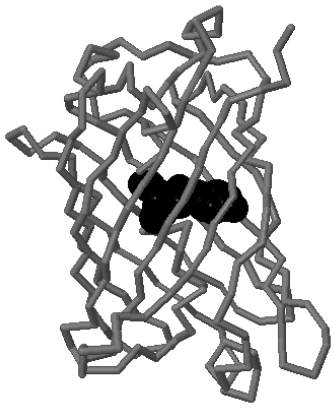
Green Fluorescent Protein

Overview

In today's lab, you will purify and study the protein "Green Fluorescent Protein" (GFP) as an illustration of protein purification and protein properties.

Background I: Green Fluorescent Protein

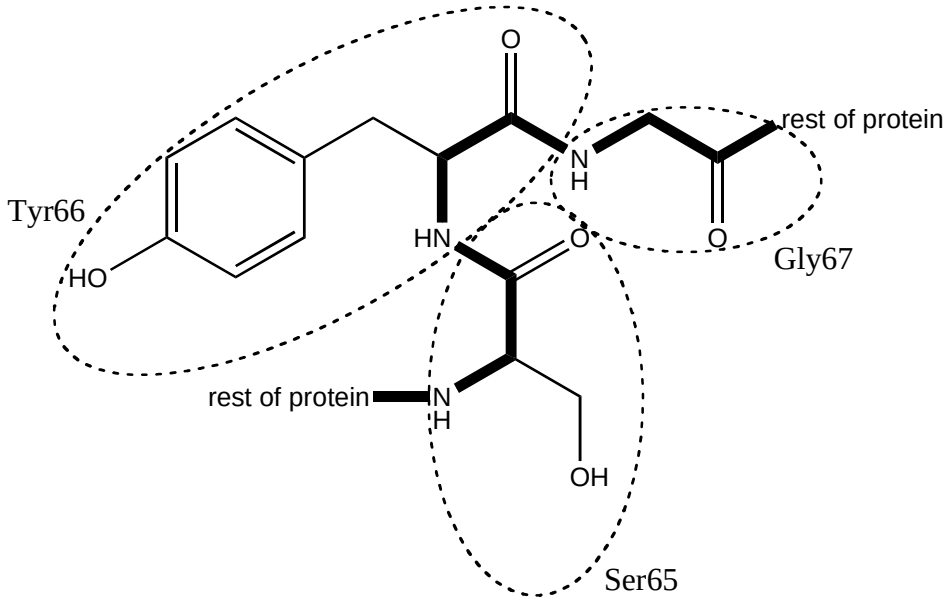
Fluorescence is a process where a molecule absorbs light at one wavelength and then gives off light at a longer wavelength. Most proteins are not fluorescent because, in order to be fluorescent, a molecule must have particular structural elements that are not normally found in proteins. However, the jellyfish *Aequoria victoria* produces a protein, called Green Fluorescent Protein (GFP), that absorbs ultraviolet (UV) light and gives off longer wavelength green light. This makes GFP an interesting protein for study. GFP has been used in a wide variety of experiments exploring protein structure and folding, gene expression, development, etc. In this lab, we will use GFP as an example of a 'typical' protein for biochemical study.



