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Quality Control Testing of Purified RFP

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ABE Master Teacher Fellowship Program

AMGEN® Biotech Experience

Scientific Discovery for the Classroom

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The projects designed by the 2022–23 ABE Master Teacher Fellows are a compilation of curricula and materials that are aligned with Amgen Biotech Experience (ABE) and prepare students further in their biotechnology education. These projects were created over the course of a 1-year Fellowship in an area of each Fellow's own interest. Each is unique and can be adapted to fit the needs of your individual classroom. Objectives and goals are provided, along with expected outcomes. Projects can be used in conjunction with your current ABE curriculum or as an extension.

As a condition of the Fellowship, these classroom resources may be downloaded and used by other teachers for free. The projects are not edited or revised by the ABE Program Office (for content, clarity, or language) except to ensure safety protocols have been clearly included where appropriate.

We are grateful to the ABE Master Teacher Fellows for sharing their work with the ABE community. If you have questions about any of the project components, please reach out to us at ABEInfo@edc.org, and we will be happy to connect you with the author and provide any assistance needed.

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Scientific Discovery for the Classroom

San Francisco Bay Area

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Versions of This Lab:

Native PAGE and SDS-PAGE

Or

SDS-PAGE

Materials Notes:

I use Bio-Rad as my primary supplier for non-ABE materials, so most chemicals and equipment referenced will be Bio-Rad products. You can use any protein standard, running buffer, Laemmli buffer, staining solution from any supplier, and whatever PAGE gels work with your vertical electrophoresis units. You can also make your own TGS buffer.

I do want to explicitly recommend using the BioSafe Coomassie Stain. The timing windows in the protocol (1-hour staining time, 1-hour destaining time) is specifically for this stain and may not apply to others. This chemical also doesn't require special storage, although it should still be collected as hazardous waste, like all protein stains.

I also want to explicitly recommend NOT using the Flynn Scientific protein stain. This requires the purchase (or making) of additional Protein Destaining solution. Both need to be stored in a flammables cabinet and both must be collected as hazardous waste. It is significantly more difficult to destain these gels and can take 24 hours or longer. A photo comparison of a gel stained with BioSafe Coomassie and Flynn Scientific Protein stain is included in the "Representative Results" portion.

If you are purchasing from Bio-rad, I recommend purchasing their "pGLO SDS-PAGE Extension Kit," which includes the following:

- Powdered DTT
- Laemmli Buffer
- Precision Plus Protein Kaleidoscope standards
- BioSafe ™ Coomassie Stain
- 10x TGS

The only consumables you would need to purchase separately would be the precast PAGE gels.

The Precision Plus Protein Kaleidoscope standard is a good choice for this lab because some of the ladder bands are UV reactive, and you will be able to see the ladder on those photos prior to staining.

The lab that follows uses the pGLO SDS-PAGE extension kit and Mini-PROTEAN TGX ™ 4-20% gels.

Advanced Preparation

SUPER and RFP tube Preparation

1. You will need to add a step to ABE Complete series Lab 6, Part B to collect a fraction of the supernatant. Have students label a second SUPER tube in Step 3. After collecting the supernatant fraction for column chromatography (200 µL, Step 8), students should transfer the remaining supernatant to the second SUPER tube to be stored until the day of the lab. A minimum quantity of 65 µL is required.
2. Collect the RFP tubes at the end of the lab, after Step 21 and store until performing this lab

Both RFP and SUPER tubes should be stored at –20°C until ready to perform this lab. It is not recommended to freeze and thaw samples multiple times, as this may affect lab results

Chemical Preparation and Aliquoting Guide **Please follow all safety precautions presented by the reagent manufacturer, all protocols or MSDSs have been linked in this document.**

1. **Prepare Laemmli Buffer (LB)** by adding DTT. Each lab group will need 20 µL of LB so multiply the total number of lab groups by 25 to get the total quantity to prepare.
   a. Ex. 16 lab groups would require 400 µL of LB
   b. Measure out 0.010 g of DTT per 1 mL of LB needed and add to the corresponding volume of 2x Laemmli buffer.
      i. Minimum qty made should be 1 mL for more accurate measurements of DTT
   c. Aliquot 25 µL of prepared Laemmli Buffer into microcentrifuge tubes (labeled LB).
      i. Note: I prefer to aliquot enough solution for all of my class periods, so I would aliquot 50 µL into 8 tubes, one per lab table for my 16 lab groups.
   d. Store this in the refrigerator if prepared ahead of time. It can stay at room temp throughout the day. If classes meet on different days, refrigerate in between
2. **Aliquot protein standard,** 24 µL per group into tubes labeled “KM”.

