



Teacher's Guide: pBLU[®] Transformation

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• As soon as you get the kit back to school or home •

If you haven't done so already, please **put the ampicillin and plasmid into a refrigerator** (keep the ampicillin dark) and the **X-gal into a freezer**. The bacterial stab culture should be stored at room temperature.

Acknowledgements and Resources

We are indebted to Suzanne Black (Inglemoor High School, Bothell WA 98011), Carter Hoffman, SEP participants, and many others for field testing, comments, illustrations, and advice. References which contributed ideas and methods to this protocol include the Gene Connection v1.5 (1995), DNA Science (1990; 1995), NABT's Working with Bacteria and DNA in Precollege Science Classrooms (1993), and the EduGen Amylase Kit. Please see the Reference List for specific citations. Also, please note that this is written for SEP participants and that materials/assistance are not guaranteed to non-participants.

Overview

The lab is written as a scenario, in which students working as teams (we suggest cooperative groups) are challenged to improve the efficiency of bacterial transformation. They're employed by a biotech company to produce a blue dye (the X-gal cleavage product) for jeans. A pre-test ("job interview") is included to assess students' prior knowledge of transformation. To become familiar with the transformation technique, students first follow the standard technique, which we derived mostly from the Gene Connection, with reference to DNA Science. Then students propose an improvement to the technique. If you have time, we highly recommend allowing students to try out their idea. Plasmid DNA can be isolated from positive bacteria, though this is optional and neither the equipment nor reagents are in this kit. Talk to us and we can provide them (to SEP teachers).

Your students may pick up on an inconsistency in the scenario. If one simply wanted as much blue dye as possible, one could pick a transformant and grow it in large quantities. Needing to improve the efficiency of transformation is valid, though. Highest efficiency is necessary in, for example, construction of a DNA library. The genome of an organism can be broken up into thousands of DNA segments, typically 15-20 Kb long, and inserted into viral hosts. Propagation of these DNA segments requires transformation of bacteria with the library. If transformation is not efficient, many transformations will have to be done, and there is an increased chance of a particularly rare, and potentially important, clone not being taken up by the host bacteria. We're working on revising the scenario to be more accurate.

Look over the inventory list for the kit and check that you have everything, several days before you intend to use the kit, if possible. Call us immediately at (206) 667-4487 if anything is missing or insufficient. Please read through this teacher guide and the student guide before you start.

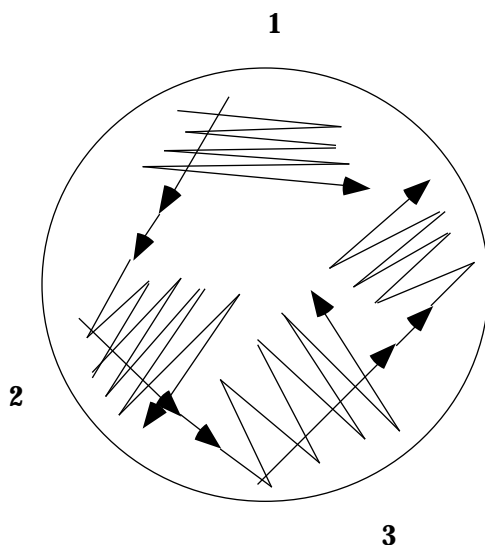
Time for lab

- **Minimum of three 50-minute periods:** two to do the lab & a third period to analyze results. If the students carry out their proposals, another three periods will be required. Alternatively, two 80-90 minute periods; the lab can be done in one period and analyzed in the next. For any class length, add time for you or your students to pour plates (~ 1 hour) and prepare stock plates of JM101 for transformation (15 minutes). Have a look at the timeline for suggested ways to do the lab within a two-week time period.

Preparation for lab

- Read pp. 51-58 and 293-310 in DNA Science (1990). Consider having your students read this too, but after the first transformation experiment, when their interest will be stimulated.

- We recommend you give the pre-employment interview 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.
- **Stock plates: Streak 3-4 stock LB plates per class with *E. coli* the day before transformations are to be done.** We include a stock of *E. coli* strain JM101; other strains can be used but must be Lac⁻, i.e. lacking endogenous β-galactosidase. You want reasonably sized colonies (2-3mm diameter), which are still in log-phase (i.e. rapid) growth. Rapidly growing bacteria appear to be more suitable for transformation by this technique. On the day of the lab, turn the stock plates upside down and draw lines dividing each plate into segments. Have each team gather its bacteria from a different segment of the plate. "Invite" students to the stock plates a few at a time to eliminate crowding. **To streak a plate, either:** flame the loop, cool in agar in an area away from bacteria; or carefully remove a sterile, disposable loop; and gather a small amount of bacteria from the surface of the provided slant culture. Streak on an LB plate, following the diagram:



- 4 Start at 1, with bacteria on the loop. Streak, then flame the loop and cool it (or use a new disposable one). Drag across the first streak and make streak 2. Flame and repeat until the plate is covered. The idea here is that if you start with very dense bacteria at 1, then by 4 (at the latest) you'll have individual, separated colonies to pick from.

