FRED HUTCHINSON CANCER RESEARCH CENTER
SCIENCE EDUCATION PARTNERSHIP

TEACHER'S GUIDE
PBLU® TRANSFORMATION KIT AND GENES R US SCENARIO

ATTENTION! Have you put the reagents that were in the freezer box in your freezer? Take care of all reagents so that they are stored properly for optimal lab use. Store the ampicillin, X-gal, and plasmid in the freezer.
X-gal and ampicillin should be protected from exposure to light.

Overview

1. This lab is written for SEP participants using SEP's bacterial transformation kit. This lab assumes that you are working with the materials supplied in the kit.
2. These transformation labs have been updated as of 2006. Please read through this guide and the lab before you get started.
3. This lab is written as a scenario where students are employed by a biotech company that uses bacterial transformation to produce an enzyme, which makes blue dye. There is a "Basic Transformation Lab" and an extension lab "Testing of Student Proposals." If you plan to do the extension lab, you will need more supplies. Please indicate this on your kit request form.
4. This document and the student lab are on a CD included in this notebook. Feel free to adapt the labs for your own use, but please credit SEP.
5. Please refer to the kit inventory list and go through the kit and equipment to make certain you have everything that is included in the kit. You will need to make solutions and aliquot other solutions for classroom use. You will need to pour plates days ahead of the lab. Further, some critical items are not supplied in the kit; e.g. **microwave oven, ice, and 10% bleach or 70% ethanol (for disinfectant solutions).**

Lab Equipment Tips and Notes

- ❑ These transformation labs require a great deal of preparation before the actual lab activities can begin. You will need to make and pour two different types of agar plates: LB agar plates and LB/amp/X-gal plates. Plates should be poured, under sterile conditions, a few days before the lab. **Preparing plates for one class will take a minimum of two hours.**
- ❑ Protect the LB/amp/X-gal plates from light by wrapping the outside of the sleeves in foil.
- ❑ Prepare classroom stock plates of JM101 the day before the lab. Fresh, overnight cultures are required. Use the JM101 slant culture provided. If the cultures fail to grow, call SEP immediately (206.667.4487) so that we can provide you with a fresh culture.
- ❑ When troubleshooting results for these labs, please refer to the Trouble Shooting Notes and Bacterial Culture Pictures in the kit notebook.
- ❑ If you have 50-minute class periods, there is a stopping point in the transformation procedure, **after** the heat shock step. It will take students between 40-50 minutes to get to this point. Bacteria can be refrigerated until the next day when you are ready to continue. Refer to the Teaching Tips and the Genes R Us lab (student's lab) in the kit notebook.

What's in the Freezer Box?

Tube Label	Tube Contents	Notes
Ampicillin	ampicillin 100 mg/ml	Thaw and mix before adding to agar.
2% X-gal	2% X-gal in DMF	Thaw and mix before adding to agar.
pBLU®	pBLU® plasmid DNA 0.01 μg/μl	Thaw and mix before aliquoting for student use.

Stock Solutions and Ingredients Needed in Lab Preparation for the Lab

(THESE STOCK SOLUTIONS ARE ALL SUPPLIED IN THE KIT. YOU WILL NEED TO PREPARE AND ALIQUOT FOR CLASSROOM USE.)

Luria Bertani (LB) Agar An agar that is high in salt and yeast extract, which provide ideal growth requirements for lab strains of *Escherichia coli*. Supplied sterilized in bottles. In our kits, our bottles contain approximately 350 ml per bottle. Make calculations of additives or pouring quantities based on the volume in the bottle.

Ampicillin An anti-microbial used to select for transformed *E. coli*. The provided ampicillin stock solution is 100 mg/ml. ***Use 1ml ampicillin stock solution per 1 liter of LB agar***. Heat sensitive---add after agar has cooled enough to pour. Light sensitive—keep agar plates covered with foil.

X-gal 5-bromo-4-chloro-3-indolyl-b-D-glactoside. A lactose analog used as a substrate for β-galactosidase, the enzyme product of the lacZ gene on the pBLU® plasmid. The provided X-gal stock solution is 2% X-gal. ***Use 0.8ml of 2%X-gal stock solution per 1 liter of LB agar***. Light sensitive---keep agar plates covered with foil.