3. **Prepare 1x TGS running buffer**
   a. Volume required will depend on your vertical gel apparatus size and class size. My gel tanks require ~600 mL each, and I use 4 per period for a total of 2.4 L per class. Solution can be reused between classes, but you will lose some volume each time
   b. Dilution factor will depend on starting concentration. I purchase 10x TGS, so I use the following quantities to make 2.5 L of buffer
      i. 250 mL of 10x TGS
      ii. 2250 mL of deionized water

Lab Day Preparation

1. Turn on dry heat block to 100°C.
2. Prepare "Used Stain" waste container for staining Step 24.
3. Thaw RFP and SUPER tubes, if frozen.
4. Distribute materials.

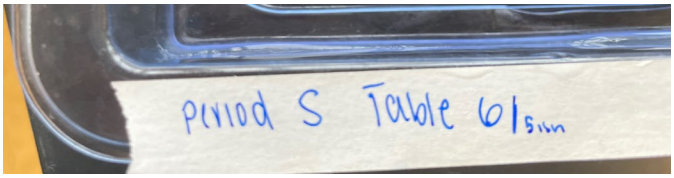
About Staining and Destaining, and Photographing Gels

On a "block" schedule (90 min period), there is typically enough time to prepare samples and run gels, but not stain. I have had limited success having students stain their own gels, and usually do this work on their behalf.

You can also have students do the rinse steps (19 through 21) and complete the staining yourself.

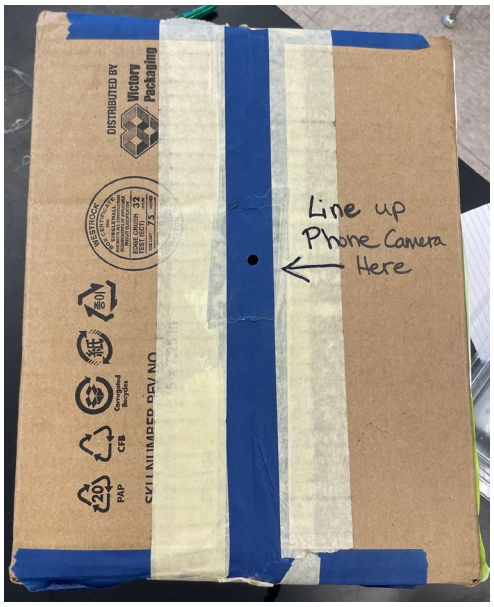
If you use the BioRad BioSafe Coomassie, staining for 1 hour is long enough. If you are using the Flynn stain, do not stain for longer than 1 hour.

When I do staining work for students, I have them label their staining trays with their table group and period number. When photographing, I always take a picture of the label prior to taking a picture of the gel, to make it easier to keep track of which gel I'm on.



For the UV light photography, I have a UV light box that I use for gel electrophoresis. One can also be built using a cardboard box and a UV flashlight, which can be purchased inexpensively as a "Pet Urine Light."

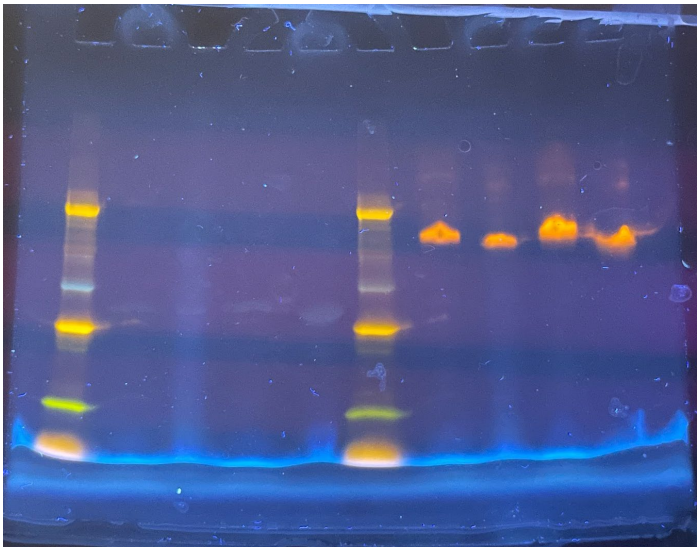
To make a dark enough contrast for photographing gels, I recommend making yourself a low-tech “gel imager.” This box goes over the entire UV light box and students (or you) can use a phone to take a picture through a hole-punch in the top. This is also another layer of protection from the UV illumination.



