Edvo-Kit # 255

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Purification and Size Determination of Green and Blue Fluorescent Proteins

Experiment Objective:

Students will use column chromatography to purify the Green (GFP) and Blue (BFP) fluorescent proteins. In an optional activity, the molecular weights of the proteins will be determined by SDS gel electrophoresis.

See page 3 for storage instructions.

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SAMPLE LITERATURE
Please refer to included weblink for correct version

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Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

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Experiment Components

	Component	Storage	Check (✓)
A	Cell Extract containing green protein (GFP)	Freezer	❑
B	Cell Extract containing blue protein (BFP)	Freezer	❑
C	Column Elution Buffer (10x)	Freezer	❑
D	Standard Protein Markers	Freezer	❑
E	Dry Matrix	Room temp.	❑
F	Protein Denaturing Solution	Freezer	❑
G	50% Glycerol Solution	Freezer	❑

This kit is designed for six (6) groups sharing three polyacrylamide gels.

All remaining components can be stored at room temperature.

- Tris Glycine SDS Electrophoresis Buffer (10x) ❑
- FlashBlue™ Protein Stain Powder ❑
- Chromatography Columns ❑
- Transfer Pipets ❑
- UV permeable microcentrifuge tubes (for fraction collection) ❑
- Screw cap microcentrifuge tubes (for protein denaturation) ❑

Experiment Requirements *(NOT included with this experiment)*

- Ring stands and column clamps
- Long U.V. lamps (Cat. #969)
- Ice Buckets and Ice
- Microcentrifuge tubes
- Vertical electrophoresis apparatus (EDVOTEK® Cat. #581 highly recommended)
- D.C. power supply
- Precast 12% SDS polyacrylamide gels (12-well gels recommended)
- Micropipette and tips (Cat #638 Fine Tip Micropipette Tips recommended)
- Hot plate
- Distilled or deionized water
- Beakers
- Aluminum foil or foam water bath float
- White Vinegar
- Ethanol (95% or higher)
- 750 mL or 1 L flask or beaker
- Small plastic tray or large weigh boat
- Plastic wrap
- White light box (recommended)
- Rocking platform (recommended)
- Latex or Vinyl Lab Gloves
- Safety Goggles

**Polyacrylamide gels are not included for the electrophoresis part of this experiment. Module II is designed for two student groups to share a gel. A total of 3 polyacrylamide gels (Precast polyacrylamide gels, Cat. #651) are required.*

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Background Information

GREEN FLUORESCENT PROTEIN (GFP) AND BLUE FLUORESCENT PROTEIN (BFP)

Bioluminescence from marine microorganisms has been observed by many summer visitors at beaches around the world. It always fascinates the observer to see the repeated parade of both color and light. This observation takes second place only to the light produced by the bioluminescent jellyfish, *Aequorea victoria*. This light is produced by green fluorescent proteins (GFPs) found in specialized photogenic cells at the base of the jellyfish's umbrella. GFP is a small protein that possesses the ability to absorb blue light and emit green light in response. This activity, known as fluorescence, does not require any additional substrates, gene products, or cofactors.

GFP was first isolated from *Aequorea victoria* in the 1970s. Once scientists identified its DNA sequence, they were able to use genetic engineering to introduce fluorescent proteins into other organisms, such as *E. coli* and the nematode *Caenorhabditis elegans*. Scientists also identified particular amino acid substitutions in GFP that altered the behavior of its 'chromophore', a special structure within the protein that is responsible for light production. Different changes bring about different patterns of light absorption and emission, allowing scientists to develop a rainbow of fluorescent proteins (Figure 1). For their discovery and development of GFP and other fluorescent proteins, Osamu Shimomura, Martin Chalfie, and Roger Tsien were awarded the Nobel Prize in Chemistry in 2008.

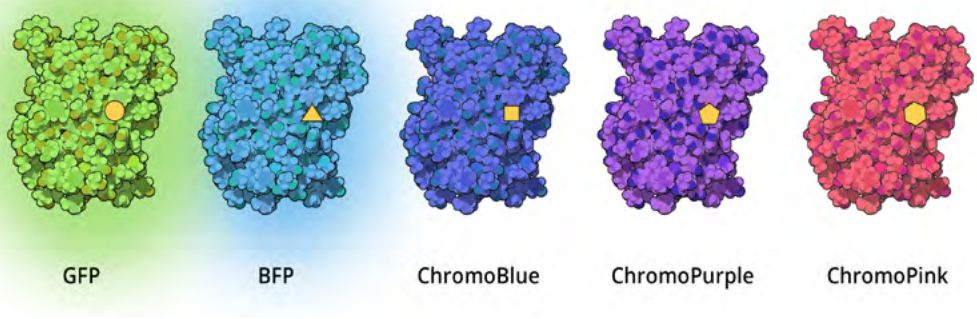

Figure 1: Structural changes can create different colored proteins.

The green fluorescent protein (GFP) has 238 amino acid residues and has a molecular weight of approximately 27,000 daltons. The chromophore responsible for the green light emission is within the primary structure of the protein and resides in a tripeptide at positions 65 to 67 which is cyclic and is composed of the amino acids Ser-Tyr-Gly. The importance of protein folding is demonstrated with GFP where the protein is fluorescent only upon proper conformational folding. The blue fluorescent protein (BFP) is a derivative variant of the GFP. It has a His-66 substitution at the Tyr-66 position and a second substitution from Tyr-145 to Phe-145.

GFP, BFP, and related fluorescent proteins have become essential tools in cell and molecular biology. Using DNA cloning strategies, proteins can be "tagged" with fluorescent proteins and then expressed in cells. Fluorescent-labeled proteins can be tracked using UV light, simplifying protein purification experiments. Additionally, researchers can determine where those proteins are normally found in the cell. Similarly, using GFP and/or BFP as a reporter, scientists can observe biological processes as they occur within living cells. For example, in the model organism zebrafish (*Danio rerio*), scientists use GFP to

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fluorescently label blood vessel proteins so they can track blood vessel growth patterns and networks. Scientists also tag regulatory DNA sequences with the fluorescent coding sequence so they can observe patterns of when and where a gene is expressed. In this way, GFP and its derivatives can reveal the role these regulatory sequences might normally play in a cell. In summary, these bright proteins have enhanced our understanding of many biological processes by allowing scientists to watch these events in real time.

In this experiment, bacterial extracts containing GFP and BFP will be fractionated by chromatography using a molecular sieve matrix. Factors that affect the separation include size, shape, and associated non-protein biologicals such as carbohydrate residues. The fluorescent proteins will be detected on the column and subsequently in the test tubes by examination under long U.V. light. For most proteins, such columns can also be used to determine apparent molecular weights. Accurate estimation of protein polypeptide composition and size(s) are performed by analyzing the fractions that contain the protein of interest by denaturing polyacrylamide gel electrophoresis.

PURIFICATION AND ANALYSIS OF PROTEINS BY COLUMN CHROMATOGRAPHY AND SDS ELECTROPHORESIS

In column chromatography, a bacterial lysate is added to a long, thin column containing a molecular matrix, a semi-solid substance used to separate proteins (Figure 2). The matrix is supported by the frit, a membrane or porous disk that retains the matrix in the column but allows buffer and dissolved solutes to pass. In size-exclusion chromatography, the method utilized in this experiment, the matrix contains microscopic pores and internal channels. The larger the molecule, the more difficult it is for it to pass through the pores. Instead, larger molecules tend to flow around and in between the beads. Smaller molecules will tend to spend more time in the maze of channels and pores in the bed. Consequently, the larger, higher molecular weight proteins will move more rapidly through the matrix and will be the first to drip out of the column, or elute.