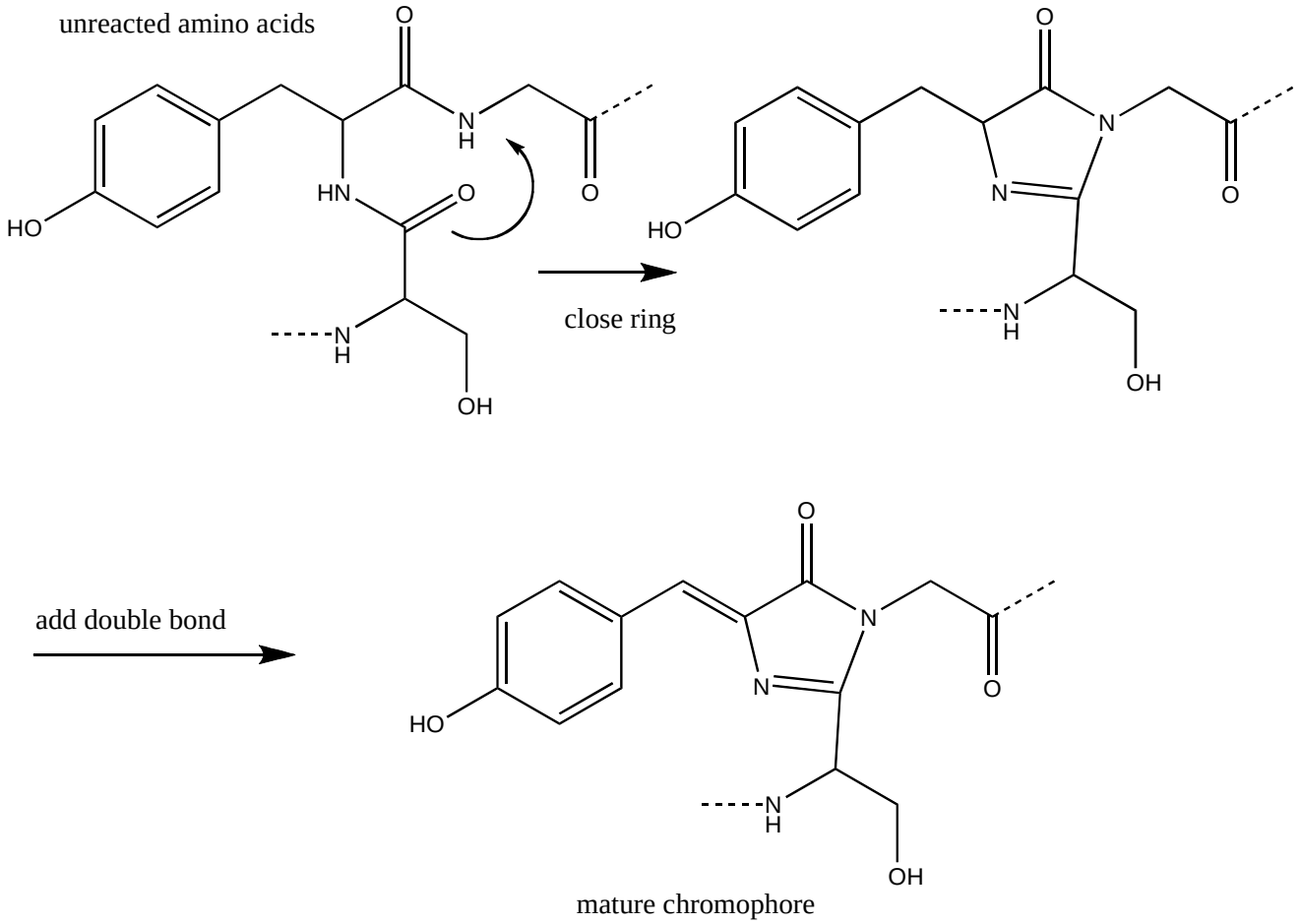
GFP is 238 amino acids in a single protein chain. It is fluorescent because it contains a *chromophore* ("color carrier"), a portion of the molecule that fluoresces. The bulk of the protein surrounds the chromophore and protects it from water, which would prevent the fluorescence.

The structure of GFP is shown at right (better views are available at the OLLM for this lab). The chromophore is shown in black spacefill representation; the remaining amino acids are shown as ribbon representations of the backbone. The protein is not hollow; it was rendered this way to show how the protein protects the chromophore from water.

Interestingly, the chromophore is formed from the side chains of three amino acids as the protein folds. The side chains of Ser65, Tyr66, and Gly67 react and link via *covalent* bonds between the side chains to form the chromophore. This unusual reaction - typically side chains only interact via *non-covalent* interactions - is shown below. First, the 3 amino acids after folding, but before the reaction, look like this (backbone shown in bold):



The reaction is shown below:



Background II: Making a "protein of interest"

In general, biologists either study the properties of proteins or use proteins as reagents to accomplish various tasks. In either case, the protein must be *pure* so that researchers can be sure that their observations are due to the protein of interest and not due to various contaminants.