The light box is under this cardboard “gel imager.” To make, punch a hole in opaque tape around the center of the box and cover any light gaps with additional opaque tape.

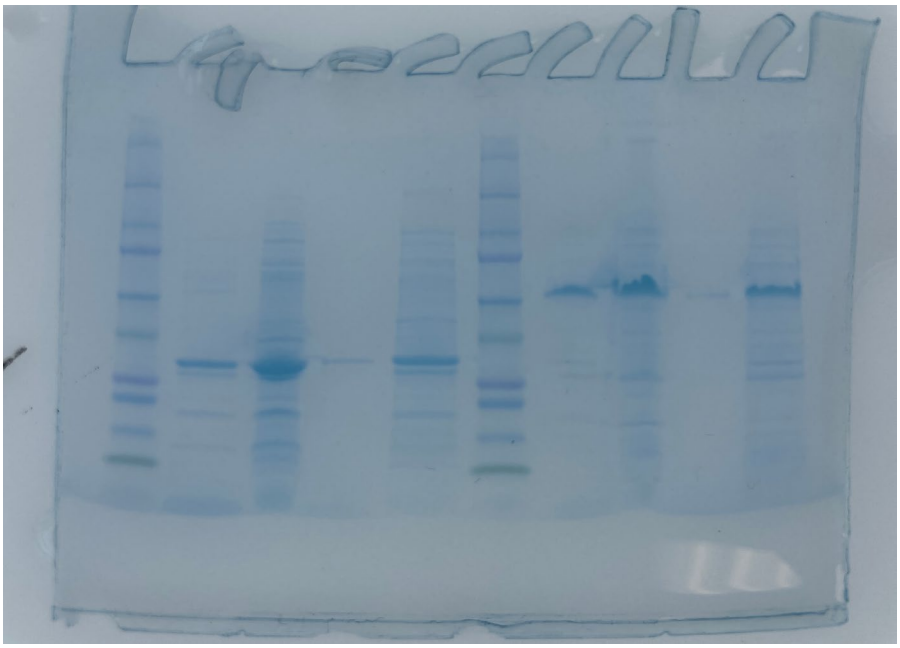
Representative Results

UV light results



Note: Denatured proteins in lanes 2 through 5 will not be visible, nor will non-fluorescent proteins. If you are going to perform native protein PAGE, use a protein ladder that is also UV reactive, like Precision Plus Protein Kaleidoscope standard.