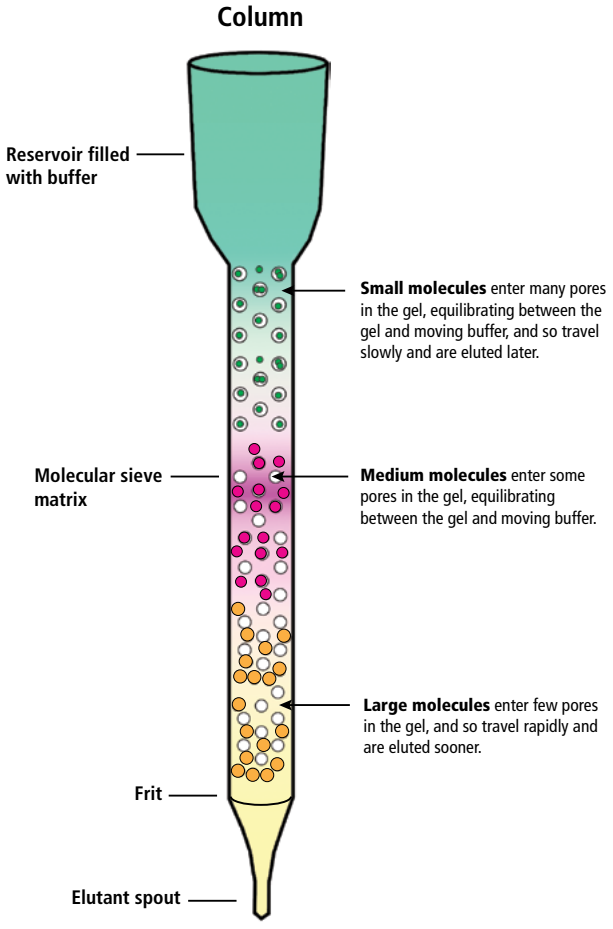

Figure 2: Size-exclusion column chromatography.

Once the protein has been purified it is ready for further investigation. To analyze proteins, researchers often make use of a technique called polyacrylamide gel electrophoresis, or PAGE. This is a simple but powerful method that provides information about the expression and purity of a molecule, along with its molecular weight. PAGE uses acrylamide and bis-acrylamide polymers to create a gel with a network of microscopic pores and channels. To perform PAGE, a gel is prepared, placed in an electrophoresis chamber, and flooded with buffer. Next, the protein samples are loaded into small indentations, or wells, at the top of the gel. Finally, an electrical current is applied to the gel box, pushing the charged proteins through the gel towards the positive electrode (Figure 3). As the proteins migrate they are forced through the pores of the gel; smaller proteins have an easier time fitting than larger proteins and will migrate further in the same amount of time.

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Proteins, however, produce a unique challenge for electrophoresis because they have complex shapes and distinct charges, both of which can change how a protein migrates through the gel. Scientists can solve these problems by using chemicals that denature the proteins, "eliminating" the complex structure and neutralizing the charge of the native protein. Sodium Dodecyl Sulfate (SDS) is a common detergent used to disrupt interactions between amino acids. The SDS molecule consists of a hydrocarbon chain bonded to a negatively charged sulfate group. When incubated with proteins and heated, SDS will unfold the protein's three-dimensional structure. To break the stronger disulfide bonds in proteins researchers use reducing agents such as ß-mercaptoethanol (ß-ME) or Dithiothreitol (DTT). Although the amino acid composition and sequence stay the same, the protein will no longer have biological activity because the specific three-dimensional shape has changed. The prepared protein sample can then be separated on a polyacrylamide gel and sized using standard markers.

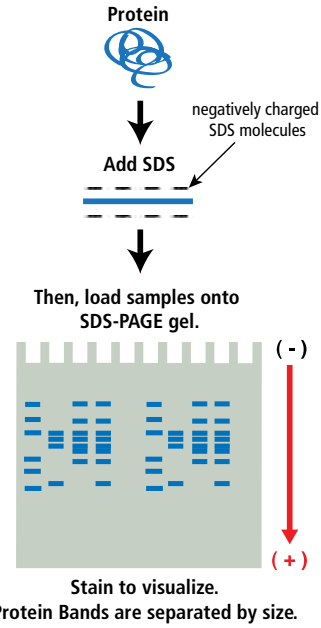

Figure 3: An Overview of SDS-PAGE.

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Experiment Overview

EXPERIMENT OBJECTIVE:

Students will use column chromatography to purify the Green (GFP) and Blue (BFP) fluorescent proteins. In an optional activity, the molecular weights of the proteins will be determined by SDS gel electrophoresis.

LABORATORY SAFETY:

Be sure to READ and UNDERSTAND the instructions completely BEFORE starting the experiment. If you are unsure of something, ASK YOUR INSTRUCTOR!

- Wear gloves and goggles while working in the laboratory.
- Exercise caution when working in the laboratory – you will be using equipment that can be dangerous if used incorrectly.
- Wear protective gloves when working with hot reagents like boiling water and melted agarose.
- DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
- Always wash hands thoroughly with soap and water after working in the laboratory.

Wear gloves and safety goggles

Acrylamide is a known neurotoxin and carcinogen and should be handled with extreme caution. Liquid acrylamide, used in the manufacture of SDS-PAGE gels, should only be handled in a chemical fume hood while wearing gloves and goggles. Polymerized acrylamide, including precast acrylamide gels, is safe but should still be handled with caution at all times.

LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:

- Record your observations.

After the Experiment:

- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

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Module I Overview

In Module I, the GFP and BFP-containing cellular lysate will be purified by column chromatography. First, columns will be packed with a molecular sieve matrix and equilibrated with a wash buffer. The cellular lysate will be run through the column and the GFP and BFP will be collected in tubes. Highly fluorescent fractions can be saved and further analyzed in Module II.

TIMING REQUIREMENTS:

