

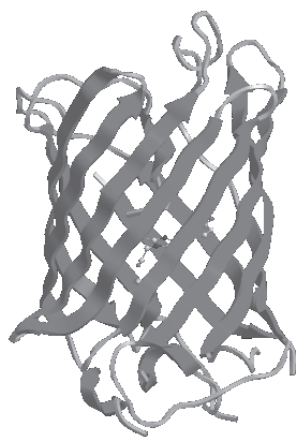
# Purification of mFP

## from an Overnight Culture

**When scientists at a therapeutics company, like Amgen, have successfully identified a promising therapeutic protein, two objectives would be to locate and isolate the gene that encodes the protein. Once isolated, the gene is inserted into a plasmid so that the gene can be cloned, as additional copies of the gene will be needed for ongoing studies. The *rfp* gene was cloned in a plasmid called pKAN-R. pKAN-R is a *cloning vector*, a plasmid that has been engineered to replicate in high numbers within the bacterial cell.**

Later, cloned genes are inserted into plasmids that have been engineered specifically for protein expression in bacteria or other suitable organism. Such plasmids are known as *expression vectors*. pARA-R is an *expression vector* and carries the cloned *rfp* gene in a specific plasmid location, which allows the bacterial cell to produce mutant fluorescent protein.

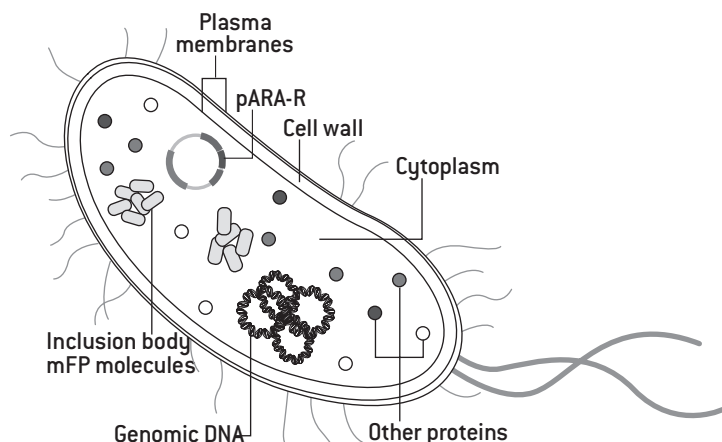
Transformed cells are allowed to express the protein in an overnight culture and then lysed (broken open) to release the newly synthesized protein from the cell. The protein is isolated from the other cytoplasmic proteins, purified and tested for activity.



You have already completed much of the work that parallels this drug discovery scenario. The bacterial cells that have been growing in the LB/amp/ara broth have been expressing mFP and are now ready to be lysed (day *one* of Lab 7) and the mFP purified (day *two* of Lab 7) using column chromatography.

Mutant fluorescent protein is a molecule that is about 238 amino acids in size. The native (as it exists in *Discosoma*) protein is shaped like a cylinder with the fluorescent region, called the fluorophore, located in the center of the cylinder.

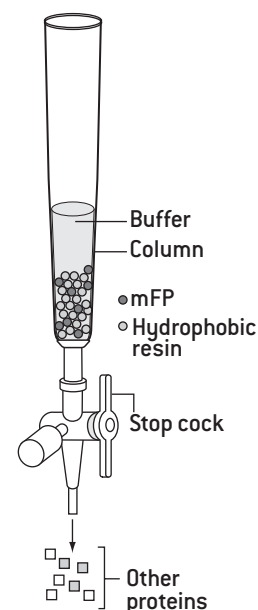
In order to purify a molecule from other proteins present in the cell, one needs to look at how groups of molecules differ from one another and how these differences can be used to effect separation.



One molecular attribute commonly used in purification is protein *hydrophobicity*. The term *hydrophobicity* is related to the behavior of a molecule in water. If a molecule is *hydrophobic*, it fears water while hydrophilic molecules love water. For example, oils, waxes and fats are hydrophobic; they do not dissolve in water. Table sugar and table salt are hydrophilic, and they dissolve quickly in water.

It is not uncommon for large molecules, such as proteins, to have regions that are hydrophobic and other regions that are hydrophilic. If these proteins are placed in water, the hydrophobic regions tend to “bend away” from water while their hydrophilic regions try to bend toward the water. To a large extent, it’s the bending of the protein’s amino acid chain that is responsible for its overall *conformation* or molecular shape, with hydrophobic regions “hiding” in the interior of the molecule and water-loving regions on the outside.

It’s important for you to know that a bacterial cell contains many different kinds of proteins. The diagram below is greatly simplified as it indicates only a few kinds. The problem, however, is how do you separate a single protein, like mFP, from all of the others? A typical bacterium may contain more than a 1000 different kinds of protein. The use of the recombinant expression



vector, pARA-R, will make mFP isolation somewhat easier: *The E. coli* cells you have cultured will have been made to produce a disproportionately high concentration of mFP.