Luria Bertani (LB) Broth (aka Luria broth) A liquid broth that is high in salt and yeast extract, which provide ideal growth requirements for lab strains of *E. coli*. Supplied in a sterilized container. Use sterile technique when aliquoting so as not to contaminate the experiments.

$CaCl_2$ Calcium chloride solution necessary to render *E. coli* cells competent for transformation experiments. Supplied sterile. Use sterile technique when aliquoting so as not to contaminate the experiments.

pBLU® A small plasmid that contains the LacZ gene and the Amp^r gene which confer the abilities to hydrolyze lactose (or in this lab, X-gal) and to break down ampicillin, respectively. Use sterile technique when aliquoting so as not to contaminate the experiments.

JM101 *E.coli* A laboratory strain of *Escherichia coli* that has been selected, based on its genotype, for several microbial and molecular biology teaching activities. We supply you with one slant of pure culture, which can be used to make 10-15 stock plates. Use sterile technique when making stock plates for your class' experiments.

Preparation for the Transformation Lab

Note: It is important to follow sterile or aseptic technique when preparing aliquots and pouring plates for this lab. Disinfect all work areas (table tops) before and after lab preparation. You can purchase a commercial disinfectant solution or make your own: use either a 10% bleach solution or a 70% ethanol solution. Label the solution for future reference.

Pouring Plates

Determine how many plates you will need.

- Each student group will need two LB agar plates and two LB/amp/X-gal plates. (The teacher adds ampicillin and X-gal to the LB agar to make these plates.) If the students test out their proposals, you will need twice as many plates.
- You will also need to pour 3-4 stock plates (LB agar) for each class.
- Each plate holds 15-25 ml media. So each class (8 groups) will require ~700 ml LB agar.
- LB agar is included in the kit. Each bottle contains about 350 ml.
- If possible, pour the plates a few days before you need them to reduce condensation. Plates can be stored up to a month if refrigerated and kept dark.

Plate pouring procedure:

1) Melt LB agar in the microwave (LOOSEN CAP FIRST!) on medium-high power until all of the agar is liquid. It's best to do this at 2-minute intervals, so the agar doesn't boil over. It takes 10-15 minutes to melt one 350 ml bottle of agar, so get an EARLY start! Hold melted agar in a 60°C water bath until you are ready to pour plates. The agar can be kept melted all day long, and plates poured after school, though you would need a large water bath. (The water bath in the kit can only hold about two bottles.)
2) When you're ready to pour, clear off and wipe down a large, flat working surface with disinfectant. Try to work in an area away from drafts.
3) Set out several rows of sterile plastic petri dishes, bottom side up; do not open the dishes
4) Label plate bottoms with the type of medium (e.g. LB or LB/amp/X-gal) and the date.
   (Alternatively, color code your plates using different colored pens (e.g. black for LB, black + blue for LB/amp/X-gal) by running a magic marker up the side of a stack of plates, making sure you mark both tops and bottoms.)

5) For LB/amp/X-gal plates:
   Add sterilized ampicillin solution and X-gal to **cooled** (**60°C**) agar after microwaving. When you can hold the warm bottle without too much discomfort, the temperature is about right. Use a micropipette to measure ampicillin and X-gal.

   Ampicillin:
   Use 1 ml ampicillin stock per 1 liter of LB agar to give a final concentration of 100 μg/ml.
   (For our bottles, use 0.35 ml of ampicillin per bottle of 350 ml LB agar.)
   Check the volume of your bottles of LB-agar to calculate how much ampicillin to add.

