

Bio314: Advanced Cell Biology

Purification of GFP



Introduction

In the previous lab, you performed a genetic transformation procedure, which bacteria were transformed to be resistant to ampicillin and to glow green. Recall that the DNA plasmid (pGLO) taken up by the bacteria contained the *bla* gene that codes for the beta-lactamase protein, which inactivates ampicillin. When bacteria were placed on plates containing ampicillin (LB/amp & LB/amp/ara), transformed bacteria survived and nontransformed bacteria did not survive. The DNA plasmid also contained the GFP gene that codes for the green fluorescent protein (GFP), which transduces blue light to green fluorescent light, and the *araC* gene that codes for the arabinose promoter protein, which regulates production of the GFP. When transformed bacteria were placed on plates containing arabinose (LB/amp/ara), the GFP gene was expressed (turned on) and the bacteria glowed green. When they were placed on plates not containing arabinose, the GFP gene was not expressed (turned off) and the bacteria did not glow green, which is an example of how many genes are regulated.

How could you set up a procedure to confirm that the original bacteria were transformed with the pGLO plasmid? What would you add to the bacterial environment? One option would be to transfer the bacteria from the LB/amp and LB/amp/ara plates onto new plates containing ampicillin and arabinose and allow them to grow. If the bacteria are transformed and survive, they will have multiplied into new colonies and glow green.

However, how can we be sure that it is the GFP that is glowing? What type of

techniques or procedures could be done to confirm that it is the GFP that is glowing? One option would be to separate the GFP from bacterial organelles, macromolecules and other proteins and observe the isolated protein under a UV light. To separate the GFP, several techniques need to be performed such as, centrifugation, the addition of Lysozyme and freezing, and protein (column) chromatography.

Background

First, transformed bacteria need to be transferred and grown in liquid cultures. The liquid media that the bacteria will grow in is an LB nutrient broth containing ampicillin and arabinose. If the bacteria have been transformed, they will survive and fluoresce green. This type of environment will yield sufficient growth in a shaking incubator at 32°C over night to allow optimal protein folding and fluorescence. Vigorous shaking of liquid cultures will deliver more oxygen to dividing cells, and allow them to grow faster and produce more GFP.

Once it has been confirmed that the bacteria have been transformed, GFP can be isolated and purified. Centrifugation is a technique used to separate molecules by high speed spinning, which forces heavier molecules to the bottom of a tube, clumped together in a pellet. The liquid remaining above the pellet is the supernatant. Depending on what is needed in further procedures, either the supernatant is discarded or the pellet. To isolate and purify GFP, centrifugation will be necessary twice, first to clump the bacteria containing the GFP into a

pellet, then to separate GFP from bacterial organelles and macromolecules into a pellet, once the bacteria have been lysed with Lysozyme. Thus the supernatant will be discarded first leaving behind bacteria containing GFP in a pellet. Then after lysing the bacteria cell walls, the pellet is discarded, which larger bacterial debris is in a pellet and the smaller proteins including GFP will be in the supernatant.

Lysozyme is an enzyme found naturally in tears, saliva and other secretions that catalyzes cleaving of polysaccharide chains in cell walls. By placing a liquid culture containing transformed bacteria and Lysozyme into a freezer, the bacterial cell membranes and cell walls will further rupture due to the osmotic forces pushing against the cell membrane and wall and the expansion of freezing components in the cell. The natural ability to “lyse” bacteria is where Lysozyme gets its name.

Once the bacteria have been lysed and centrifuged, the supernatant contains a mixture of many small proteins including the GFP. To separate GFP from these other proteins, column chromatography is used, which a column or cylinder is densely filled with microscopic beads forming a matrix that proteins must pass through before being collected. The matrix has an “affinity” for the molecule of interest, which in this case is GFP, and not the other bacterial proteins in the mixture. GFP will then stick to the column, while the other proteins pass through.