Protein purification can use hydrophobicity to separate and purify protein molecules. One common purification procedure that uses differences in hydrophobicity to separate proteins is called column **chromatography**. Column chromatography uses a plastic or glass cylinder into which a separating medium, referred to as “resin,” is placed. The specific type of resin used will vary depending on what type of protein is being purified. In this lab, we will be using a resin bed consisting of small hydrophobic beads. Mutant fluorescent protein is highly hydrophobic and when mFP is placed into a solution of *high salt concentration*, the mFP molecule is distorted in a way that will cause the hydrophobic regions of the molecule to adhere to the hydrophobic resin in the chromatography column. The hydrophilic proteins made by the cell continue down the column, through the resin

without sticking to the resin bed and are flushed away.

Once the mFP is trapped in the resin bed, the column can be washed with a solution of lower salt concentration to elute (wash out) moderately hydrophobic molecules from the column. This column *wash buffer* will have a slightly *lower salt* concentration than the solution used to bind mFP to the resin but not so low as to wash the mFP from the resin. Finally, we can use a solution of *very low salt* concentration to elute or release the mFP from the resin beads. Under low salt concentration, the hydrophobic regions of the mFP molecule point toward the interior of the molecule, thus releasing the mFP from the hydrophobic resin in the column.

Industrial protein purification is much more complex than this mFP purification protocol, but the principles employed by industry are similar. The mFP sample that you obtain from this purification does contain other proteins. The procedure, however, has removed many of the other proteins present in the bacterium’s cytoplasm.

## Materials

### REAGENTS

2 mL LB/amp/ara culture of *E. coli* (Lab 6)  
 Lysis buffer (TE, NaCl, SDS)  
 Binding buffer, 4 M  $(\text{NH}_4)_2\text{SO}_4$   
 Column equilibration buffer, 2 M  $(\text{NH}_4)_2\text{SO}_4$   
 Column wash buffer, 1.3 M  $(\text{NH}_4)_2\text{SO}_4$   
 Elution buffer, 10 mM TE  
 10% Bleach or other disinfectant  
 TE (same as elution buffer)

### EQUIPMENT & SUPPLIES

Centrifuge  
 P-200 pipette and tips  
 P-1000 pipette and tips  
 Chromatography column  
 Microfuge tube rack  
 1.5 mL microfuge tubes  
 Permanent marker  
 6 mL waste collection tube  
 Cell-contaminated waste bag

## Methods

### Preparation of cell lysate from the overnight liquid culture

- 1 Obtain 1 mL LB/amp/ara culture from your teacher.
- 2 Examine this culture. What color is the culture?
- 3 Place this tube into the centrifuge.  
**Important:** You or your teacher will need to make certain the tubes have been placed in the rotor in a balanced configuration before the centrifuge is turned on. Centrifuge the microfuge tubes for **5 minutes**.
- 4 After the rotor has stopped, **carefully** remove your tube to avoid disturbing the cell pellet.
- 5 **Determine the location** of the mFP. Is it in the bacterial cell pellet, or in the supernatant (the liquid **above** the cell pellet)?
- 6 Once you've determined the location of the mFP, carefully decant (pour off) the supernatant into the beaker containing disinfectant. Do this without disturbing the cell pellet.
- 7 Obtain a second 1 mL aliquot of the overnight culture and repeat steps 3–6.
- 8 Pick up a tube of “Elution buffer” and “Lysis buffer” from your teacher.
- 9 Invert the microfuge tube containing the cell pellet and, using a small piece of paper towel, try to wick away as much of the liquid as you can from your microfuge tube **without touching** the cell pellet. Discard the used towel in the “cell-contaminated waste” bag.
- 10 Using the P-200 pipette (set at “1-5-0”) and a clean tip, transfer 150  $\mu$ L of elution buffer to the cell pellet. Close the cap tightly.
- 11 Resuspend the cells by dragging the tightly capped microfuge tube briskly across the surface of the microfuge tube rack. You may need to do this several times to resuspend the cells. Examine the tube carefully to make certain there are no visible clumps of cells.
- 12 Using the P-200 pipette (set at “1-5-0”), transfer 150  $\mu$ L of lysis buffer to the resuspended cells. Lysis buffer will dissolve *E. coli*'s plasma membrane which helps to break open the cells. After adding the lysis buffer to the cells, cap the tube tightly and drag the tube vigorously across the plastic tube rack several times to mix.
- 13 Check to see if you have labeled this tube with your group number and class period. Give the tube to your teacher. The cells will be left to incubate at room temperature overnight.