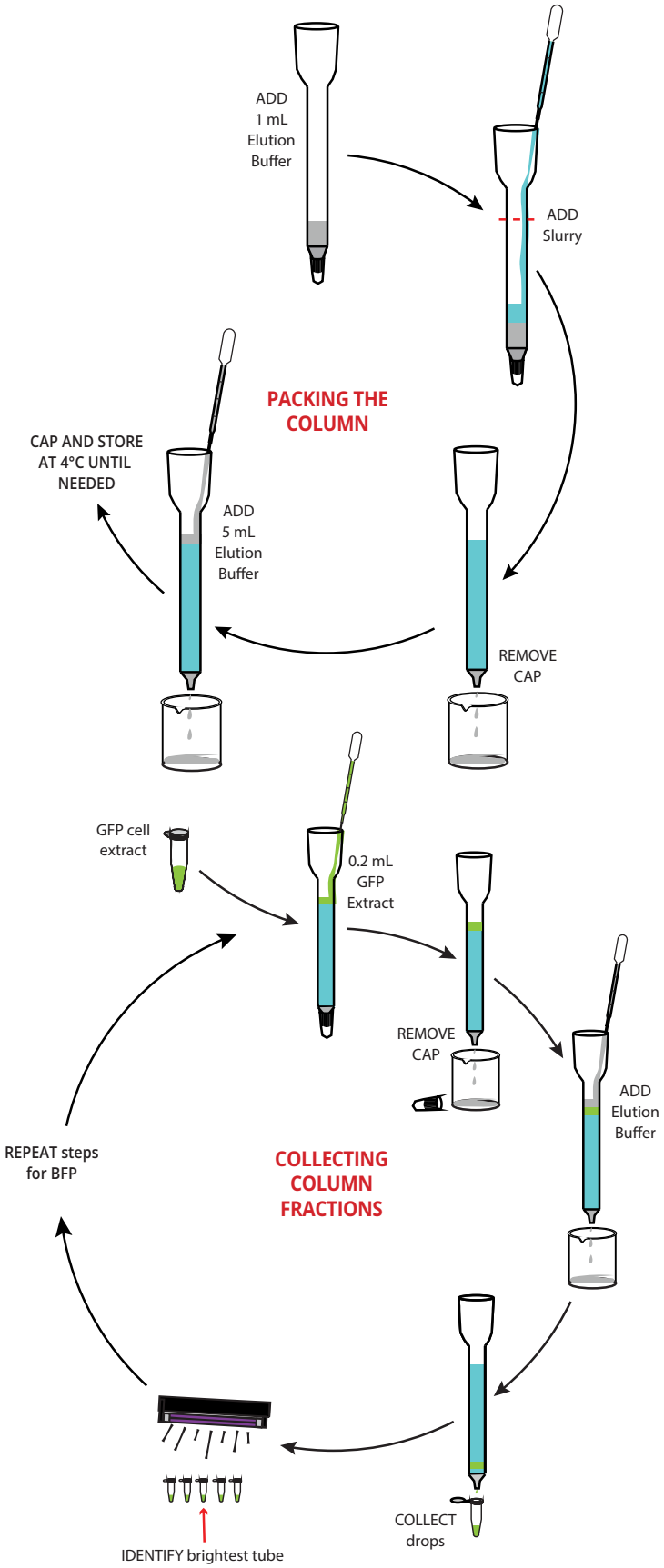
PACKING COLUMN
30 min.

↓

PURIFYING GFP AND BFP
30 min.

↓

STOPPING POINTS
Packed column can be stored at 4°C, GFP and BFP fractions can be stored at -20°C until needed.



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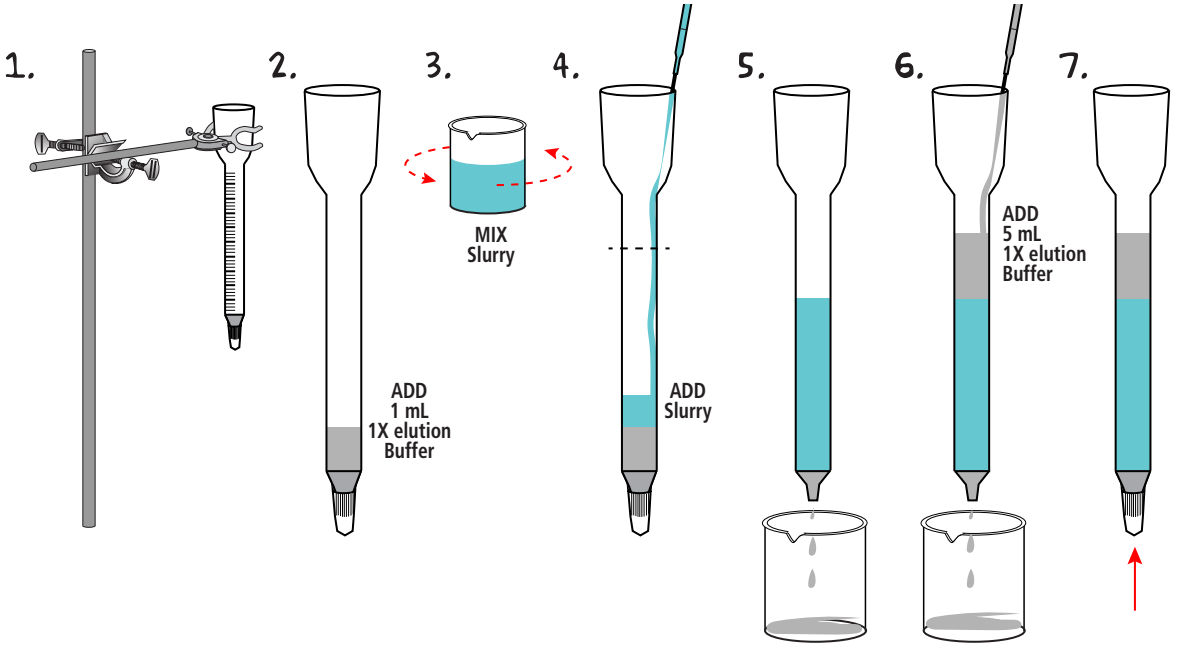
Module I: Purification of GFP & BFP by Column Chromatography



PACKING AND EQUILIBRATING THE COLUMN

1. Vertically **MOUNT** the column on a ring stand. Make sure it is straight and that the white cap is firmly attached to the bottom of the column.
2. **ADD** 1 mL of 1X elution buffer to the column and confirm that the bottom cap is sealed.
3. **MIX** the slurry (molecular sieve matrix) thoroughly by swirling or gently stirring.
4. Carefully **PIPET** approx. 5 mL of the mixed slurry into the column by letting it stream down the inside walls of the column.
   If the flow is stopped by an air pocket, stop adding the slurry and firmly tap the column until the air is removed and the slurry continues to flow down the side of the column.
5. **PLACE** an empty beaker under the column to collect 1X elution buffer. **REMOVE** the cap from the bottom of the column and allow the matrix to pack into the column. Some fine sediment may drip through the column but the majority will form a uniform solid matrix.
   NOTE: The matrix should fill ~2/3 of the narrow part of the column. If the packed matrix is too low, additional slurry can be added to raise the volume.
6. **WASH** the packed column with 5 mL of 1x elution buffer. Always keep a thin layer of elution buffer on top of the packed matrix to prevent drying.
7. **SLIDE** the cap onto the spout and make sure it does not drip.

NOTE:

The loading of the column and subsequent elution will be done at room temperature. The elution buffers and the fractions collected will be stored on ice as they elute from the column.

Do not allow the column to dry!

OPTIONAL STOPPING POINT:

Packed columns can be stored at 4°C until needed. Ensure that the column is capped and sealed to prevent the slurry from drying out.

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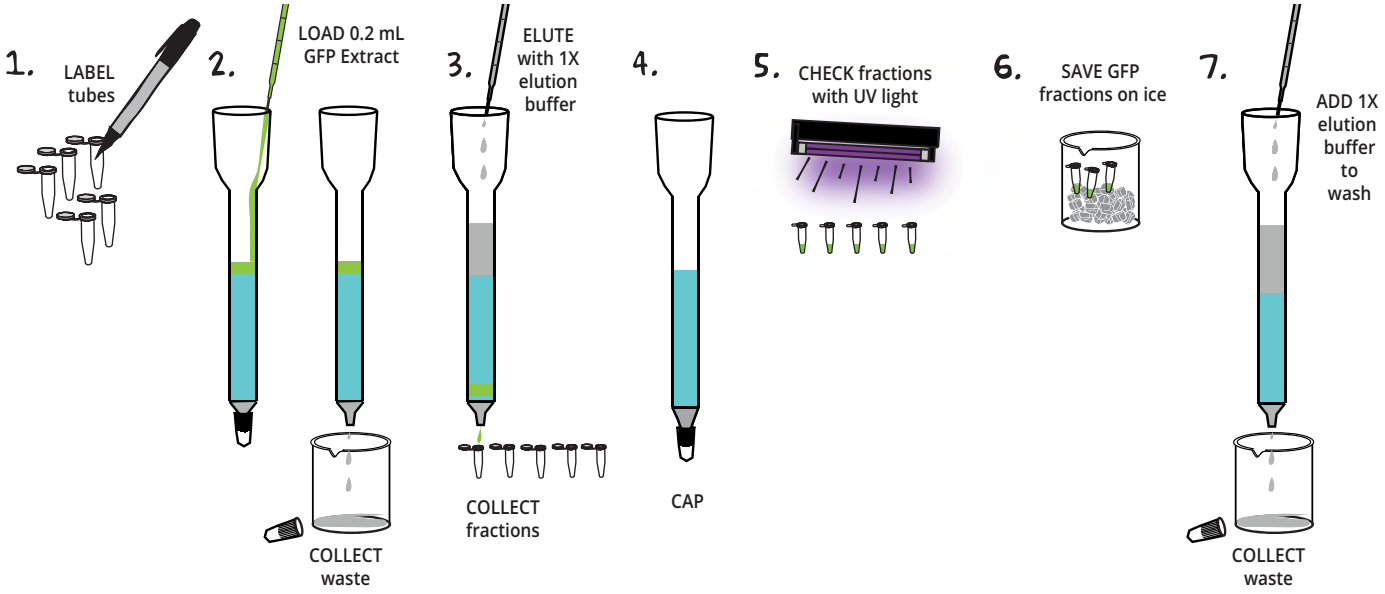
Module I: Purification of GFP & BFP by Column Chromatography, continued



