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## GFP protein separation lab introduction

### 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 (this code is transferred to RNA which can move out of the nucleus and actually encodes for the protein). Human cells contain around 40,000 genes, but many more different proteins.

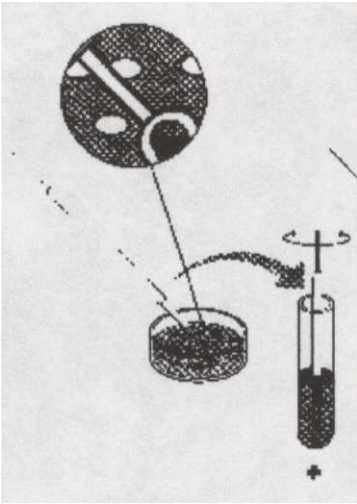
The process of separating the bacteria on the surface of a plate so that each individual cell can grow up into a clump of identical cells (a colony), is called cloning. Because all the cells in a single colony are genetically identical, they are called clones. During the lab, you will select an individual colony of bacteria you transformed in an earlier lab. Then when you have grown a tube full of these transformed bacteria, you will bust open the bacteria and isolate one protein, Green Fluorescent Protein from all the other molecules that were inside the bacterial cells.

Follow the procedure on the back. Answer the questions.

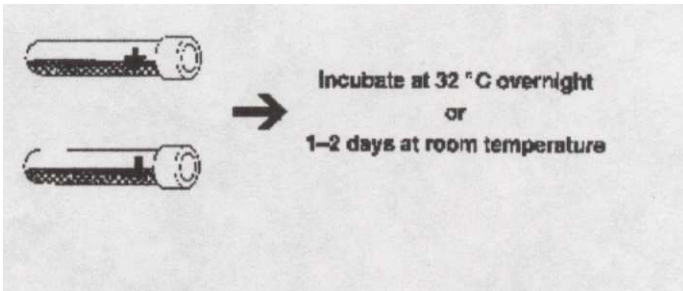
### Questions:

1. What is a protein?
  
  
  
  
  
  
  
  
  
  
2. List three examples of proteins found in your body.
  
  
  
  
  
  
  
  
  
  
3. Explain the relationship between genes and proteins.
  
  
  
  
  
  
  
  
  
  
4. Using your own words, define or describe cloning.
  
  
  
  
  
  
  
  
  
  
5. Describe how the bacterial cells in a library are different from the cells of a single colony.
  
  
  
  
  
  
  
  
  
  
6. Describe how you might isolate the GFP from the bacterial cells.

## GFP protein separation lab day 1 (Lesson 1)



1. Obtain a culture tube containing the growth media LB/amp/ara. Label the tube with your group name. Using a sterile loop, lightly touch the loop to a single green colony and immerse the loop in the tube. It is important to pick only a single colony. Spin the loop between your index finger and thumb to disperse the entire colony.



2. Place the tubes on 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.

Questions:

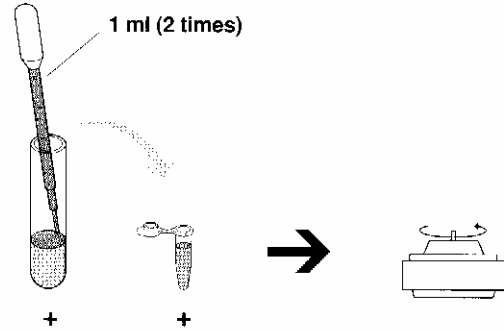
1. What is a bacterial colony?
  
3. How are these items helpful in this cloning experiment?
  - a. Ultraviolet light
  
  - b. Incubator
  
  - c. Shaking platform
  
4. Explain how placing cloned cells in nutrient broth to multiply relates to your overall goal of testing the fluorescent protein.