Unfortunately, it is not possible to synthesize large quantities of proteins chemically in a pure form; only cells can make proteins easily and cheaply. This means that producing a pure protein involves three steps:

1) **Get the cells to make or "express" the protein**. Any given cell will not necessarily make the desired protein. In our case, it is difficult to obtain jellyfish that make GFP, so we will use the bacterium *E. coli*, which are easy to grow in large quantities. Since normal *E. coli* don't contain the gene for GFP, we will use a strain of *E. coli* to which we have added the GFP gene by *transformation*.
2) **Break open the cells to let the protein out**. Bacterial cells are enclosed by a tough cell wall and a cell membrane. Both must be broken to let the protein out. We will digest away the cell wall with the enzyme lysozyme. We will then break the cell membrane with repeated cycles of freezing and thawing - the ice crystals formed rupture the membrane. These techniques are fierce enough to release the GFP without damaging it.
3) **Purify the protein from the other cell contents**. Cells contain thousands of other proteins, DNA, RNA, small molecules, etc. We will separate GFP from all this other 'gunk' in two steps. First, we will discard any material that is not water soluble. We will then separate the GFP from the water-soluble material using *chromatography* that takes advantage of its hydrophobic properties (see next section).

Background III: Hydrophobic Interaction Chromatography (HIC)

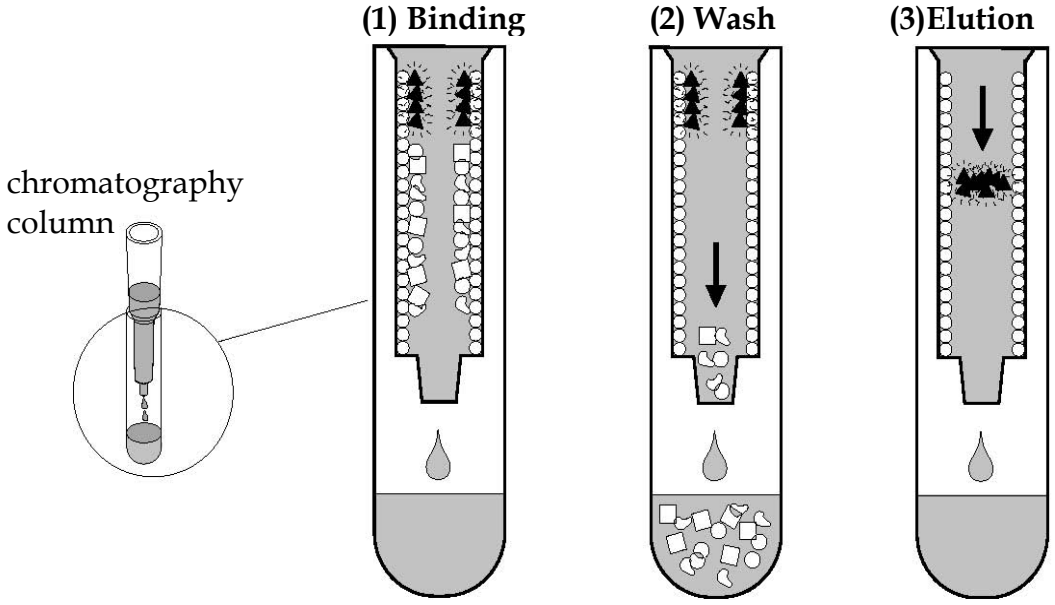
Chromatography is a term that describes a huge and diverse set of methods for separating molecules based on their different properties. We are using affinity chromatography today. In it's simplest form, it consists of three steps:

1) **Binding**. A mixture of molecules is exposed to a solid material. The molecule of interest binds to the solid via some non-covalent interaction; the others do not.
2) **Wash**. The solid is rinsed with a solution (wash buffer) which allows the molecule of interest to remain bound to the solid while washing off the unwanted molecules.
3) **Elution**. The solid is rinsed with a different solution (elution buffer) which releases the molecules of interest from the solid. The resulting solution contains (ideally) only the molecule of interest.