- There are two suggested ways to approach this lab:
 - (1) Plate transformants onto LB/amp/X-gal plates. Surviving cells will be amp resistant and also blue. This way only takes one day of plating, but students often have difficulty understanding that the ampicillin (not the X-gal) is responsible for the selective growth of the Amp^r transformants, and that the β-gal gene "tagged along" in the plasmid.
 - (2) Plate transformants onto LB/amp plates to first select for transformants. On the next day, toothpick or replica plate transformants to LB/amp/X-gal plates to show that the β-gal gene "tagged along" in the plasmid. Students better understand the use of ampicillin as a selection agent by this method. You will need to prepare and use more plates with this variation.

- **Pouring plates:** You will need to pour the plates for this lab. Do it a few days before you need them, if possible, so they can dry out a little bit. Assuming variation 1 from above, you'll need two LB-agar plates and two LB-agar/ampicillin/X-gal plates per student group, for each transformation experiment. You'll need at least twice as many if the students test out their proposals. See below for the LB-agar recipe; LB-agar is included with the kit. Each LB-agar bottle contains approximately 350ml; you'll need 15-25ml per plate. Plates can be stored up to a month if refrigerated and kept dark. LB refers to the liquid; LB-agar refers to the liquid plus agar, a solid at room temperature.

Plate pouring tips:

- (1) Clear off and wipe down a large, flat working surface with 70% alcohol or 10% bleach, away from drafts.
- (2) Set out several rows of sterile plastic petri dishes in stacks of 3-4. Label plate bottoms with the type of medium (e.g. LB or LB/A/X) and the date. (Alternatively, color code your plates (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.)
- (3) Microwave LB-agar (**LOOSEN CAP FIRST!**) on medium-high power until liquid. Hold melted agar in a 60°C water bath until you are ready to pour plates. It takes 5-10 minutes to melt one 350-ml bottle of agar, so get an **EARLY** start! You might microwave bottles all day long, and pour plates after school, though you would need a large waterbath.
- (4) When bottle is cool enough to be held, commence pouring plates. Each plate holds about 15-25 ml of medium; pour in enough to just cover the bottom of the plate. Pour plates in stacks of 3-4. Some condensation will occur mostly in the top plate of each stack. Try to avoid air bubbles. Bubbles, if numerous or large, can be popped with a sterile pipet or with a quick pass of an inverted, lighted bunsen burner over the surface of the agar.
- (5) When solidified (allow at least half an hour), place plates upside down in a closed box or bag to minimize drying out and keep at room temperature. You may also store them in the refrigerator (in the sleeves the plates came in or wrapped in Parafilm), but unsterile condensation may drip into the plates and contaminate them.
- (6) **To pour LB/ampicillin/X-gal plates**, repeat steps #1-5 but 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 the provided micropipette to measure ampicillin and X-gal. **Ampicillin:** The provided ampicillin stock solution, at 100mg/ml, should be stored in the refrigerator. Use it at a final concentration of 100µg/ml, e.g. add 1ml to 1 liter of LB-agar. Check the volume of your bottles of LB-agar to calculate how much ampicillin to add. **X-gal:** Add 0.8ml

2% X-gal stock per liter of 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 dark as light will break down ampicillin and X-gal.

- On the day of transformation, warm the water bath to 42°C for the heat-shock step; warm the incubator to 37°C for cultures. Both should already be adjusted to the correct temperature but **check** to be sure.
- Set out all supplies. Tips and tubes must be sterile, and students should use sterile technique. Depending on your class size, some reagents/equipment may need to be shared between groups. We suggest groups of 2-4 students; the kit is designed for a maximum of 8 groups of 4 students per class.
- Design room layout to minimize congestion and foot traffic near water baths & incubators.
- You may decide to aliquot LB, sterile water, and calcium chloride for teams, or you may appoint a few students in each class to do all the dispensing. NOTE: the LB has to be sterile. Inspect the bottle! If it's cloudy or there are molds floating in it, treat with bleach and discard it.