## GFP protein separation lab day 2

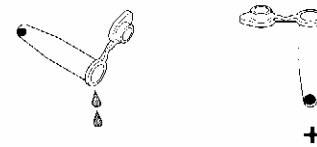
So far you have produced living cultures of a bacterial clone which contains the gene that produces the green fluorescent protein (GFP). Now is the time to extract the GFP from the bacterial cells. First, we need to collect a large number of these bacterial cells.

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)?

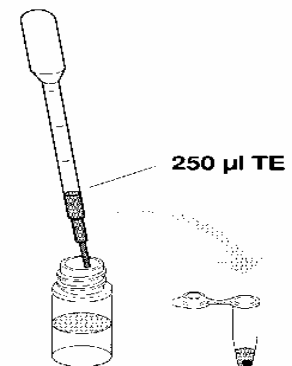
1. Label two microtubes with your lab table number and class period. Remove your liquid cultures from the shaker and observe with the UV light. Using a new sterile pipette, transfer as much of the cell culture as you can to each microtube. Spin the tubes for 5 minutes in a centrifuge at maximum speed. The pipette used in this step can be repeatedly rinsed in a beaker of water and used for all following steps of this lab period.



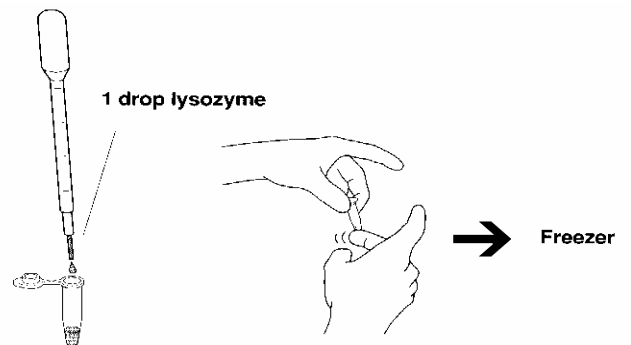
2. Pour out the supernatant and observe the pellet under UV light. It should be glowing green. If not, repeat step 1 again.



3. Using a rinsed pipette, add 250  $\mu$ l of TE buffer to the each 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 UV light. If it doesn't glow, you need to repeat the above steps.



5. Place your microtubes in the freezer until the next lab period.

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Questions:

1. You have used a bacterium to propagate a gene that produces a special protein that fluoresces (remember that it is a gene that originally came from a jellyfish but you transformed or put it into a bacterium). Identify the function of these items you need in lab day 2:

a. Centrifuge

b. Lysozyme

c. Freezer

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

3. 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?

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

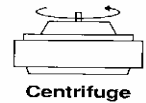
### GFP Protein separation lab day 3

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.

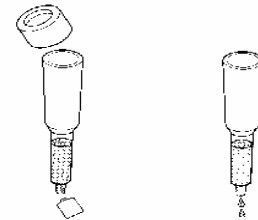
GFP has surfaces that are very hydrophobic (water hating). In salt water, these parts of the protein tend to stick tightly to other hydrophobic surfaces. Protein mixtures containing GFP can be poured through a column packed full of hydrophobic beads. When the beads are in salt water, hydrophobic proteins—like 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.

1. Remove the microtubes from the freezer and thaw using hand warmth. Place the tubes in the centrifuge and pellet the insoluble bacterial debris by spinning for 10 minutes.

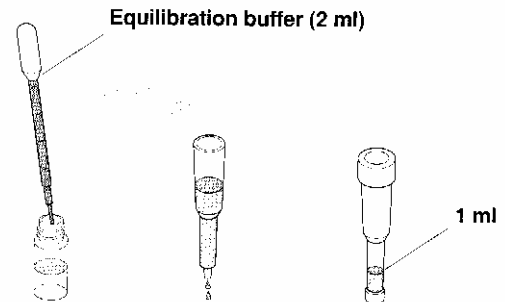
Thaw



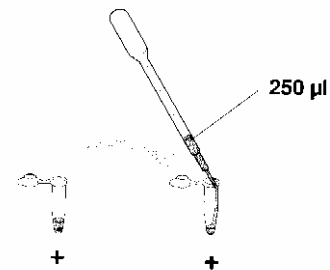
2. While your tubes are spinning, prepare the chromatography column. Remove the cap and snap off the bottom (if necessary). Allow all of the liquid buffer to drain from the column (3-5 min).