COLLECTING COLUMN FRACTIONS OF (GFP) PROTEIN

1. **LABEL** the first set of five microcentrifuge tubes #1-5.
2. Slowly **LOAD** the column with 0.2 mL of the GFP extract. **REMOVE** the cap and allow the extract to completely enter the column, collecting the "flow-through" waste in your beaker.
3. Begin to **ELUTE** the column with 1X elution buffer. **ADD** buffer slowly (several drops at a time) to avoid diluting the protein sample. **COLLECT** 0.5 mL fractions in the labeled micro-centrifuge tubes beginning with tube 1 and using the graduated marks on the side of the tube to estimate volume. **STORE** fractions on ice.
4. After collecting all 5 fractions **CAP** the column and set aside.
5. **CHECK** the fractions by using the long wave UV light and **IDENTIFY** the tubes that contain fluorescent proteins. **PHOTOGRAPH** and/or **RECORD** fluorescent tube numbers in your lab book.
6. (Optional) **SAVE** the fractions containing GFP proteins for further analysis by SDS gel electro-phoresis.
7. Slowly **ADD** 10 mL of 1x Elution buffer. **PLACE** the waste beaker underneath and **REMOVE** the cap. **ALLOW** the elution buffer to filter through the column and wash it. **DO NOT AL-LOW THE COLUMN TO DRY.**
8. **LABEL** a second set of five microcentrifuge tubes #6-10.
9. **REPEAT** steps 2 through 6 using the BFP extract and tubes #6-10.

WEAR UV SAFETY GOGGLES

Monitor the elutions of GFP and BFP by shining a long wave UV light on the side of the column. Predict the peak tube that will contain each protein! Do not use short wave UV light, which can cause burns and serious damage to the eyes.

OPTIONAL STOPPING POINT:

If time does not permit you to continue with the SDS Gel Electrophoresis, you may freeze the fractions at -20°C and perform the assays at a later date.

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Module II Overview

In Module II, GFP and BFP fractions from Module I will be analyzed using SDS-PAGE. "Native" samples will be mixed with a glycerol mixture, while "Denatured" samples will be mixed with a denaturing solution and boiled. Both samples will then be run on an SDS-PAGE Gel and analyzed.

TIMING REQUIREMENTS:

PREPARING SAMPLES
10 minutes

↓

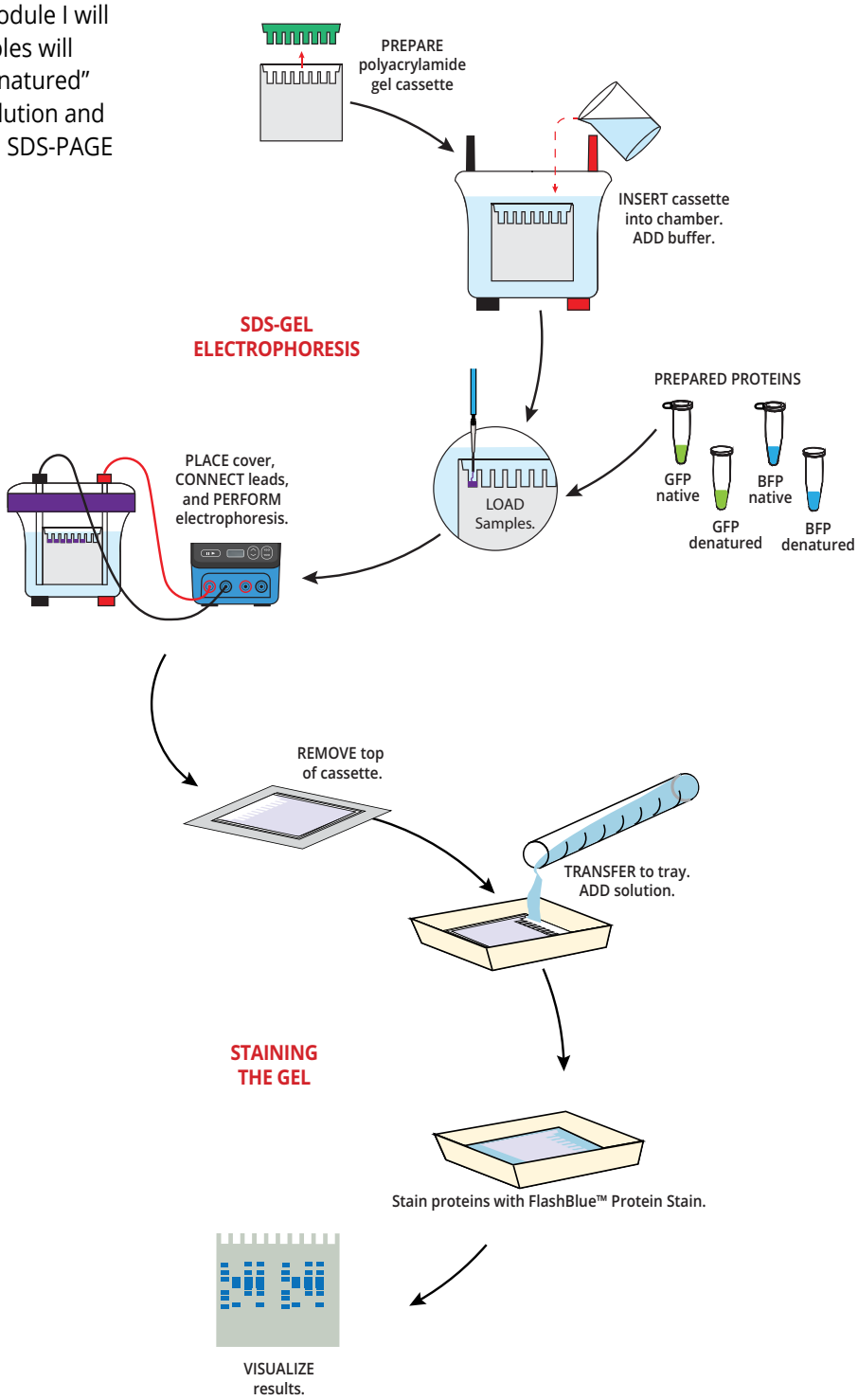
RUNNING
60 minutes +

↓

STAINING
3 hours - overnight

↓

STOPPING POINTS
Stained gels can be stored in water for up to 24 hours.