X-gal:

Use 0.8 ml X-gal stock per 1 liter of LB-agar to give a final concentration of 16 μg/ml. (For our bottles, use 0.28 ml of 2% X-gal per bottle of 350 ml LB agar.) Check the volume of your bottles of LB agar to calculate how much X-gal to add. *Handle X-gal carefully, wearing gloves and avoiding the vapors. The solvent, N, N-dimethylformamide, is a potential teratogen and carcinogen. See the included Materials Safety Data Sheet for more information.*

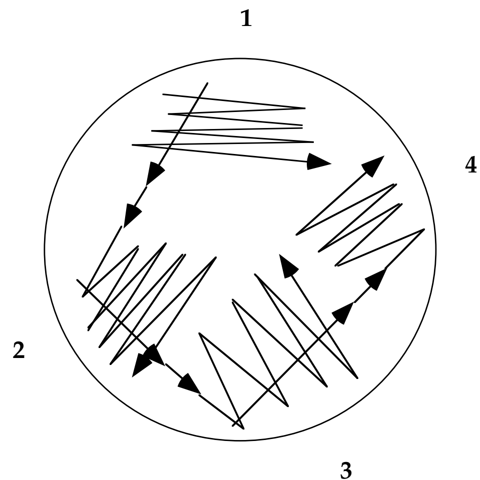
Mix thoroughly, pour plates, and store in the dark, as light will break down ampicillin and X-gal.

6) Turn plates over so that the bottom is down. Keep lids on plates until you are ready to pour. When the bottles are cool enough to hold, commence pouring plates. If you have a Bunsen burner or alcohol burner available, it is best to sterilize the opening of the bottle and then pour. Each plate holds about 15-25 ml of medium; pour in enough to just cover the bottom of the plate. Cover each plate immediately after pouring. Some condensation will occur, mostly in the top of each plate. As you pour, try to avoid air bubbles. Bubbles, if numerous or large, can be popped with a sterile pipet tip, the flame of a match or with a quick pass of an inverted, lighted bunsen burner over the surface of the agar.

7) When solidified (allow at least half an hour), **place plates upside down** and allow condensation to dry. If you haven't labeled the plates, you should do so now. Let the excess moisture in the plates dry overnight or over a weekend. Then store plates in the plastic sleeves either at room temperature or in the refrigerator. Store bottom side up! Plates with ampicillin and X-gal should be protected from light; use foil or a box.

Making Stock Plates (for Student Lab Use)

- Set up incubator and be sure it is set for 37°C.
- Streak the stock plates on the day before transformations are to be done so that you have reasonably sized colonies (2-3 mm diameter), which are still in log-phase (i.e. rapid) growth. Rapidly growing bacteria appear to be more suitable for transformation by this technique.
- Prepare 3-4 LB stock plates per class.
  For each plate:
  - Carefully remove a sterile, disposable loop. Gather a small amount of bacteria from the surface of the provided slant culture. We use a stock of *E. coli* strain JM101 (other strains can be used but must be Lac$^-$, i.e. lacking endogenous β-galactosidase).
  - Streak on an LB plate, following the diagram:
    - Start at (1), with bacteria on the loop.
    - Streak, then stop.
    - Using a new loop, drag across the first streak and make streak (2). Stop.
    - Using a new loop, drag across (2) and make streak (3). Stop.
    - Using a new loop, steak from (3) to (4) without touching the first streak (1).



Note: The purpose of the streak plate is to isolate individual bacterial cells that will grow into pure colonies of identical cells. The idea is, if you start with very dense bacteria at (1), then by (4) you'll have individual, separated colonies from which to select.

- Incubate overnight at 37°C.
- Remove from incubator the next morning, before colonies grow into each other. You should have isolated colonies in streak area (3) and (4).

Preparation for the Day of the Transformation Lab

- Set out all of the agar plates. (If they were stored in the refrigerator, pull out the night before so that there is little or no condensation. Clearly label the stacks of plates so the students can tell the difference. Keep the LB/amp/X-gal plates covered, protected from light.
- Turn on the water bath and maintain it at 42°C for the heat shock step (if you are doing the procedure in one day, remember to turn down the water bath to 37°C for the recovery step).
- Turn on the incubator and maintain it at 37°C for the incubation step.
- Remind students about using sterile technique to minimize contamination. All table tops and work areas should be disinfected before and after each lab session.
- Design room layout to minimize congestion around water bath.
- To minimize bottlenecks, aliquot LB broth, sterile water, pBLU® plasmid and $CaCl_2$ for groups—based on how you arranged groups in your class.
- Obtain ice and store properly until needed for the heat shock step.
- Remind students to clearly label their culture tubes and their plates so that they can discern their group's experiment from other groups'.