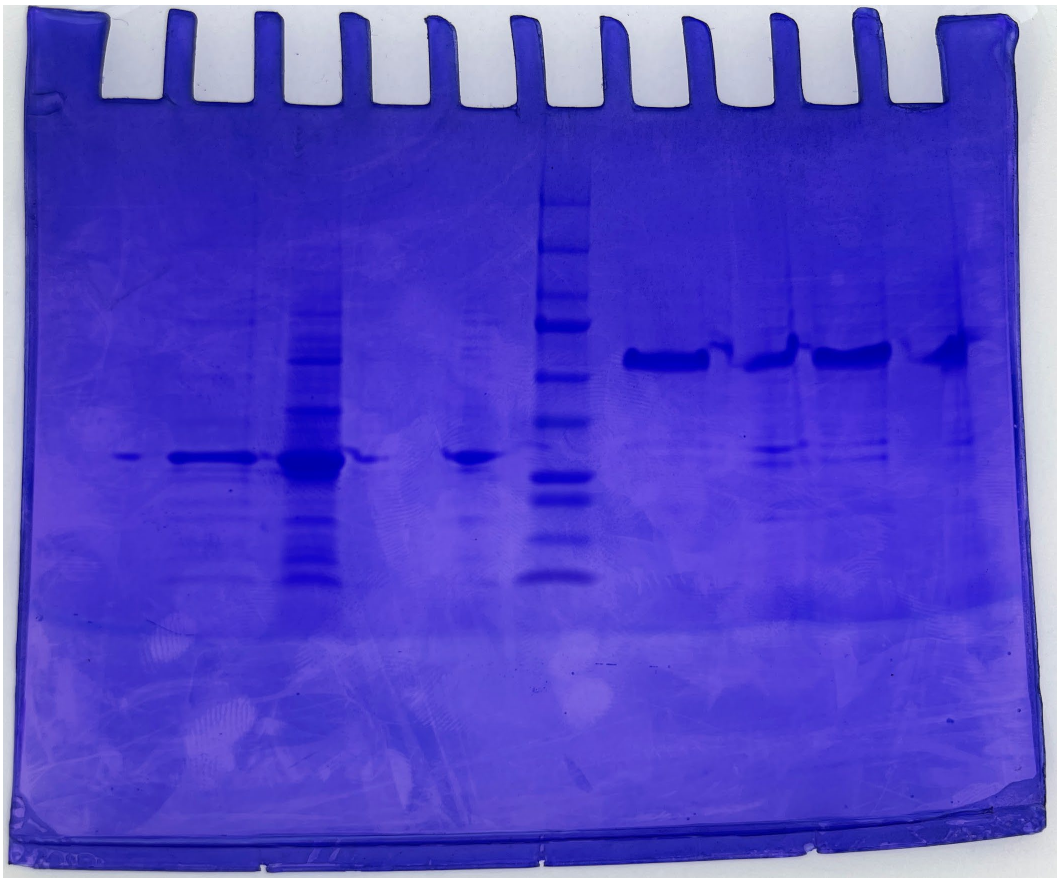
Gel stained with BioSafe Coomassie



Note that the right side of the gel is the native proteins, which maintain their 3-D structures. As a result, proteins are sorted by both size and shape and do not migrate as far down the gel as the denatured proteins.

Lanes 2 and 4 contain the RFP after column chromatography. Note that there are significantly fewer proteins present than in the SUPER lanes (3 and 5). This demonstrates that the column chromatography has succeeded in reducing the number of other proteins but is not truly "purified" yet.

Gel stained with Flynn Protein Staining Solution



This gel still gives readable results; however, it took an additional 2 hours of destaining and an additional destaining solution to reach this level of contrast. I used commercially available destaining solution (Flynn); however, it is possible to make this solution from glacial acetic acid and methanol.

Biotechnology Name_____ Per_____

Quality Control Testing of Red Fluorescent Protein Purification

Background

Recall that during ABE Lab 6, your team purified the red fluorescent protein (RFP) sample using column chromatography. This is a necessary step in the production of medicine because other proteins may prevent the medicine from working as intended. The procedures for purifying biologic pharmaceuticals, like insulin, are highly regulated and precise to ensure each batch of medicine will function properly.

One of these regulation measures is called "quality control," and all companies that make medical devices must have a strict plan in place for quality control (QC) testing and record keeping. This is overseen by the U.S. Food and Drug Administration (FDA), which does regular audits of companies' practices to ensure the safety and effectiveness of medical products. How QC testing is done will depend on the type of medical device or medicine produced. A pacemaker, which is an artificial device to signal the heart to beat, may be tested for producing the correct electrical signal. An HIV positive control, for use in detecting the presence of HIV in a blood sample, would be used in the QC lab using the same test a doctor's office uses to make sure it yields a positive result.

QC testing for a pharmaceutical like insulin would likely involve two tests. One test would be for the appropriate concentration of medicine in a dose (e.g., 1 mg of insulin per 1 mL of medication). The other test would be for the purity of the medicine, to ensure that other proteins from the process have been effectively removed.

In this lab, we will be doing the purity testing portion of the QC process of our model molecule, red fluorescent protein. The technique we will be using to characterize the proteins present is a type of protein electrophoresis called PAGE: Polyacrylamide Gel Electrophoresis. While the same principles of electrophoresis apply, there are some key differences in PAGE.

Recall that the type of electrophoresis we have been doing separates DNA by size and shape. We saw different configurations of the same plasmid make multiple bands in the gels for Lab 4, when we checked for successful ligation. That said, DNA configuration is not typically a factor in electrophoresis, especially with products from PCR.

