Green Fluorescent Protein (GFP) Purification

Student Manual

"Bioengineered DNA was, weight for weight, the most valuable material in the world. A

single microscopic bacterium, too small to see with the human eye, but containing the gene for a heart attack enzyme, streptokinase, or for "ice-minus" which pre- vented frost damage to crops, might be worth 5 billion dollars to the right buyer."

Michael Crichton - Jurassic Park

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Lesson 1 Finding the Green Fluorescent Molecule

Genetic Transformation Review

With the pGLO Bacterial Transformation kit, you performed a genetic transformation of E. coli bacterial cells. The results of this procedure were colonies of cells that fluoresced when exposed to ultraviolet light. This is not a normal phenotype (characteristic) for E.coli. You were then asked to figure out a way to determine which molecule was becoming fluorescent under UV light. After determining that the pGLO plasmid DNA was not responsible for the flu- orescence under the UV light, you concluded that it was not the plasmid DNA that was fluo- rescing in response to the ultraviolet light within the cells. This then led to the next hypothesis that if it is not the DNA fluorescing when exposed to the UV light, then it must be a protein that the new DNA produces within the cells.

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, describe cloning.

3. Describe how the bacterial cloned cells on your LB/amp plate differ from the cells on

your LB/amp/ara plate. Can you design an experiment to show that both plates of cloned cells behave similarly and do contain the same DNA?

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

Laboratory Procedure for Lesson 2

Picking Colonies and Growing a Cell Culture

Examine your two transformation plates under the ultraviolet (UV) lamp. On the LB/amp plate pick out a single colony of bacteria that is well separated from all the other colonies on the plate. Use a magic marker to circle it on the bottom of the plate. Do the same for a single green colony on the LB/amp/ara plate. Theoretically both white and green 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 culure tubes and let them grow and multiply overnight.

Your Task

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.

Workstation Daily Inventory Check (✔) List

Your Workstation. Materials and supplies that should be present at your student worksta- tion site prior to beginning this lab activity are listed below.

Instructors (Common) Workstation. Materials, supplies, and equipment that should be present at a common location that can be accessed by your group during each lab activity are also listed below.

Your workstation Number (✔) Transformation plates from pGLO Bacterial Transformation kit (LB/amp/ara and LB/amp) 2 ❑ Inoculation loops 2 ❑ Culture tubes, containing 2 ml growth media 2 ❑ Marking pen 1 ❑ Test tube holder 1 ❑

Instructors workstation

Shaking incubator, shaking water bath, tube roller or rocking platform (optional) 1 ❑ UV light 1 ❑

Laboratory Procedure for Lesson 2 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 culture tubes containing 2 ml 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.

LB/amp LB/amp/ara

+ -

LB/amp LB/amp/ara

4. Cap your tubes and place them in the shaking incubator, shaking water bath, tube roller

or rocker. Let the tubes incubate for 24 hr 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 sec. Then place them in an incubator oven for 24 hr. Lay the tubes down horizontally in the incubator. (If a rocking table or tube roller is available, but no incubator, tape the tubes to the platform or insert in tube roller and let them rock or spin at maximum speed for 24 hr at 32°C or at room temperature for 48 hr. We do not recommend room temperature incubation without rocking or shaking.)

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

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

30 ➔

Cap the tubes. Incubate at 32°C overnight or 48 hr at room temperature.

Lesson 2 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)? Why do you think you picked one of each color? 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 purifying the fluorescent protein.

Lesson 3

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?

Workstations Check (✔) List

Your Workstation. Make sure the correct materials listed below are present at your work- station prior to beginning this lab experiment.

Instructors (Common) Workstation. Materials that should be present at a common loca- tion to be accessed by your group are also listed below.

Your workstation Number (✔) Microcentrifuge tubes 1 ❑ Pipets 1 ❑ Microcentrifuge tube rack 1 ❑ Marking pen 1 ❑ Beaker of water for rinsing pipets 1 ❑

Instructors workstation TE buffer 1 bottle ❑ Lysozyme (rehydrated) 1 vial ❑ Centrifuge 1 ❑ UV light 1–4 ❑

Laboratory Procedure for Lesson 3 1. Using a marker, label one new microcentrifuge tube with your name and period. 2. Remove your two liquid cultures from the shaker or incubator and observe them in nor-

mal room lighting and then with the UV light. Note any color differences that you observe. Using a clean pipet, transfer the entire contents of the (+) liquid culture into the 2 ml microcentrifuge tube also labeled (+), then cap it. You may now set aside your (–) culture for disposal.