Safety Concerns and Sterile Technique

- ♦ Explain sterile or aseptic technique to your students. It is important to keep the laboratory environment clean and free of contamination---from both the experimenters and from the experiments! When dealing with microorganisms, whether pathogenic or non-pathogenic, the same general safety procedures should be followed.
- ♦ Do not permit food or drink in the laboratory.
- ♦ Wash hands before and after each lab.
- ♦ Disinfect all working surfaces before and after each lab.
- ♦ Used sterilized materials for everything that comes in contact with the bacteria, e.g. pipet tips, toothpicks, culture media, tubes, and spreaders.
- ♦ Avoid touching any part of these materials that come in contact with the bacteria.

Safe Handling of E.coli Cells Before and After Transformation

The strain of *E. coli* we're using is called JM101, after Joachim Messing and his colleagues; the people who originally developed it.
The genotype of JM101 (a listing of particular genes it has we're interested in) is:
F' *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q}*Δ(lacZ)M15*/ *Δ (lac-proAB) glnV thi*

All of this is a kind of shorthand, or code, to explain what form of a gene this strain has, or whether it has that gene at all. For us, the key one is *Δ (lacZ)*. This means the bacteria's original β-galactosidase gene is gone. The Δ symbol, Greek for the letter delta, stands for "deleted" (get it, starts with d!). So, when we add a β-galactosidase gene through transformation with pBLU, we restore the bacteria's original genotype and make them lac+.

Important information about *E. coli*, pathogenicity, and antibiotic resistant strains of bacteria:

It is recommended that you give your students a little background about the commensal bacterium *E. coli* that resides in the human gastrointestinal tract. One of many natural and beneficial flora in humans, this commensal strain is harmless. There are pathogenic strains of *E. coli*, which are harmful to humans, and can cause severe illness, e.g. O157:H7, which is a strain that lives in bovine intestines. However, the *E. coli* cells used in this experiment are a laboratory variety, derived from the wild type strain K-12. Several lab strains from K-12 have been passed in vitro since the mid-1940s. They are not considered pathogenic. In fact, several experiments have been conducted to test whether these K-12 derived strains could colonize the human gut. So far this has not occurred, which is why K-12 derived strains (e.g. JM101, MM294, and HB101) are considered safe for laboratory use. One hypothesis for this non-pathogenicity phenomenon is that because these cells have lived in laboratory culture for so long, they have lost some of their original, wild type traits. In particular, these strains have lost the "O" antigen domain of the lipopolysaccharide that composes the outer membrane. The "O" antigen is thought to be necessary for infection in mammals.

Safe handling of any bacteria, regardless of pathogenicity, is a prerequisite for working in a shared laboratory environment. Even harmless bacteria can enter the body through cuts in the skin and later cause infections. Follow sterile technique and keep your students, and you, safe!

The results of a successful transformation experiment are cultures of antibiotic resistant strains of *E. coli*. After counting colonies and collecting the data necessary to complete the lab, these cultures should be destroyed and disposed of properly. These new strains of bacteria should not be released into the environment; after all, your students have genetically engineered new *E. coli* cells! However, it is important to note that these cultures probably would not survive in the wild, nor would they be able to pass on their antibiotic resistance to other bacteria. In this lab experiment, it was necessary to treat the cells chemically ($CaCl_2$) and physically (heat shock) to allow the plasmid to be taken up by the cells. And even then, few cells actually take up the plasmid.

In the natural world of *E.coli*, plasmid DNA is passed from cell to cell via a pilus, which acts as a bridge between two cells. One cell acts as the donor, the other cell acts as the recipient. The plasmid DNA must also be able to "move" from one cell, through the pilus, to the other cell. The plasmid itself has the genes which, when expressed, allow it be moved into the recipient cell. The plasmids, which are commonly used as "teaching plasmids", (e.g. pBLU, pAMP, and pKAN) have been genetically altered so they do not express the mobility protein required for transport.

