Biotechnology Explorer™

Secrets of the Rain Forest

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Biotechnology Explorer Program

Technical advances over the past several decades have created a new branch of science, called biotechnology, which has transformed and revolutionized biological and life science research. Powerful methods to isolate, analyze, and manipulate DNA, the basic building block of life, have already allowed many breakthroughs in understanding biological processes, human disease states, and therapeutic methodologies. So, it is becoming increasingly important for schools to expose students to these concepts. In the coming decades, where a routine visit to a family doctor might include a battery of DNA diagnostic tests, and DNA fingerprints will become the definitive form of personal identification, an understanding of these principles will be as important as learning about hygiene and nutrition.

In order to provide students at the high school, junior college, and college levels with exposure to these new technologies, Bio-Rad has developed a series of instructional classroom kits supported by curricula, equipment, and supplies which allows students to conduct experiments and have hands-on experience in the basic techniques of DNA technology. Called the Biotechnology Explorer, this program was developed in collaboration with the San Francisco Bay Area Biotechnology Educational Consortium, Rutgers University, Maxygen Inc., (a commercial biotechnology company), and The Stanford Human Genome Center.

The Biotechnology Explorer provides laboratory-based activities that capture the imagination while enhancing student awareness and understanding of the science behind biotechnology which will increasingly influence their lives and affect their personal and community decisions. It is important for students to learn about DNA but it is also important to show them how the science behind biotechnology can be applied in the real world to help solve human problems. The Biotechnology Explorer program performs these functions.

How Can Jellyfish Shed Light on Biotechnology Education?

One of the biggest challenges for those studying molecular biology for the first time has been that many of the events and processes they study are invisible. The Biotechnology Explorer offers a novel solution: a unique gene and its Green Fluorescent Protein (GFP) which glows with a brilliant green color when viewed with the light of an inexpensive hand held ultraviolet lamp. The gene for the fluorescent green protein was originally isolated from a bioluminescent jellyfish. The jellyfish gene has recently been cloned into Bio-Rad's exclusive pGLO plasmid specifically for use in Biotechnology Explorer kits.

Using the pGLO system, students become genetic engineers, transforming bacteria (Bio-Rad Kit 1) and producing and purifying fluorescent recombinant proteins (Bio-Rad Kit 2). They can directly observe the results of gene transfer, gene regulation, gene expression, and the process of protein purification as they are occurring. Bio-Rad's pGLO system is unique, and generates excitement and creative teaching applications among science educators.

Secrets of the Rain Forest

Secrets of the Rain Forest provides a two-week biotechnology curriculum which takes the students on an adventure starting in the rain forests in the Andes, on to a biotechnology company engaged in developing new pharmaceutical compounds, and finally to the Food and Drug Administration.

The adventure begins as Tisha is hiking through the rain forest, looking for unique plant species, when she meets a native boy who tells her about an old medicine man with mysterious green leaves which have the power to treat and cure stomach cancer. The boy gives Tisha a bottle with a few of the leaves which she takes back to Biotex, a fictitious biotechnology company working on developing new compounds to treat human diseases.

At Biotex, Tisha notices that the mysterious leaves glow bright green under ultraviolet lights. Other scientists at Biotex determine that the fluorescent green substance is a protein with possible therapeutic powers. During the first week of this curriculum, students play the role of Biotex researchers and learn about removing the DNA that produces the protein from the leaves and inserting it into bacteria cells.

Students purify a green fluorescent protein (GFP) from bacteria containing a cloned gene for GFP. The gene is said to come from mysterious fluorescent green leaves which can cure stomach cancer.

In the second week (Lessons 7–10) students simulate the real world process of taking this medicinal protein to market as a treatment for stomach cancer. They will examine the needs and viewpoints of advocacy groups, the biotechnology industry, and the Food and Drug Administration (FDA). Given real life scenarios, students will develop possible strategies for problem-solving. This portion simulates true biotechnology in action.

In order for students to gain the most from this module, they should know what a gene is and understand the relationship between genes and proteins. It is not necessary for students to understand the details of cloning to be successfully involved in this module.

This curriculum was developed by the Santa Clara County Biotechnology Education Partnership (SCCBEP), a participant in the Bay Area Biotechnology Education Consortium (BABEC). A portion of the purchase price of this manual will be awarded to these educational groups to help support education in their region.

Biotechnology Explorer kits provide real-life, comprehensive investigations designed to replace cookbook teaching labs. The focus is not so much on the answer or result, but rather how the result was obtained and supported by careful observation and analysis of data. Bio-Rad's GFP-based curriculum has generated an unprecedented level of excitement among science educators and their students. We strive to continually improve our curriculum and products. Your input is extremely important to us. We welcome your stories, comments, and suggestions.

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Instructors Guide

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Kit Inventory (✔) Checklist

This section lists the components provided in the Rain Forest Kit. It also lists required accessories. Each kit contains sufficient materials to outfit eight student workstations. Use this as a checklist to inventory your supplies before beginning the experiments.

Components Provided with the Kit	Number/Kit	(✔)
Module 1 Lessons 2 and 3—Cloning and Screening		
Bacterial Library—lyophilized	1 vial	❑
LB-agar tablets	5 tablets	❑
Petri dishes—60 mm, sterile	20	❑
Inoculation loops—packs of 10 loops	1 pack	❑
Pipettes—sterile, individually wrapped	10	❑
Module 2 Lessons 4, 5, and 6 - Purification		
Ampicillin—lyophilized	1 vial	❑
Arabinose—lyophilized	1 vial	❑
LB-broth tablet (to make 50 milliliters)	1 tablet	❑
Inoculation loops—packs of 10 loops	2 pack	❑
Pipettes—sterile, individually wrapped	40	❑
Microtubes—2.0 milliliters, clear	30	❑
Culture tubes—15 milliliters, sterile (pack of 25)	1 pack	❑
Collection tubes—5 milliliters, polystyrene	25	❑
TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0; sterile)	1 bottle	❑
Lysozyme—lyophilized	1 vial	❑
Binding buffer (4 M $(NH_4)_2SO_4$)/TE/pH 8.0)	1 bottle	❑
Equilibration buffer (2 M $(NH_4)_2SO_4$)/TE/pH 8.0)	1 bottle	❑
Wash buffer (1.3 M $(NH_4)_2SO_4$)/TE/pH 8.0)	1 bottle	❑
HIC chromatography columns	8	❑
Column end caps	1 bag	❑
Foam microtube rack	8	❑

Accessories Not Included in this Kit	Number/Class	(✔)
UV lamp—Long Wavelength* Bio-Rad catalog number 166-0500-EDU	1–8	❑
Microwave oven	1	❑
1 liter flask	1	❑
250 milliliter flask	1	❑
100 milliliter and 250 milliliter graduated cylinder	1	❑
Distilled water	1 gallon container from supermarket	
Thermometer	1	❑
Centrifuge	1	❑
Beaker of water for rinsing pipettes	1	❑
Bleach**	10 milliliters	❑
Scissors	8 pairs	❑
Colored markers	8 sets	❑
Butcher paper	8 sheets	❑
Refrigerator Freezer	1	❑
Optional Accessory Equipment – Not Included in the Kit		
Shaking/Rocking Platform or Incubator Use of a rocker or shaker will speed bacterial growth in liquid cultures, but is not required.	1	❑

*UV lamp

Ultraviolet radiation can cause damage to eyes and skin. Short-wave UV is more damaging than long-wave UV light. The Bio-Rad UV lamp recommended for this module is long-wave. If possible, use UV-rated safety glasses or goggles.

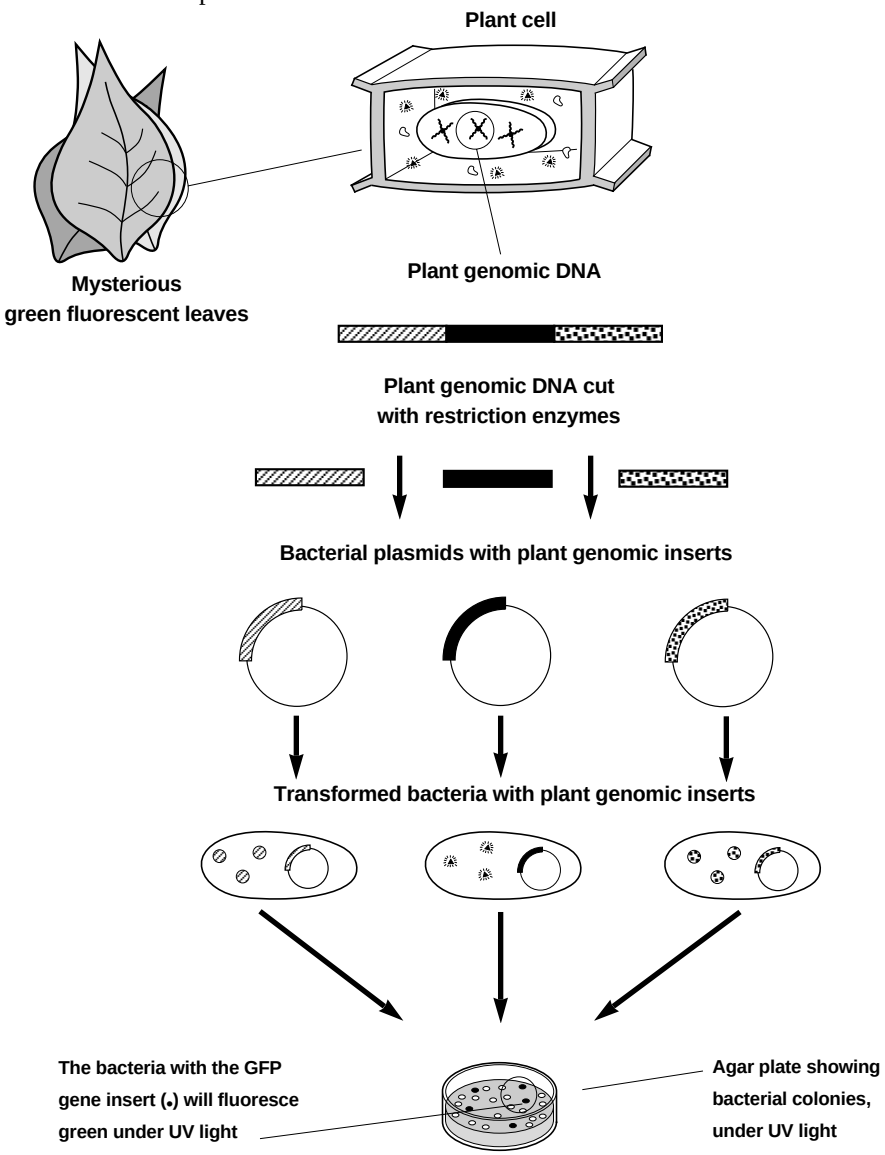
**See Appendix C about disposal of biological waste.

Lesson 1 The Mysterious Green Fluorescent Leaves

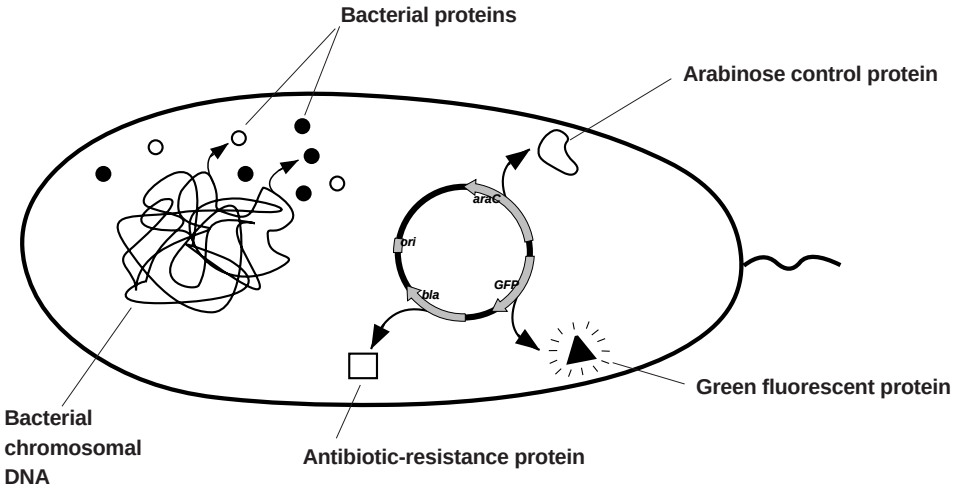
While hiking through the Andean Rain Forest, Tisha obtains medicinal green leaves which she brings back to Biotex, a biotechnology company working on a cure for cancer. Biotex scientists determine that the leaves contain a fluorescent green protein, so DNA from the leaves is inserted into bacterial cells.

Lessons 2 and 3 Cloning the Gene from the Mysterious Leaves

DNA from the medicinal leaves has been randomly cut and pasted into plasmid vectors, which have been inserted into bacteria. Only those bacteria which contain the GFP gene will fluoresce a brilliant green color under long-wave ultraviolet light. In Lessons 2 and 3, your students will need to find and separate these bacteria from the others. The flow chart below summarizes the process:



In order to find the bacteria containing the GFP gene, students must first streak-out the bacterial library onto an agar plate containing bacterial food. This process of separating the bacteria on the surface of the plate so each individual cell can grow up into a clump of identical cells (a colony) is called cloning. Refer to the schematic diagram below.



Genetically engineered plasmid used to insert new genes into bacteria.

Green fluorescent protein.

Beta-lactamase. A protein which gives bacteria resistance to the antibiotic, ampicillin.

araC, a protein which regulates production of the green fluorescent protein.

Lessons 4, 5, and 6 Purifying the Green Fluorescent Protein

Biotex technicians need to separate the GFP from other bacterial proteins. In this step, students grow the bacterial cells and use a simple procedure to make a bacterial extract which is then loaded onto a chromatography column to separate contaminating bacterial proteins from the GFP. The result is a purified GFP protein that can be tested for its ability to cure stomach cancer.

Lesson 7 Testing and Placebos

Before the purified protein is tested on humans, your students must first test it on rats or mice with stomach cancer. To determine if the protein really works, students learn to use a control, or placebo. The U.S. Food and Drug Administration (FDA) will examine Biotex's experiments to determine if the protein is safe and actually cures stomach cancer without negative side effects.

Lessons 8 and 9 Ethics and Economics

In this section students learn how a protein becomes a commercial product. Scenarios acquaint students with four main viewpoints: the FDA, social conscience, patient advocacy groups, and employees of Biotex. The real life dilemmas presented in this lesson must be resolved, as students take their new protein to market.

Lesson 10 Student Presentations

In this final lesson, students use the knowledge they have gained from lessons 1–9 to develop a solution to one of the dilemmas presented in Lesson 10.

Implementation Time Line

Student Lessons

There are ten lessons in Secrets of the Rain Forest, including five active student laboratory sessions. All lessons are designed to be carried out in consecutive 50 minute periods. For continuity, the core lab sessions (Lessons 2–6) can be conveniently started on a Monday and completed on a Friday.