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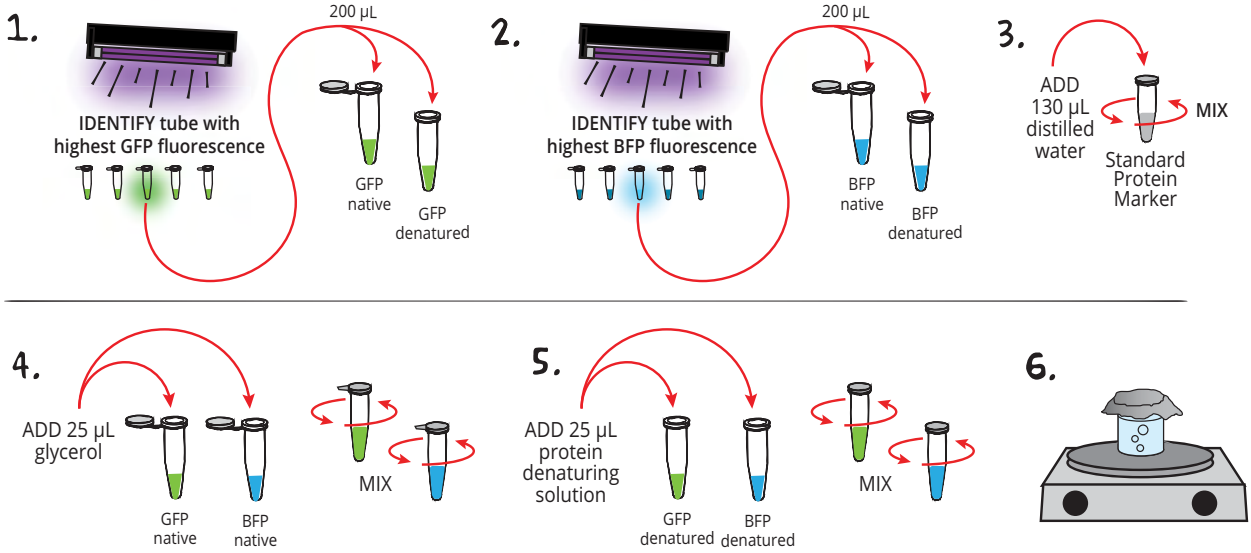
Module II: Analysis of GFP & BFP by Denaturing SDS-Gel Electrophoresis



SAMPLE PREPARATION

1. **IDENTIFY** the tube with the highest fluorescence of the GFP. **TRANSFER** 200 µL each of the peak extract into two clean microcentrifuge tubes – one screw cap and one snap cap. **LABEL** the snap top tube "GFP native" and the screw cap tube "GFP denatured".
2. **IDENTIFY** the tube with the highest fluorescence of the BFP. **TRANSFER** 200 µL each of the peak extract into two clean microcentrifuge tubes – one screw cap and one snap cap. **LABEL** the snap cap tube "BFP native" and the screw cap tube "BFP denatured".
3. (Optional) If your standard protein marker has not been rehydrated and boiled by your instructor, **ADD** 130 µL of distilled or deionized water to it and allow the sample to **REHYDRATE** for several minutes. Vortex or **MIX** vigorously. Then, denature the standard protein marker along with the two denatured proteins in step 5, below.

PREPARING THE NATIVE AND DENATURED PROTEINS

4. To each of the tubes labeled "GFP native" and "BFP native", **ADD** 25 µL of 50% glycerol. **MIX** and set aside for later electrophoresis.
5. To each of the tubes labeled "GFP denatured" and "BFP denatured", **ADD** 25 µL of protein denaturing solution. **MIX** well. The denaturing solution contains sodium dodecylsulfate (SDS) and 2-mercaptoethanol.
6. Bring a beaker of water, covered with aluminum foil, to a **BOIL** on a hot plate.

NOTE: A 95-100°C water bath can be used in place of a hot plate for heating protein samples. One beaker or water bath can be shared by multiple groups. For faster heating, heat water in the microwave before covering with aluminum.

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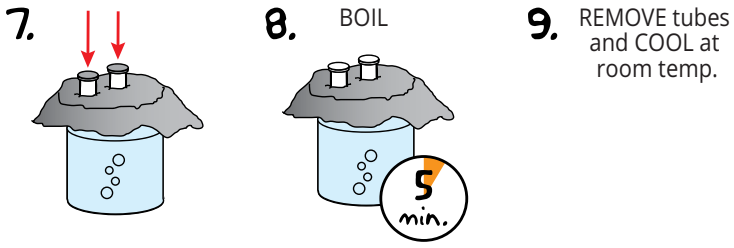
Module II: Analysis of GFP & BFP by Denaturing SDS-Gel Electrophoresis, continued



7. **CONFIRM** that GFP and BFP denatured tubes are tightly capped and thawed. **PUSH** bottom of both tubes through the foil and **IMMERSE** in the boiling water. The tube should be kept suspended by the foil.
8. **BOIL** the tubes for 5 minutes.
9. **REMOVE** the sample tube from the beaker and allow it to **COOL** for a few minutes at room temperature.

CAUTION:

Always use screw-cap tubes for boiling denatured samples.

QUICK REFERENCE:

Proteins unfold and lose their tertiary structures by boiling for 5 minutes in the presence of denaturing solutions which contain SDS and 2-mercaptoethanol. In the absence of boiling, regions of the protein can remain intact in their native state. You can confirm that the proteins have been denatured by viewing the samples on a UV or blue light transilluminator or hand held UV light. Unlike native GFP and BFP, the denatured sample will not glow.

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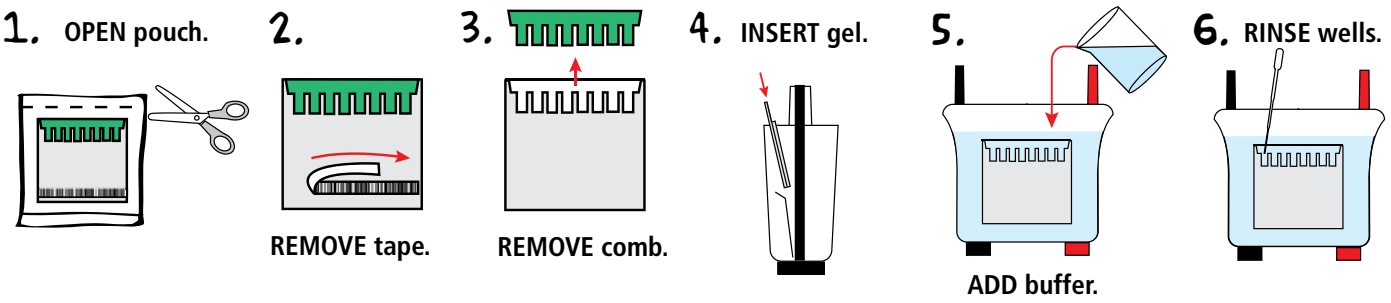
Module II: Analysis of GFP & BFP by Denaturing SDS-Gel Electrophoresis, continued



PREPARING GEL AND CHAMBER

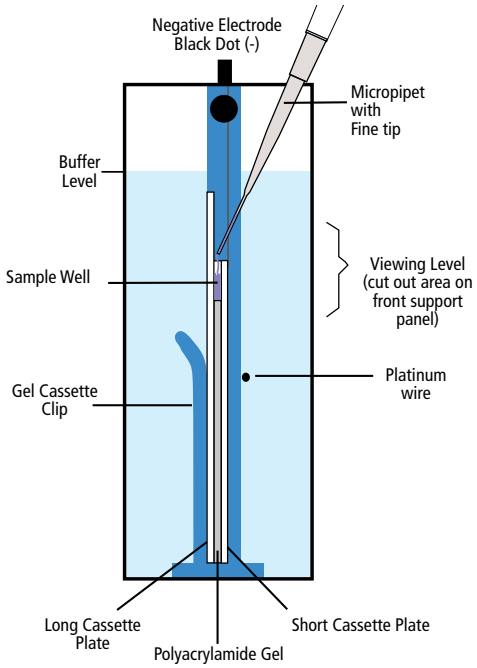
NOTE: Although precast polyacrylamide gels and protein chambers will vary slightly in design, the procedure for their use will be similar.