Safely destroy and dispose of bacteria from the experiment by soaking culture plates in a 10% bleach bath. Soak for at least 30 minutes, then dispose of in the garbage. If you have access to an autoclave, place items in a biohazard bag and autclave for 15 minutes at 121°C. Dispose of bag in the garbage. Make sure that your custodian knows that you have killed the bacteria and that it is no longer a biohazard. (You may want to cross out the biohazard symbol on the bag!)

- ❑ If you need to keep an experiment to show students an example—seal the plates (wrap parafilm around the circumference) and store plates, bottom side up, in the refrigerator.

- ❑ DO NOT KEEP CULTURES GROWING IN THE 37°C INCUBATOR! This will allow all kinds of microbes to proliferate and contaminate the culture. These contaminants ARE a biohazard as they could be pathogenic.

Acknowledgements and References

We are indebted to Suzanne Black (Inglemoor High School, Bothell WA), Carter Hoffman, Mark Hertle, SEP participants, and many others for field testing, comments, illustrations, and advice. References that contributed ideas and methods to this protocol include the Gene Connection v1.5 (1995), *DNA Science* (1990), *Laboratory DNA Science*, (1996), *Working with Bacteria and DNA in Precollege Science Classrooms* (1993), and the EduGen Amylase Kit. Please see the reference list for specific citations.

Working with Bacteria and DNA in Precollege Science Classrooms, 1989, by Toby Horn, is a comprehensive safety and appropriate use guide. (Out of print.)

An Introduction to Biotechnology: A Unit for Seventh and Eighth Grade Students, 1995, by The Biotechnology Education Project of the St. Louis Mathematics and Science Education Center. Kendall/Hunt Publishing Co., Dubuque, Iowa.

An Introduction to Biotechnology: A Unit for High School Students, 1995, by The Biotechnology Education Project of the St. Louis Mathematics and Science Education Center. Kendall/Hunt Publishing Co., Dubuque, Iowa.

Laboratory DNA Science: An introduction to recombinant DNA techniques and methods of genomic analysis, 1996, by Mark Bloom, Greg Freyer, and Dave Micklos. The Benjamin/Cummings Publishing Company, Inc., Menlo Park, California.

Sweeney, Diane, "The Amylase Project: Creating a classroom of biotechnologists," *American Biology Teacher*, 2:122-126 February, 1998

PBLU® TRANSFORMATION KIT TEACHING TIPS & TROUBLE SHOOTING NOTES

Teaching Tips

- Transformation efficiency can be a difficult concept. Go through it with your students.
  - Use a creative demonstration to explain what transformation is and how it is done. See the kit notebook for one suggestion (Lesson 13 of An Introduction to Biotechnology: A Unit for Seventh and Eighth Grade Students). There are also several overheads included in the kit notebook that demonstrate the transformation process.
- The pre-employment interview is actually a type of pre-assessment for you to use. It is designed to fit with the Genes R Us Scenario. You can use this to assess your students' prior knowledge of transformation. It can be oral or written, before or after reading the scenario. You could also modify it and use it at the end of the lab.
- Demonstrate how to pick bacteria from a plate, and how to resuspend cells in $CaCl_2$ by repeated, slow pipetting.
- Go through a dry run of the ice⇒heat shock⇒ice steps.
- Remind students to label their culture tubes and plates so as not to confuse them with other groups' experiments.
- The first part of the transformation lab is designed for one 80-minute period; however, it can be divided up into two 50-minute periods. If you have 50-minute periods, follow these steps.
  - After the heat shock step, the tubes can be stored in the refrigerator overnight.
  - The next day, have students incubate the tubes at 37°C for 15 minutes so that the cells can recover before plating.
  - Spread bacteria on plates and incubate overnight at 37°C.

Discussion of Results and Trouble Shooting

- Encourage students to come up with their own explanations for problems before you offer a solution. Resist offering explanations until they have studied their results and made their own observations.
- Be prepared to explain the existence of *satellite colonies.* (These are different from the actual transformed colonies that the students should count.) These are tiny colonies appearing around a larger, central colony on an LB/amp/X-gal plate. The large colony is an amp-resistant transformant; the smaller ones are usually not! They are living in an area where ampicillin was inactivated by β-lactamase secreted by the large colony.