Lesson 1	**Friday**	Introduction to Secrets of the Rain Forest. Lecture and discussion
Lesson 2	**Monday** (Lab)	Streak bacterial libraries on agar plates
Lesson 3	**Tuesday** (Lab)	Screen libraries and propagate selected clones in liquid culture. (May require 2 days if a shaker is not available.)
Lesson 4	**Wednesday** (Lab)	Concentrate bacteria by centrifugation
Lesson 5	**Thursday** (Lab)	Lyse (break open) bacteria to release proteins
Lesson 6	**Friday** (Lab)	Purify the Green Fluorescent Protein using protein chromatography
Lesson 7	**Monday**	Testing. Compare trial results of the fluorescent protein and a placebo.
Lesson 8	**Tuesday**	Dilemmas. Identify viewpoints and examine financial considerations.
Lesson 9	**Wednesday**	Prepare for presentations to classmates on selected scenarios
Lesson 10	**Thursday/Friday**	Presentation of student solutions to classmates

Advance Preparation Instructors Overview

This section outlines the recommended schedule for advance preparation on the part of the instructor. A detailed advanced preparation guide begins on page 8.

Prep activity	**When**	**Time required**
Read through Rain Forest manual	Immediately	1 hour
Prepare and Pour Agar Plates (20)	2–5 days before Lesson 2	1 hour
Prepare Liquid Culture Media (broth)	2–5 days before Lesson 2	15 minutes
Rehydrate Bacterial Library	Day of Lesson 2	5 minutes
Set up Workstations	Day of each lesson	5 minutes/day

Workstations Daily Inventory Check (✔) List

Student Workstations

Materials and supplies that should be present at each student workstation site prior to beginning each lab activity are listed below. The components provided in this kit are sufficient to outfit 8 complete student workstations.

Instructors (Common) Workstation

Materials, supplies, and equipment that should be present at a common location that can be accessed by all students during each lab activity are also listed below. It is up to the discretion of the teacher as to whether students should access common buffer solutions/equipment, or whether the teacher should aliquot solutions and operate equipment.

Lesson 2

Student workstations	**Number**
Inoculation loops—Sterile	1
Poured agar plates—Sterile	2
Marking pen	1
Instructors workstation	
Rehydrated bacterial library	1
37 °C incubator oven	1

Lesson 3

Student workstations	
Streaked bacterial plates	2
Inoculation loops	2
Culture tubes (growth medium)	2
Marking pen	1
Test tube holder	1
Instructors workstation	
Shaking incubator or platform (optional)	1
UV light	1–4

Lesson 4

Student workstations	
Microtubes	1
Pipette	1
Microtube rack	1
Marker	1
Beaker of water for rinsing pipettes	1
Instructors workstation	
TE buffer	1 vial
Lysozyme (rehydrated)	1 vial
Centrifuge	1
UV light	1–4

Lesson 5

Student workstations	**Number**
Microtubes	1
Pipette	1
Microtube rack	1
Marker	1
Beaker of water for rinsing pipettes	1
HIC chromatography column	1
Column end cap	1
Instructors workstation	
Binding buffer	1 vial
Equilibration buffer	1 vial
Centrifuge	1
UV light	1–8

Lesson 6

Student workstations	
Collection tubes	3
Pipette	1
Microtube rack	1
Beaker of water for rinsing pipettes	1
HIC chromatography column	1
Column end cap	1
Instructors workstations	
Wash buffer	1 vial
Equilibration buffer	1 vial
TE buffer	1 vial
UV light	1–8

Lessons 7, 8, 9, and 10

Student workstations	
Internet access and computer	1 min
Telephone book	1 min

Instructors Advance Preparation for Labs

This section describes preparation to be performed in advance by the instructor for Lessons 2 through 6.

Lesson 2 Advance Preparation

Objectives

- Prepare agar plates
- Prepare liquid culture media
- Rehydrate bacterial library
- Set up student and instructors workstations

Time required One hour and 15 minutes

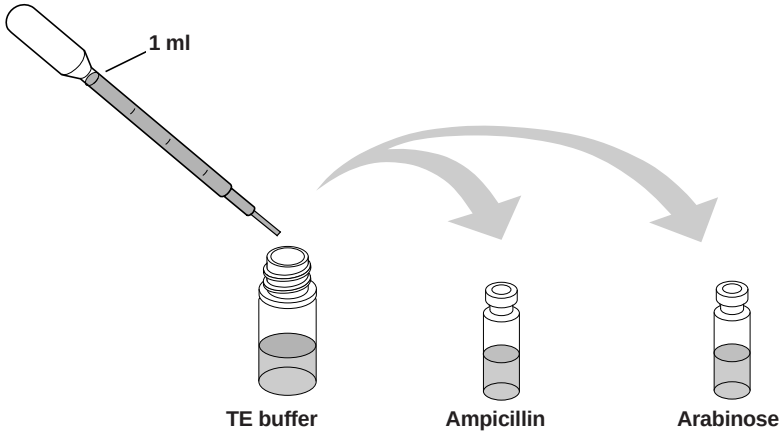
Note: Observe sterile technique while preparing the following materials. See Appendix C.

For students to begin Lesson 2 they will need 2 pre-poured agar plates at each workstation and access to the rehydrated bacterial library.

1. Prepare Ampicillin and Arabinose Solutions

Ampicillin and arabinose are shipped dry in small vials. After being rehydrated, both are added to the molten agar (before plates are poured) and to the liquid growth media. Ampicillin is an antibiotic which inhibits growth of bacterial contaminants which may be introduced from the environment. Arabinose is a sugar which promotes the overexpression of the Green Fluorescent Protein in cloned cells. Refer to Appendix A and B for more details on the functions of these two components.

Using a sterile pipette, add 3 milliliters of TE buffer directly to the vial containing the ampicillin. Using another sterile pipette, add 3 milliliters of TE buffer to rehydrate the arabinose. Mix the vials and gently vortex to aid in rehydration. Put the vials aside and begin preparing the agar solution.



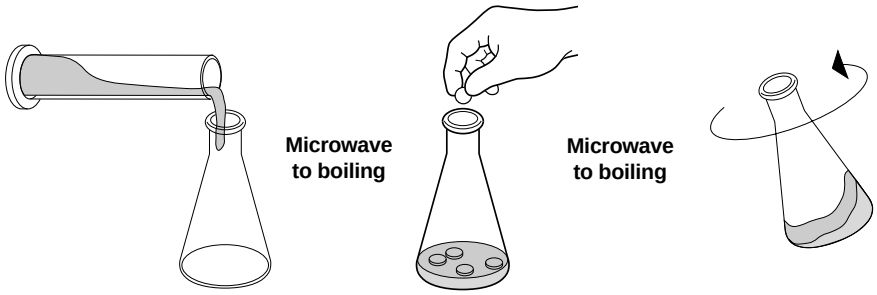
Note: Rehydrate ampicillin and arabinose the day you pour agar plates. Arabinose requires 10 minutes to dissolve—be patient.

2. Prepare LB Agar (for pouring 20 agar plates)

Note: Agar plates should be prepared at least 2 days before Lesson 2 begins.

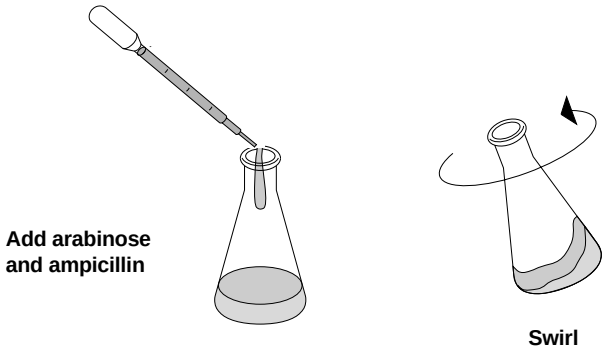
This can conveniently be done on a Friday, with the covered plates left out over the weekend to adequately dry. Two days at room temperature allows the agar to dry out (cure) sufficiently to readily take up the bacterial library. Plates that are prepared more than 2 days before the laboratory sessions should be refrigerated until used. Plates can be poured and stored in the refrigerator for up to 3 months.

A. Prepare the agar by adding 260 milliliters of distilled water to a 1 liter Erlenmeyer flask and heat to boiling in a microwave oven. Add 5 LB-agar tablets to the flask and let the tablets soak in the hot water for 20 minutes—this will aid in dissolving and prevent clumping of agar particles. Heat the flask again to boiling in the microwave, then swirl. **Allow the flask to cool for 30 seconds before swirling so that the molten agar does not suddenly bump or boil over onto your hand when swirled. Use gloves or a folded paper towel to hold the flask and protect your hand when swirling.** Repeat heating and swirling until all the agar is dissolved and the solution no longer appears cloudy. Taking evaporation into consideration, the volume should now be about 250 milliliters.



B. Before pouring plates, ampicillin and arabinose must be added to the hot liquid agar. Because excessive heat (>50 °C) will destroy ampicillin and arabinose, allow the agar to cool so that the outside of the flask is just barely comfortable to hold (~50 °C). When the agar has cooled to 50 °C, use a new sterile pipette and add 2.5 milliliters of the ampicillin solution you prepared to the flask of agar. Using a new pipette, transfer 2.5 milliliters of the arabinose solution to the 250 milliliters of cooled liquid agar. Swirl to mix. Remember that the agar will solidify at 27 °C, so after the ampicillin and arabinose are added, you should pour all 20 plates from start to finish without interruption.

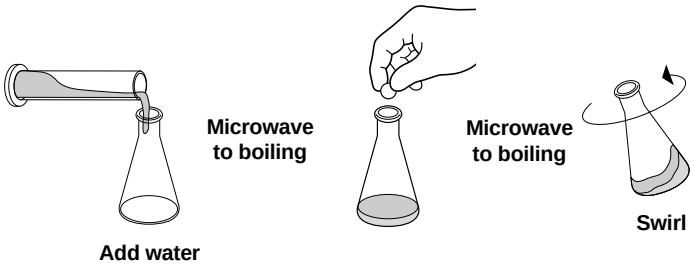
The remaining 0.5 milliliters of ampicillin and arabinose will be used for the preparation of the liquid nutrient broth in the next step.



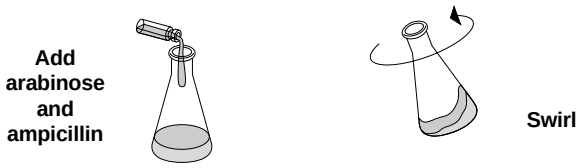
3. Prepare Liquid LB Media

Note: Observe sterile technique when preparing broth. Liquid nutrient media is used in Lesson 3. We put this section here because it is most conveniently prepared on the same day as the nutrient agar. The procedures are very similar and can be done in parallel.

In Lesson 3, each student workstation will require two culture tubes containing 2 milliliters of liquid nutrient media. These will be used to grow bacterial cultures. To prepare the liquid media, add 55 milliliters of distilled water to a 250 ml Erlenmeyer flask and heat to boiling in a microwave. Then, add the single LB tablet to the flask. Let the tablet soak in the hot water for several minutes; this will aid in dissolving. Heat the flask again to boiling in the microwave. Swirl the flask to dissolve the tablet. Repeat heating and swirling several times until the entire tablet is dissolved, but be careful to allow the flask to cool a little before swirling so that the hot medium does not boil over onto your hand.



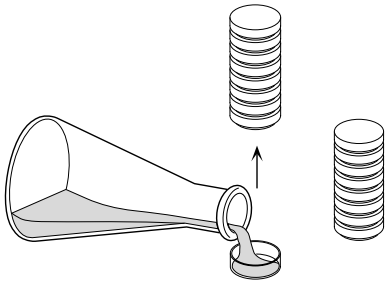
When the entire tablet has dissolved, allow the LB to cool so that the outside of the flask is comfortable to hold, or below 50 °C. While the media is cooling, get the ampicillin and arabinose solutions that were prepared in step 1 above. When the media has cooled, transfer the remaining 0.5 milliliters of arabinose and ampicillin into the flask.



Using a new pipette, aliquot 2 milliliters of the liquid media into 16 of the culture tubes. (This can be accomplished by transferring the media in two 1 milliliter aliquots from a sterile pipette.) Store the culture tubes in a refrigerator until the day of use. Nine extra culture tubes are provided. You may fill these extra tubes with media and use them as back-up cultures in Lesson 3.

4. Pour Agar Plates

Stack the empty plates (with lids on) in piles 4 to 8 high. Starting with the bottom plate, lift the lid (and the upper plates) up and to the side with one hand and pour the agar using your other hand. Fill the plate about one-third (<12 milliliters) with agar, replace the lid, and continue up the stack. Pour 20 plates in this fashion, or, you may choose to employ your own pouring technique. The agar should solidify within 15–20 minutes.



Note: After the plates are poured, do not disturb them until the agar has solidified.

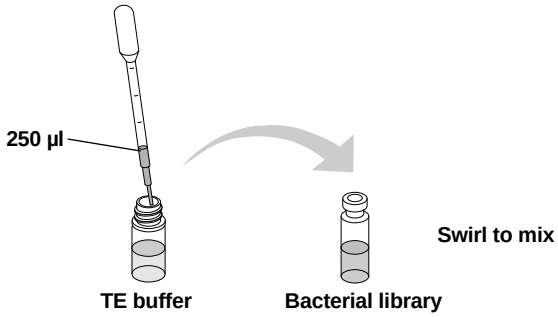
5. Storing Agar Plates

After the plates have cured for 2 days at room temperature, they can be stacked (twenty high) and the plastic sleeve slipped back over them. The stack should then be inverted and the sleeve taped closed. Plates can be stored upside-down in a refrigerator for up to 3 months.

6. Rehydrate Bacterial Library

Note: Rehydrate on the day of Lesson 2.

The bacterial library is shipped dehydrated and should be rehydrated the day of Lesson 2. Because of the ever present possibility of external contamination, it is important the rehydration of the library be done the day of Lesson 2. Using a new sterile pipette, add 250 µl of TE buffer to the bacterial library vial. Swirl gently to resuspend. The bacteria in the library settle quickly; the vial should be thoroughly mixed before student teams streak their plates.



Lesson 3 Advance Preparation

Objective
- Set up workstations
- Set up rocking table, shaking incubator, or incubator oven

Time required 10 minutes

For Lesson 3, each student workstation will need two 15 milliliter culture tubes each containing 2 milliliters of liquid culture media prepared in step 3 above.

Lesson 4 Advance Preparation

Objectives Set up workstations
Rehydrate lysozyme

Time required 10 minutes

The day of Lesson 4, rehydrate the vial of lyophilized lysozyme with 1 milliliter of TE buffer using a new pipette. Mix gently to aid in resuspension. Keep the vial of lysozyme on ice or in a refrigerator until use.

Lesson 5 Advance Preparation

Objectives Set up workstations (the only preparation needed)

Time required 10 minutes

Lesson 6 Advance Preparation

Objectives Set up workstations (the only preparation needed)

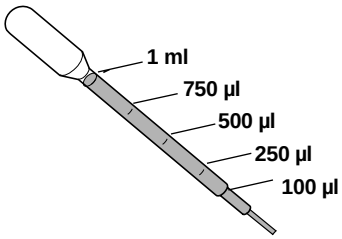
Time required 10 minutes

Lesson Points to Highlight

This section describes steps in the experimental protocols which may be technically challenging or which are extremely important to the overall outcome and understanding of the experiments. Instructors should alert their students to these points, and when possible, demonstrate the technique before the students attempt the procedure.

