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.

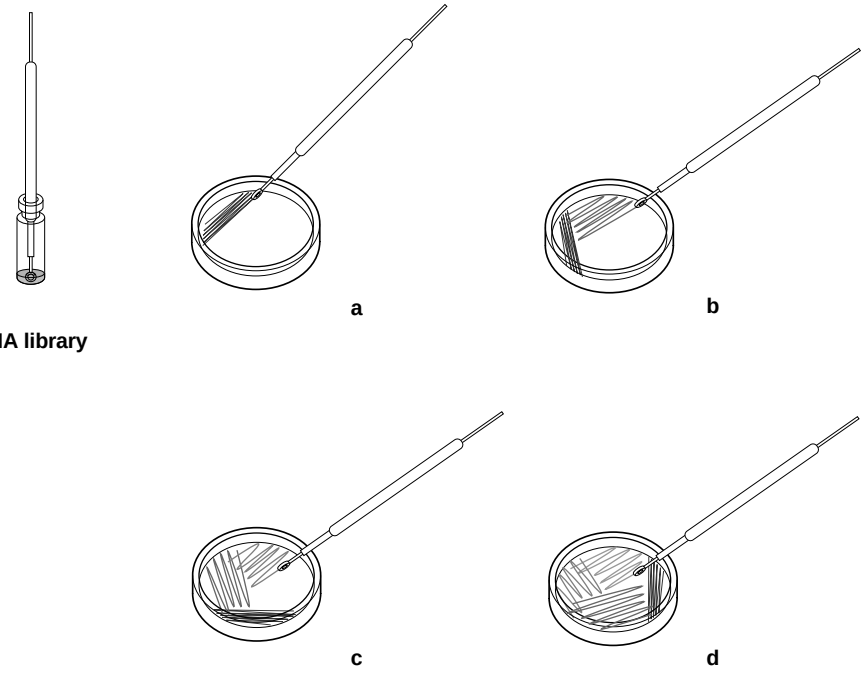
    a. Insert a sterile inoculation loop straight into the vial of the bacterial library without tilting the vial. Remove the loop and streak for single colonies as illustrated below. Streaking takes place sequentially in four quadrants on each plate. The first streak is to just spread the cells out a little. 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.

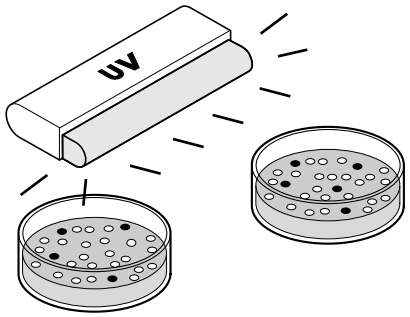
Workstations Check (✔) List

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

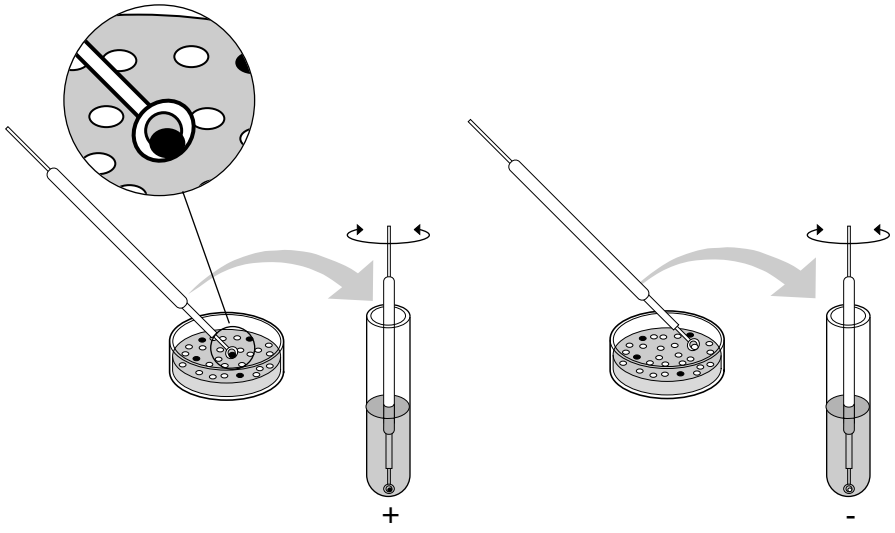
1. Remove your streaked plates from the incubator and examine them. First use normal room lighting, then use an ultraviolet light in a darkened area of your laboratory. Note your observations.

   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.

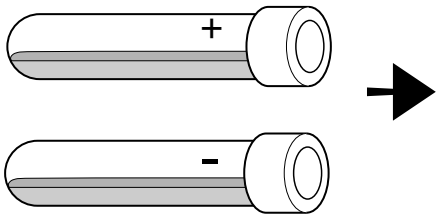


2. Identify several green colonies that are not touching other colonies on one plate. Turn the plate over and circle several of these green colonies. On the other plate, identify and circle several white colonies that are also well isolated from other colonies on the plate.

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.