In this lab, we will use the fact that GFP has significantly more hydrophobic amino acids on its surface than most proteins. Therefore, it will bind - via the hydrophobic effect - to a solid that also has a hydrophobic surface more tightly than most other proteins.

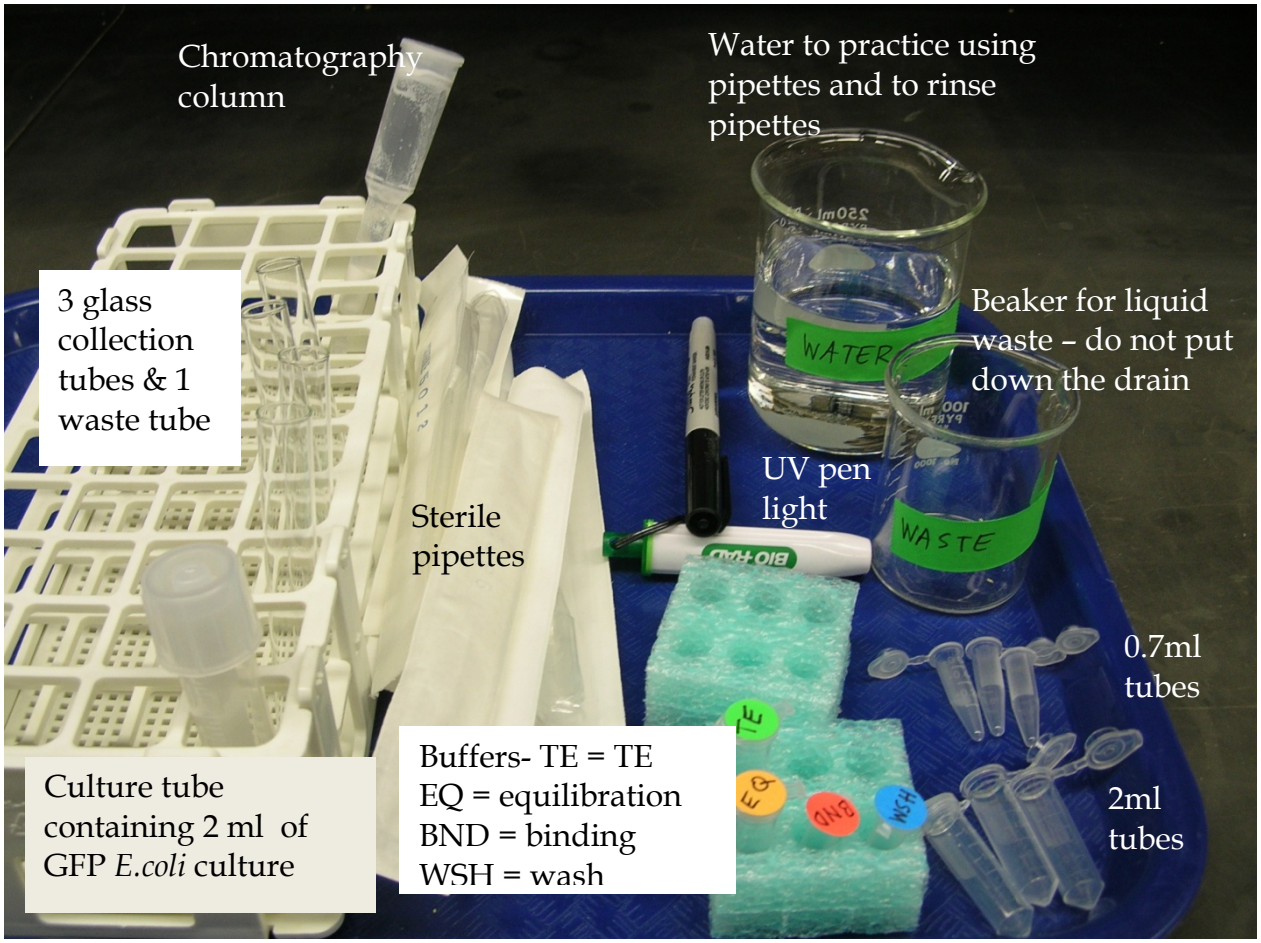
We will also use another important property of the hydrophobic effect to selectively bind and elute GFP. In solutions with a *high* salt concentration, the hydrophobic effect is *strengthened*; in solutions with *low* salt concentrations, the hydrophobic effect is *weakened*. It turns out that it doesn't matter what the salt is; we will use ammonium sulfate ($(NH_4)_2SO_4$) because it is very soluble in water and does not harm most proteins.