In this lab, Hydrophobic Interaction Chromatography (HIC) is the specific method of chromatography that will be used. Columns in HIC are packed with hydrophobic beads, called a hydrophobic interaction matrix. When a sample containing proteins is loaded onto the matrix in salt water, the hydrophobic proteins will stick to the beads in the column. Recall that hydrophobic substances do not mix well with water and when dropped into salty water, they tend to stick together. When proteins such as GFP with hydrophilic surfaces are dropped into highly

salted solutions, the three dimensional structure of the protein changes so that the hydrophobic regions of the protein are more exposed on the surface of the protein and the hydrophilic regions are more shielded inside the protein. When salt is removed from the solution, proteins will change back to a more hydrophilic surface with a hydrophobic interior. The more hydrophobic a protein is, the more tightly it will stick to the hydrophobic column of beads. GFP is largely hydrophobic and will stick to the HIC beads, while other proteins that are less hydrophobic pass through.

Four buffers are used in HIC. First, a medium salt Equilibration Buffer is pipetted down the column to prime or equilibrate the column for the binding of GFP. Second, a high salt Binding Buffer is added to the bacterial lysate that will cause the GFP to stick to the column as it is loaded onto the column, which the supernatant containing the GFP will have the same concentration of salt as the column has. You should begin to see a green fluorescing ring form around the top of the column. Third, a medium salt Wash Buffer is applied to the column to wash away weakly bound proteins from the column. Finally, the fourth buffer, which is a low salt Elution buffer, is used to wash GFP from the column, which it should move down the column as a fluorescing ring. Low salt buffers have a higher concentration of water molecules, so the three dimensional structure of the GFP will change back to a more hydrophilic surface, which allows it to pass down the column.

Objectives

- You will transfer transformed bacteria from plates that contain arabinose and from plates that do not contain arabinose into a liquid media that does contain arabinose and grow new cell cultures.
- You will separate and purify the GFP from the transformed bacteria grown in

the media containing arabinose, in three phases.

- Phase 1 – bacterial concentration and lysis
- Phase 2 – remove bacterial debris
- Phase 3 – protein chromatography

Procedures

- You will use a UV light throughout this lab.
- Create a data table that will contain a list of samples in one column, predictions in the second column and observations in the third column. This will be a rough draft, which a nicely finished table will be turned in with answers to the questions.
- Before you obtain your plates from the previous lab, predict which of the plates will contain bacteria that will survive and glow.

Grow cell cultures

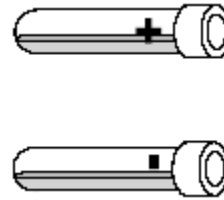
Materials

- 2 - Transformation plates from kit 1 (LB/amp/ara & LB/amp)
- 2 – Inoculation loops
- 2 – 15 ml Culture tubes, containing 2 ml LB growth media.
- 1 – Test tube holder
- 1 – Shaking incubator

Procedure

1. Using a UV light, examine both plates containing bacteria. Record your observations.
2. Label one culture tube “+” and one tube “-“, and your initials on both tubes.
3. Identify one single colony of bacteria on the LB/amp/ara plate and using a sterile inoculation loop, scoop it up and immerse the loop into the culture tube marked “+”. Gently spin the loop to remove the bacteria. Using a new sterile inoculation loop, repeat for a single colony from the LB/amp plate into the culture tube marked “-“.

4. Cap the tubes and place them into a shaking incubator and incubate for 24 hours at 32°C.



Phase 1 – bacterial concentration and lysis

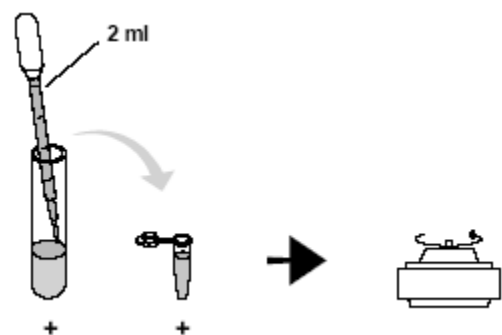
Materials

- Both culture tubes from shaking incubator
- 1 – Microtube
- 1 – Microtube rack
- 2 – Pipettes
- Beaker of water for rinsing pipettes
- Beaker for waste
- TE solution from instructor
- Lysozyme (rehydrated) from instructor
- Centrifuge

Procedure

First predict which of the tubes will contain transformed bacteria that will have survived and now glow. Fill in table.