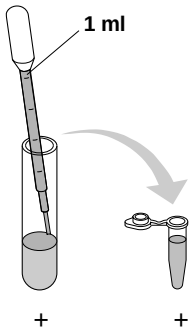
A good way to concentrate a large number of cells is to place a tube containing the liquid cell culture into a centrifuge and spin it. As you spin the cell culture, where would you expect the cells to collect, in the liquid portion (supernatant) or at the bottom of the tube (pellet)?

Workstations Check (✔) List

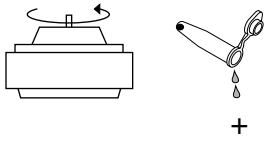
Your workstation	Number	(✔)
Microtubes	1	❑
Pipette	1	❑
Microtube rack	1	❑
Marker	1	❑
Beaker of water for rinsing pipettes	1	❑
Instructors workstation		
TE buffer	1 vial	❑
Lysozyme (rehydrated)	1 vial	❑
Centrifuge	1	❑
UV light	1–4	❑

Laboratory Procedure for Lesson 4

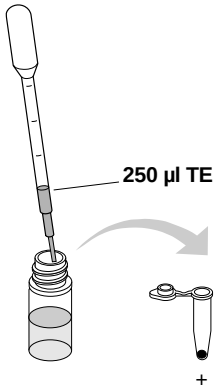
1. Using a marker, label one new microtube with your name and period.
2. Remove your two liquid cultures from the shaker or incubator and observe them in normal room lighting and then with the UV light. Note any color differences that you observe. Using a clean pipette, transfer the entire contents of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



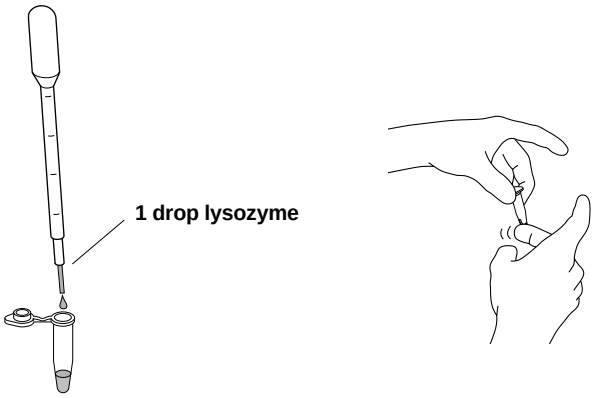
3. Spin the (+) microtube for 5 minutes in the centrifuge at maximum speed. Be sure to balance the tubes in the machine. If you do not know how to balance the tubes, do not operate the centrifuge. Ask the teacher for instructions.
4. After the bacterial liquid culture has been centrifuged, open the tube 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

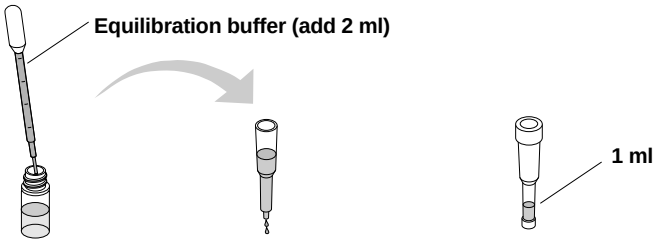
Student workstation items	**Number**	**(✔)**
Microtubes	1	❑
Pipette	1	❑
Microtube rack	1	❑
Marker	1	❑
Beaker of water for rinsing pipettes	1	❑
HIC chromatography column	1	❑
Column end cap	1	❑
Waste beaker or tube	1	❑
Instructors workstation items		
Binding buffer	1 vial	❑
Equilibration buffer	1 vial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

Laboratory Procedure for Lesson 5

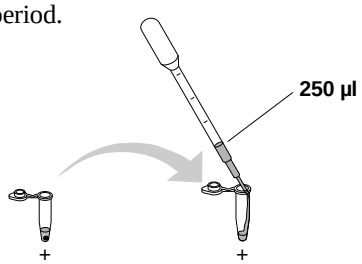
1. Remove your microtube from the freezer and thaw it out using hand warmth. Place the tube in the centrifuge and pellet the insoluble bacterial debris by spinning for 10 minutes at maximum speed. Label a new microtube with your team's initials.

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).

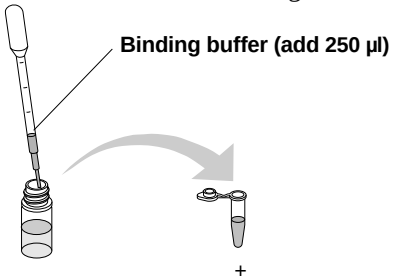
3. Prepare the column by adding 2 milliliters of Equilibration buffer to the top of the column, 1 milliliter at a time using a well a rinsed pipette. Drain the buffer from the column until it reaches the 1 milliliter mark which is just above the top of the white column bed. Cap the top and bottom of 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 $(NH_4)_2SO_4$)

