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)

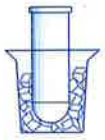
▼ See Appendix 4 for Cautions list.

METHODS

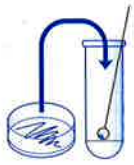
Prepare *E. coli* Colony Transformation (40 minutes)

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

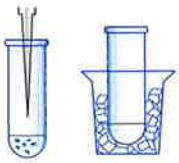
1. Use a permanent marker to label one sterile 15-ml tube +plasmid. Label another 15-ml tube –plasmid. Plasmid DNA will be added to the +plasmid tube; none will be added to –plasmid tube.



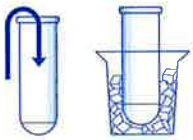
If there are no separate colonies on the starter plate, scrape up a small cell mass from a streak. Transformation efficiency decreases if too many cells are added to the calcium chloride.



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.



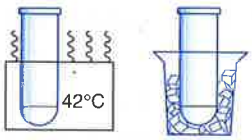
To save plates, different experimenters may omit either the +LB or the –LB plate.

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.



12. Following the 15-minute incubation, heat shock the cells in the +plasmid and –plasmid tubes. *It is critical that cells receive a sharp and distinct shock.*
    a. Carry the ice beaker to the water bath. Remove the tubes from ice, and *immediately* immerse them in the 42°C water bath for 90 seconds.
    b. Immediately return both tubes to ice for at least 1 additional minute.

If time permits, allow +plasmid and –plasmid cells to recover for 5–30 minutes at 37°C. Gentle shaking is also helpful.

STOP An extended period on ice following the heat shock will not affect the transformation. 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.

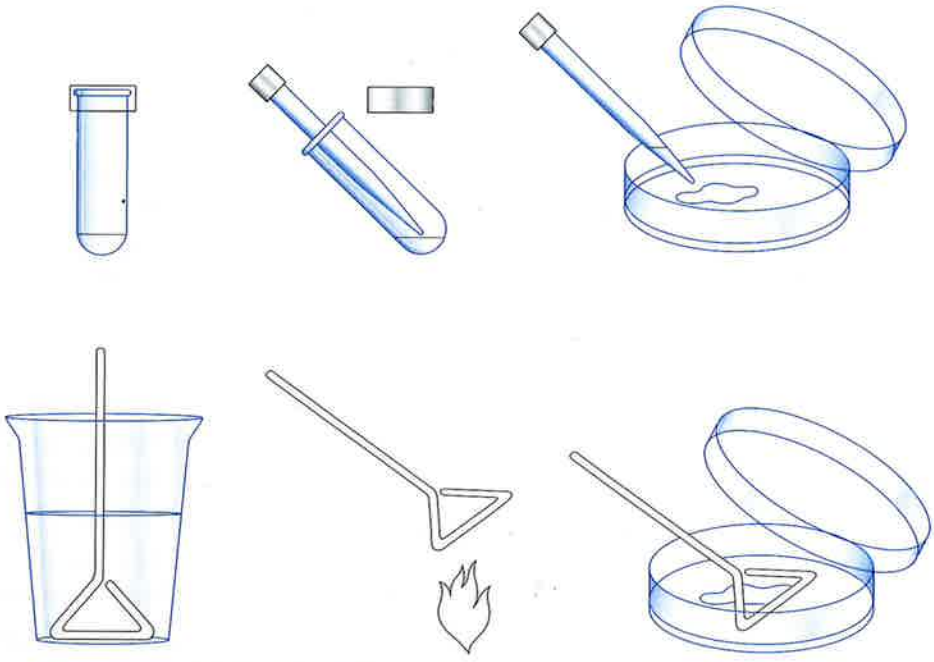
13. Place +plasmid and –plasmid tubes in the test tube rack at room temperature.
14. Use a 100–1000-µl micropipettor and sterile tip (or sterile transfer pipette) to add 250 µl of LB broth to each tube. Gently tap tubes with finger to mix.
15. Use the matrix below as a checklist as +plasmid and –plasmid cells are spread 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 cell suspension from the –plasmid tube onto the –LB plate, and another 100 µl onto the –LB/amp plate. *Do not allow the suspensions to sit on the plates too long before proceeding to Step 17.* Spread cells using one of the methods described in Steps 17 and 18.



Sterile Spreading Technique (Steps 16 and 17)

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.

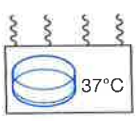
    c. Close plate lids and use a swirling motion to move glass beads around the entire surface of the plate. This evenly spreads the cell suspension on the agar surface. Continue swirling until the cell suspension is absorbed into the agar.

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