Safety Issues

- Read pp. 197-8 in DNA Science, and NABT's Working with Bacteria and DNA in Precollege Science Classrooms (included with this notebook).
- Make sure students understand the differences between the E. coli in the experiment (and their vital role in the human digestive tract) and the pathogenic strains associated with contaminated food.
- Kit 1 includes alcohol lamps for flaming loops and spreaders. If you prefer not to use flames with your students, Kit 2 has disposable and reusable items.
- If this is the first lab with live bacteria, go over the use of disinfectant to decontaminate spills. Students (and you!) **must wash hands before and after the lab**. You should have a sink available in the classroom. Instruct students to **disinfect tabletops thoroughly**, before *-and* after plating. There must be absolutely no food or drink consumed or left on tabletops.
- When pipetting suspension culture, be careful not to breathe any aerosol which is formed. Keep your nose and mouth away from the end of the pipet. Never mouth pipet!

- Keeping cultures growing for long periods (>3 days), even at room temperature, can encourage contaminants — which may be pathogenic — to proliferate. Refrigerate cultures or dispose of promptly (see below).
- If you have an autoclave, this is the best way to dispose of contaminated plates and solutions. Place the items in a biohazard bag and autoclave for at least 15 minutes at 121°C. Bags can then be safely disposed of in the trash. Make sure the janitors understand the process; the biohazard symbol can be worrisome. You can try crossing out the biohazard symbol with a marker after autoclaving. If you don't have an autoclave, soak plates in 10% household bleach, or add 10% bleach to contaminated solutions. Disinfect for at least 15 minutes, then dispose of in normal trash or pour down the drain.

What to demonstrate/explain.

- Use a creative demonstration to explain what transformation is and how it is done. See the kit notebook for one suggestion (Lesson 13 of the Biotechnology Unit for Middle School Students, from the St. Louis Mathematics and Science Education Center (314) 516-5650)
- 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 when you have manipulated something. Positive controls help you e.g. 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 which were transformed with water only (a negative control) form blue colonies, something's wrong and you need to explore what has happened. Until then you cannot trust your experimental results.
- Transformation efficiency can be a difficult concept. Go through it with your students. An alternative (less graphical) way to calculate the efficiency is on the last page of this.
- Remind students to CLEARLY LABEL their culture tubes and plates.
- Demonstrate how to pick bacteria from a plate, and how to resuspend cells in CaCl_2 by repeated, slow pipetting.

- View the video with your students which demonstrates flaming (if appropriate), colony picking, transferring bacteria from the plate into a microcentrifuge tube, pipetting, and spreading of bacteria.
- Make a dry run of the ice→heat shock→ice steps.
- Review fire safety and locations of fire blanket and extinguisher.
- Demonstrate how to plate and uniformly spread cells.
- Each colony represents a single bacterium which was transformed, then grew clonally to form a visible colony.

Troubleshooting and Technique Tips

- Encourage your students to suggest answers to problems before you offer a solution. They will learn a great deal more by puzzling through the steps. For example, resist offering an explanation for satellite colonies until students have tried to explain them.
- Other bacteria, yeast, mold, or fungi may contaminate your plates. This isn't usually a problem unless you incubate the plates for more than three days. The numbers of contaminant cells are very low compared to the millions of intended cells on the plate. Yeast colonies are often yellow and dull. Fungus or mold may have a filamentous appearance, with irregular-edged colonies. What may be very confusing, though, is contamination with bacteria which naturally express the β -galactosidase gene (it has been specifically deleted from the JM101 bacteria we provide). This will give the *appearance* of transformation having been successful when in fact it has not been.
- If bacterial colonies fail to grow anywhere, it may be:
 - bacteria were killed by a "too hot" wire loop during spreading?
 - heat shock step was too harsh?
- If bacteria given pBLU[®] plasmid do NOT grow on the LB/amp/X-gal plates, there is poor transformation efficiency due to
 - poor quality plasmid?
 - a mistake in timing (e.g., a 9 sec. heat shock instead of 90 sec)?
 - cells not competent enough? e.g. old stock plate used
- If bacteria grow on the "wrong" plates, perhaps
 - antibiotic solutions or plates were old or have lost their effectiveness? or
 - poor sterile technique may have allowed contaminants to grow on plates, or
 - plates were over-incubated (incubator temps over 40°C destroy antibiotic in plates), or

- a huge inoculum was put on the plate to start with (antibiotics kill dividing cells only), or
- error was made in mixing media and/or pouring plates?
- Devise an efficient means of sharing class results (oral reports? product meetings? shareholder meeting?).
- Do not let students open petri dishes on which bacteria are growing, unless picking a colony.
- You can store your best plates in the refrigerator for a few weeks if wrapped in Parafilm (included in kit).