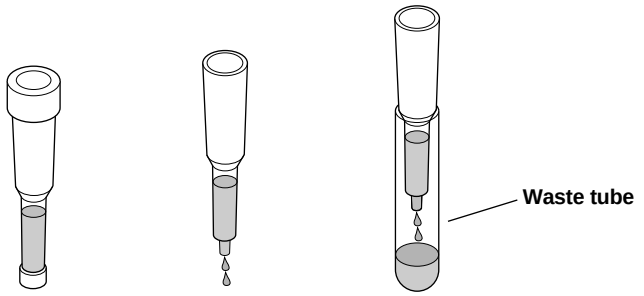
TE (Elution) buffer—A very low salt solution (10 mM Tris/EDTA)

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	❑

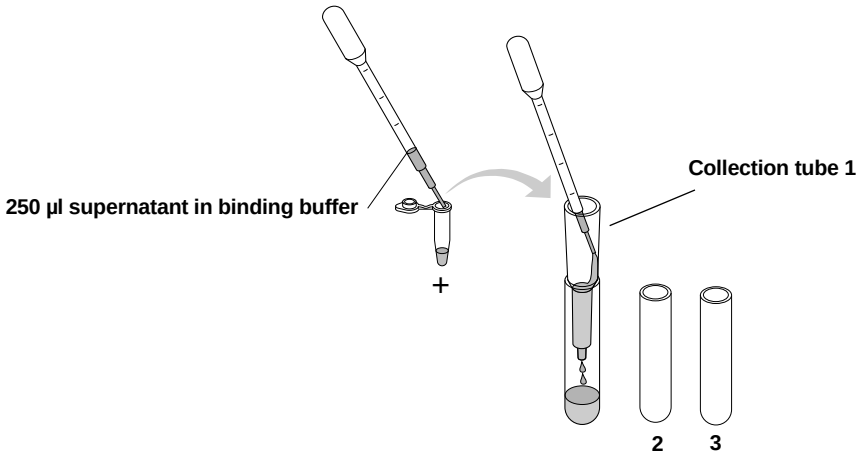
Lesson 6 Laboratory Procedure

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.

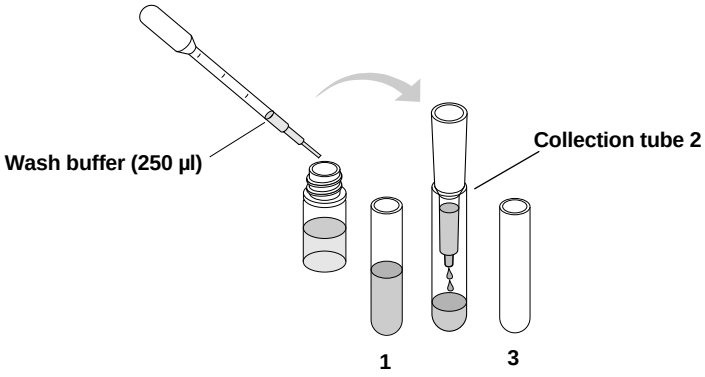


2. Predict what you think will happen for the following steps and write it along with your actual observations in the data table on page 42.

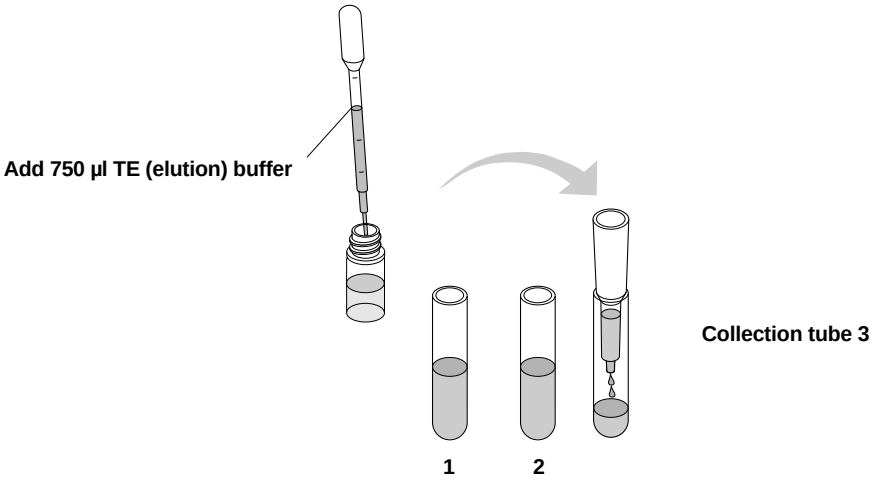
3. Using a new pipette, carefully load 0.25 milliliters (250 µl) of the supernatant (in Binding buffer) into the top of the column by resting the pipette tip against the side of the column and letting the supernatant drip down the side of the column wall. Examine the column using the UV light. Note your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



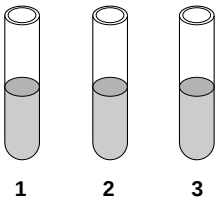
4. Transfer the column to collection tube 2. Using the rinsed pipette and the same loading technique described above, add 250 µl of Wash buffer and let the entire volume flow into the column. As you wait, predict the results you might see with this buffer. Examine the column using the UV light and list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