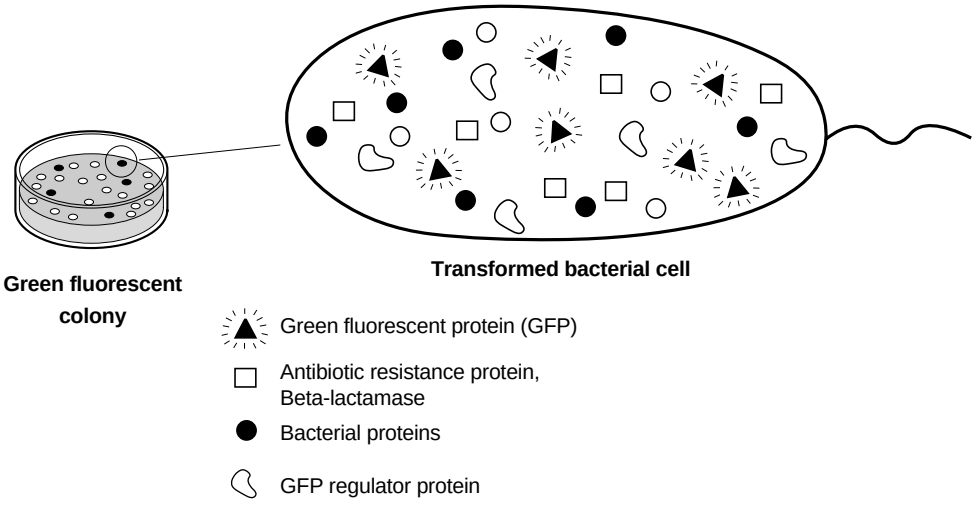
The Student Manual contains the detailed descriptions and drawings of all laboratory steps and the techniques employed in Lessons 2–6. Refer to it for questions about specific steps in the experimental protocols used in lab.

Use of the pipette. Before beginning the laboratory sessions, point out the graduations on the pipette to the students. Both the 250 µl and 1 milliliter marks will be used as units of measurement throughout Lessons 4–6.



Lesson 2 Streaking Agar Plates to Produce Single Colonies

The purpose of streaking the library is to generate single colonies on an agar plate, starting from a concentrated suspension of bacteria. The Student Manual contains a detailed description of this procedure. You should stress to your students that they may not see any liquid on the inoculation loop after they stick it into the rehydrated bacterial library, but that a tiny amount of the bacterial suspension goes a long way. Under favorable conditions, one cell multiplies to become millions of genetically identical cells (clones) in just 24 hours. There can be greater than a million individual bacteria in a single bacterial colony.



There are four extra plates included in the kit. These plates may be used to demonstrate streaking, or they can be used as back up plates for those student groups that may have difficulty generating single colonies on their own plates.

Lesson 3 Isolating Clones to Propagate in Liquid Culture Media

In this activity, students will pick one white and one green colony from their agar plates for propagation in separate liquid cultures. It is extremely important that single isolated colonies (separated from other colonies on the agar) are chosen for transfer to the culture media. A bacterial colony arises from a single bacterial cell.

It is important to observe sterile technique during the picking of colonies. You may want to prepare back-up culture tubes, inoculated with green colonies, for any student team that has difficulty generating green cultures due to poor technique.

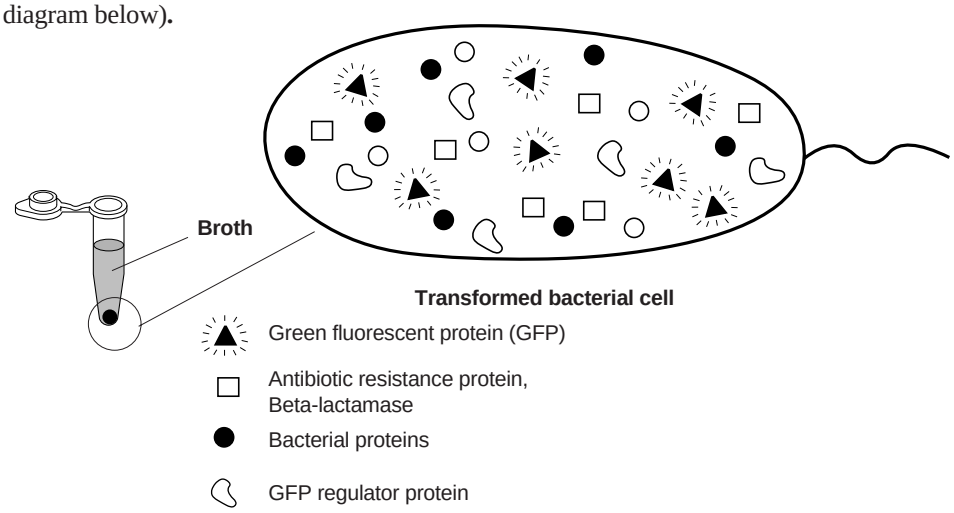
The green colonies of bacteria growing on the agar plates have been genetically transformed with a plasmid that contains the gene for GFP. It is these bacteria that the students will be selecting for propagation. In addition to the GFP gene, this plasmid contains a gene which is responsible for giving the bacteria resistance to the antibiotic, ampicillin. Finally, the plasmid contains a gene for a protein which regulates (turns on or off) the production of GFP. (See appendix B for more details on gene regulation.)

Overnight liquid cultures. The GFP gene requires a 32 °C (or lower) incubation temperature for proper protein folding and fluorescence. If a 32 °C incubator is unavailable, the bacteria can be cultured by shaking at room temperature, but this may require a longer culture period. In this lesson, we have found that liquid cell bacterial cultures will yield sufficient growth and production of protein simply by incubating overnight at 32 °C—without shaking. However, vigorous shaking of the liquid cell culture delivers more oxygen to the dividing cells, allowing them to grow faster. The cultures will grow best if the tubes are placed on their sides. Following incubation, the (+) liquid culture should fluoresce bright green upon exposure to UV light.

Lesson 4 Concentration of Bacteria from Liquid Culture Media

Centrifugation is a technique used to separate particles by mass using a high speed spin (somewhat analogous to the spin cycle in a washing machine where the clothes become compacted against the walls of the washer). In this lab session, centrifugation results in a pellet of bacteria found at the bottom of the tube, and a liquid supernatant above the pellet.

Using a disposable pipette, students carefully transfer 2 milliliters from the culture tube into a 2 milliliter microtube. They then spin the bacterial pellet down to the bottom of the microtube and pour off (discard) the supernatant. *Make sure the students know how to properly load and balance the centrifuge.* At this stage, the pellet will fluoresce bright green upon exposure to UV light because the green protein is being expressed within the bacteria (see diagram below).

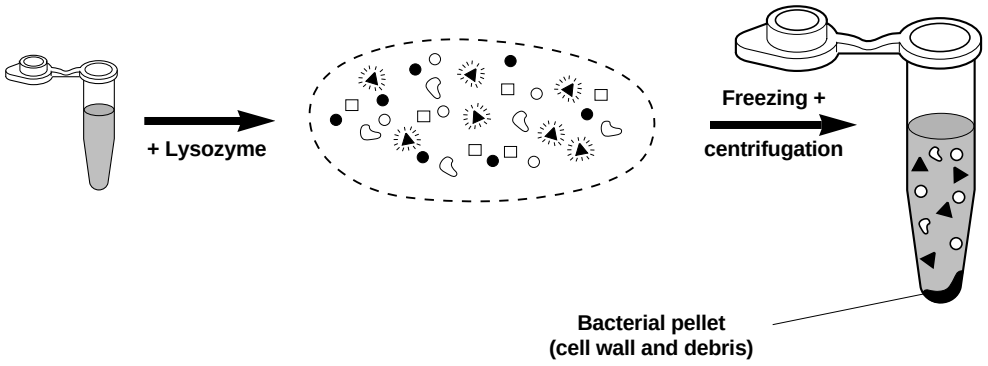


Lesson 5 Prepare a Bacterial Lysate for Protein Chromatography

Resuspension of the bacterial pellet. In this step of the protocol, it is essential that the entire bacterial pellet, concentrated in the previous lab period, is resuspended in TE buffer. The students should add 250 µl of TE buffer to the pellet of bacteria, and then resuspend the pellets by carefully but rapidly pipetting up and down with the 250 µl volume. (If vortexes are available, gentle vortexing will also aid in resuspension.) The liquid in the tube can be visually inspected: if large chunks of unsuspended bacteria are observed, continue to pipette up and down to fully resuspend the bacteria.

Lysozyme. Lysozyme is an enzyme that functions to degrade (or lyse) the bacterial cell wall. The freeze-thaw step used in this lesson aids in the complete disruption of the wall and internal membrane (lysis), releasing soluble components, including GFP.

Centrifugation of bacterial debris. This final centrifugation step serves to separate the large bacterial debris from the proteins, including GFP, which are much smaller. The debris forms a pellet in the bottom of the microtube, while the proteins remain in the supernatant (see figure below). Since the supernatant contains GFP, it will fluoresce bright green upon exposure to UV light. Using a clean 1 milliliter pipette, students should then carefully remove the supernatant away from the pellet and transfer it to a new, labelled, 2.0 milliliter microtube.



Lesson 6 Protein Chromatography

Chromatography is a powerful technique for separating proteins in a complex mixture. Bacteria contain thousands of bacterial proteins from which the GFP must be separated.

Hydrophobic Interaction Chromatography (HIC). In lesson 6, the soluble GFP in the supernatant is purified using HIC. GFP has surfaces that are very hydrophobic (water hating). In salt water, these parts of the protein tend to stick tightly to other hydrophobic surfaces. Protein mixtures containing GFP can be poured through a column packed full of hydrophobic beads. When the beads are in salt water, hydrophobic proteins—like GFP—passing through the column will stick to the beads, while the other proteins will drip through. When the salt is removed, the shape of the GFP protein changes so that the hydrophobic surfaces are less exposed than before. The result is that GFP no longer sticks to the beads and will drip out the bottom of the column. In this way GFP can be separated from the other bacterial proteins.

Buffers. Four different buffers are used in the HIC procedure.

Equilibration buffer. A salt buffer (2.0 M $(NH_4)_2SO_4$) which is used to equilibrate or prime the chromatography column for the binding of GFP.

Binding buffer. An equal volume of high salt binding buffer (4.0 M $(NH_4)_2SO_4$) is added to the bacterial lysate, the end result is a GFP containing lysate which has the same salt concentration as the equilibrated column (2.0 M $(NH_4)_2SO_4$).

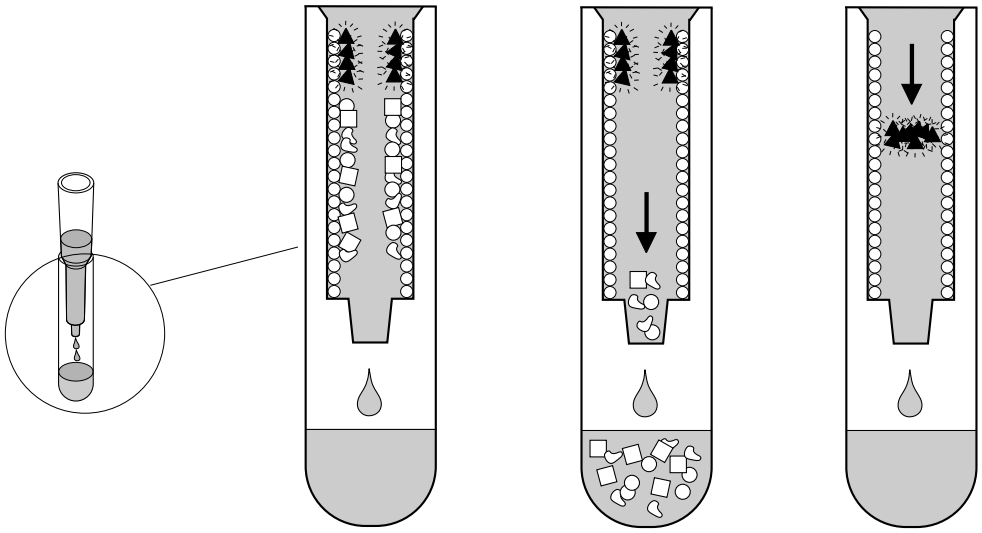
Wash buffer. A medium salt wash buffer (1.3 M $(NH_4)_2SO_4$) is used to wash weakly associated proteins from the column; proteins which are strongly hydrophobic (such as GFP) remain bound to the column. When the wash buffer is applied to the column, a ring of GFP should begin to penetrate the upper surface of the column bed.

Elution buffer. A low salt buffer (TE Solution; 10 mM Tris/EDTA) is used to wash GFP from the column. The 0.75 milliliters of TE buffer is applied to the column as described above. The GFP should pass down the column as a green fluorescent ring (observed under UV light). If successful, collection tube 3 should fluoresce bright green. If all of the GFP has not eluted completely, students can add an additional 250 µl of TE.

Storage of tubes. All collection tubes and their contents can be tightly covered and stored for approximately 1–2 weeks in the refrigerator.

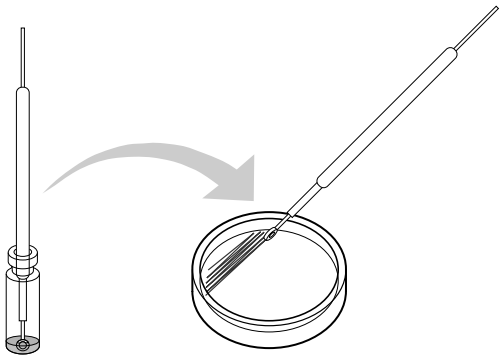
Important Hints for Chromatography

1. Place the column gently into the collection tubes. Jamming the column tightly into the collection tubes will create an air tight seal and the sample will not flow through. You can create a paper crutch by folding a small piece of paper, about the size of a match stick, and wedging it between the column and the collection tube. This crutch makes it impossible for an air tight seal to form, and insures that the column will flow.
2. The flow rate of the column can be increased in the elution step by placing the top cap tightly back onto the column. This creates air pressure which pushes on the column bed, causing the sample to flow faster.
3. The columns are designed to drip slowly. The entire chromatography procedure should take 20 to 30 minutes. It is important not to remove the column more than needed from collection tube to collection tube, as motion can cause major disturbance to the column bed.



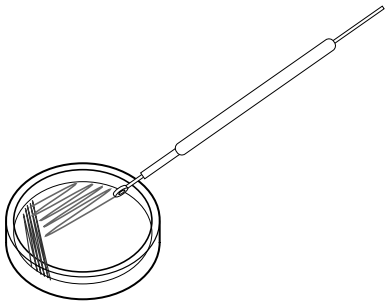
Lesson 2 Cloning

1. Insert a sterile inoculation loop into the rehydrated bacterial library. Insert the loop straight into the vial and do not tilt the vial. Remove the loop and streak for single colonies as illustrated at right.

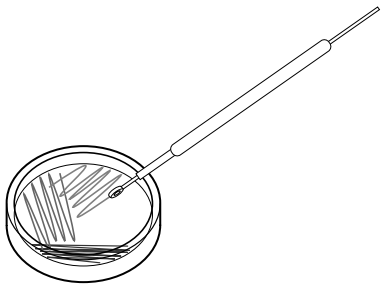


2. Streaking takes place sequentially in four quadrants. The first streak is to just spread the cells out a little. Go back and forth with the loop about a dozen times in the small area shown.