Wear gloves and safety goggles

1. **OPEN** the pouch containing the gel cassette. Remove the cassette and place on bench with the shorter front plate facing up.
2. Gels may feature a sticker or tape at the bottom of the front plate. **REMOVE** the tape (if present) to expose the bottom of the gel.
3. Carefully **REMOVE** the comb by gently pulling upwards. Pull the comb straight up to prevent damage to the wells of the gel.
4. **INSERT** the gel into the electrophoresis chamber. Orient the gel according to the manufacturer's instructions. ***NOTE: For EDVOTEK® vertical electrophoresis chambers, the short plate should face the interior.***
5. **ADD** diluted electrophoresis buffer to the chamber. The buffer should cover the top of the front, shorter plate.
6. **RINSE** each well by squirting electrophoresis buffer into the wells using a transfer pipet.

The gel is now ready for sample loading or practice gel loading (see Appendix B).

IMPORTANT:
EDVOTEK® Cat. #638, Fine Tip Micropipette Tips are recommended for loading samples into polyacrylamide gels. A regular micropipette tip may damage the cassette and result in the loss of protein samples.



A polyacrylamide gel cassette in the EDVOTEK® Vertical Electrophoresis Apparatus, Model #MV10.

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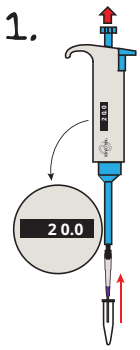
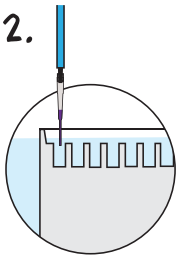
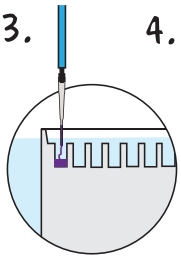
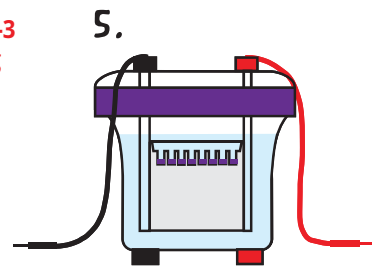
Module II: Analysis of GFP & BFP by Denaturing SDS-Gel Electrophoresis, continued

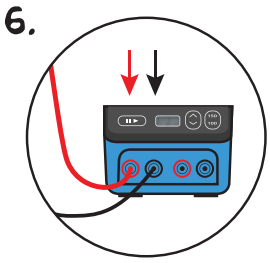


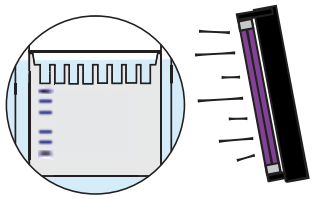


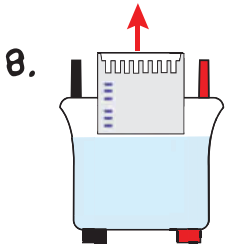


4. **Repeat steps 1-3 for remaining samples.**









Wear gloves and UV safety goggles!

LOADING THE PROTEIN SAMPLES

Two student groups can share one gel. Some of the samples contain denaturing solution which contains SDS and 2-mercaptoethanol. Wear gloves and UV safety goggles.

1. Using a fresh *fine tip* micropipette tip, **MEASURE** 20 µL of the first sample as indicated in Table 1.
2. **PLACE** the pipette tip under the buffer and directly above the sample well, resting gently against the back plate of the gel cassette.
3. Slowly **DISPENSE** the sample by depressing the plunger.
4. **REPEAT** steps 1-3 for remaining samples in Table 1, changing the tip between each new sample.

NOTE: Be sure to change pipette tips between loading each sample!

5. Once all samples have been loaded, carefully **PLACE** the cover onto the electrode terminals.
6. **CONNECT** the electrical leads to the power supply.
7. **SET** the voltage of the power supply and **PERFORM** electrophoresis (See Table A for time and voltage guidelines). Allow the proteins to separate on the gel for the recommended length of time, or until the tracking dye reaches the bottom of the gel.

NOTE: Shine the long wave UV light on the gel while the native proteins are separating. Be sure wear UV safety goggles!

8. After the electrophoresis is finished, **TURN OFF** the power supply, disconnect the leads, and carefully **REMOVE** the cover. The gel can now be removed from the chamber. Immediately proceed to staining instructions on page 16.

Table 1: Gel Loading

Lane	Sample	Time Boiled
FIRST STUDENT GROUP		
1	20 µL Standard Protein Markers	Boiled 5 min.
2	20 µL GFP Native	Not Boiled
3	20 µL GFP Denatured	Boiled 5 min.
4	20 µL BFP Native	Not Boiled
5	20 µL BFP Denatured	Boiled 5 min.
SECOND STUDENT GROUP		
6	20 µL Standard Protein Marker	Boiled 5 min.
7	20 µL GFP Native	Not Boiled
8	20 µL GFP Denatured	Boiled 5 min.
9	20 µL BFP Native	Not Boiled
10	20 µL BFP Denatured	Boiled 5 min.

Table A Time and Voltage Guidelines

	Recommended Time	
Volts	Minimum	Optimal
100	70 min.	90 min.
125	50 min.	60 min.
150	40 min.	50 min.

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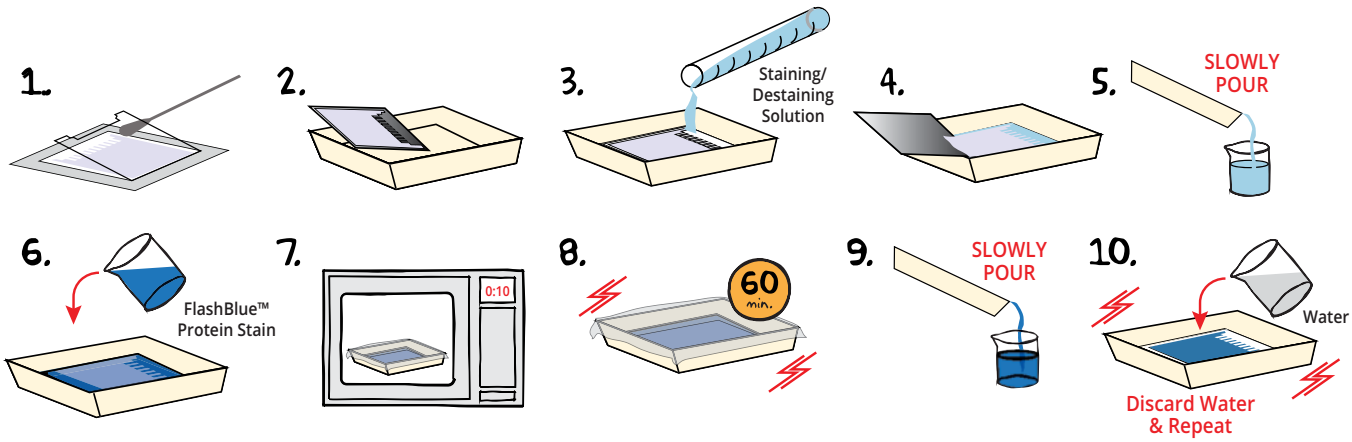
Module II: Analysis of GFP & BFP by Denaturing SDS-Gel Electrophoresis, continued

GEL STAINING WITH FLASHBLUE™ PROTEIN STAIN

Although the protein samples are provided in a pre-stained format it is possible to increase the intensity of the bands by using FlashBlue™ Protein Stain. Staining is rapid and sensitive. Student groups that shared a polyacrylamide gel during electrophoresis should also stain this gel together.