Extensions:

- Be prepared to explain the existence of *satellite colonies* (though you should ask your students for their hypotheses first; they usually come up with the right answer). These are tiny colonies appearing around a larger, central colony on an LB amp 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. Students may want to toothpick colonies onto LB and LB-amp plates to test this further...
- To confirm amp resistance, toothpick the main colony and its satellites onto LB and LB-amp plates.
- 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.
- Calculate the transformation efficiency, if students haven't already, and use it to quantify student-initiated experiments. See the student lab sheets.
- Collect samples from the classroom, homes, or outside and smear samples on LB plates (try 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.
- Make a DNA miniprep from a small liquid culture of pBLU[®]-transformed bacteria. This is described in Lab 6 of DNA Science, and we can provide you with the reagents and equipment necessary. This is best done in conjunction with the Electrophoresis Kit, which will allow you to analyze your isolated plasmid, though you can store isolated plasmid, or bacterial plates, in the refrigerator for months until you have the Electrophoresis Kit. You will, however, have to use micropipets to isolate the plasmid (accuracy is critical with this technique).
- Pages 308-9 of DNA Science have several extensions for their pAMP lab; these can be used for pBLU[®]. We also have pAMP available if you want it. Ambitious students may want to cut the β -galactosidase gene out of the plasmid, isolate it, and ligate it back into the plasmid. Or we can prepare the β -galactosidase gene and the remainder of the plasmid, and your students can ligate the gene back into the plasmid, then check if they were successful. These are time-consuming experiments and you should check with us well ahead if you anticipate doing this.

- The Amylase Project has a good and more extensive microbiology/transformation curriculum for identifying, characterizing, and transforming an amylase plasmid (allowing *E. coli* to digest starch). They also sell the reagents necessary, though in our experience the shipping and packaging has had problems. Contact Diane Sweeney at EduGen Inc. Phone (415) 348-5761.
- You may also use one of several "pUC" plasmids that contain the alpha-peptide fragment of beta-galactosidase gene (pUC 8, 18 and 19 are some that have been successfully used), though without addition of IPTG, the β -gal gene will not be induced and the blue color will be faint. pBLU[®] has the β -gal permanently turned on.
- The disk included with the notebook contains the pBLU DNA sequence and a shareware program, DNA Strider, to analyze the sequence. On a Macintosh, start up DNA strider and load the pBLU sequence into it. Students can do hypothetical restriction digests, translate the sequence into protein, and more.
- A math extension: 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.

Assessment

Some suggestions:

- Give the pre-employment interview before the activity to assess students' prior knowledge of transformation.
- Have students create a flow chart of the lab before they do it. There can be text but the majority of it should be pictorial. It doesn't have to be fancy but it must be accurate. Students may resist doing this but it helps greatly in understanding the steps of the transformation. See [DNA Science](#) and the kit notebook for examples of flow charts.
- Have students label/explain the drawing of the steps of transformation; before the activity (they should be able to identify some of the features), while they are formulating their proposal to increase transformation efficiency, or after the activity. A version with some possible answers is included. Note that this drawing only represents the uptake and expression of the ampicillin resistance gene. Students could add in the events associated with the β -galactosidase gene.
- Watch the students as they work on the activity; by listening to their reasoning and discussions you should get an idea of how well they understand the material.
- Ask your students to come up with ways to summarize the lab. One way:

Medium	Transformed Cells (+ pBLU)	Bacteria only (no pBLU)
LB/amp/X-gal	Experiment (# of colonies)	Negative control (# of colonies)
LB	Positive control (# of colonies)	Positive control (# of colonies)

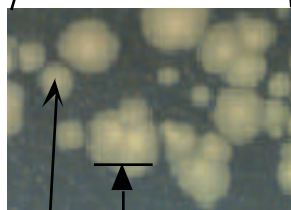
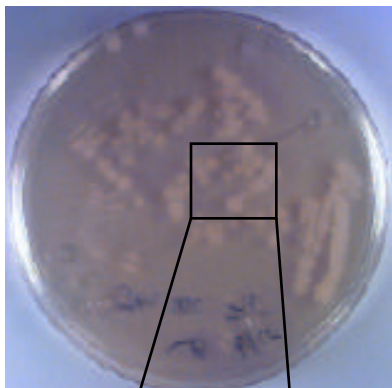
- Consider giving a "lab practical" test, allowing students to work in groups or individually.

- Present students with plates from an experiment and ask them to explain what has happened (e.g. white bacteria on a plate: transformed? control?).
- Without telling the students, give them a plasmid such as pAMP (we can provide it) to transform bacteria with. It will confer ampicillin resistance, but has no β -gal gene to generate the blue product with X-gal. Students may deduce that there is no β -gal, or suggest good controls to check the system (e.g. using a known pBLU[®] plasmid to check that the X-gal is active; confirming that pAMP transformants are genuinely amp resistant (by picking colonies and spreading on a new ampicillin plate)).