To make the process easier to carry out, the solid material called the "resin" will be packed into a tube called a *chromatography column*. Here's how it will work (the hydrophobic solid resin is indicated by circles; GFP is indicated by black triangles; other molecules are indicated by the other shapes):



1) **Binding** - a solution containing the cell contents, including GFP, is run through the column in *high salt*. GFP binds via the hydrophobic effect; most other molecules don't. Those that don't stick *flow through* the column and are discarded.
2) **Wash** - a solution of *medium salt* is run through the column. GFP remains bound because it has more hydrophobic amino acids on its surface while any remaining other molecules wash out. The molecules in the wash are discarded.
3) **Elution** - a solution of *low salt* is run through the column. This disrupts the hydrophobic interaction between the column and GFP so GFP comes off the column in relatively pure form for study.

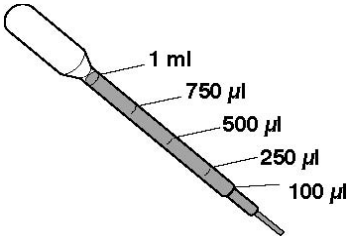
Kit of Parts:



Procedure I: Pipette Practice

In order that your experiment work, it is very important to measure the quantities of various reagents carefully. Although the pipettes we will be using look easy to use, you will need to learn several tricks to using them properly. For this reason, we will start with a little practice.

The pipette you will be using is shown at right. It measures volumes from 100µl (micro-liter; 1 millionth of a liter) to 1000µl or 1ml (milli-liter; 1 thousandth of a liter). It is calibrated with marks on the side.



To use one, you **first** squeeze out the air **before** you put it in the liquid (otherwise you make bubbles, mix your sample, and cause chaos). You then put it in the liquid and release the bulb **slowly** to draw up the desired amount of liquid. You then **gently** squeeze the bulb to release the measured volume of liquid. You should do the following until all the members of your group can do this with ease.

a) One person puts 500µl of water in a microtube.
b) Another person takes out 250µl, discards it, and takes the remaining 250µl. There should be no bubbles in the pipette (indicating that less than 250µl remained) nor should there be any liquid left (indicating that more than 250µl remained).

Procedure II: Purification of GFP

WARNINGS:

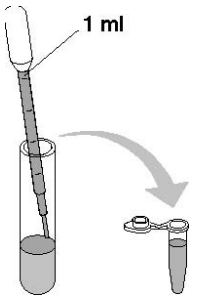
1. In general, the lab is unforgiving of mistakes like using the wrong solution or taking the wrong amount. The construction folks at "This Old House", say "Measure twice; cut once." We'll adapt this to "*Check twice; pipette once*".
2. Although the *E. coli* strain we use is non-pathogenic (it is not known to cause disease in healthy individuals), you should be careful with it. *Always wear gloves, don't eat or drink in lab, and wash your hands thoroughly when you are all done.*
3. We will be using ultraviolet light in the lab. *Never shine the UV light directly into anyone's eyes.*
4. We will be using liquid nitrogen in this lab. It is very cold (-321° F, -196° C).
   - *Always use insulating gloves when working with liquid nitrogen.*
   - *Always use liquid nitrogen with your TA's supervision.*
   - *Do not get liquid nitrogen on your hands or other body parts.*

Last night, the set up staff started 2ml cultures of *E. coli* that contain the GFP gene. They have been growing and producing GFP until there are roughly 10 billion cells in the culture tube. You will start with this culture.

• <u>Concentrate the bacterial cells in a suitable buffer</u>

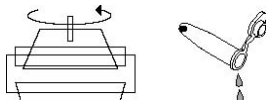
1) Using a marker, label one new microtube with your group initials.