Proteins, however, always have a 3-dimensional shape that can affect how they will migrate through a gel, which makes it harder to compare proteins by size. PAGE of native (not denatured) proteins can be informative, but may not tell the whole story. A specific type of

PAGE, called SDS-PAGE has accounted for this challenge by adding a denaturing process, which will unfold proteins to their primary structure. Denatured proteins run through PAGE will then separate by size only, and allow for easy comparison.

In this lab, we will be running both native PAGE and SDS-PAGE of our samples in the same gel. For the denatured proteins, we will use Laemmli Buffer, which contains SDS, and heat as our primary denaturing agents.

Protein size is reported as a molecular weight, using the unit kilodaltons (kDa). Proteins usually have molecular weights between 10 and 300 kDa. We will determine the sizes of proteins present in our gel using the same principles of DNA electrophoresis, using a protein ladder and the distance migrated by proteins to determine the size. Smaller proteins will travel further than larger ones.

PAGE uses different equipment than DNA electrophoresis. The gels themselves are made from polyacrylamide, rather than agarose, and sandwiched between two plastic plates. These are purchased rather than made in the classroom, because the starting materials are hazardous until the gel is fully formed. Gels come in a variety of concentrations, between 4 and 20%, and some have a gradient of concentrations to help proteins separate further, called a discontinuous polyacrylamide gel. Samples are loaded with the gel still inside the plastic cassette and are only removed once completed. PAGE gels use a vertical, rather than horizontal, gel chamber and a different running buffer, but use the same power supplies.

Finally, finished gels must be stained in order to view the proteins. DNA electrophoresis also requires staining, but we can add the stain into the gel itself or into the loading dye to image DNA gels right away. In order to visualize the proteins, we will remove the gel from the cassette, and place in Commassie Blue protein stain (same active ingredient as in Bradford Reagent), and then “destain” in diH_2O to remove any Coomassie that is stuck to the gel put isn’t bonded to any proteins.

To evaluate how successful our purification process was, we will run PAGE on both the RFP fractions from ABE Lab 6 Part B and on the supernatant produced after lysing cells in ABE Lab 6 Part A.

Pre-lab Questions:

1. What is the purpose of QC testing?

2. Give an example of a product or substance that you believe should be QC tested.

3. Fill in the chart below comparing and contrasting PAGE and DNA electrophoresis.

DNA Electrophoresis	Similarities	PAGE

4. What do you expect will be the difference between the supernatant and purified RFP samples on the gel (i.e., which do you expect will have more protein bands? How will your gel tell you which one is purer?)?

Materials:

Sample Preparation:
4 screwcap microcentrifuge tubes
2x Laemmli Buffer with added DTT(LB) (25 µL)
Supernatant fraction (SUPER) from lab 7
Purified RFP (RFP) fraction from lab 7
Dry Heat block (shared)

Gel Loading:
Vertical Gel Electrophoresis Unit with power supply (1 per 2 groups)
Buffer dam and loading guide
Protein Standard Ladder (KM)
1X TGS Running Buffer
Protein loading tips

Staining and Data Collection:
Coomassie Blue staining solution
Staining Tray (1 per 2 groups)
Deionized water or destaining solution
Rocker
White light box
Camera and imaging software

Sample Preparation

1. Label 4 screwcap microcentrifuge tubes with Table #/Period and the following marks:
   a. Label one tube SD (Supernatant, Denatured)
   b. Label one tube SN (Supernatant, Native)
   c. Label one tube PD (for Purified, Denatured)
   d. Label one tube PN (for Purified, Native)
2. Transfer 30 µL of the supernatant from the SUPER to the SD tube.
3. Transfer 30 µL of the supernatant from the SUPER to the SN tube.
4. Transfer 30 µL of the purified RFP fraction (RFP) into the PD tube.
5. Transfer 30 µL of the purified RFP fraction (RFP) into the PN tube.
6. Add 10 µL of Laemmli buffer (LB) to the denaturing tubes (SD and PD).
7. Centrifuge all tubes for 20 seconds.
8. Place SD and PD tubes in the dry heat bath for 5 minutes at 100^{o}C.
   a. NOTE: Do not heat the SN and PN tubes. We do not want to denature these proteins.

Setting Up and Loading the Protein Gel

Two groups will load their samples onto the same gel. Denatured proteins go on the left and native proteins on the right.