3. For subsequent streaks the overall idea is to use up as much of the surface area of the plate as possible. Rotate the plate approximately 45° (so that the streaking motion is comfortable for your hand) and start the second streak. Go into the previous streak about two times and then back and forth as shown for a total of about 10 times.

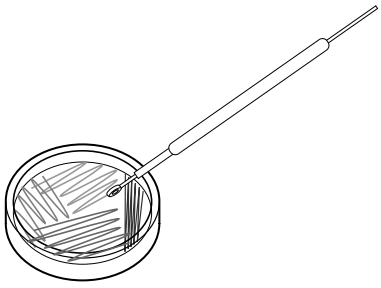


4. Rotate the plate again and repeat streaking.



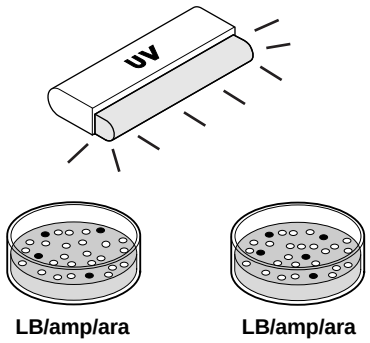
5. Rotate the plate for the final time and make the final streak. Repeat steps 1–5 with the second LB/amp/ara plate and the same inoculation loop.

6. Culture the plates overnight in a 37° incubator. Place the plates upside down in the incubator.

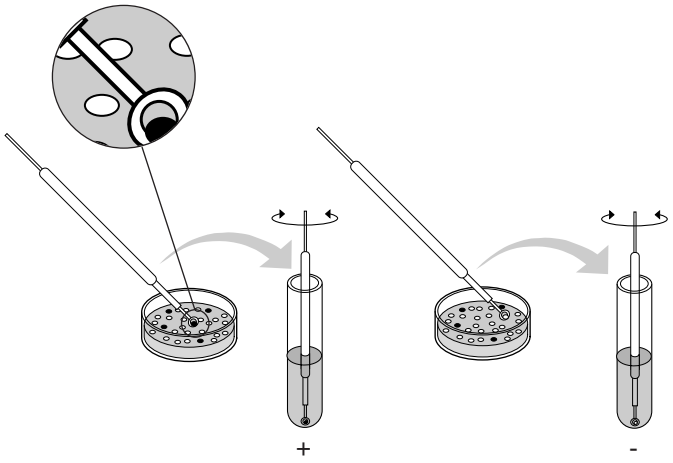


Lesson 3 Screening and Liquid Cultures

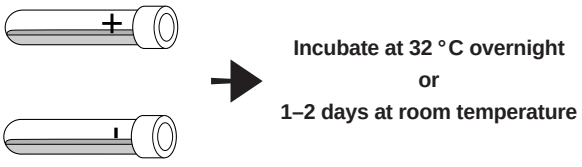
1. Remove the plates from the incubator and examine using the UV light. Identify several green colonies that are not touching other colonies on the plate. On the bottom of the plate, circle several isolated green colonies. Likewise, underline several white colonies that are also separated from other colonies on the plate.



2. Obtain two culture tubes containing the growth media LB/amp/ara. Label one "+" and one "-". Using a sterile loop, lightly touch the loop to a circled green colony and immerse it in the "+" tube. Using a new sterile loop repeat for an underlined white colony and immerse it in the "-" tube (it is very important to pick only a single colony). Spin the loop between your index finger and thumb to disperse the entire colony.

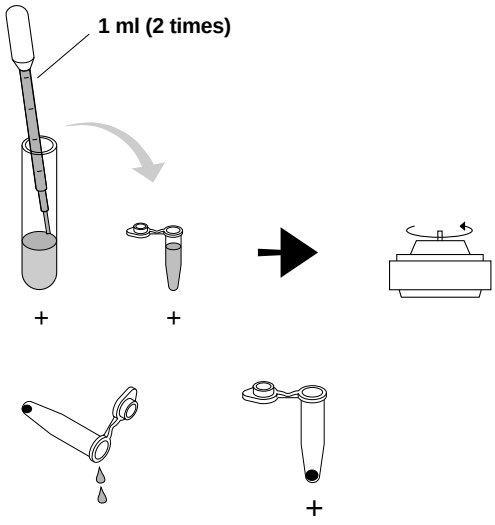


3. Place the tubes in the shaking incubator or on the shaking platform and culture overnight at 32 °C or 1–2 days at room temperature. If a shaker is not available, shake the tubes vigorously, then place horizontally in the incubator and culture for 1–2 days.



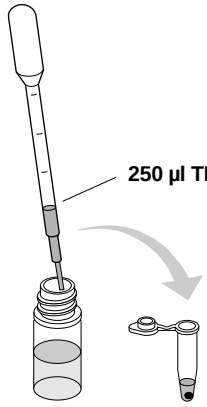
Lesson 4 Purification Phase 1
Bacterial Concentration

1. Label one microtube "+" with your name and class period. Remove your liquid cultures from the shaker and observe with the UV light. Note any color differences between the two cultures. Using a new pipette, transfer 2 ml of "+" liquid culture into the "+" microtube. Spin the microtube for 5 minutes in the centrifuge at maximum speed. The pipette used in this step can be repeatedly rinsed in a beaker of water and used for all following steps of this laboratory period.

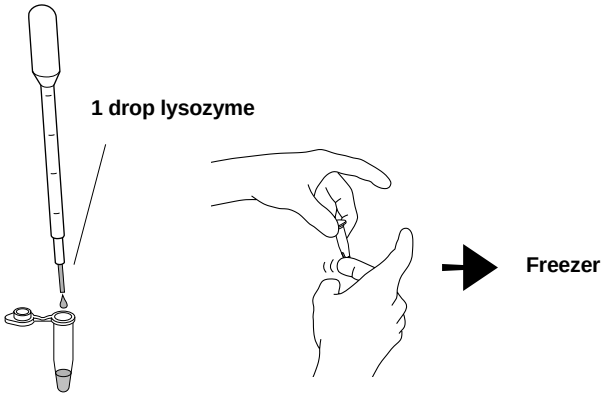


2. Pour out the supernatant and observe the pellet under UV light.

3. Using a rinsed pipette, add 250 µl of TE buffer to the tube. Resuspend the pellet thoroughly by rapidly pipetting up and down several times.



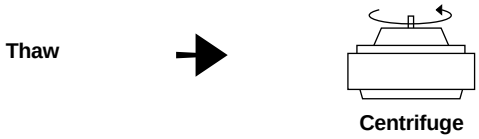
4. Using a rinsed pipette, add 1 drop of lysozyme to the resuspended bacterial pellet to initiate enzymatic digestion of the bacterial cell wall. Mix the contents gently by flicking the tube. Observe the tube under the UV light.



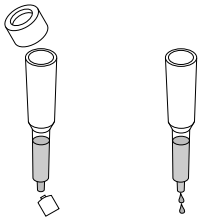
5. Place the microtube in the freezer until the next laboratory period. The freezing causes the bacteria to rupture completely.

Lesson 5 Purification Phase 2
Bacterial Lysis

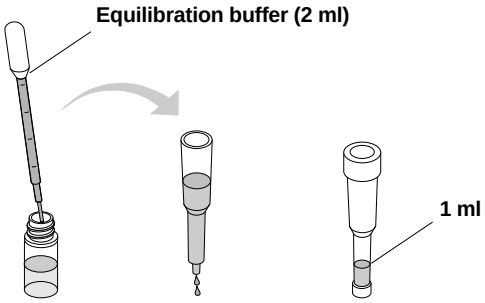
1. Remove the microtube from the freezer and thaw using hand warmth. Place the tube in the centrifuge and pellet the insoluble bacterial debris by spinning for 10 minutes at maximum speed.



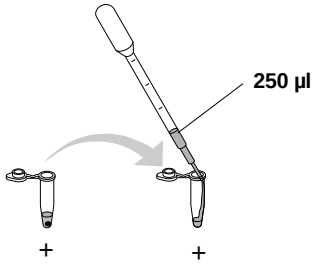
2. While your tube is spinning, prepare the chromatography column. Remove the cap and snap off the bottom from the prefilled HIC column. Allow all of the liquid buffer to drain from the column (~3–5 minutes).



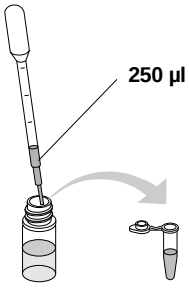
3. Prepare the column by adding 2 ml of Equilibration buffer to the top of the column. This is done by adding two 1 ml aliquots with a rinsed pipette. Drain the buffer to the 1 ml mark on the column. Cap the top and bottom and store the column at room temperature until the next laboratory period.



4. After the 10 minute spin, immediately remove your tube from the centrifuge. Examine the tube with the UV light. Using a new pipette, transfer 250 µl of the "+" supernatant into a new microtube labeled "+". Again, rinse the pipette well for the rest of the steps of this lab period.

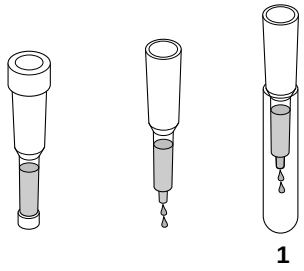


5. Using a well rinsed pipette, transfer 250 µl of Binding buffer to the "+" supernatant. Place the tube in the refrigerator until the next laboratory period.

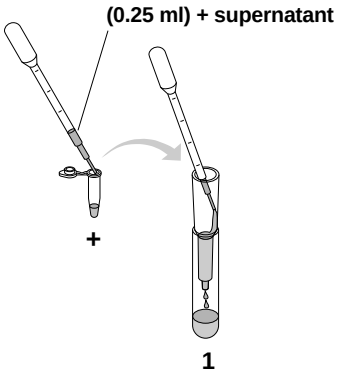


Lesson 6 Purification Phase 3
Protein Chromatography

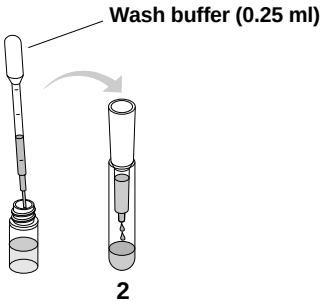
1. Label 3 collection tubes 1–3 and place the tubes in the foam rack or in a rack supplied in your laboratory. Remove the caps from the top and bottom of the column and place the column in collection tube 1. When the last of the buffer has reached the surface of the HIC matrix, proceed to the next step below.



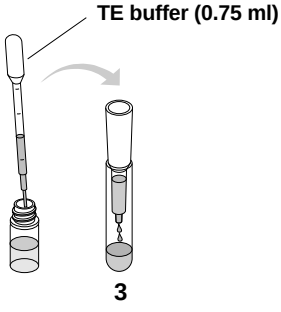
2. Using a new pipette, carefully and gently load 250 µl of the "+" supernatant onto the top of the column. Hold the pipette tip against the side of the column wall, just above the upper surface of the matrix and let the supernatant drip down the side of the column wall. Examine the column using a UV light. Note your observations. After it stops dripping, transfer the column to collection tube 2.



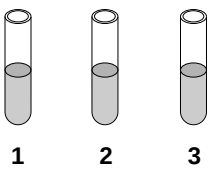
3. Using the rinsed pipette, add 250 µl of Wash buffer and let the entire volume flow into the column. Examine the column using the UV light. Note your observations. After the column stops dripping, transfer it to tube 3.



4. Using the rinsed pipette, add 750 µl of TE (Elution) buffer and let the entire volume flow into the column. Examine the column using the UV light. Note your observations.



5. Examine all three collection tubes and note any differences in color between the tubes. Parafilm® or Saran Wrap® the tubes and place in the refrigerator until the next laboratory period.



Secrets of the Rain Forest

Student Manual

Contents

Secrets of the Rain Forest Lesson 1

The Mysterious Green Fluorescent Leaves

Tisha was hiking through the Andean Rain Forest looking for unique plant species when she met a young boy named Ramon who told her about an old medicine man with mysterious leaves. Ramon said that his own sister had once suffered stomach pains and been diagnosed by a town doctor as having stomach cancer, a painful and usually fatal disease. Ramon's family could not afford the doctor's treatments, so they approached the medicine man who made a small cut in Ramon's sister's arm and placed his mysterious leaves on it.

A few months later, when Ramon's sister was examined by the town doctor, she was told that her cancer was cured. Ramon went back to thank the old medicine man and found him very ill. Soon, the old man died and, with him, the secret of the 'mysterious leaves'.

Ramon gave Tisha a bottle with a few of the mysterious leaves. Tisha returned to Biotex, a biotechnology company working on developing new medicines to treat human disease.

At Biotex, Tisha noticed that the mysterious leaves glowed bright green under her ultraviolet light. Other Biotex scientists determined that the shiny green substance in the leaves was a protein which caused special effects. To make more of this protein, DNA from the leaves was removed and inserted into bacterial cells. Some of these bacteria now contain the gene for the special green protein.

Biotex is taking a gamble on developing a cure for stomach cancer. It is a long shot, costing over $100 million dollars in testing over about 10 years, but the potential for curing the disease and making a profit is good. Right now there is enough money in the bank to pay Tisha and the other employees at Biotex for a few more years, but Biotex urgently needs a product so it can survive beyond that. On top of all this, Tisha has received a desperate letter from Ramon. His recurrent stomach aches have been diagnosed as stomach cancer.

Secrets of the Rain Forest Lesson 1 Name _____

Review Questions The Mysterious Green Leaves

You have been hired by Biotex to help Tisha identify the bacteria which now carry the gene for the green fluorescent protein, and to extract this protein from the bacteria for further testing as treatment for stomach cancer. You may also encounter several dilemmas in getting your cancer cure to market and you will need to find ways to overcome these obstacles.

1. Who is Tisha and why is she in the Andes?

2. List two problems that Tisha and Biotex need to solve.

3. Why were the genes from the mysterious leaves inserted into bacterial cells?

4. What clues will help you determine if the Biotex technicians were successful in placing the special cancer-curing protein into bacterial cells?

Secrets of the Rain Forest Lesson 2

Cloning

Our bodies contain thousands of proteins which perform many different tasks. Each protein is encoded by a unique gene, a section of DNA which contains the code for making a protein. Human cells contain 80–100,000 genes.

Biotex scientists have found a gene for a cancer-curing protein in the mysterious leaves. They have randomly cut and pasted all of the genes from Ramon's leaves into a group of bacterial cells. Only some of these bacterial cells now contain the gene for the cancer-curing protein. You need to find and separate these bacteria from the others.

You must first streak out or spread a sample of this bacterial library onto a petri plate containing a special blend of bacterial food. In a day or two, you will be able to see individual colonies on the plate. This process of separating the bacteria on the surface of the plate so that each individual cell can grow up into a clump of identical cells (a colony), is called cloning. Because all the cells in a single colony are genetically identical, they are called clones.

Follow the procedure outlined below to streak out the bacterial library.

Your Workstation Check (✔) List

Your workstation	**Number**	**(✔)**
Inoculation Loops—Sterile	1	❑
Poured agar plates—Sterile	2	❑
Marking pen	1	❑
Instructors workstation		
Lyophilized bacterial library	1	❑
37 °C Incubator Oven	1	❑

Laboratory Procedure for Lesson 2

1. Turn your petri dishes upside down. Using a magic marker, label your two agar plates with your team name and period.

2. Using the *E. coli* library, streak two plates to begin Lesson 2. The purpose of streaking is to generate single colonies from a concentrated suspension of bacteria. A minute amount of the bacterial suspension goes a long way. Under favorable conditions, one cell multiplies to become millions of genetically identical cells in just 24 hours.

