Biology 206
Laboratory 6
Purification of Green Fluorescent Protein (GFP)

Introduction

One of the basic tools of modern biotechnology is DNA splicing, cutting DNA and linking it to other DNA molecules. The basic concept behind DNA splicing is to remove a functional DNA fragment—let's say a gene—from one organism and combine it with the DNA of another organism in order to make the protein that gene codes for. The desired result of gene splicing is for the recipient organism to carry out the genetic instructions provided by its newly acquired gene. For example, certain plants can be given the genes for resistance to pests or disease, and in a few cases to date, functional genes can be given to people with nonfunctional or mutated genes, such as in a genetic disease like cystic fibrosis.

Genes can be cut out of human, animal, or plant DNA and placed inside bacteria. For example, a healthy human gene for the hormone insulin can be put into bacteria. Under the right conditions, these bacteria can make authentic human insulin. When allowed to multiply in gigantic vats (fermenters) these bacteria can be used to mass produce the human insulin protein. This genetically engineered insulin is purified using protein chromatography and used to treat patients with the genetic disease, diabetes, whose insulin genes do not function normally.

A common problem in purifying genetically engineered "designer" proteins from transformed bacteria is contamination by endogenous bacterial proteins. Chromatography is a powerful method used in the biotechnology industry for separating and purifying proteins of interest from bacterial proteins. Proteins purified in this manner can then be used, for example, as medicines to treat human disease, or, for household agents such as natural enzymes to make better laundry detergents.

The cloning and expression of the GFP gene (pGLO Bacterial Transformation), followed by the purification of its protein (in Purification of GFP), is completely analogous to the processes used in the biotechnology industry to produce and purify proteins with commercial value. The real-life source of the Green Fluorescent Protein gene is the bioluminescent jellyfish *Aequoria victoria*.

Day 1: Picking Colonies and Growing a Cell Culture

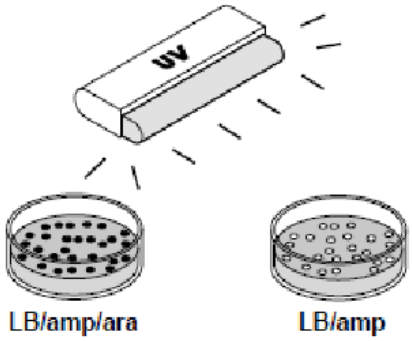
Examine your two transformation plates under the ultraviolet (UV) lamp. On the LB/amp and LB/amp/ara plates, a well-separated single colony of bacteria has been marked with an arrow. Theoretically both white (LB/amp plate) and green (LB/amp/ara plate) colonies were transformed with the pGLO plasmid? How can you tell? Both colonies should contain the gene for the Green Fluorescent Protein. To find out, you will place each of the two different bacterial colonies (clones) into two different culture tubes and let them grow and multiply overnight.

In this lab, you will pick one white colony from your LB/amp plate and one green colony from your LB/amp/ara plate for propagation in separate liquid cultures. Since it is hypothesized that the cells contain the Green Fluorescent Protein, and it is this protein we want to produce and purify, your first consideration might involve thinking of how to produce a large number of cells that produce GFP. You will be provided with two tubes of liquid nutrient broth into which you will place cloned cells that have been transformed with the pGLO plasmid.

Procedure:

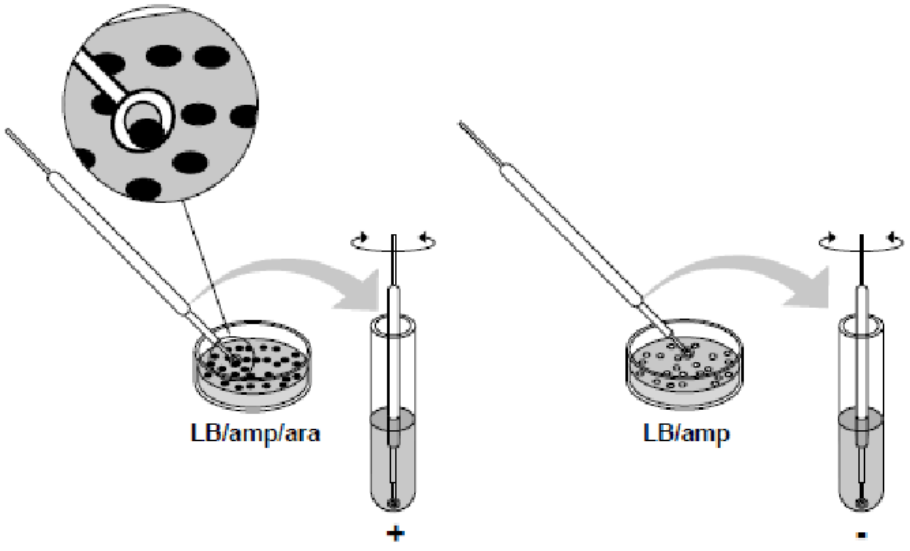
1. Examine your LB/amp and LB/amp/ara plates from the transformation lab. First use normal room lighting, then use an ultraviolet light in a darkened area of your laboratory. Note your observations.

   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.



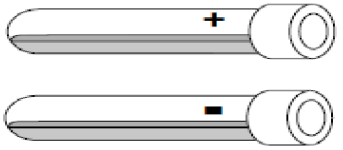
2. Identify several green colonies that are not touching other colonies on the LB/amp/ara plate. Turn the plate over and circle several of these green colonies. On the other LB/amp plate identify and circle several white colonies that are also well isolated from other colonies on the plate.

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 2 days at room temperature. If a shaker is not available, shake your two tubes vigorously, like you would shake a can of spray paint, for about 30 seconds. Then place them in an incubator oven for 24 hours. Lay the tubes down horizontally in the incubator. (If a rocking table is available, but no incubator, tape the tubes to the platform and let them rock at maximum speed for 24 hours at 32° or at room temperature for 48 hours. We do not recommend room temperature incubation without rocking or shaking.)

Be sure your tubes are tightly capped before shaking!!



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

Culture Condition	Days Required
32 °C—shaking	1 day
32 °C—no shaking	1–2 days*
Room Temperature—shaking	2 days
Room Temperature—no shaking	Not recommended

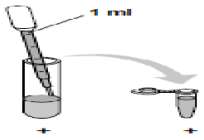
* Periodically shake by hand and lay tubes horizontally in incubator.

Day 2: Purification Phase 1 - Bacterial Concentration and Lysis

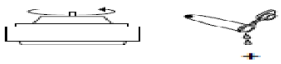
So far you have mass produced living cultures of two cloned bacterium. Both contain the gene which produces the green fluorescent protein. Now it is time to extract the green protein from its bacterial host. Since it is the bacterial cells that contain the green protein, we first need to think about how 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 concentrate, in the liquid portion or at the bottom of the tube in a pellet?

Procedure:

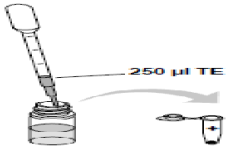
1. Using a marker, label one new microtube with your name and period.
2. Remove your two liquid cultures from the shaker or incubator and observe them in normal room lighting and then with the UV light. Note any color differences that you observe. Using a clean pipette, transfer the entire contents of 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.
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 Solution to each tube. Resuspend the bacterial pellet thoroughly by rapidly pipetting up and down several times with the pipette.



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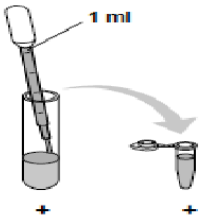

Pour off supernatant into WASTE BEAKER, not the sink!!