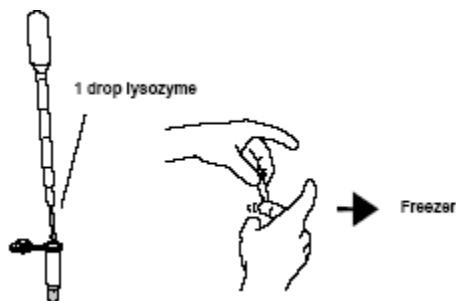
1. Using a UV light, examine both microtubes. Record your observations.
2. Using a clean pipette, transfer the entire contents of the “+” liquid culture into a new 2 ml microtube and label it “+”.
3. Spin the microtube for 5 minutes in the centrifuge at maximum speed. Be sure to balance the tubes in the machine.



- Remove the tube from the centrifuge and carefully pour off only the supernatant above the pellet. Leave the pellet in the tube.



- Observe the pellet under UV light and record your observations.
- Using a new pipette, add 250 μ l of TE solution to the microtube with the pellet. Resuspend the bacterial pellet thoroughly by rapidly pipetting up and down several times with the pipette.
- Rinse the pipette and add 1 drop of Lysozyme to the resuspended bacterial pellet. Cap the microtube and gently flick the tube with your index finger to mix the contents.
- Place the microtube into the freezer for 15-20 minutes.



Phase 2 – remove bacterial debris

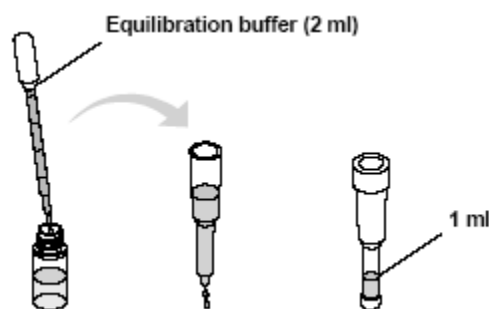
Materials

- 1 – Microtube
- 1 – Pipette
- 1 – HIC chromatography column with caps
- Binding buffer from instructor
- Equilibration buffer from instructor

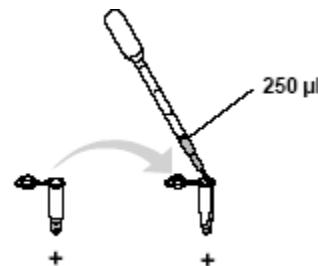
Procedure

- Remove your microtube from the freezer and thaw by rolling between your hands.

- Place the microtube into the centrifuge and spin for 10 minutes at maximum speed.
- While the centrifuge is spinning, prepare the chromatography tube. Shake the column vigorously to resuspend the beads inside the tube. Then tap the tube on the counter to bring the beads to the bottom of the tube. Remove the cap and snap of the bottom tab of the column and drain the liquid buffer into the waste beaker.
- Using a well rinsed pipette, add 2 ml of Equilibration buffer to the top of the column, 1 ml at a time. Drain the buffer from the bottom until it reaches the 1 ml marker. Cap the bottom and top of the column until needed.



- Which of the contents in the microtube will be glowing, the pellet or the supernatant? Record your predictions.
- Remove the tube from the centrifuge and observe the contents under a UV light. Record your observations.
- Using a new pipette, immediately transfer 250 μ l of the supernatant into the new microtube. Rinse the pipette for the rest of the steps in this lab.