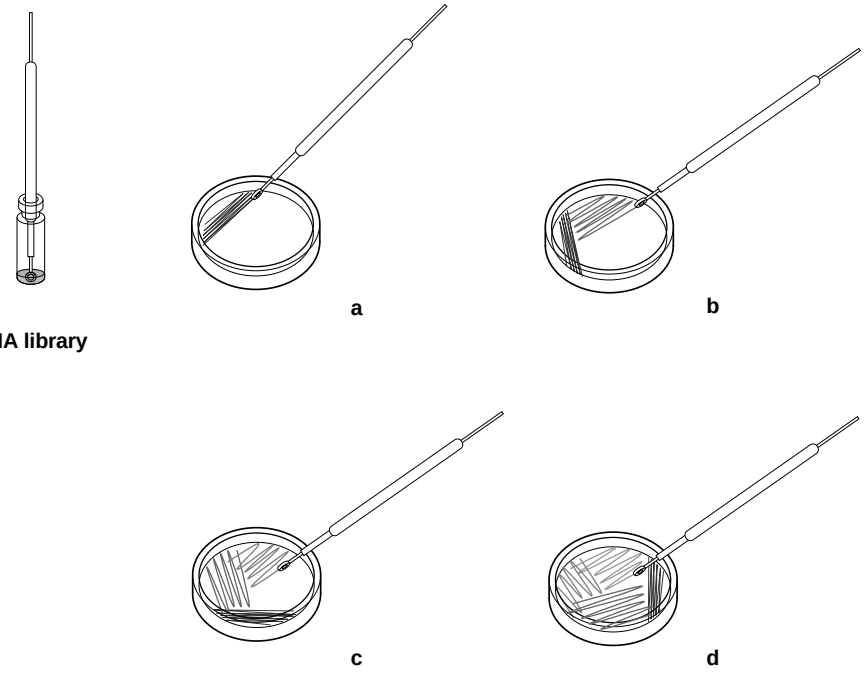
    a. Insert a sterile inoculation loop straight into the vial of the bacterial library without tilting the vial. Remove the loop and streak for single colonies as illustrated below. Streaking takes place sequentially in four quadrants on each plate. The first streak is to just spread the cells out a little. Go back and forth with the loop about a dozen times in each of the small areas shown. In subsequent streaks the cells become more and more dilute increasing the likelihood of producing single colonies.

    b. For subsequent streaks, the goal is to use as much of the surface area of the plate as possible. Rotate the plate approximately 45° (so that the streaking motion is comfortable for your hand) and start the second streak. Go into the previous streak about two times and then back and forth as shown for a total of about 10 times.

    c. Rotate the plate again and repeat streaking.

    d. Rotate the plate for the final time and make the final streak. Repeat steps a–c with the remaining agar plate. Use the same inoculation loop for both plates. When you are finished with each plate cover it immediately to avoid contamination.

    e. Place the plates upside down inside the incubator overnight at 37 °C. Use within 24–36 hours and do not refrigerate before use.



Secrets of the Rain Forest Lesson 2 Name_____

Review Questions

1. Proteins
   a. What is a protein?

   b. List three examples of proteins found in your body.

   c. Explain the relationship between genes and proteins.

2. Using your own words, define or describe cloning.

3. Describe how the bacterial cells in a library are different from the cells of a single colony.

4. Describe how you might isolate the cancer-curing protein from the bacterial cells.

Secrets of the Rain Forest Lesson 3

Screening

Your bacterial colonies are ready for examination under an ultraviolet (UV) lamp. Remember, Tisha noticed something special about Ramon's mysterious leaves under a UV light.

Pick out two colonies of bacteria: one that contains the gene for the green fluorescent protein (green) and another which does not (white). You will transfer the two types of colonies into culture tubes and let them grow overnight.

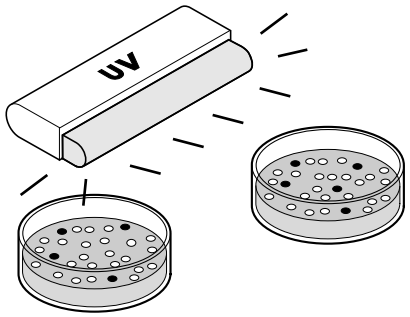
Workstations Check (✔) List

Your workstation	**Number**	**(✔)**
Streaked bacterial plates	2	❑
Inoculation loops	2	❑
Culture tubes—containing 2 ml growth media	2	❑
Marking pen	1	❑
Test tube holder	1	❑
Instructors workstation		
Shaking platform or incubator	1	❑
UV light	1 or more	❑

Laboratory Procedure for Lesson 3

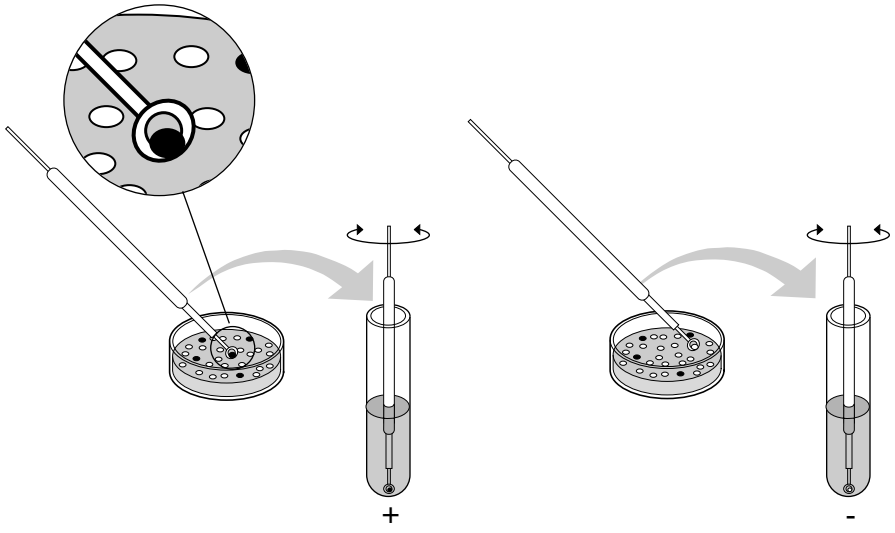
1. Remove your streaked plates from the incubator and examine them. First use normal room lighting, then use an ultraviolet light in a darkened area of your laboratory. Note your observations.

   To prevent damage to your skin or eyes, avoid exposure to the UV light. Never look directly into the UV lamp. Wear safety glasses whenever possible.

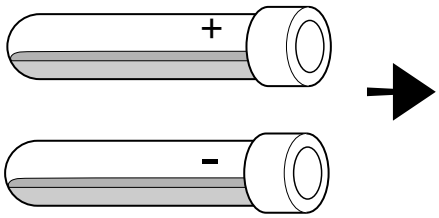


2. Identify several green colonies that are not touching other colonies on one plate. Turn the plate over and circle several of these green colonies. On the other plate, identify and circle several white colonies that are also well isolated from other colonies on the plate.

3. Obtain two 15 milliliter culture tubes containing 2 milliliters of nutrient growth media and label one tube "+" and one tube "-". Using a sterile inoculation loop, lightly touch the loop end to a circled single green colony and scoop up the cells without grabbing big chunks of agar. Immerse the loop in the "+" tube. Spin the loop between your index finger and thumb to disperse the entire colony. Using a new sterile loop, repeat for a single white colony and immerse it in the "-" tube. It is very important to pick cells from a single bacterial colony.



4. Cap your tubes and place them in the shaker or incubator. Let the tubes incubate for 24 hours at 32 °C or for up to 2 days at room temperature. If a shaker is not available, lay the tubes down horizontally in the incubator. If a shaker is available, but no incubator, tape the tubes to the platform and let them shake for 24 hours at 32° C or at room temperature for up to 48 hours.



Cap the tubes.
Incubate at 32 °C overnight
or
1–2 days at room temperature.

Culture condition	Days required
32 °C - shaking	1 day
32 °C - no shaking	1–2 days*
Room temperature - shaking	1–2 days
Room temperature - no shaking	Not recommended

* Periodically shake by hand and lay tubes horizontally in incubator.

Secrets of the Rain Forest Lesson 3 Name_____

Review Questions

1. What is a bacterial colony?

2. Why did you pick one green colony and one white colony from your agar plate(s)? What could this prove?

3. How are these items helpful in this cloning experiment?

   a. ultraviolet (UV) light—

   b. incubator—

   c. shaking incubator—

4. Explain how placing cloned cells in nutrient broth to multiply relates to your overall goal of testing the fluorescent protein as a cure for cancer.

Secrets of the Rain Forest Lesson 4

Purification Phase 1—Bacterial Concentration

So far you have produced living cultures of two bacterial clones. One contains the gene which produces the green fluorescent protein (GFP) while one does not. Now it is time to extract the GFP from the bacterial cells. First, we need to collect a large number of these bacterial cells.

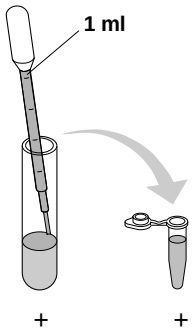
A good way to concentrate a large number of cells is to place a tube containing the liquid cell culture into a centrifuge and spin it. As you spin the cell culture, where would you expect the cells to collect, in the liquid portion (supernatant) or at the bottom of the tube (pellet)?

Workstations Check (✔) List

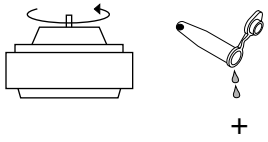
Your workstation	Number	(✔)
Microtubes	1	❑
Pipette	1	❑
Microtube rack	1	❑
Marker	1	❑
Beaker of water for rinsing pipettes	1	❑
Instructors workstation		
TE buffer	1 vial	❑
Lysozyme (rehydrated)	1 vial	❑
Centrifuge	1	❑
UV light	1–4	❑

Laboratory Procedure for Lesson 4

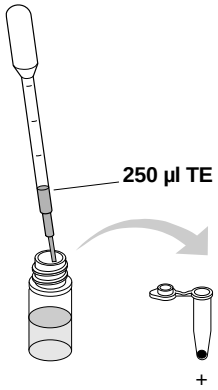
1. Using a marker, label one new microtube with your name and period.
2. Remove your two liquid cultures from the shaker or incubator and observe them in normal room lighting and then with the UV light. Note any color differences that you observe. Using a clean pipette, transfer the entire contents of the (+) liquid culture into the 2 milliliter microtube also labeled (+), then cap it. You may now set aside your (-) culture for disposal.



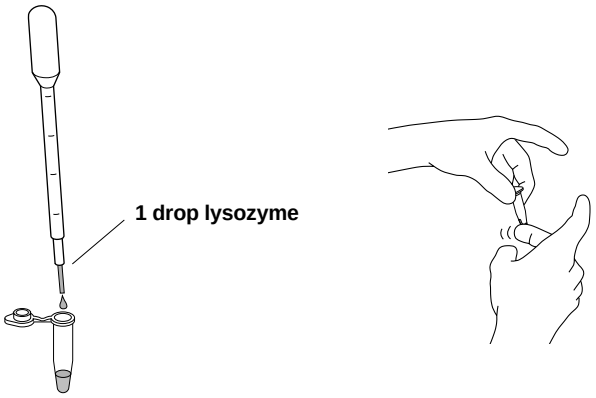
3. Spin the (+) microtube for 5 minutes in the centrifuge at maximum speed. Be sure to balance the tubes in the machine. If you do not know how to balance the tubes, do not operate the centrifuge. Ask the teacher for instructions.
4. After the bacterial liquid culture has been centrifuged, open the tube and slowly pour off the liquid supernatant above the pellet. After the supernatant has been discarded, there should be a large bacterial pellet remaining in the tube.



5. Observe the pellet under UV light. Note your observations.
6. Using a new pipette, add 250 µl of TE buffer to each tube. Resuspend the bacterial pellet thoroughly by rapidly pipetting up and down several times with the pipette.



7. Using a rinsed pipette, add 1 drop of lysozyme to the resuspended bacterial pellet. Cap and mix the contents by flicking the tube with your index finger. The lysozyme will start digesting the bacterial cell wall. Observe the tube under the UV light. Place the microtube in the freezer until the next laboratory period. The freezing will cause the bacteria to explode and rupture completely.



Secrets of the Rain Forest Lesson 4 Name _____

Review Questions

1. You have used a bacterium to propagate a gene that produces a special plant protein that may hold a cure for stomach cancer. Identify the function of these items you used in Lesson 4.
   a. Centrifuge—
   b. Lysozyme—
   c. Freezer—

2. Why did you discard the supernatant in this part of the protein purification procedure?

3. Why did you discard the white liquid from the "-" tube but keep the green one?

4. Can you explain why the bacterial cells' outer membrane ruptures when the cells are frozen? What happens to an unopened soft drink when it freezes?

5. What was the purpose of rupturing or lysing the bacteria?

Secrets of the Rain Forest Lesson 5

Purification Phase 2—Bacterial Lysis

News of your stomach cancer research has reached the public. During the past month, stomach cancer victims and their families have been trying to contact you, wanting to know more about your GFP.

Meanwhile, Biotex scientists have developed a technique to separate the GFP from other bacterial proteins by chromatography. Chromatography is a powerful technique for separating proteins in a mixture. Bacteria contain thousands of bacterial proteins from which the GFP must be separated.

GFP has surfaces that are very hydrophobic (water hating). In salt water, these parts of the protein tend to stick tightly to other hydrophobic surfaces. Protein mixtures containing GFP can be poured through a column packed full of hydrophobic beads. When the beads are in salt water, hydrophobic proteins—like GFP—passing through the column will stick to the beads, while the other proteins will drip through. When the salt is removed, the shape of the GFP protein changes so that its hydrophobic surfaces are less exposed than before. The result is that GFP no longer sticks to the beads and will drip out the bottom of the column. In this way GFP can be separated from the other bacterial proteins.

Workstations Check (✔) List

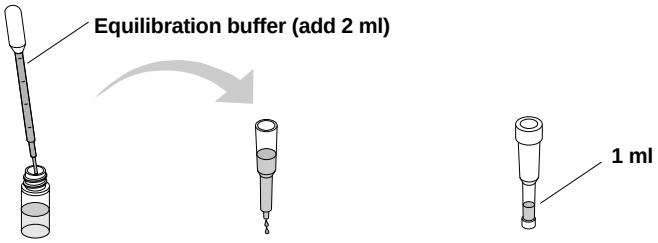
Student workstation items	**Number**	**(✔)**
Microtubes	1	❑
Pipette	1	❑
Microtube rack	1	❑
Marker	1	❑
Beaker of water for rinsing pipettes	1	❑
HIC chromatography column	1	❑
Column end cap	1	❑
Waste beaker or tube	1	❑
Instructors workstation items		
Binding buffer	1 vial	❑
Equilibration buffer	1 vial	❑
Centrifuge	1	❑
UV light	1–4	❑

Laboratory Procedure for Lesson 5

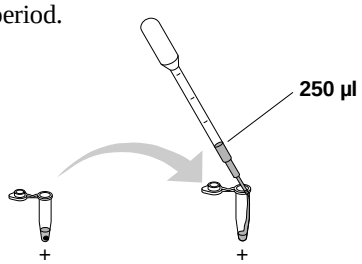
1. Remove your microtube from the freezer and thaw it out using hand warmth. Place the tube in the centrifuge and pellet the insoluble bacterial debris by spinning for 10 minutes at maximum speed. Label a new microtube with your team's initials.