2) Remove your liquid culture from the shaker or incubator and observe it in normal room lighting and then with the UV light. The GFP in the bacteria should show green fluorescence. Using a clean pipette, transfer <u>the entire contents</u> of the liquid culture into the microtube, then cap it.



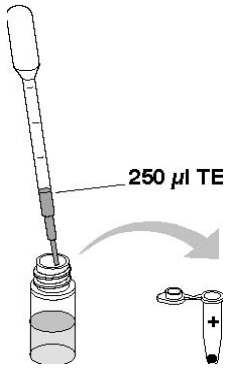
3) Spin the microtube for 5 minutes in the centrifuge at maximum speed. Be sure to balance the tubes in the machine (your TA will help with this). If you do not know how to balance the tubes, do not operate the centrifuge.

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 to the waste beaker, 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 the tube (check twice; pipette once). Resuspend the bacterial pellet thoroughly by vortexing the tube (your TA will demonstrate). You are done when the pellet is no longer visible on the side of the tube.



• <u>Break open ("lyse") the bacterial cells</u>

7) Using a rinsed pipette, your TA will add 1 drop of lysozyme solution 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.

8) Let tube sit 5 minutes at room temperature.

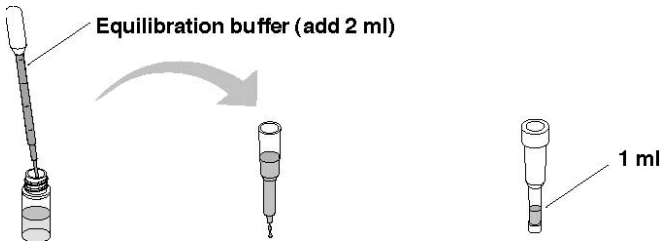
9) Freeze and thaw the cells. Dip the tube in dry-ice/ethanol or liquid nitrogen - BE CAREFUL NOT TO DIP YOUR FINGERS! Hold it there until it freezes - about 10 seconds. Take the tube out and thaw it in your hands until it is fully melted. Freeze and thaw one more time to be sure the bacteria are fully lysed.

• <u>Purification Part 1: Remove insoluble material</u>

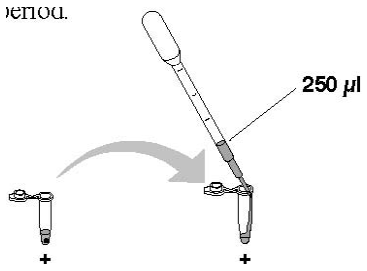
10) After making sure that the suspension is completely melted, place the tube in the centrifuge, have your TA balance the tubes, and pellet the insoluble bacterial debris by spinning for 10 minutes at maximum speed. Label a new microtube with your team's initials.

11) While you are waiting for the centrifuge, prepare the chromatography column. Before performing the chromatography, shake the column vigorously to resuspend the beads of resin. 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 then the bottom cap of the chromatography column. Allow all of the liquid buffer to drain from the column (this will take ~3–5 minutes). **Keep track of both the top can bottom cap, these columns are reusable.**

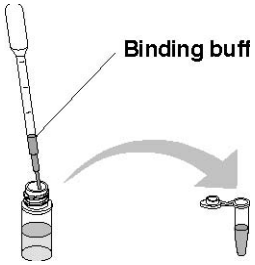
12) Prepare the column by adding 2 milliliters of **Equilibration Buffer** (check twice; pipette once) to the top of the column, 1 milliliter at a time using a well rinsed pipette. Drain the buffer from the column until it reaches the 1 milliliter mark which is just above the top of the white column bed. Cap the top and bottom of the column while you wait for the centrifuge to stop.



13) 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.

