a. Transform *E. coli* using DNA concentrations list in Question 6 above.
   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 experiment with concentrations clustered on either side of the presumed saturation point to produce a fine saturation curve.
5. Repeat the experiment in Step 4 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.

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

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?

6. 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?