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.
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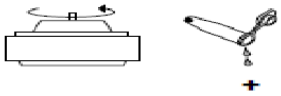
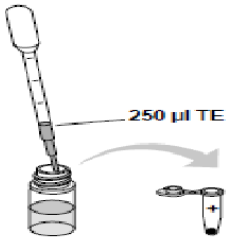
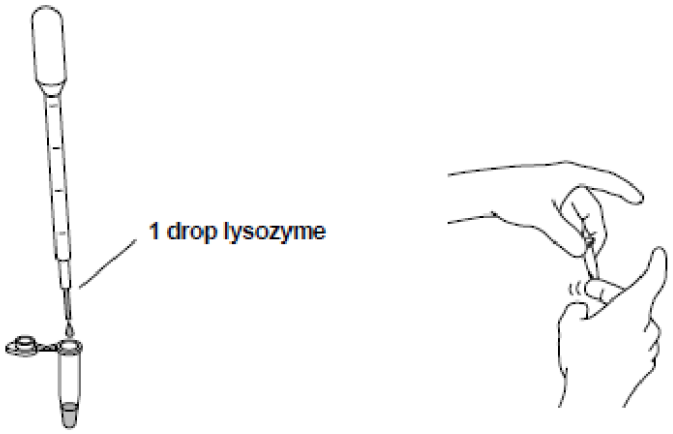


5. Observe the pellet under UV light. Note your observations.
6. Using a new pipette, add 250 µl of TE Solution to each tube. Resuspend the bacterial pellet thoroughly by rapidly pipetting up and down several times with the pipette.



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.



Day 3: Purification Phase 2 - Bacterial Lysis

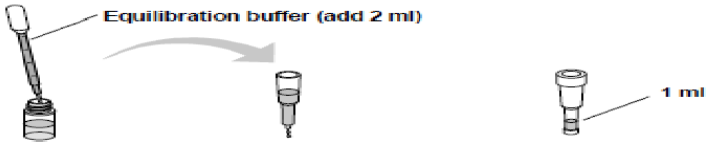
The bacterial lysate that you generated in the last lab contains a mixture of GFP and endogenous bacterial proteins. Your goal is to separate and purify GFP from these other contaminating bacterial proteins. Proteins are long chains of amino acids, some of which are very hydrophobic or "water-hating." GFP has many patches of hydrophobic amino acids, which collectively make the entire protein hydrophobic. Moreover, GFP is much more hydrophobic than most of the other bacterial proteins. We can take advantage of the hydrophobic properties of GFP to purify it from the other, less hydrophobic (more hydrophilic or "water-loving") bacterial proteins.

Chromatography is a powerful method for separating proteins and other molecules in complex mixtures and is commonly used in biotechnology to purify genetically engineered proteins. In chromatography, a column is filled with microscopic spherical beads. A mixture of proteins in a solution passes through the column by moving downward through the spaces between the beads. You will be using a column filled with beads that have been made very hydrophobic — the exact technique is called hydrophobic interaction chromatography (HIC). When the lysate is applied to the column, the hydrophobic proteins that are applied to the column in a high salt buffer will stick to the beads while all other proteins in the mixture will pass through. When the salt is decreased, the hydrophobic proteins will no longer stick to the beads and will drip out the bottom of the column in a purified form.

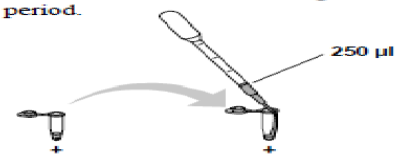
Procedure:

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).

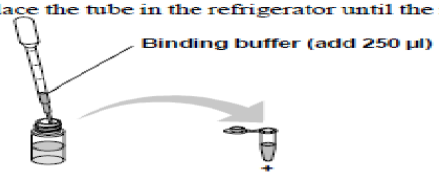
3. Prepare the column by adding 2 milliliters of Equilibration Buffer to the top of the column, 1 milliliter at a time using a well rinsed pipette. Drain the buffer from the column until it reaches the 1 milliliter mark which is just above the top of the white column bed. Cap the top and bottom of the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



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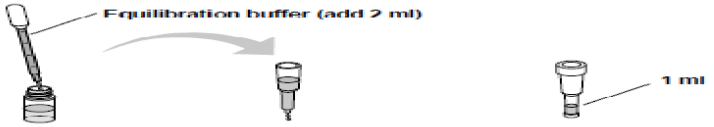