3. Spin the (+) microcentrifuge tube 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 pipet, add 250 µl of TE buffer to each tube. Resuspend the bacterial pellet

thoroughly by rapidly pipetting up and down several times with the pipet.

33

1 ml

+ +

250 µl TE

+

+

7. Using a rinsed pipet, 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 micro- centrifuge tube in the freezer until the next laboratory period. The freezing will cause the bacteria to explode and rupture completely.

34

1 drop lysozyme

Lesson 3 Name _____

Review Questions

1. You have used a bacterium to propagate a gene that produces a green fluorescent protein. Identify the function of these items you need in Lesson 3. a. Centrifuge - b. Lysozyme - c. Freezer -

2. Can you explain why both liquid cultures fluoresce green?

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

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?

Lesson 4

Purification Phase 2 Removing Bacterial Debris

The bacterial lysate that you generated in the last lab contains a mixture of GFP and endoge- nous bacterial proteins. Your goal is to separate and purify GFP from these other contami- nating 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 proper- ties of GFP to purify it from the other, less hydrophobic (more hydrophilic or "water-loving") bac- terial 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 sperical 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.

Workstations Check (✔) List

Student Workstations. Make sure the materials listed below are present at your worksta- tion prior to beginning this lab experiment.

Instructors (Common) Workstation. Materials that should be present at a common loca- tion to be accessed by your group are also listed below.

Student workstation items Quantity (✔) Microcentrifuge tubes 1 ❑ Pipets 1 ❑ Microcentrifuge tube rack 1 ❑ Marking pen 1 ❑ Beaker of water for rinsing pipets 1 ❑ HIC chromatography column 1 ❑ Column end cap 1 ❑ Waste beaker or tube 1 ❑ Instructors workstation items Binding buffer 1 bottle ❑ Equilibration buffer 1 bottle ❑ Centrifuge 1 ❑ UV light 1–4 ❑

Laboratory Procedure for Lesson 4 1. Remove your microcentrifuge tube 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 microcentrifuge tube with your team's initials. 2. While you are waiting for the centrifuge, prepare the chromatography column. Before per- forming 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 bot- tom. 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 ml of equilibration buffer to the top of the column, 1 ml at

a time using a well rinsed pipet. Drain the buffer from the column until it reaches the 1 ml 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 min centrifugation, immediately remove the microcentrifuge tube 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 pipet, transfer 250 µl of the supernatant into the new microcentrifuge tube. Again, rinse the pipet well for the rest of the steps of this lab period.

5. Using the well-rinsed pipet, transfer 250 µl of binding buffer to the microcentrifuge tube

containing the supernatant. Place the tube in the refrigerator until the next laboratory peri- od.

Equilibration buffer (add 2 ml)

1 ml

250 µl

+ +

Binding buffer (add 250 µl)

+

Lesson 4 Name _____

Review Questions

1. What color was the pellet in this step of the experiment? What color was the supernatant? What does this tell you?

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

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

Lesson 5

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 at your workstation contains a matrix of microscopic hydropho- bic 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 structure 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)

Workstation Check (✔) List

Your Workstation. Make sure the materials listed below are present at your workstation prior to beginning this lab experiment.

Instructors (Common) Workstation. Materials that should be present at a common location to be accessed by your group are also listed below.

Your workstation Number (✔) Collection tubes 3 ❑ Pipets 1 ❑ Microcentrifuge tube rack 1 ❑ Marking pen 1 ❑ Beaker of water for rinsing pipets 1 ❑ HIC chromatography column 1 ❑ Column end cap 1 ❑ Beaker to collect waste 1 ❑ Instructors workstation Wash buffer 1 vial ❑ Equilibration buffer 1 vial ❑ TE buffer 1 vial ❑ UV light 1–4 ❑

Lesson 5 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 pipet, carefully load 250 µl of the supernatant (in binding buffer) into the

top of the column by resting the pipet 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.

250 µl supernatant in binding buffer

40

+

Collection tube 1

2 3

Waste tube

4. Transfer the column to collection tube 2. Using the rinsed pipet 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 pipet, add 750 µl of TE (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 plastic wrap the tubes and place in the refrigerator until the next laboratory period.

Collection tube 2 Wash buffer (250 µl)

1 3

Add 750 µl TE (elution buffer)

1

2

1

3

2

Collection tube 3

Lesson 5 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.

	Observations Under UV Light	Collection Tube Number	Prediction (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.