9. Remove gel from the package and take off green tape from the bottom edge. If your gel does not have the wells clearly marked, dry the front of the gel with a Kimwipe and use a sharpie to put a line on at the bottom of each well for easier loading.
10. Place the gel comb side up into the holder and put the buffer dam on the other side. Fix clamps. Remove the comb carefully and insert the yellow loading guide.
11. Fill the inner compartment with 1X TGS Running Buffer up to the top. Fill the remainder of the chamber with the same buffer up to a height that completely covers the gel.
12. Pulse spin all samples.
13. Using the long protein electrophoresis tips, load your samples according to the loading map.
14. Mark in the table both where your table's samples are and where your partner table's samples are.

Gel Loading Map

Left

Lane	Contents	Tube Label	Quantity	Table/Period #
1	Standard Protein Marker	KM	10 µL	N/A
2	Purified protein, Denatured (Group A)	PD	15 µL	
3	Supernatant, Denatured (Group A)	SD	15 µL	
4	Purified protein, Denatured (Group B)	PD	15 µL	
5	Supernatant, Denatured (Group B)	SD	15 µL	
6	Standard Protein Marker	KM	10 µL	N/A
7	Purified Protein, Native (Group A)	PN	15 µL	
8	Supernatant, Denatured (Group A)	SN	15 µL	
9	Purified Protein, Native (Group B)	PN	15 µL	
10	Supernatant, Denatured (Group B)	SN	15 µL	

Right

15. Remove the sample loading guide and place the lid on the gel chamber.

16. Connect to power supply (red to red, black to black).
17. Run gel at 200 volts for at least 30 minutes. Use the blue tracking dye to determine how far samples have traveled and stop the gel while the buffer is still visible.
    a. Start time: _____
    b. End time: _____
18. While the gel is running, label a staining tray with period and both table group numbers.

Staining and Data Collection

Note: Some of this may be done for you.

19. Open the gel cassette and carefully remove one of the plastic panels. Pour a small amount of diH_2O into the staining tray. Place the cassette gel-side down onto the surface of the water. Touching the gel to the water's surface should help pull it off of the remaining plastic panel. Use a spatula if necessary to completely remove the plastic case.
20. If a rocker is available, rock the gel in water for a total of 15 minutes, pouring off the water and replacing it twice.
21. Using a spatula, move the gel carefully to a UV light box and image the gel under UV light. The RFP should be visible in the native sample lanes.
22. Pour any remaining water out of the staining tray and transfer the gel back into the tray.
23. Add enough Biosafe Coomassie Stain to just cover the gel, about 50 mL. Place on the rocker to stain for 1 hour. Staining can continue for up to 24 hours, but cover the staining tray with plastic wrap if left overnight
24. Pour off the staining solution into the "used stain" bottle.
25. Cover gel with destaining solution, approximately 100 mL, or dH_2O for at least 1 hour, changing the solution several times.
    a. If the solution is visibly blue, it is probably time to pour the solution out and replace it.
    b. Laying a Kimwipe flat on the surface of the destaining solution can help absorb staining solution, which should be changed if the paper is visibly blue.
26. Once the background color has lifted enough to clearly see protein bands, move the gel to the white light box and photograph.

PAGE of RFP Analysis and Conclusion

Click to access an editable version of this Analysis and Conclusion page

1. Copy the image of native proteins under the UV light below.

2. Use the chart below to identify the samples in each lane.

Lane #	Contents	Lab Group
6		
7		
8		
9		
10		

3. According to the protein ladder, how big is RFP?

4. Is the method for visualizing this protein an effective tool for quality control? Explain. *Hint: Can you see a difference between the supernatant and RFP tubes?*

5. Copy the image of your denatured, stained proteins on the white light box below.

6. Use the chart below to identify the samples in each lane.

Lane #	Contents	Lab Group
1		
2		
3		
4		
5		

7. According to the protein ladder, how big is RFP?

8. Is there a difference in the size of RFP between the UV picture and the white light box picture? What could explain the difference if there is one?

9. Is the method used for this image an effective tool for quality assurance? Explain.
   Hint: can you see a difference between the supernatant and RFP tubes?

10. Is your purified RFP ready to be used as medicine? Explain **citing** data from your gels.