1. After electrophoresis, **LAY** the cassette down and **REMOVE** the front plate by placing a thin spatula or screwdriver at the side edge and gently lift it away from the larger back plate. In most cases, the gel will stay on the back plate. If it partially pulls away with the front plate, let it fall onto the back plate. ***Handle very carefully as the thin gels are extremely fragile.***
2. **TRANSFER** the gel on the back plate to a clean tray.
3. **ADD** a sufficient volume (approximately 50-75 mL) of the staining/destaining solution into the tray to **COVER** the gel and back plate.
4. Carefully **REMOVE** the back plate from the tray, leaving just the gel in the tray containing the staining/destaining solution. ***NOTE: If the gel sticks to the plate, gently nudge the gel off the plate using two GLOVED fingers.***
5. **DISCARD** the staining/destaining solution. ***Pour slowly to keep the gel in the container.***
6. **ADD** 30 mL of prepared FlashBlue™ Protein Stain.
7. (OPTIONAL) **COVER** the container with plastic wrap and **MICROWAVE** for 10 seconds to gently heat the solution.
8. **INCUBATE** for 60 minutes at room temperature, **SHAKING** occasionally.
9. **DISCARD** the FlashBlue™ Protein Stain solution. ***Pour slowly to keep the gel in the container.***
10. **WASH** the gel by partially filling container with water and gently rocking back and forth several times. **DISCARD** the used water and **REPEAT** with fresh water.

WEAR GLOVES AND SAFETY GOGGLES

Gloves must be worn during this procedure. Avoid touching the gel without gloves.

Polyacrylamide gels are very thin and fragile. Use care in handling to avoid tearing the gel.

continued

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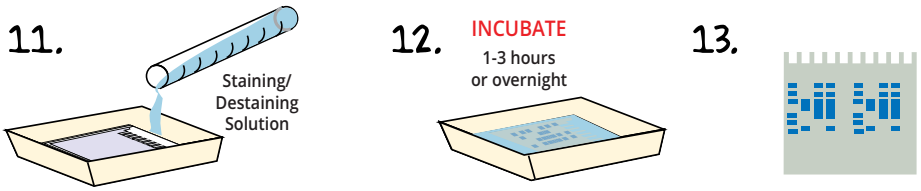
Module II: Analysis of GFP & BFP by Denaturing SDS-Gel Electrophoresis, continued



11. **ADD** 30 mL of staining/destaining solution to the gel.

12. **INCUBATE** for 1-3 hours at room temperature. Optionally, you can let the gel stain overnight. **EXAMINE** the gel.

13. After staining, protein bands will appear medium to dark blue against a light background. A white light box can be used to better visualize the protein bands.

STORING THE GEL

- Gel may be left in deionized water for several hours with no loss in sensitivity and band intensity. This step should be performed once a desired background and stained protein bands are obtained. Pour off the destaining solution from Step 12 and add a sufficient amount of deionized water to cover the gel.

- For permanent storage, the gel can be dried between two sheets of cellophane (saran wrap) stretched in an embroidery hoop. Air dry the gel for several days until the gel is paper thin. Cut the "extra" saran wrap surrounding the dried gel. Place the dried gel overnight between two heavy books to avoid curling. Tape it into a laboratory book.

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Module II: Analysis of GFP & BFP by Denaturing SDS-Gel Electrophoresis, continued

DETERMINATION OF MOLECULAR WEIGHTS

1. **MEASURE** the migration distance, in centimeters (to the nearest millimeter) of every major band in the gel. All measurements should be from the bottom of the sample well to the bottom of the protein band.

2. Using semilog graph paper, **PLOT** the migration distance or relative mobility (Rf) of each standard protein on the non-logarithmic x-axis versus its molecular weight on the logarithmic y-axis. **CHOOSE** your scales so that the data points are well spread out.

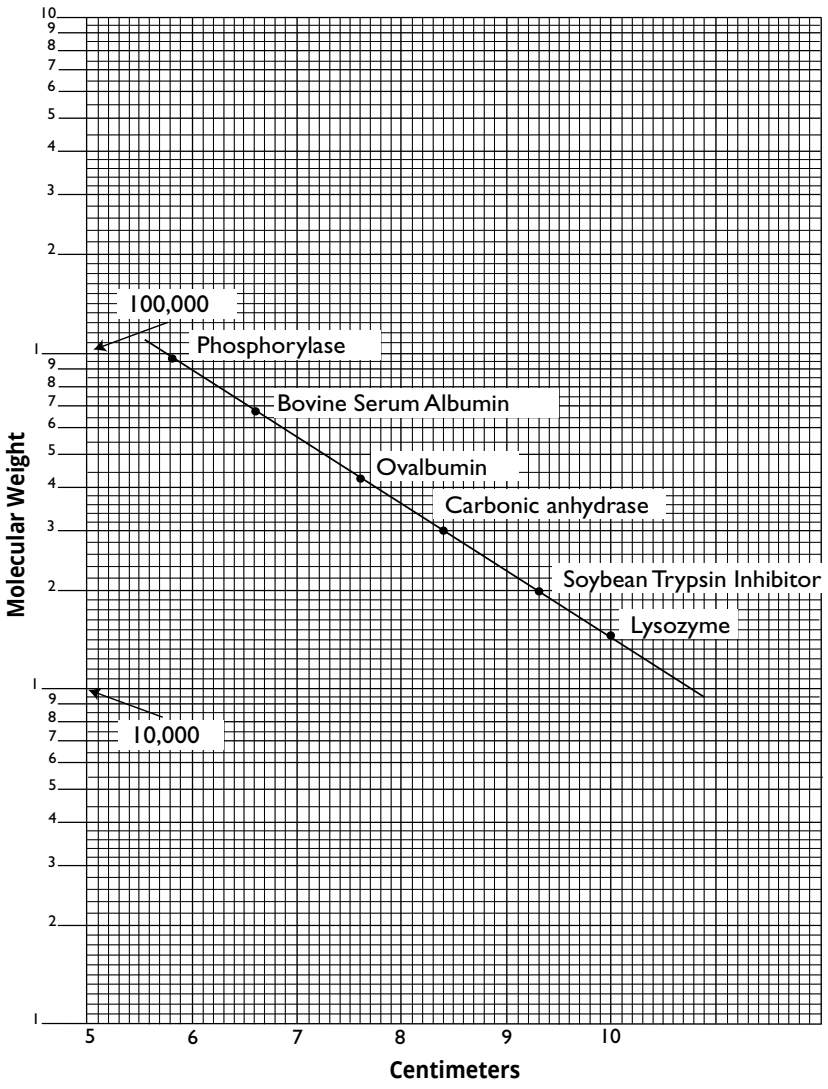
3. **DRAW** the best average straight line through all the points. This line should roughly have an equal number of points scattered on each side of the line. As an example, refer to the figure at left. This method is a linear approximation.