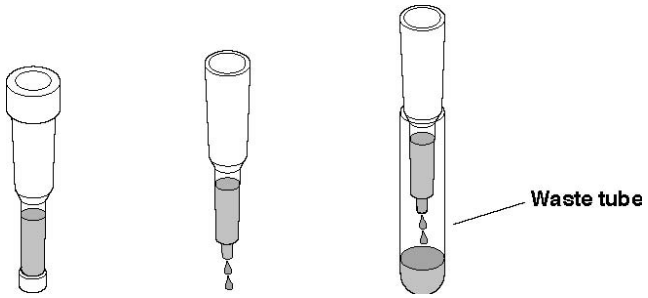


14) Using the well-rinsed pipette, transfer 250 µl of **Binding Buffer** (check twice; pipette once) to the microtube containing the supernatant.



• Purification Part 2: Hydrophobic Interaction Chromatography

15) 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 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.

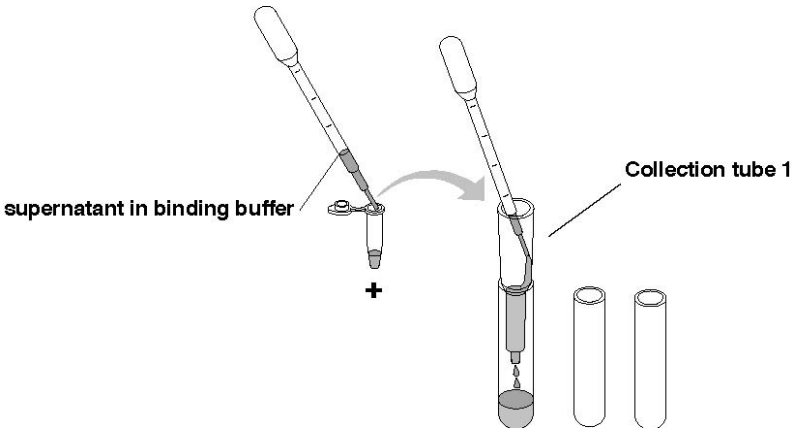


You can create a "paper crutch" by folding a small piece of paper, about the size of a match stick, and wedging it between the column and the collection tube. This crutch makes it impossible for an air tight seal to form, and insures that the column will flow.

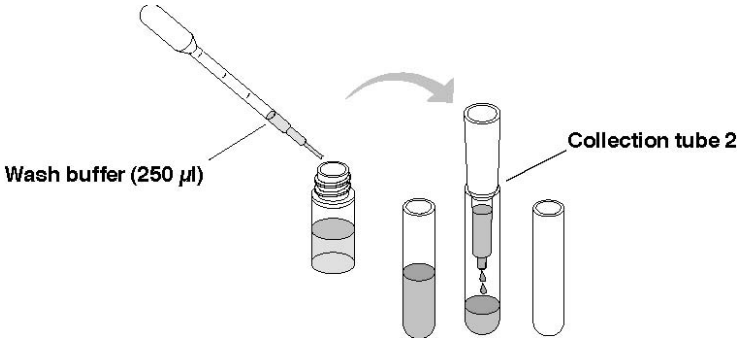
16) Based on the description in the background section of the lab manual, predict which tube the GFP will end up in. Explain your reasoning.

1 2 3

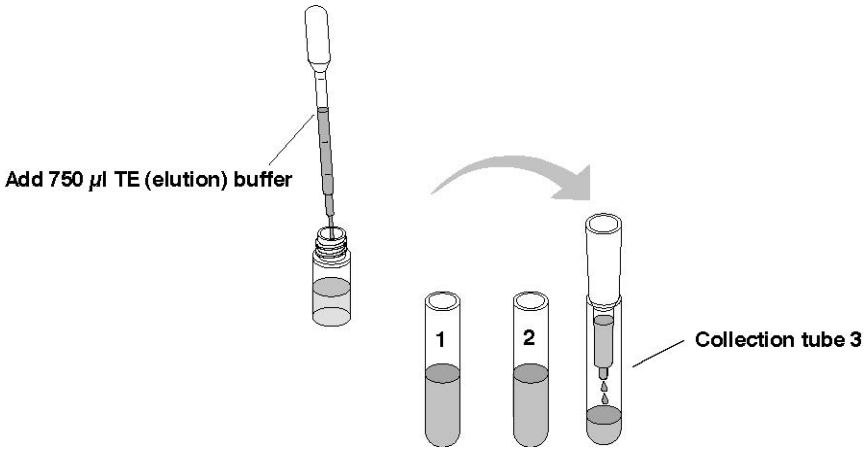
17) Using a new pipette, carefully load **all** of the supernatant into the top of the column by resting the pipette tip against the side of the column and letting the supernatant drip down the side of the column wall. Let the entire volume of supernatant flow into tube 1. Examine the column using the UV light. Where is the GFP?