You should be seeing this page in color.

Bacterial Culture Pictures

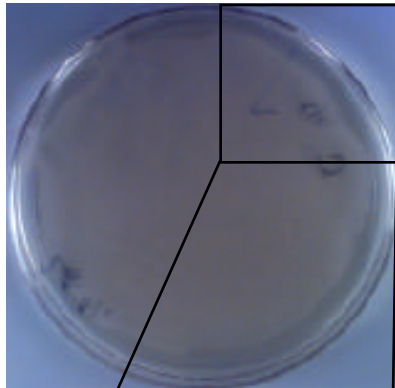
Stock, streaked culture plate



single colony

four colonies

Bacterial lawn

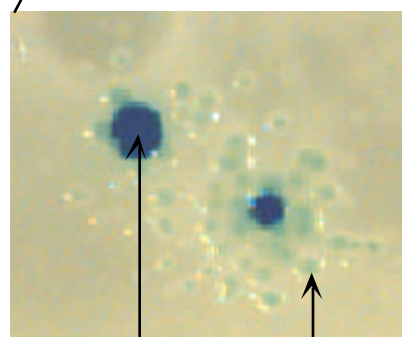
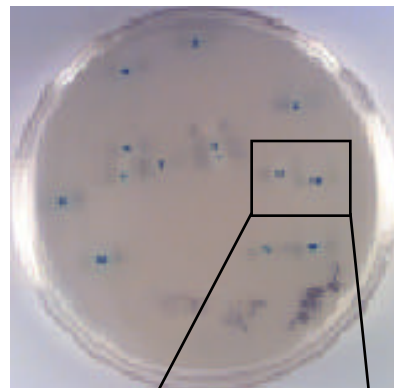


edge of lawn

dense bacterial colonies

no bacteria

Transformed, positive colonies, with satellites



blue, positive colony

satellite colony (normally whiter than this)



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Recipes

Note: we provide these reagents to you ready to use. The recipes are provided for your information and if you wish to make up your own solutions.

Luria Broth (LB); for liquid culture

per liter:

5g yeast extract

10g NaCl

10g tryptone (trypsin digest of milk casein)

caution: the yeast extract and tryptone are very powdery and will send up a huge cloud of dust if you're not careful.

Add the dry ingredients to distilled water (800ml), while stirring. If you put the water onto a pile of the dry reagents, an air bubble forms over them and makes dissolution much slower. Adjust the pH to 7.5 using a pH meter or pH paper, using 4M NaOH. Dispense into aliquots of 350ml for liquid cultures/plate preps, or 100ml for small cultures/transformations. You can sterile filter into sterile containers, or autoclave the LB in bottles for 15 minutes at 121°C. Aim for the smallest practical bottle size, since once you open a bottle the chances of it becoming contaminated are high. Always check for bacterial or fungal contamination by swirling the bottle and examining for any cloudiness. Discard if this is the case.

LB-agar; for plates

Start with prepared liquid LB (above), or add all the dry ingredients for LB plus:

15 g agar (also called Bacto-agar, agar-agar...) per liter. Adjust pH as above if necessary and autoclave. Can be stored up to 3 months either in bottles or in plates.

Ampicillin

Using the sodium salt of ampicillin (we use Sigma Chemical Co.; #A-9518), make a 100mg/ml stock. Sterile filter and dispense into sterile containers (usually 1ml); store frozen for no more than 6 months.

X-gal

Full name is 5-bromo-4-chloro-3-indolyl- β -D-galactoside. It's very expensive; \$75/gram is the best we've found, from Research Organics Inc., (800) 321-0570. Using a hood, if possible, and minimizing contact, make a solution of 2% X-gal in N,N-dimethylformamide. DMF is toxic and mutagenic. Dispense into sterile containers and store dark and in a freezer (-20°C).

CaCl₂

Make 100ml of a 1M stock of CaCl₂. You can use either the anhydrous or dihydrate form, but make sure to calculate the amount to use accordingly. Store at room temperature indefinitely. Dilute 1M stock to 50mM, then either sterile filter or autoclave. Store in sterile tubes or bottles at 4°C so that it's already cold and ready for use.

pBLU[®] is available from Carolina Biological. Small quantities for transformation are relatively inexpensive.

Reference List

Gene Connection v1.5, 1995, originally from the San Mateo (CA) County Office of Education; written by Suzanne Black, Kathy Liu, and Stan Ogren. An excellent collection of molecular biology lab activities. Available as a Macintosh disk for \$50 from Fotodyne, 1-800-362-3686.