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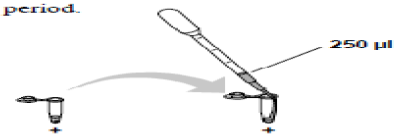
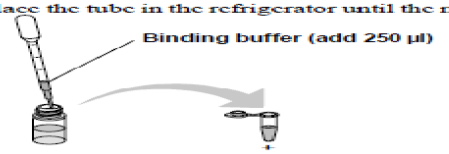


5. Using the well-rinsed pipette, transfer 250 µl of Binding Buffer to the microtube containing the supernatant. Place the tube in the refrigerator until the next laboratory period.



Day 3: Purification Phase 3 - Protein Chromatography

In this final step of purifying the Green Fluorescent Protein, the bacterial lysate you prepared will be loaded onto a hydrophobic interaction column (HIC). Remember that GFP contains an abundance of hydrophobic amino acids making this protein much more hydrophobic than most other bacterial proteins. In the first step, you will pass the supernatant containing the bacterial proteins and GFP over an HIC column in a highly salty buffer. The salt causes the three-dimensional structure of proteins to actually change so that the hydrophobic regions of the protein move to the exterior of the protein and the hydrophilic ("water-loving") regions move to the interior of the protein.

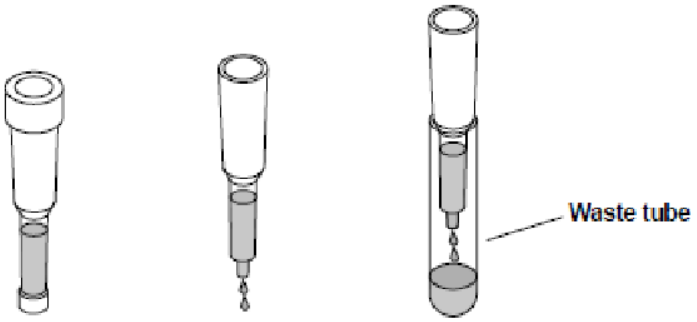
The chromatography column contains a matrix of microscopic hydrophobic beads. When your sample is loaded onto this matrix in very salty buffer, the hydrophobic proteins should stick to the beads. The more hydrophobic the proteins, the tighter they will stick. The more hydrophilic the proteins, the less they will stick. As the salt concentration is decreased, the three-dimensional structures of proteins change again so that the hydrophobic regions of the proteins move back into the interior and the hydrophilic ("water-loving") regions move to the exterior.

You will use these four solutions to complete the chromatography:

Equilibration Buffer—A high salt buffer (2 M $(NH_4)_2SO_4$)
Binding Buffer—A very high salt buffer (4 M $(NH_4)_2SO_4$)
Wash Buffer—A medium salt buffer (1.3 M $(NH_4)_2SO_4$)
Elution Buffer—A very low salt buffer (10 mM Tris/EDTA)

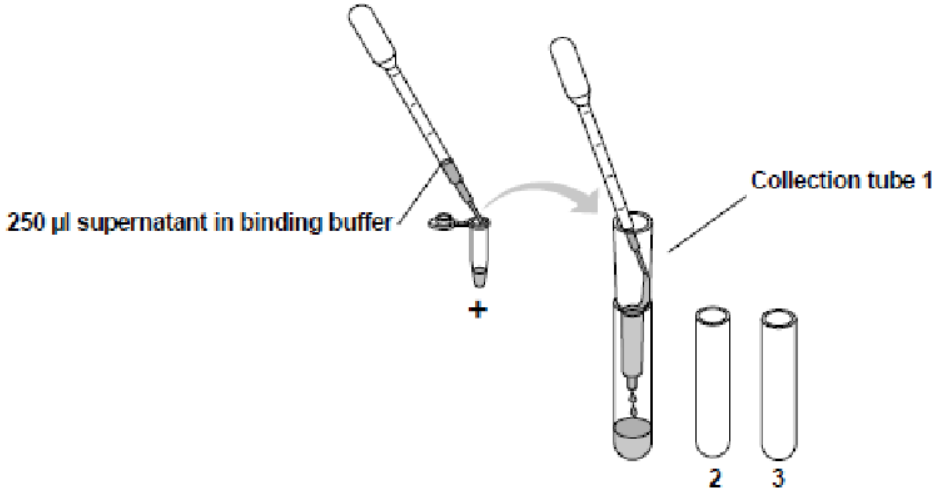
Procedure:

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.