2. While you are waiting for the centrifuge, prepare the chromatography column. Before performing the chromatography, shake the column vigorously to resuspend the beads. Then shake the column down one final time, like a thermometer, to bring the beads to the bottom. Tapping the column on the table-top will also help settle the beads at the bottom. Remove the top cap and snap off the tab bottom of the chromatography column. Allow all of the liquid buffer to drain from the column (this will take ~3–5 minutes).

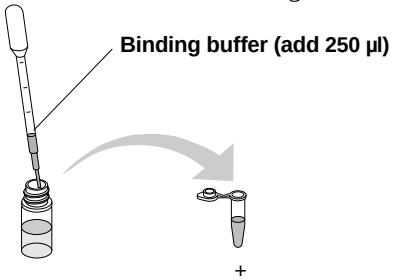
3. Prepare the column by adding 2 milliliters of Equilibration buffer to the top of the column, 1 milliliter at a time using a well a rinsed pipette. Drain the buffer from the column until it reaches the 1 milliliter mark which is just above the top of the white column bed. Cap the top and bottom of the column and store the column at room temperature until the next laboratory period.



4. After the 10 minute centrifugation, immediately remove the microtube from the centrifuge. Examine the tube with the UV light. The bacterial debris should be visible as a pellet at the bottom of the tube. The liquid that is present above the pellet is called the supernatant. Note the color of the pellet and the supernatant. Using a new pipette, transfer 250 µl of the supernatant into the new microtube. Again, rinse the pipette well for the rest of the steps of this lab period.



5. Using the well-rinsed pipette, transfer 250 µl of Binding buffer to the microtube containing the supernatant. Place the tube in the refrigerator until the next laboratory period.



Secrets of the Rain Forest Lesson 5 Name _____

Review Questions

1. (a) What color was the pellet in this step of the experiment? (b) What color was the supernatant? (c) Explain why you think this is so.

2. Why did you discard the pellet in this part of the protein purification procedure?

3. Briefly describe protein chromatography and identify its purpose in this lab.

Secrets of the Rain Forest Lesson 6

Purification Phase 3—Protein Chromatography

In this final step of purifying the GFP, the bacterial lysate you prepared will be loaded onto a hydrophobic interaction (HIC) column. The HIC column contains tiny hydrophobic beads. GFP should stick to the beads while the other bacterial proteins should pass straight through. Later, when the salt is removed, the GFP will no longer stick to the beads and will drip out the bottom of the column.

You will use these four solutions to perform the chromatography.

Equilibration buffer—A high salt solution (2 M $(NH_4)_2SO_4$)

Binding buffer—A very high salt solution (4 M $(NH_4)_2SO_4$)

Wash buffer—A medium salt solution (1.3 M $(NH_4)_2SO_4$)

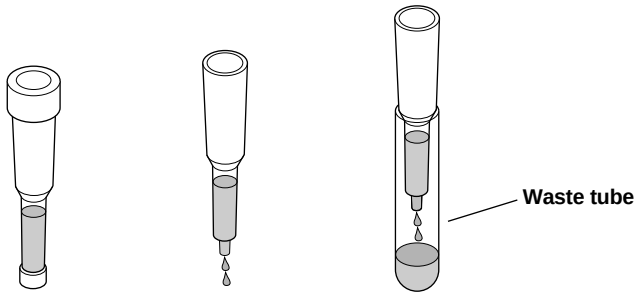
TE (Elution) buffer—A very low salt solution (10 mM Tris/EDTA)

Workstation Check (✔) List

Your workstation	Number	(✔)
Collection tubes	3	❑
Pipette	1	❑
Microtube rack	1	❑
Marker	1	❑
Beaker of water for rinsing pipettes	1	❑
HIC chromatography column	1	❑
Column end cap	1	❑
Test tube or beaker to collect waste	1	❑
Instructors workstation		
Wash buffer	1 vial	❑
Equilibration buffer	1 vial	❑
TE buffer	1 vial	❑
UV light	1–4	❑

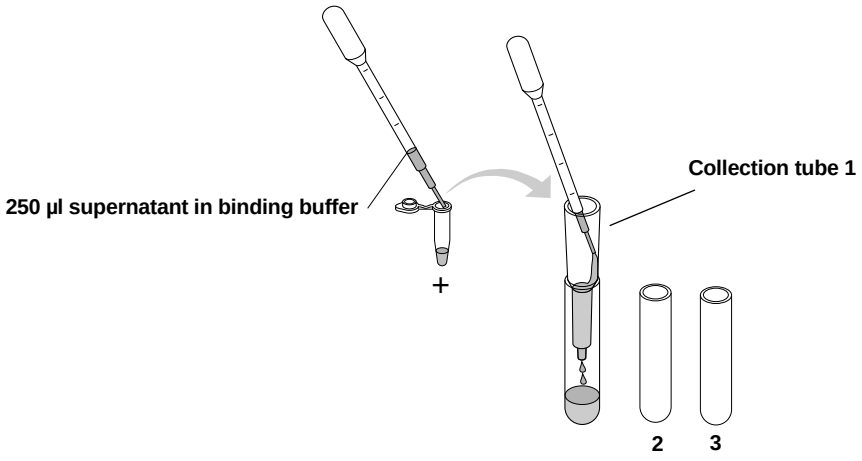
Lesson 6 Laboratory Procedure

1. Obtain 3 collection tubes and label them 1, 2, and 3. Place the tubes in a rack. Remove the cap from the top and bottom of the column and let it drain completely into a liquid waste container (an extra test tube will work well). When the last of the buffer has reached the surface of the HIC column bed, gently place the column on collection tube 1. Do not force the column tightly into the collection tubes–the column will not drip.

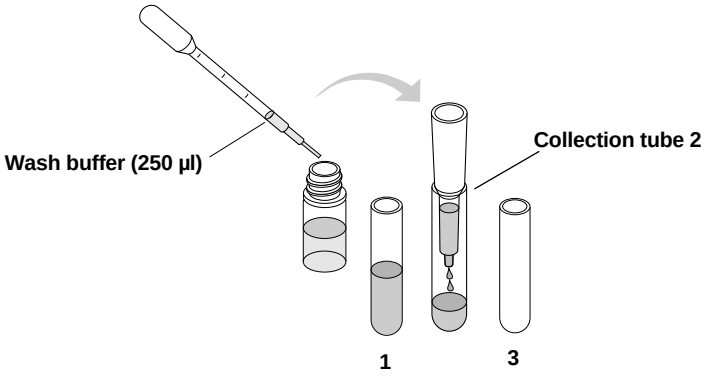


2. Predict what you think will happen for the following steps and write it along with your actual observations in the data table on page 42.

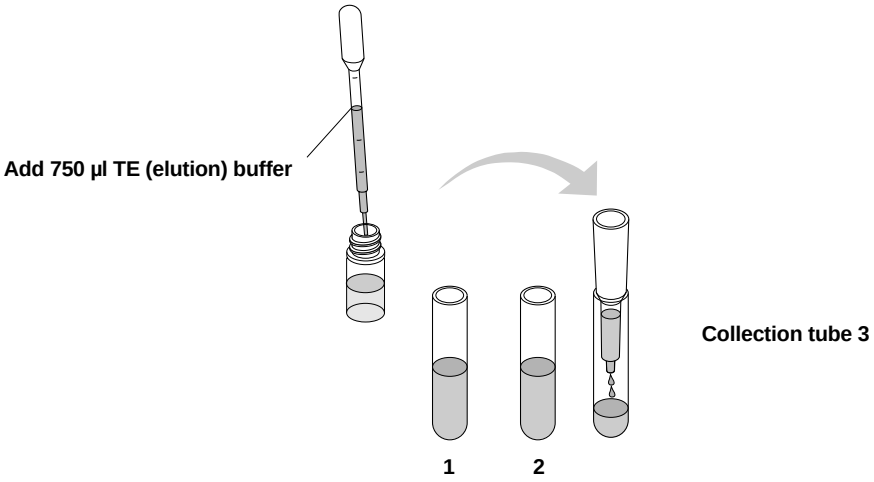
3. Using a new pipette, carefully load 0.25 milliliters (250 µl) of the supernatant (in Binding buffer) into the top of the column by resting the pipette tip against the side of the column and letting the supernatant drip down the side of the column wall. Examine the column using the UV light. Note your observations in the data table. Let the entire volume of supernatant flow into tube 1.



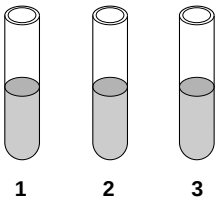
4. Transfer the column to collection tube 2. Using the rinsed pipette and the same loading technique described above, add 250 µl of Wash buffer and let the entire volume flow into the column. As you wait, predict the results you might see with this buffer. Examine the column using the UV light and list your results on page 42.



5. Transfer the column to tube 3. Using the rinsed pipette, add 0.75 milliliters (750 µl) of TE buffer (Elution buffer) and let the entire volume flow into the column. Again, make a prediction and then examine the column using the UV light. List the results in the data table on page 42.



6. Examine all of the collection tubes using the UV lamp and note any differences in color between the tubes. Parafilm or Saran Wrap the tubes and place in the refrigerator until the next laboratory period.



Secrets of the Rain Forest Lesson 6 Name _____

Review Questions

1. List your predictions and observations for the sample and what happens to the sample when the following buffers are added to the HIC column.

Collection tube number	**Prediction**	**Observations under UV light** (column and collection tube)
Tube 1 Sample in Binding buffer		
Tube 2 Sample with Wash buffer		
Tube 3 Sample with Elution buffer		

2. Using the data table above, compare how your predictions matched up with your observations for each buffer.

   a. Binding buffer—

   b. Wash buffer—

   c. Elution buffer—

3. Based on your results, explain the roles or functions of these buffers. Hint: how does the name of the buffer relate to its function?

   a. Equilibration buffer—

   b. Binding buffer—

   c. Wash buffer—

   d. TE (Elution) buffer—

4. Which buffers have the highest salt content and which have the least? How can you tell?

5. Were you successful in isolating and purifying GFP from the cloned bacterial cells? Identify the evidence you have to support your answer.

Secrets of the Rain Forest Lesson 7

Testing and Placebos

Before your protein is tested on humans, you must first test it on rats or mice with stomach cancer. You must determine if these animals are cured and, if so, how much of your protein it takes to do it. You also need to find out about possible side effects or problems that your protein may cause.

In order to determine if your protein really works you must use a control, or placebo. A placebo can be any inert substance, like water, which has no medicinal value. The U.S. Food and Drug Administration (FDA), will examine Biotex's experiments to determine if your protein is safe and actually cures stomach cancer without negative side effects. The FDA also requires that you test all drugs on two species of animals, before you conduct human studies. If you do not comply with these FDA regulations, you will lose millions of dollars in wasted research and never be able to help Ramon and other stomach cancer victims.

One of Biotex's technicians has injected 1, 2, or 3 drops of your purified protein into rats with stomach cancer. She has also similarly tested the placebo on another set of animals. After a time, she has counted the number of stomach tumors still left in the animals. Examine her observations shown in the table below and answer Questions 1–3.

Sample tested	# of drops	# of tumors found
Green Protein	1	11
Green Protein	2	7
Green Protein	3	1
Placebo	1	14
Placebo	2	17
Placebo	3	13

1. Based on the results above, does it appear that the green protein controls stomach cancer? Explain your answer.

2. What amounts of our protein would you now recommend to be tested on larger animals like monkeys or humans?

3. Having heard about your testing, animal activists from People for Ethical Treatment of Animals (PETA) are in Biotex's lobby while others are sending faxes and letters requesting that you find alternatives to animal testing. Because some individuals feel so strongly about treating animals humanely, they are threatening to boycott all of your future products. Bad publicity could keep potential investors away and threaten additional research. Matthew, an activist from PETA, is in Tisha's office. What would you say or do, if you were Tisha, to help Matthew with his concerns and mend this explosive situation?

Secrets of the Rain Forest Lesson 8

Marketing—Advocacy Groups and Viewpoints

Consider the following groups and how their opinions might influence your efforts to get your green protein to the market.

Phase I Identifying the Players

For each of the groups below, describe their goals and how they might respond to your cancer-curing protein research. Who are they and what do they want? Explain in your own words. You may need to do some research. Try the internet or the resources in your library.

A. **FDA** (Food and Drug Administration)

B. **Ramon** and the other stomach cancer victims

C. **Matthew** and other animal activists from **PETA** (People for the Ethical Treatment of Animals)

D. **Tisha** and other employees of **Biotex**

Secrets of the Rain Forest Lesson 9

Marketing—Ethics and Economics

Currently Biotex has 50 employees and $40 million in the bank. Since your protein has not completed all the FDA-required testing, your protein cannot be marketed yet, so you have no income. During the first two years of testing, you used over $16 million. Examine the table below to help you decide how much Biotex will need to charge customers once you market this cancer-curing protein.

Estimated expenses for Biotex: Years 1 through 6

Year	Number of employees	Estimated expenses (millions $)	Testing and commercialization events
1	50	8	Rodent testing w/placebos.
2	50	8	Monkey testing w/placebos.
3	70	11	Submit animal testing results to FDA and start Human testing: Phase I—identify side effects in healthy humans.
4	85	13	Human testing: Phase II—determine if protein works, best dose, and side effects are tolerable to actual cancer patients. If this testing is successful, expect investors to give Biotex an additional $70 million.
5	100	45	Human testing : Phase III—many stomach cancer patients involved and if successful, send in Product License Application (PLA) to FDA.
6	100	18	FDA Approval? If yes, Biotex will continue to spend $18 million yearly to produce and market the protein.

Assume that it costs Biotex $400 (per treatment) to manufacture the drug. The selling price of the medicine determines how many people can afford the treatment. After FDA approval the market might look like this:

Type of cancer treated	Number of patients who can afford treatment	If cost of treatment is
Stomach cancer	10,000	$2,000
(Medicare will not cover above	30,000	$1,000
$1,000 per treatment)	60,000	$600
Lung cancer	6,000	$2,000
(Medicare will cover, if priced	15,000	$1,000
$500 or less, since it is currently paying	300,000	$500
$500 for a competing treatment)		

Secrets of the Rain Forest Lesson 9 Name_____

Review Questions Marketing—Ethics and Economics

Given the financial situation of Biotex and what the current market will bear, answer questions 1–3.

1. Which type of cancer, lung or stomach, will receive the most financial support from Medicare?

2. Which type of cancer, lung or stomach, will enable Biotex to make the largest profit? Explain your answer.

3. If Biotex were to market the green protein for both types of cancer, at what price should the company sell the medication: the same price for both, or different prices? Explain your answer.