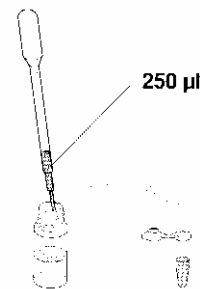
3. Prepare the column by adding 2 ml of Equilibration buffer to the top of the column. This is done by adding two 1 ml aliquots with a rinsed pipette. Drain the buffer to the 1 ml mark on the column. Cap the top and bottom and store the column at room temperature until the next lab period.



4. After the 10 minute spin, immediately remove your tubes. Examine the tubes with the UV light. If it does not glow, discard the tube. Using a new pipette, transfer 250  $\mu$ l of the supernatant into a new microtube labeled with your lab station and your class period. Rinse the pipette well for the rest of the steps of this lab period.



5. Using the well-rinsed pipette, transfer 250  $\mu$ l of Binding Buffer to the supernatant. Place the tube in the refrigerator until the next lab period.



Questions:

1. What color was the pellet in this step of the experiment? What color was the supernatant?
2. Explain why you think the pellet and the supernatant were the colors observed.
3. Why did you discard the pellet in this part of the protein purification procedure?
4. Briefly describe protein chromatography and identify its purpose in this lab.

## GFP Protein separation lab day 4

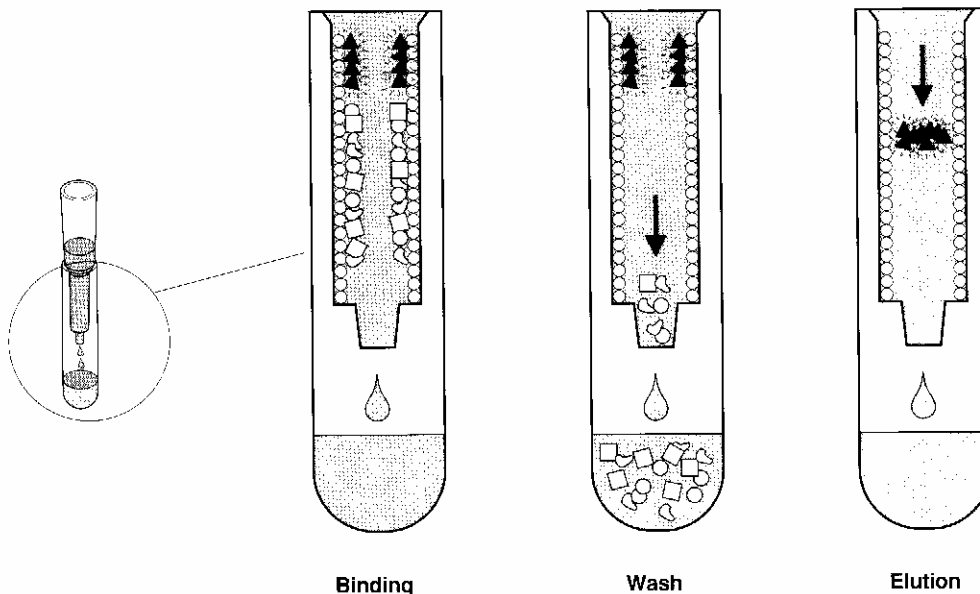
In this final step of purifying the GFP, the bacterial lysate you prepared will be loaded onto a hydrophobic interaction (HIC) column. The HIC column contains tiny microscopic hydrophobic beads. GFP should stick to the beads while the other bacterial proteins should pass straight through. Later, when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