18) 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. Examine the column using the UV light. Where is the GFP?



19) 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. Examine the column using the UV light. Where is the GFP?



20) Examine all of the collection tubes using the UV lamp and note any differences in color between the tubes. Do these results match your prediction from step (16)?

21) You now have purified GFP in one of your tubes. You can then study it in the remainder of the lab.

Procedure III: Studies of Purified GFP

• Temperature denaturation of GFP

If you heat GFP (or any protein), eventually you reach a temperature where the protein *denatures* - where the energy of the molecules is sufficient to disrupt the *non-covalent* interactions that give the protein its shape. The protein is then said to be *denatured*. Different proteins denature at different temperatures depending on the particular non-covalent bonds that give them their shape. When GFP denatures, the chromophore is exposed to the surrounding water and it is no longer fluorescent.

22) Take three small samples of your purified GFP - about 100µl each - and put each in a new tube. Label each tube with your initials.

23) Put one tube at in the 50°C water bath, one in the 75°C water bath, and one in the boiling (100°C) water bath. Let them sit for 5 minutes.

24) Observe the tubes under UV light. Which are fluorescent; which are not? What does this tell you about the temperature required to denature GFP?

• Digestion with protease enzymes or other solutions provided:

Enzymes called *proteases* break peptide bonds and are used by all living organisms to digest proteins into amino acids. As GFP is digested into amino acids, the chromophore will become exposed to water and stop fluorescing.

25) Make a solution of a protease or other solution provided - meat tenderizer or dietary supplement. Put a little of the protease powder - about the size of a grain of rice - in 200µl TE and dissolve it completely. Add about 100µl of this to the remainder of your GFP and observe it under UV light. How long does it take to digest the GFP?

26) Clean up; you are done.

GFP Follow-Up Assignment

Although you will perform these experiments as a group, each member of the group must turn in an individual write-up , which must be in your own words.

You will need to look at the 3-d structure of GFP. You can find this on the web--see the "GFP 3-d structure" link for this lab.

Answer the following questions:

1) In which tube (1, 2 or 3 from Step 20) would you expect to find the GFP if you had used TE (low salt) buffer in Step 14 instead of Binding Buffer and used Binding Buffer (high salt) instead of TE in Step 19? Explain your reasoning.

2) Look at View (2) of GFP on the GFP 3-d Structure web site. It shows hydrophilic amino acids in white and hydrophobic amino acids in red. Note the large number of hydrophobic amino acids on the surface. Suppose you engineered a "GFP-x" protein that had no hydrophobic amino acids on its surface; in which tube (1, 2 or 3 from Step 20) would you expect to find the GFP-x? Explain your reasoning.

3) Look at View (3) of GFP on the GFP 3-d Structure web site. It shows three amino acids in purple. These three amino acids were altered from the original GFP sequence isolated from the jellyfish *Aequoria victoria* to give the version we used in lab today. The original *Aequoria victoria* GFP had Phe at position 99, Met at position 153, and Val at position 163. This protein was not useful in the lab because it was not very water-soluble. The version we used today has Ser at position 99, Thr at position 153, and Ala at position 163 and is much more water-soluble than the original. Explain why these changes would make the protein more water-soluble.

4) Look at Views (6), (7), and (8) of GFP on the GFP 3-d Structure web site. They show the locations of various amino acids in the GFP molecule. Based on this, explain why mutations that alter amino acids 48 or 80 have no effect on the color of GFP while changing 66 to histidine changes the color dramatically (in fact, it makes it into blue fluorescent protein, but you don't have to explain why it's blue - just why you'd expect it to change color).