Secrets of the Rain Forest Lesson 10

Student Presentations

Here are some real-life dilemmas which you may encounter while bringing a new medicine to market. You and your team will select one dilemma to research and develop a well-rounded solution that considers these four main viewpoints:

A. FDA

B. Patient Advocacy Groups—Ramon and Other Cancer Patients, American Cancer Society

C. Social Conscience Groups—Sierra Club, PETA, National Resource Defense Council of Concerned Scientists, *et al.*

D. Biotex Employees

Dilemma 1

During preclinical testing, in year 2, Matthew and PETA protesters become concerned about the use of monkeys in testing. Biotex tries to get the FDA to waive the requirement, but FDA refuses (now, why would it do that?) and the whole process gets bogged down. Biotex calls a group meeting attended by all four groups, including cancer patients who are desperate to break the impasse and get things moving again. Devise a way to resolve the issue and get your protein to market as soon as possible.

Dilemma 2

During Phase II human testing, doctors refuse to use a placebo treatment on their patients. These doctors believe that the green protein can cure cancer, so why should half their patients receive a placebo and therefore be allowed to die ? Determine a way to convince the doctors to get your protein tested properly so the FDA will allow Biotex to market this cure.

Dilemma 3

Year 6 is half gone. The Phase III results were very good and Tisha and Ramon can hardly wait for FDA approval. But the FDA calls for a public meeting, to which Matthew and other PETA members are invited. The FDA is concerned about possible long-term side effects of the drug on reproduction, since none of the information gathered in the trials has addressed that issue. This would involve doing more studies using monkeys. Come up with a compromise strategy to deal with this new issue and still get the medicinal protein to market quickly.

Dilemma 4

In year 7, Biotex starts selling your cure for stomach cancer. Using the financial data provided in Lesson 7, decide on a pricing policy. Note that if you take in less than your annual expense of $18 million, you will either need to lay off employees or go out of business. Come up with a way to keep Biotex competitive and financially stable without losing employees or its ability to make and sell its products.

Dilemma 5

Imagine that a new study in year 8 at Biotex shows that your green protein can also cure lung cancer. There is a huge market for this product, if only Medicare will reimburse hospitals for the treatment. But Medicare will only pay if you promise to charge patients $500 (or less). You are already charging more than that for the same medicine to treat stomach cancer patients. What can you do to keep your stomach cancer patients and still receive the benefits of financial support from Medicare?

Dilemma 6

In year 9, a patent is issued to an obscure scientist in Massachusetts for the same cancer-curing protein. Apparently, he had been studying this protein independently just before Tisha's fateful journey to the Andes. He now has the legal right to demand payment from Biotex. He asks for $20 million. What can you offer this scientist in order to avoid going out of business?

Dilemma 7

Members of Ramon's village as well as other Andean patients are unable to pay more than $40 for the use of the cancer-curing protein that costs Biotex $400 to produce. (a) Given that your product was originally found in their country, can you afford to help these patients, and if so, how? If you cannot help Ramon and the other Andean villagers, what can they do to receive this needed treatment? (b) Assume you decide to provide your product to these villagers below cost. Six months later, a thriving black market for your drug appears in Los Angeles — at reduced prices! Meanwhile, a remarkable number of Ramon's village folks seem to have come down with stomach cancer, and you are obligated to help them all. What do you do now?

Dilemma 8

Apparently, your green protein may also help prevent or control a rare skin disorder called Splotchy Skin. This disease affects approximately 250 to 300 individuals worldwide. Should Biotex determine if the green protein also cures Splotchy Skin so that these unfortunate people no longer have to hide themselves from their neighbors? If Biotex takes on this study it must follow all of the FDA testing requirements and spend an additional $12 million to verify if the green protein can safely treat this condition. Explain your answer.

Secrets of the Rain Forest Lesson 10 Name_____

Review Questions

To create an outline for your presentation analyze one of the eight dilemmas, and describe the controversies or problems created by it. Then design two possible questions and answers. In each case, identify the viewpoint of the person asking the question:

A. FDA

B. Patients like Ramon

C. Mathew and Other PETA Protesters

D. Biotex

Dilemma _____ Your response

What is the controversy?

Question 1 Possible answer
(Viewpoint I, II, III, or IV?)

Question 2 Possible answer
(Viewpoint I, II, III, or IV?)

Secrets of the Rain Forest Lesson 10

Students Presentation Criteria

Group Members _____
_____
_____
_____

Dilemma _____Check Off Sheet

Item	Points possible	Your points
Brief Description of Scenario	8	
View Points *From*		
* FDA	8	
* Patient Advocacy Group	8	
* Social Conscience Group (Sierra Club, PETA, National Resource Defense Council Union of Concerned Scientists, or ?)	8	
* Biotex Employees	8	
Solution and Rationale	30	
Graphics	10	
* Representing Each Viewpoint		
* Solution and Rationale		
* Or _____		
Oral Presentation	20	
Total points	100	

Appendix A Glossary of Terms

Agar	Provides a solid support for bacterial growth. A nutrient mixture of carbohydrates, amino acids, salts, and vitamins is added to the agar.
Arabinose	A sugar, used as food by bacteria.
Bacterial Library	A collection of bacterial cells that has been transformed with plasmid vectors carrying random DNA inserts from a single species. In this case, the DNA came from the species of plant with the mysterious green leaves.
Bacterial Lysate	Material released from inside a lysed bacterial cell. Includes proteins, DNA and other cell ingredients.
Biotechnology	Application of biology in the real world, by genetically manipulating living organisms to produce useful products.
Chromatography	A process for separating ingredients of complex mixtures. A (liquid) mixture is poured through a column packed with tiny beads. The properties of the beads cause some ingredients to stick to the beads, while others pass through.
Cloning	When a population of cells is prepared by growth from a single cell, all the cells in the population will be genetically identical *i.e.* they will contain identical DNA. The process of creating a clonal population is called "cloning".
Colony	A clump of genetically identical bacterial cells growing on an agar plate. Because the cells in a colony are genetically identical they are clones.
Centrifugation	Spinning a mixture at high speed to separate heavy and light particles. In this case, centrifugation results in a "pellet" found at the bottom of the tube, and a liquid "supernatant" that resides above the pellet.
Culture Media	The liquid and solid media (LB broth and LB agar) are made from an extract of yeast and meat byproducts which provides nutrients for bacterial growth. Agar, which is very similar to Jell-O, provides a solid support on which to culture the bacteria.
Food and Drug Administration (FDA)	A government organization which protects consumers by determining if a new food or drug really works as the manufacturer claims.
Genetic Engineering	The manipulation of an organism's DNA by introducing or eliminating specific parts of it.
Green Fluorescent Protein	Green Fluorescent Protein (GFP) was originally isolated from the jellyfish, *Aequorea victoria*. When exposed to ultraviolet light, it gives off energy in the form of visible green light.

Lysozyme	Enzyme which can lyse, or break open, bacteria cell walls.
Pellet	After centrifugation, the heavier particles such as bacteria or the debris of lysed bacteria are found at the bottom of a microfuge tube in a pellet.
Plasmid	A circular DNA molecule, capable of independent replication, sometimes carrying genes for antibiotic resistance.
pGLO	Plasmid containing the GFP gene under arabinose control. Also contains a gene for antibiotic resistance.
Screening	Process of identifying desired bacteria from a bacterial library.
Sterile Technique	Minimizing the possibility of outside bacterial contamination during an experiment by using careful technique. See Appendix C.
Streaking	Process of passing an inoculating loop with bacteria on it across an agar plate, in order to separate the cells.
Supernatant	Liquid, containing cell ingredients, which are lighter than the pellet formed after centrifugation.
Vector	An independently replicating DNA molecule (*e.g.* a plasmid) into which foreign DNA fragments can be inserted for propagation.

Appendix B

Basic Concepts

Cells are the smallest living units capable of reproducing independently. Cells can be gathered from their natural locations and grown inside laboratory containers. Appropriate food and environment must be provided for the cells to grow. After growth is complete, cells in culture can be harvested for study.

Cloning and Streaking. When a population of cells is prepared by growth from a single cell, all the cells in the population will be genetically identical. The process of creating such a population is called cloning. The purpose of streaking bacteria on agar is to generate single colonies, each arising from a single cell. We can now examine their properties. Cells containing the GFP gene, for instance, will glow bright green under UV light because the GFP gene makes GFP protein inside these cells, but only if there is arabinose in the growth medium.

DNA and genes. DNA is a long, stringy chemical found inside cells. Its job is to carry information. When cells divide, they pass on a copy of their DNA to their offspring.

DNA is like a file cabinet. The information in it is divided into files, called genes. Most genes contain information for making a protein. There are 80,000–100,000 proteins in the human body, each encoded by a different gene. In the jellyfish *A. victoria*, the GFP gene carries the information for assembling the GFP protein. It is possible to assemble GFP protein somewhere else (like, inside a bacterial cell), if the gene for GFP is moved into that location.

How arabinose can control a gene. Genes are carefully regulated to allow adaptation to different environments. For example, bacteria can use the sugar arabinose as a source of carbon. The bacterial enzymes needed to digest arabinose are only made when arabinose is present. In other words, arabinose turns on the genes for these digestive enzymes. When the arabinose is utilized, these genes are turned off.

The pGLO plasmid has been engineered to combine these controlling elements with the GFP gene. Therefore, in the presence of arabinose, GFP protein is produced. Cells fluoresce a brilliant green color as they accumulate GFP protein. In the absence of arabinose, the GFP gene is shut off. When the GFP protein is not made, bacterial colonies will appear white with no fluorescence.

Plasmids and transformation. Plasmids are small circular DNAs found inside some bacterial cells. They replicate on their own, and thus can persist indefinitely inside these cells. Because of their small size, plasmid DNAs are easy to extract and purify from bacterial cells. They can be re-introduced into bacterial cells by a procedure called transformation. Some plasmids contain antibiotic-resistance genes. When such plasmids are introduced into bacterial cells by transformation, it is easy to select those bacteria that have received the plasmid—because only these bacteria have acquired the ability to survive the antibiotic.

DNAs can be cut and pasted. Scientists can cut and paste DNA using enzymes. One common technique is to make a random cut of all the DNA from a given source, then paste the pieces—one at a time—into plasmids which can then be put back into bacterial cells. Each bacterial cell receives a different piece. In each case, the plasmid perpetuates the foreign piece of DNA. A collection of such bacteria is called a bacterial library. If the original source of the DNA was a ‘mysterious green leaf’ then all the genes from that leaf will be found somewhere in the bacterial library.

Appendix C

Safety, Sterile Technique, and Waste Disposal

Sterile Technique

Because contaminating bacteria are ubiquitous and are found on fingertips, bench tops, etc., it is important to minimize contact with these contaminating surfaces. When students are working with the inoculation loops and agar plates, you should stress that the round circle at the end of the loop, the tip of the pipetter, and the surface of the agar plate should not be touched or placed onto contaminating surfaces.

Working with *E. coli*

The host organism, an *E. coli* K-12 strain and, the plasmid containing the GFP gene are not pathogenic organisms like the *E. coli* O157:H7 strain that has been in the news. However, handling of the *E. coli* K-12 strains of the Rain Forest Kit requires the use of Standard Microbiological Practices. These practices include but are not limited to the following: Work surfaces are decontaminated with 10% bleach once a day and after any spill of viable material. All contaminated liquid or solid wastes are decontaminated before disposal. Persons wash their hands: (i) After they handle materials involving organisms containing recombinant DNA molecules, and (ii) before exiting the laboratory. All procedures are performed carefully to minimize the creation of aerosols. Mechanical pipetting devices are used; mouth pipetting is prohibited. Eating, drinking, smoking, and applying cosmetics are not permitted in the work area.

Decontamination and Disposal

If an autoclave is not available, all solutions and components (loops and pipettes) that have come in contact with bacteria can be placed in a fresh 10% bleach solution for at least 20 minutes for sterilization. A shallow pan of this solution can be placed at every lab station. Sterilize Petri dishes by covering the agar with 10% bleach solution. Let it stand for 1 hour or more. Once sterilized, the agar plates can be double bagged and treated as normal trash. Safety glasses are recommended when using bleach solutions.

Appendix D

Facts About Stomach Cancer

Epidemiology and Risk Factors

There are about 25,000 cases of stomach cancer each year in the U.S. It is twice as common among males as females, and twice as common among blacks as whites. The peak age at incidence is 50–59. Despite declining incidence in the U.S., stomach cancer continues to be a major cancer worldwide.

Suspected causes are predominantly food related. Sodium nitrates and nitrites (the preservatives found in most hot-dogs and prepared meats) are converted by stomach acids to nitrosamines, a powerful carcinogen. Apparently, vitamin C protects against the chemical reaction involving nitrosamines.

Lower socioeconomic status, cigarette smoking and alcohol are associated with increased risk. Stomach cancer is sometimes misdiagnosed as an ulcer.

Prevention and Early Detection

A cancer-risk-reducing diet is the most obvious precaution against stomach cancer, along with not smoking or drinking.

Symptoms and Diagnosis

The symptoms are vague, usually beginning with minimal discomfort in the region of the stomach and some weight loss. Later symptoms are accelerated weight loss, an earlier feeling of being full upon eating, trouble keeping food down, blood in the stool, anemia, a palpable mass and a swollen abdomen. If an ulcer in the stomach does not improve after 4 to 6 weeks of treatment, it may be cancer.

Treatment and Prognosis

Stomach cancer is hard to treat. In half of the cases, the surgeon opens the abdomen and finds that the cancer is too widespread to attempt removal. If surgery is attempted, part or all of the stomach is removed. The overall five-year survival rate is only about 10%.

Reference

Understanding Cancer, 3rd ED., 1989, Pages 176-177, ISBN 0-915950-86-3.

Appendix E

Advocacy Groups and Their Viewpoints

View point	Organization	Who are they and what do they want?
I	**FDA** Food and Drug Administration	A governmental agency responsible for evaluating a product's safety and determining if the product really works as the manufacture claims. All manufacturer claims must be verified with animal and human testing.
II	**Ramon** & other Stomach cancer victims	Cancer victims and their families want relief now no matter the cost or animal testing involved.
III	**Matthew** & other animal activists from **PETA** (People for the Ethical Treatment of Animals)	Animal Rights activists want to protect all living things, including mice and monkeys, from inhumane treatment. Some activists feel that animal testing is not necessary.
IV	**Tisha** & other employees of **Biotex**	Biotechnology employees want to find a cure and market it, preventing patient suffering or death, at the same time maintaining company profitability.

Appendix F

Instructors Answer Guide

Lesson 1
Review Questions

1. Who is Tisha and why is she in the Andes?

   Tisha is a biologist in the Andean Rain Forest looking for unusual plant species.

2. List two problems that Tisha and Biotex need to solve.

   They need to determine how to propagate and isolate the bacteria that have taken up the gene for the green protein. They also need to figure out how to grow large amounts of the bacteria and purify the green protein from the culture.

3. Why were the genes from the magic leaves inserted into bacterial cells?

   The bacterial cells act as hosts which can harbor and propagate the plant genes. The scientists were specifically interested in propagating the green fluorescent protein gene.

4. What clues will help you determine if the Biotex technicians were successful in placing the special cancer-curing protein into bacterial cells?

   The bacteria should fluoresce brightly like the leaves if the cloning was successful.

Lesson 2
Review Questions

1. Proteins

   a. What is a protein?

      A macromolecule which consists of chains of amino acids.

   b. List three examples of proteins found in your body.