DNA Science: A first course in recombinant DNA technology, by Micklos and Freyer. The 1990 edition has a narrative history and good explanations of molecular biology concepts, along with molecular biology laboratories. The 1995 edition has labs only: updated versions of the 1990 labs plus new ones such as the polymerase chain reaction. The 1990 edition is \$35 and available from Carolina Biological (800) 334-5551 or CSH Press (800) 843-4388.

Working with Bacteria and DNA in Precollege Science Classrooms, 1989, by Toby Horn, is a comprehensive safety and appropriate use guide. Cost is \$8 for NABT members. Order from National Association of Biology Teachers, 11250 Roger Bacon Drive, #19, Reston VA 22090. Telephone (703) 471-1134.

The Amylase Project has a good and more extensive microbiology/transformation curriculum for identifying, characterizing, and transforming an amylase plasmid (allowing *E. coli* to digest starch). They also sell the reagents necessary, though in our experience the shipping and packaging has had problems. Contact Diane Sweeney at EduGen Inc. Phone (415) 348-5761.

Calculation of transformation efficiency

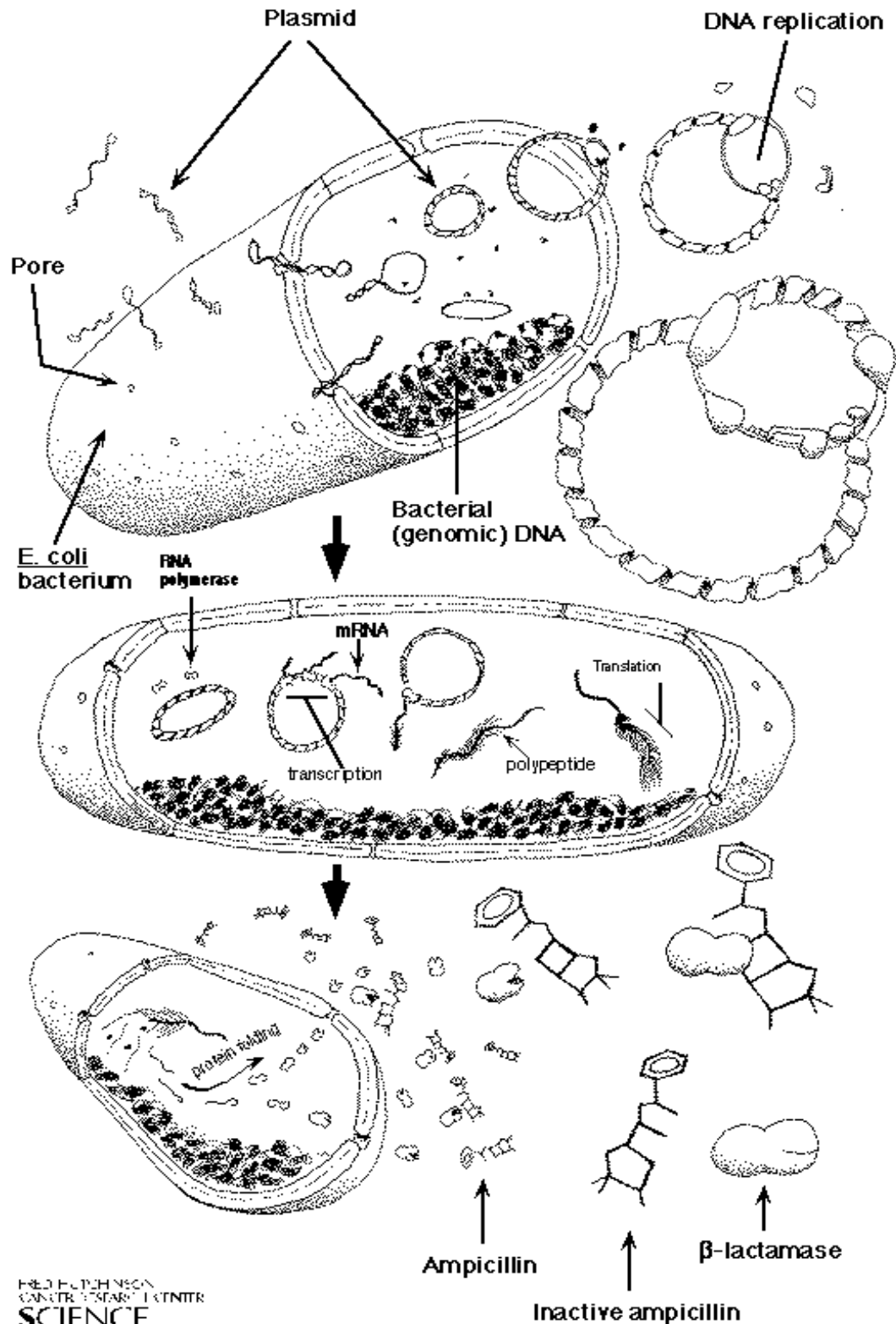
We can calculate the efficiency of transformation to get a quantitative assessment of how successful your transformation was. Transformation efficiency is expressed as the number of antibiotic-resistant colonies per microgram of pBLU DNA. The object is to determine exactly how much DNA (inside the *E. coli*) was spread on the plate and relate that to the amount of DNA added to the *E. coli* at the beginning of the experiment. It seems more complicated than it is.