**Incubate cells overnight at room temperature.  
Cells can then be frozen until the next lab.**

# Purification of mutant fluorescent protein from the cell lysate

## Getting the materials

- 1** Organize your group for multi-tasking.
  - **Person A** checks to see if the following reagents are at your workstation. These reagents will be shared with another group.
    - Binding buffer
    - Equilibration buffer
    - Wash buffer
  - **Person B** collects the lysed cells from your teacher; these cells were frozen overnight. This person should take the cells to the centrifuge to pellet the cell debris.
  - **Person C** collects the following supplies:
    - 2 • 1.5 mL microfuge tubes. Label one tube “*mFP*” and the other “*super*”
    - 1 • 6 mL waste collection tube (This may be already in the plastic tube rack.)

## Preparing the column

- 2** Set up your chromatography column as directed by your teacher, being careful not to dislodge the stopcock attached to the lower portion of this tube.
- 3** Set the waste collection tube or container under the stopcock. Carefully open the column by turning the stopcock valve and allow the equilibration buffer to begin draining from the column. **Leave about 1mm of this liquid above the resin bed to avoid drying out the resin in the column.**
- 4** Your chromatography column is now ready for the mFP sample. While you are waiting for the mFP sample, be certain that the fluid is not draining from the column. If the waste collection tube is filled with liquid, this is a good time to dump the liquid down the sink.

## Preparing the mFP sample

- 5** Centrifuge the cell lysate for **five minutes** to pellet the cell debris. You or your teacher will need to check the rotor to be certain it is balanced before closing the lid and spinning. **Balancing these tubes before centrifugation is very important.**
- 6** After centrifugation, pick up your microfuge tube. Examine the microfuge tube. Where is the mFP: supernatant or cell pellet?
- 7** **Without disturbing the cell debris pellet**, carefully remove 200 $\mu$ L of supernatant using the P-200 pipette (set at “2-0-0”) and a clean tip. Do this without transferring any cell debris. If you dislodge the debris pellet, you will have to centrifuge the tube again. Dispense the 200 $\mu$ L of clean, debris-free supernatant into a 1.5 mL microfuge tube labeled “*super*” (one of your group members should have labeled this tube).
- 8** Replace the pipette tip on the P-200 and add 200 $\mu$ L of **binding buffer** to the supernatant you dispensed in the tube labeled “*super*.” Mix the binding buffer with the supernatant by gently pumping the solutions in and out using this pipette.
- 9** Using the p-1000 pipette (set at “0-4-0”) and a clean tip, add 400 $\mu$ L of this solution, mFP supernatant/binding buffer, to the prepared column using the same pipette you used to mix the solutions. Do this without disturbing the surface of the resin bed by dispensing the solution down the side of the column.
- 10** Without allowing the column to run dry, open the stopcock and allow the solution in the column to drain into the waste collection tube. **Leave about 1 or 2 mm of buffer above the resin bed.**

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- 11 Examine the column and locate the mFP. Is the mFP spread throughout the resin bed or does it appear to be restricted to a single band?
- 12 Using the P-1000 pipette (set at "1-0-0"), add 1000 $\mu$ L (=1ml) of **wash buffer** gently down the side of the column. Without allowing the column to run dry, allow this buffer to drain from the column, **leaving 1 or 2 mm of buffer above the resin bed.**
- 13 Examine the column and locate the mFP. Has the location of the mFP changed in the resin bed? The wash buffer will elute some of the less hydrophobic proteins off the column. The wash buffer's salt concentration is less than the binding buffer but not so low as to cause the mFP to release from the resin.
- 14 Using the P-1000 pipette (set at "1-0-0") and a clean tip, add 2 x 1000 $\mu$ L (=2ml total) of **elution buffer** gently down the side of the column. As the mFP begins to drip from the tip of the stopcock, collect the protein in the tube labeled "*mFP*." Collect only the red eluate into this tube. Cap the tube when you have collected all the mFP.
- 15 After all the mFP has been collected, add 2000 $\mu$ L (=2ml) of **equilibration buffer** to the column using the P-1000 pipette and a clean tip. This will help prepare the column for the next class.
- 16 Cap the column tightly.
- 17 The solution in the waste collection tube can be discarded down the sink.
- 18 All microfuge tubes, except the one containing your mFP, should be discarded in the cell-contaminated waste bag.
- 19 Compare your tube with mFP tubes from other groups. Is there a difference in intensity of color from sample to sample?

# Conclusions

- 1** What characteristic of mFP is used as the basis for separation by column chromatography?  
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- 2** Following centrifugation of the cell lysate, was the mFP localized in the supernatant or in the cell debris pellet?  
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- 3** When would the hydrophilic proteins have been eluted from the column?  
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- 4a** Does the eluate containing your mFP appear less bright or brighter than it did in the cell lysate following centrifugation?  
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- 4b** If there is a noticeable difference in the intensity of the red color, what might account for the difference?  
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- 5** How might the column be adjusted or modified to increase the *purity* of the mFP sample?  
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- 6** Although this laboratory involved the expression of a sea anemone gene, cite some examples of human proteins that could potentially be expressed and purified using similar methods.  
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