- ❑ You may have other bacteria, yeast, or mold on your plates. Be prepared to discuss possible causes of contamination.
  - o Plates that were left in the incubator longer than the recommended incubation period allow for any contaminants to flourish. Even though the plates might be contaminated to begin with, the less than 24 hour incubation allows for the intended cells (the millions of *E.coli* plated!) to grow. Longer incubation times allow for the contaminants to appear.
  - o Fungal contaminants may have a filamentous appearance. Yeast colonies are often a dull, yellow color. Other bacteria may have grown as well; these could be different colors and shapes, depending on the type.

- ❑ If bacteria fail to grow, or if there was growth on plates where there should not have been---there are several variables that could cause these results.
  - o Was the heat shock too harsh?
  - o Was there ampicillin in all of the plates?
  - o Were the bacteria dead on the stock plate?
  - o Was there no ampicillin in the plates? Or was the ampicillin not working?
  - o Were the plates labeled accurately?

  If the transformed bacteria do not grow on the LB/amp/X-gal plates.
  - o Were the cells made competent? Did you use $CaCl_2$?
  - o Was the heat shock performed correctly?
  - o Poor quality plasmid?

- ❑ There are different ways to approach this lab to help students understand gene function and expression. Here are some considerations and suggestions for thinking about the lab by using a one-step plating method (as in the protocol) or a two-step method.

One-Step plating

- Plate transformants onto LB /amp/X-gal. Surviving cells will be amp resistant and also blue.
- **Advantage**: takes only one day of plating.
- **Disadvantage**: students often have difficulty understanding that the ampicillin (not the X-gal) is responsible for the selective growth of the Amp^r (ampicillin resistant) transformants, and that the β-gal gene "tagged along" in the plasmid.

Two-Step Plating

- Plate transformants onto LB/ampicillin plates first to select for transformants, then the next day toothpick transformants onto LB/X-gal plates to see the blue color.
- **Advantage**: Students better understand the use of ampicillin as a selection agent by this method.
- **Disadvantage**: You will need to prepare and use more plates with this variation, and it will require an extra day. Notify SEP if you decide to do the two-step plating; you will need more supplies.

- [ ] Give students some possible experimental results and have students interpret sample data prior to interpreting their own. Have students suggest positive and negative controls. It's essential to emphasize the importance and role of controls to help interpretation of experimental results. Positive controls are samples included that you expect to work, that you expect to give you positive results. Positive controls help you check the effectiveness of your reagents. As much as possible, the treatment of your negative controls should match your experimental sample, ideally differing in only one respect (your variable). Negative controls are samples included which should not work, or show no effect, if everything goes as expected. A negative control helps to put results in context. For example, if bacteria transformed with water only (a negative control) form blue colonies, something is wrong and you need to explore what has happened. Until then you cannot trust your experimental results.

Extension activities

- Calculate the transformation efficiency, if students haven't already, and use it to quantify student-initiated experiments. (See the student lab sheets.) You may need to explain that ideally, transformation efficiencies achieved with the colony protocol used in this lab should be approximately 5×10^3 to 5×10^4 colonies per microgram of plasmid. Have students consider ways to improve efficiencies for commercial use.

- Discuss the challenges of antibiotic-resistant strains of bacteria, which are becoming prevalent; the appropriate use of antibiotics; and the challenges of developing new antibiotics.

- Collect samples from the classroom, homes, or outside and smear samples on LB plates (you could make some LB/amp plates too). Caution: some bacteria, molds, yeasts, and fungi, which you may collect, can be pathogenic. Be particularly careful handling these plates, do not culture for more than 3 days, and dispose of correctly and promptly.

- Calculate the number of bacteria in a colony, given that the generation time is typically 20 minutes and that the colony started from a single bacterium.

- Have students devise a way to mass produce a protein of their choice by searching gene sequence databases, researching possible vectors, and finding methods to design an experiment that their own biotech company could use.