- (1) **Equilibration Buffer**—A high salt solution (2 M  $(\text{NH}_4)_2\text{SO}_4$ ) used to equilibrate or prime the chromatography column for the binding of GFP.
- (2) **Binding Buffer**—A very high salt solution (4 M  $(\text{NH}_4)_2\text{SO}_4$ ) added to the bacterial lysate, the end result is a GFP containing lysate which has the same salt concentration as the equilibration buffer.
- (3) **Wash Buffer**—A medium salt solution (1.3 M  $(\text{NH}_4)_2\text{SO}_4$ ) is used to wash weakly associated proteins from the column; proteins which are strongly hydrophobic (such as GFP) remain bound to the resin beads in 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.
- (4) **TE (Elution) Buffer**—A very low salt solution (10mM Tris/EDTA) is used to wash GFP from the column. The 0.75 ml 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, only one of your collection tubes should fluoresce bright green.

### Important Hints for Chromatography

1. Place the column gently into the collection tubes. Jamming the column tightly into the collection tubes will create a vacuum 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 20-30 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.



**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 | Observation under UV Light (column and collection tube) |
|--------------------------------------|------------|---|
| TUBE 1<br>Sample in Binding Buffer   |            |   |
| TUBE 2<br>Sample with Wash Buffer    |            |   |
| TUBE 3<br>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. (How does the name of the buffer relate to its functions?)

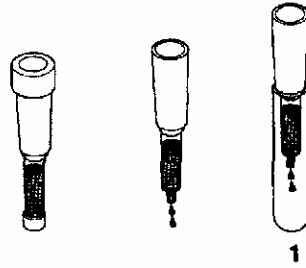
- a. Equilibration buffer
  
- b. Binding buffer
  
- c. Wash buffer
  
- d. TE (Elution buffer

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

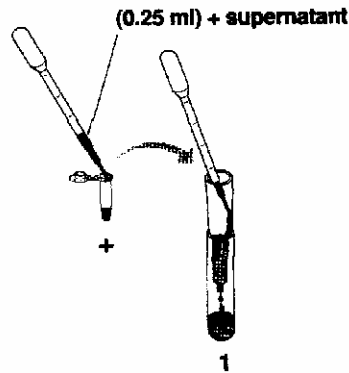
5. Were you successful in isolating and purifying GFP from the cloned bacterial cells? Identify the evidence you have to support your answer.

## Protein Chromatography

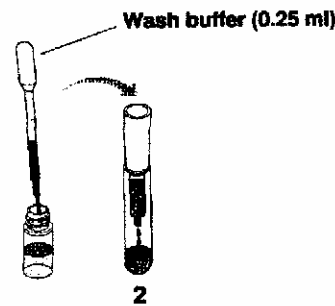
1. Label 3 collection tubes 1–3 and place the tubes in the foam rack or in a rack supplied in your laboratory. Remove the caps from the top and bottom of the column and place the column in collection tube 1. When the last of the buffer has reached the surface of the HIC matrix, proceed to the next step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



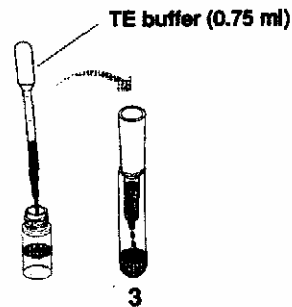
2. Using a new pipette, carefully and gently load 250  $\mu$ l of the “+” supernatant onto the top of the column. Hold the pipette tip against the side of the column wall, just above the upper surface of the matrix and let the supernatant drip down the side of the column wall. Examine the column using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



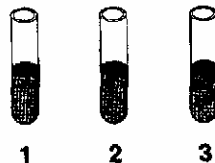
3. Using the rinsed pipette, add 250  $\mu$ l of Wash buffer and let the entire volume flow into the column. Examine the column using the UV light. Note your observations. After the column stops dripping, transfer it to tube 3.



4. Using the rinsed pipette, add 750  $\mu$ l 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.



6. Allow another 750  $\mu$ l of TE buffer to flow into the column. When there is just a small layer of fluid above the resin bed, cap off the bottom and top of the column. Return to the teacher for storage. **DO NOT THROW THE COLUMNS AWAY!!!**