Note: symbols connect the places where the same answer is used

1. Determine the total mass, in micrograms (μg), of pBLU DNA used in step 4.
Concentration of the DNA \times volume of DNA solution used = mass of pBLU*.
2. Determine how much of the cell suspension you spread, in microliters (μl), onto the LB/amp/X-gal plate. Volume of suspension spread (step 14)/total volume of suspension (steps 1, 4, + 11) = fraction of cell suspension spread♣.
3. Determine the mass of pBLU contained in the cell suspension spread onto the LB/amp/X-gal plate. Mass of pBLU* \times fraction of cell suspension spread♣ = mass of pBLU spread♦.
4. Determine the number of colonies per microgram of pBLU. Express the answer in scientific notation. Colonies counted/mass of pBLU spread♦ = transformation efficiency.

After "DNA Science," by Micklos & Freyer, 1990, pp. 308-309.

Bacterial Transformation

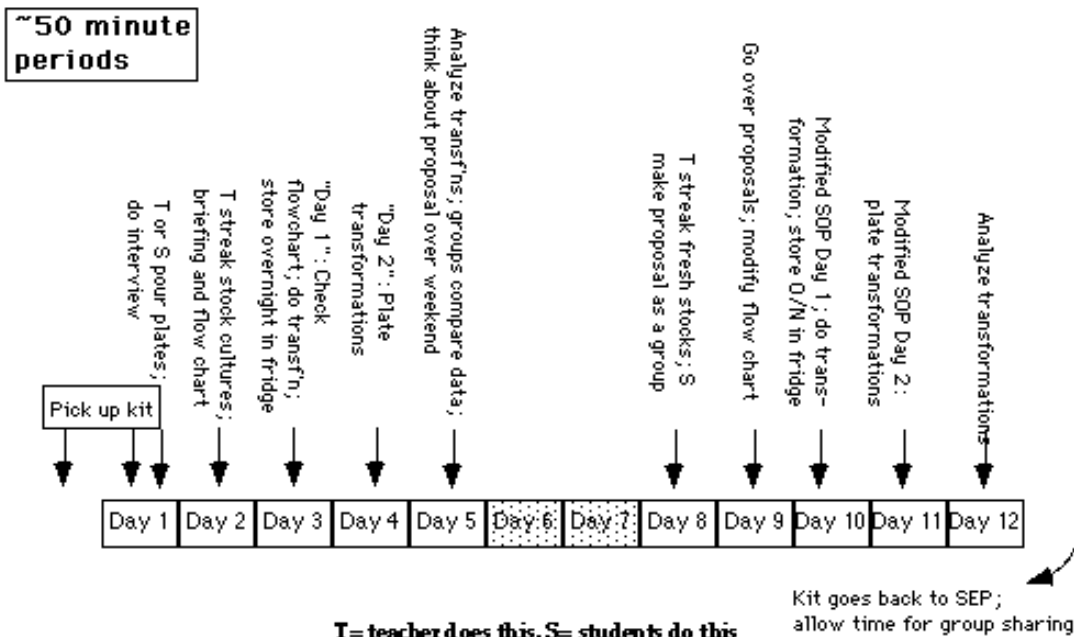


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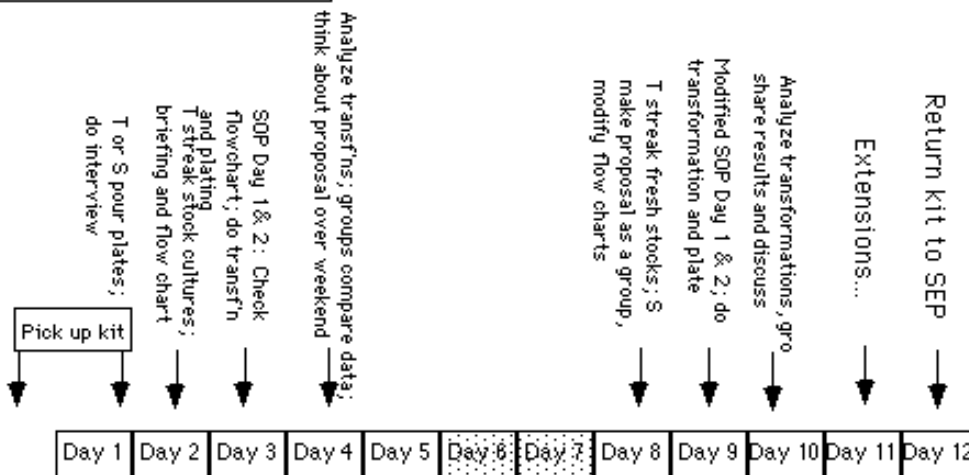
By Carter Hoffman, 1995