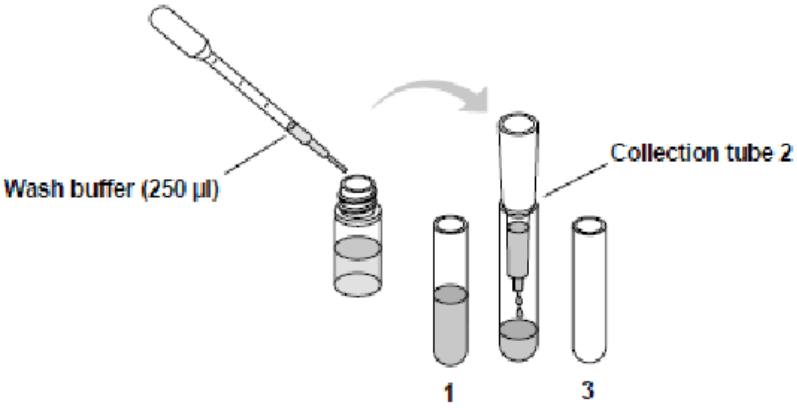


2. Predict what you think will happen for the following steps and write it along with your actual observations in the data table on page 42.

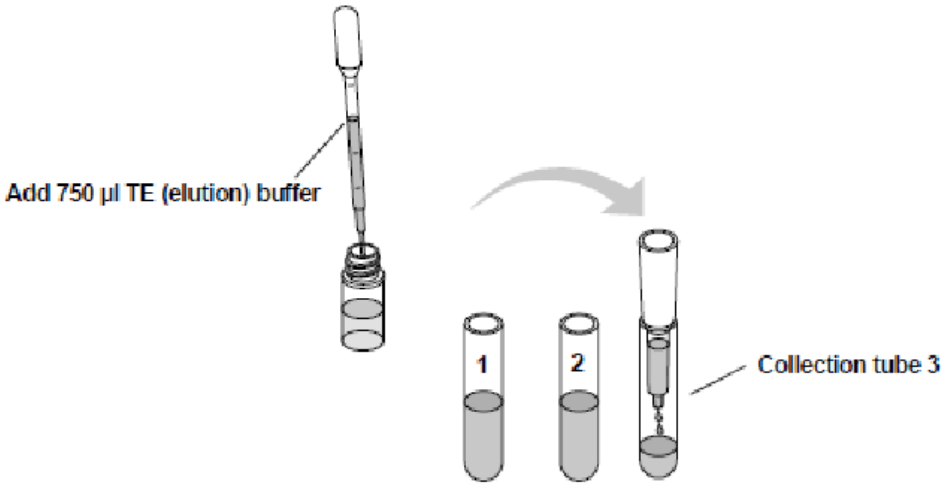
3. Using a new pipette, carefully load 250 µl of the supernatant (in Binding Buffer) into the top of the column by resting the pipette tip against the side of the column and letting the supernatant drip down the side of the column wall. Examine the column using the UV light. Note your observations in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



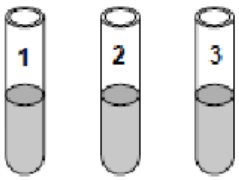
4. Transfer the column to collection tube 2. Using the rinsed pipette and the same loading technique described above, add 250 µl of Wash Buffer and let the entire volume flow into the column. As you wait, predict the results you might see with this buffer. Examine the column using the UV light and list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



5. Transfer the column to tube 3. Using the rinsed pipette, add 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.



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-