8. Using the rinsed pipette, transfer 250 μ l of Binding Buffer to the microtube containing the supernatant.

Phase 3 – Protein chromatography

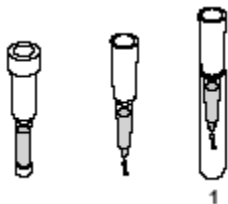
Materials

- 3 – Collection tubes
- 1 – Well rinsed pipette
- 1 – Prepared HIC chromatography column
- Wash Buffer – from instructor
- Elution Buffer (TE) – from instructor

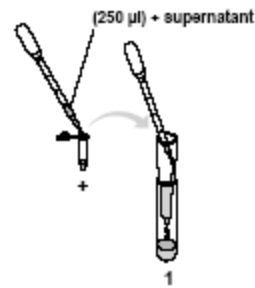
Procedure

*****Important!** To load the supernatant and buffers onto the column, rest the pipette tip against the side of the column, and pipette carefully and slowly, letting the solutions drip down the side of the column.

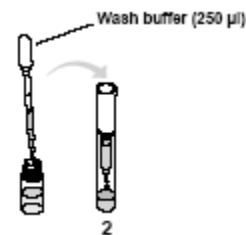
1. Predict what will happen before you complete each of the following steps (4-6) and record your predictions as you go.
2. Label the collection tubes 1, 2, and 3.
3. Remove the caps from the column and let the Equilibration buffer drain into collection tube #1. When the buffer has reached the surface of the HIC matrix, proceed to the next step.



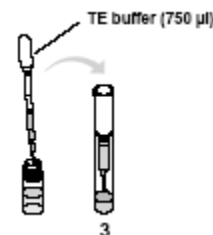
4. Using a new pipette, carefully load 250 μ l of the supernatant with the Binding Buffer into the column. Let the entire volume of supernatant flow into tube 1. Observe the column and tube with a UV light and record your observations.



5. Transfer the column to tube #2. Using a rinsed pipette add 250 μ l of the Wash Buffer to the column. Allow the entire volume to flow into the collection tube. Again, observe the column and tube and record your observations.



6. Transfer the column to tube #3. Using a rinsed pipette, add 750 μ l of the Elution (TE) Buffer and allow the entire volume to flow into the tube. Observe the column and tube one last time and record your observations.



Assignment

Answer questions 1-11 and turn in by _____

1. Provide a completely finished data table that includes a list of samples (8), predictions, observations, and label the table "Table 1" (above table) with a title that describes the contents of the data that is found in the table. (See examples of tables in your text.)
2. Predict what would happen to a single colony of glowing bacteria grown on the LB/amp/ara plates if the colony were transferred to an LB/amp plate.
3. Briefly explain why both liquid cultures fluoresce green?
4.
 - a. What is an operon?
 - b. Explain the relationship between a promoter and an operon.
5.
 - a. What is a clone?
 - b. What are cloning vectors? Provide an example.
 - b. How are cloning vectors used?
6.
 - a. What is Lysozyme?
 - b. Explain how Lysozyme causes a bacterial cell wall to weaken.
 - c. How does freezing aid in rupturing the cell wall?
7.
 - a. What is the purpose or function of using a centrifuge?
 - b. Explain why the supernatant was discarded after the first centrifugation and why the pellet was discarded after the second centrifugation?
8. Briefly describe hydrophobic interaction chromatography and identify its purpose in this lab.

9. Briefly explain the functions of each of the four buffers used in this lab.

10. Explain why it was necessary to use liquid cultures to perform these procedures?

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

Literature Cited

1. Alberts, B., et al, 2002. Molecular Biology of The Cell, 4th Ed. NY, Garland Science.
2. Green Fluorescent Protein (GFP) Purification Kit, Biotechnology Explorer, Instruction Manual, Bio-Rad Laboratories, Inc.

Illustrations

Purification Kit – Quick Guide. Bio-Rad Laboratories. Retrieved October 2, 2005 from <http://www.bio-rad.com>