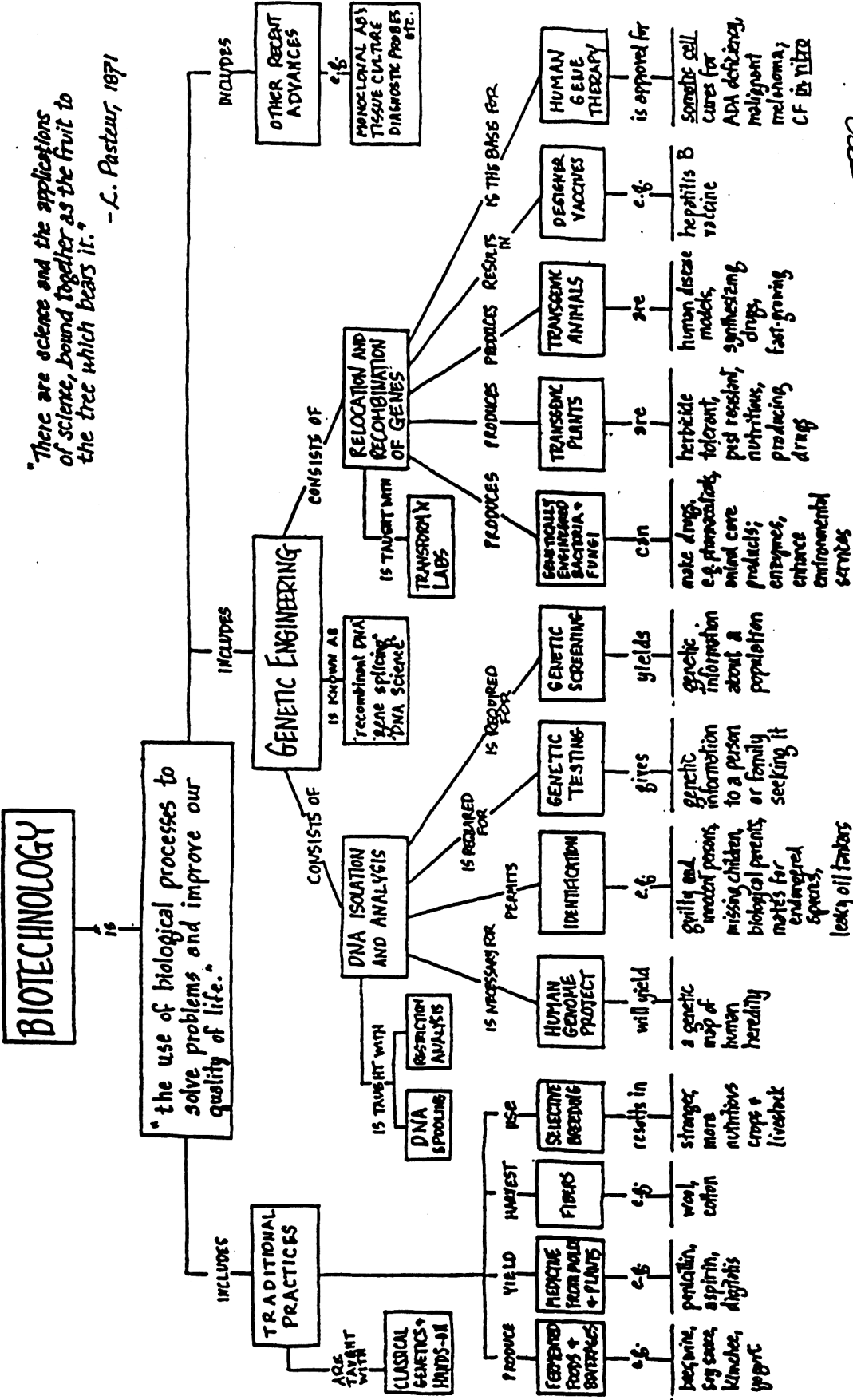


pBLU Transformation: Suggested Timelines



80/90-minute periods:





SBA

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