      Antibodies, digestive enzymes, hair proteins, hormones, and hemoglobin are all examples of proteins.

   c. Explain the relationship between genes and proteins.

      Genes contain the genetic code which determine the amino acid composition of a protein. There is a unique gene for each protein within all of the cells of the body.

2. Using your own words, define or describe cloning.

   The duplication and propagation of a cell or organism.

3. Describe how bacterial cells in a library are different from the cells of a single colony.

   A library of bacterial cells contains a diverse mixture of bacterial types which contain a diverse mixture of genes. A single colony of bacteria originates from an individual clone which only contains a single gene.

4. Describe how you might recover the cancer-curing protein from the bacterial cells.

   One can isolate a single fluorescent green colony of bacteria and grow large amounts of the bacteria in a liquid growth media. Bacteria in liquid media can be concentrated by centrifugation. After the bacterial cells are lysed to release the cancer curing protein, the protein can be isolated by passage through a chromatography column which has an affinity for the cancer-curing protein.

Lesson 3
Review Questions

1. What is a bacterial colony?

   A bacterial colony is a large group or cluster of bacterial cells that originated from a single, clonal cell.

2. Why did you select one green and one white bacterial colony from your agar plate(s)? What could this prove?

   A green colony was picked in order to propagate or produce large amounts of the green cancer-curing protein. A white colony was chosen as a "negative control" to show that only the green colonies contain and produce the green cancer-curing protein.

3. How are these items helpful in this cloning experiment?

   a. ultraviolet (UV) light—**The green protein fluoresces, and is thus visible, when exposed to the UV light.**

   b. incubator—**The incubator provides a warm temperature which enables the bacteria to grow.**

   c. shaking incubator—**A shaking incubator provides a warm temperature and provides aeration which oxygenates the bacterial cultures. Increased oxygen accelerates the growth rate of bacteria.**

4. Explain how placing cloned cells in nutrient broth to multiply relates to your overall goal of testing the fluorescent protein as a cure for cancer.

   Cloned cells of bacteria which contain the green protein must first be grown on a larger scale to produce adequate amounts of GFP which can be purified. When a large amount of GFP-containing bacteria is available, the cells can be concentrated and the GFP can be released and purified from the concentrated pellet.

Lesson 4
Review Questions

1. You have used a bacterium to propagate a gene that produces a special plant protein that may hold a cure for stomach cancer. Identify the function of these items you used in Lesson 4.

   a. Centrifuge—**Functions to pellet the bacteria and separate the bacteria from the growth media.**

   b. Lysozyme—**Functions to enzymatically digest the bacterial cell well, which in turn weakens the cell wall so that it will rupture upon freezing.**

   c. Freezer—**Functions to freeze the bacteria which causes the cytoplasm to expand, which completely ruptures the weakened cell wall.**

2. Why did you discard the supernatant in this part of the protein purification procedure?

   The supernatant contains the bacterial growth media and does not contain the desired GFP.

3. Why did you discard the white liquid from the "-" tube but keep the green one?

   The white culture of bacteria does not contain the GFP and is not needed for the subsequent purification step.

4. Can you explain why the bacterial cells' outer cell wall ruptures when the cells are frozen? What happens to an unopened soft drink when it freezes?

   When a bacterial cell freezes, the volume of cytoplasm expands. The expansion puts pressure on the weakened cell wall, which then ruptures from the pressure.

5. What was the purpose of rupturing or lysing the bacteria?

   The bacteria need to be ruptured in order to release the GFP, which can then be purified using column chromatography.

Lesson 5
Review Questions

1. (a) What color was the pellet in this step of the experiment? (b) What color was the supernatant? (c) Explain?

   (a) The pellet should be a whitish or pale green color. (b) The supernatant should fluoresce bright green. (c) The fluorescent green color of the supernatant indicates that the green protein was released from the bacteria and remained in the supernatant. The much lighter color of the bacterial pellet suggests that the GFP was released form the bacteria upon lysis.

2. Why did you discard the pellet in this part of the protein purification procedure?

   The pellet contains unwanted bacterial debris- bacterial cell walls, membranes, and chromosomal DNA. The pellet contains little, if any, GFP and can be discarded.

3. Briefly describe protein chromatography and identify its purpose in this lab.

   Protein chromatography is a technique which can be used to separate or purify proteins from other molecules. This lab used hydrophobic interaction chromatography to purify GFP based upon its hydrophobic properties.

Lesson 6
Review Questions

1. List your predictions and observations for the sample and what happens to the sample when the following buffers are added to the HIC column.

Collection tube number	**Prediction**	**Observations under UV light** (column and collection tube)
Tube 1 Sample in Binding buffer	**GFP should stick to the column**	**GFP resides as a band at the top of the column**
Tube 2 Sample in Wash buffer	**GFP should stick to the column**	**GFP remains as a broad band on top of the column**
Tube 3 Sample with Elution buffer	**GFP should elute from the column**	**GFP travels down the column as a ring and elutes into tube 3.**

2. Using the data table above, compare how your predictions matched up with your observations for each buffer.

   a. Binding buffer—**GFP binds to the top of the chromatography column.**

   b. Wash buffer—**GFP remained bound to the top of the column.**

   c. Elution buffer—**GFP is eluted from the column.**

3. Based on your results, explain the roles or functions of these buffers. Hint: how does the name of the buffer relate to its function?

    a. Equilibration buffer—**This buffer prepares the column for the application of GFP. Equilibration buffer raises the salt concentration of the column to match that of the bacterial GFP lysate.**

    b. Binding buffer—**This buffer raises the salt concentration of GFP which causes a conformational change in GFP, exposing the hydrophobic regions.**

    c. Wash buffer—**Wash buffer functions to wash away less hydrophobic, contaminating proteins from the column.**

    d. TE (Elution) buffer—**This buffer functions to remove GFP from the column.**

4. Which buffers have the highest salt content and which have the least? How can you tell?

    Binding buffer >>Equilibration buffer>>Wash buffer>>Elution buffer

    Binding buffer has the highest concentration of salt because it is needed to raise the salt concentration of the GFP lysate. The hydrophobic patches of proteins are exposed in high salt buffer. TE Elution Buffer has the lowest salt concentration because it causes GFP to elute from the column. The hydrophobic patches of proteins re-orient to the interior, and the hydrophilic regions are exposed in low salt buffer.

5. Were you successful in isolating and purifying GFP from the cloned bacterial cells? Identify the evidence you have to support your answer.

    If tube 3 fluoresces green, the student was successful in purifying GFP. If GFP is not present in tube 3, examine the column- application of an incorrect buffer would prevent the elution. Alternatively, if the student did not start with a bright green culture, tube 3 will not be extremely bright.

Lesson 7
Testing and Placebos

1. Based on the results above, does it appear that the green protein controls stomach cancer? Explain your answer.

    Yes. The number of tumors decreases as the amount of GFP administered increases. Also, the placebo has no effect on the number of tumors, which further supports the effect of GFP on stomach cancer.

2. What amounts of our protein would you now recommend to be tested?

    The amounts would probably depend on the animals weight. If an average rat weighs 0.25 kg and an average human weighs 72 kg, than you would probably need ~288 drops to treat a human if there is a linear relationship of dose. However, dose testing would need to be done to confirm this.

3. Having heard about your testing, animal activists from People for Ethical Treatment of Animals (PETA) are in Biotex's lobby while others are sending faxes and letters requesting that you find alternatives to animal testing. Because some individuals feel so strongly about treating animals humanely, they are threatening to boycott all of your future products. Bad publicity could keep potential investors away and threaten additional research. Matthew, an activist from PETA, is in Tisha's office. What would you say or do, if you were Tisha, to help Matthew with his concerns and mend this explosive situation?

   You could try to explain to Matthew that animal studies are crucial to developing GFP as a treatment for human stomach cancer; it is difficult to know what doses to administer to humans without having tested it on animals first. You could also suggest that you will explore non-animal testing mechanisms, such as cell culture systems, to test the effect of the drug.

Lesson 8
Questions

A. FDA. Food and Drug Administration

The FDA will want thorough testing of GFP both in animals and humans, before it will be authorized for use as a marketed drug in humans.

B. Ramon and the other stomach cancer victims

They are looking for an effective cure or treatment for stomach cancer.

C. Matthew and other animal activists from PETA (People for the Ethical Treatment of Animals)

They are looking out for the best interests of animals and would like to avoid animal testing.

D. Tisha and other employees of Biotex

They obviously want their purified GFP to be effective and get approval from the FDA to go to market. The use of GFP as an effective agent against stomach cancer will substantiate their scientific research efforts.

Lesson 9
Review Questions

1. Which type of cancer will receive the most financial support from Medicare?

   Medicare will provide the most support to lung cancer: $150 million dollars (300,000 X $500 = 150 million dollars).

2. Which type of cancer will enable Biotex to make the largest profit? Explain your answer.

   Biotex will make more of a profit treating stomach cancer, since the differential between the cost of the drug and the cost of treatment is $200 for stomach cancer (as compared to $100 for lung cancer).

3. If Biotex were to market the green protein for both types of cancer, at what price should the company sell the medication: the same price for both, or at different prices? Explain your answer.

   Debatable question. Ethically, one would think they should sell the drug at the same price, but Biotex is a biotechnology company that needs to make a profit to survive, so charging more for stomach cancer could be argued for company viability.

Lesson 10
Dilemmas

Teachers should expect a wide variety of answers and solutions to the dilemmas posed in this section.

Appendix G

Gene Regulation. One Gene: One Protein.

Our bodies contain thousands of different proteins which perform many different jobs. Digestive enzymes are proteins; some of the hormone signals that run through our bodies and the antibodies protecting us from disease are proteins. The information for assembling a protein is carried in our DNA. The section of DNA which contains the code for making a protein is called a gene. There are over 100,000 genes in the human genome. Each gene codes for a unique protein: one gene-one protein. The gene which makes a digestive enzyme in your mouth is different from one which makes an antibody or the pigments that color your eyes.

Organisms regulate expression of their genes and ultimately the amounts and kinds of proteins present within their cells for a myriad of reasons including developmental, cellular specialization and adaptation to the environment. Gene regulation not only allows for adaptation to differing conditions, but also prevents wasteful overproduction of unneeded proteins which would put the organism at a competitive disadvantage. The genes involved in the transport and breakdown (catabolism) of food are good examples of highly regulated genes. For example, the sugar arabinose is both a source of energy and a source of carbon. *E. coli* bacteria produce three enzymes (proteins) needed to digest arabinose as a food source. The genes which code for these enzymes are not expressed when arabinose is absent, but they are expressed when arabinose is present in their environment. How is this so?

Regulation of the expression of proteins often occurs at the level of transcription from DNA into RNA. This regulation takes place at a very specific location on the DNA template, called a promoter, where RNA polymerase sits down on the DNA and begins transcription of the gene. In bacteria, groups of related genes are often clustered together and transcribed into RNA from one promoter. These clusters of genes controlled by a single promoter are called operons.

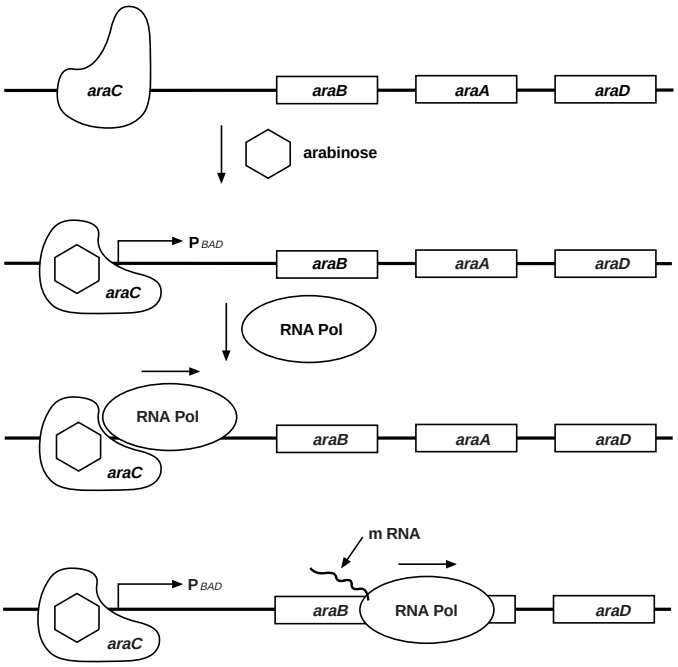
The three genes (*ara*B, *ara*A and *ara*D) that code for three digestive enzymes involved in the breakdown of arabinose are clustered together in what is known as the arabinose operon.[3] These three proteins are dependent on initiation of transcription from a single promoter, (P_{BAD}). Transcription of these three genes requires the simultaneous presence of the DNA template (promoter and operon), RNA polymerase, a DNA binding protein called *ara*C and arabinose. *Ara*C binds to the DNA at the binding site for the RNA polymerase (the beginning of the arabinose operon). When arabinose is present in the environment, bacteria take it up. Once inside, the arabinose interacts directly with *ara*C which is bound to the DNA. The interaction causes *ara*C to change its shape which in turn promotes (actually helps) the binding of RNA polymerase and the three genes B, A and D, are transcribed. Three enzymes are produced, they do their job, and eventually the arabinose runs out. In the absence of arabinose the *ara*C returns to its original shape and transcription is shut off.

The DNA code of the pGLO plasmid has been engineered to incorporate aspects of the arabinose operon. Both the promoter (P_{BAD}) and the *ara*C gene are present. However, the genes which code for arabinose catabolism, *ara*B, A and D, have been replaced by the single gene which codes for the Green Fluorescent Protein (GFP). Therefore, in the presence of arabinose, *ara*C protein promotes the binding of RNA polymerase and GFP is produced. Cells fluoresce a brilliant green color as they produce more and more protein. In the absence of arabinose, *ara*C no longer facilitates the binding of RNA polymerase and the GFP gene is not transcribed. When the GFP protein is not made, bacteria colonies will appear to have a wild type (natural) phenotype - of white colonies with no florescence.

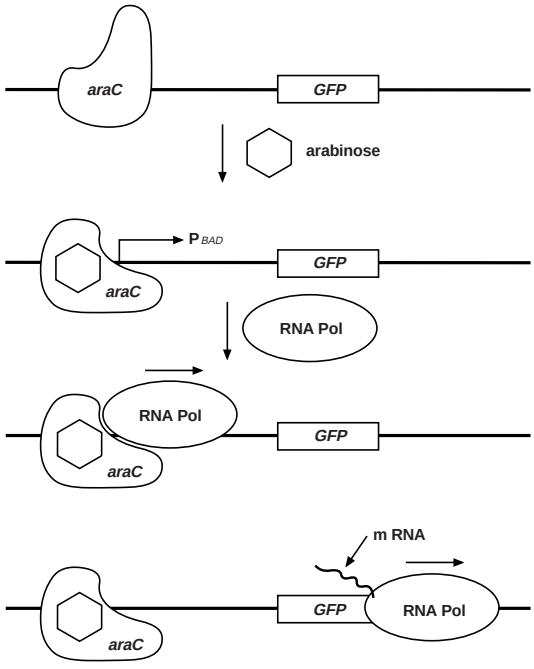
This is an excellent example of the central molecular framework of biology in action:

DNA>RNA>PROTEIN>TRAIT.

The Arabinose Operon



Expression of Green Fluorescent Protein



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