4. Using your standard graph, **DETERMINE** the molecular weight of the native and denatured GFP. This can be done by finding the Rf (or migration distance) of the unknown band on the x-axis and drawing a straight vertical until the standard line is intersected.

5. A straight line is then made from the intersection across to the y-axis where the approximate molecular weight can be determined.

In this experiment, the standard molecular weights are:

94,000	30,000
67,000	20,000
38,000	14,000



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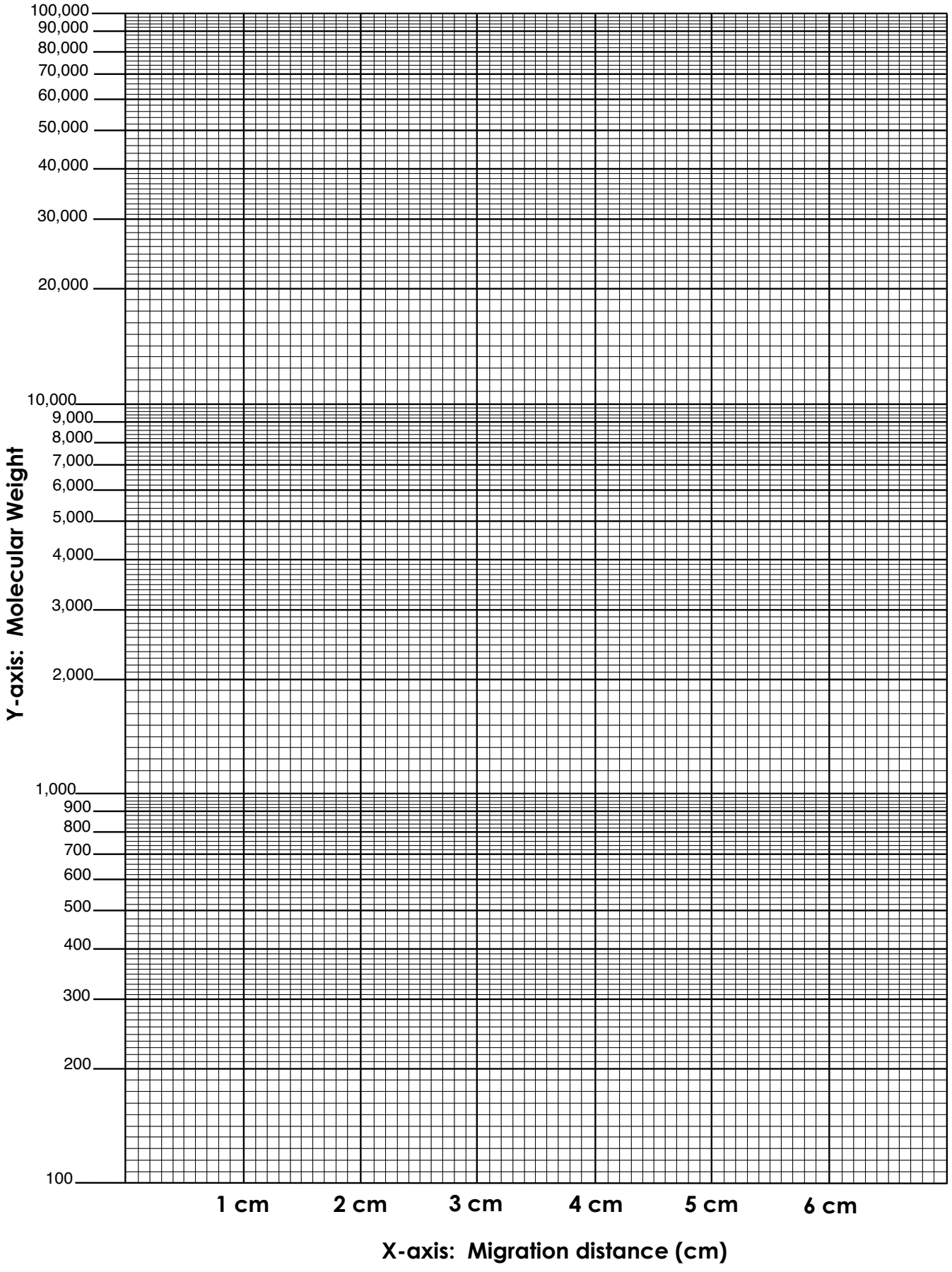
100,000
90,000
80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
900
800
700
600
500
400
300
200
100
Y-axis: Molecular Weight
1 cm
2 cm
3 cm
4 cm
5 cm
6 cm
X-axis: Migration distance (cm)

Study Questions

1. Why is the molecular sieve matrix swelled prior to packing the column?

2. What is the basis of molecular sieve chromatography?

3. Why might the native and denatured versions of the same protein show large differences in migration on a PAGE gel?

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Instructor's Guide

HOW THIS EXPERIMENT IS ORGANIZED

This experiment module contains biologicals and reagents for six (6) groups sharing three (3) polyacrylamide gels (2 groups per gel). Enough buffer is included for three (3) vertical electrophoresis units (Model MV10 or equivalent). Additional electrophoresis buffer and stain is required for more than three units and gels. ***Note: Polyacrylamide gels are not included. You may choose to purchase precast gels (Cat. #651 or #652).***

A variety of factors, such as class size, length of laboratory sessions, and availability of equipment, will influence the implementation of this experiment with your students. These guidelines can be adapted to fit your specific set of circumstances. To facilitate implementation, this experiment can be divided into general experimental activities with logical stopping points. These optional stopping points can occur after each series of procedures. Your personal preference and schedule will determine how and when the gels will be prepared for use in this experiment.

This experiment kit contains practice gel loading solution. If your students are unfamiliar with vertical gel electrophoresis, it is suggested that they practice loading the sample wells before performing the actual experiment. See Appendix B.

APPROXIMATE TIME REQUIREMENTS

	WHAT TO DO:	WHEN:	TIME REQ.
MODULE I	Prepare and aliquot Elution Buffer	Anytime before performing the experiment	10 min.
	Prepare and aliquot Molecular Sieve Matrix	60 mins before performing the experiment	60 min.
MODULE II	Aliquot Glycerol and Protein Denaturation Solution	Anytime before performing the experiment	10 min.
	Reconstitute & aliquot Molecular Weight Standards	Before or during the experiment	30 min.
	Prepare Electrophoresis buffer	Anytime before performing the experiment	10 min.
	Prepare Staining solutions	Anytime before performing the experiment	10 min.

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Pre Lab Preparations

MODULE I

Column Elution Buffer

1. **DILUTE** the Column Elution Buffer by mixing 30 mL of 10X Column Elution Buffer (C) with 270 mL of Distilled water.
2. **SAVE** 40 mL of the diluted column elution buffer for the preparation of the molecular sieve matrix (next step) and **DISPENSE** the remaining buffer for 6 student groups, 40 mL per group. **LABEL** this buffer "1X Column Elution Buffer". **STORE** on ice until needed or freeze for up to one week.

FOR MODULE I
Each Group Requires:

- 1 Chromatography column
- 1 Ring stand with clamp
- Adjustable volume micropipette & tips
- 10 Microcentrifuge tubes
- 1x Column Elution Buffer (40 mL)
- Molecular Sieve Matrix (6 mL)
- 1 tube "GFP extract" (220 µL)
- 1 tube "BFP extract" (220 µL)

Preparation of the Molecular Sieve Matrix

3. **ADD** 40 mL of the 1x Column Elution Buffer to the bottle containing Dry Molecular Sieve Matrix (E).
4. Gently **SWIRL** or stir the bottle of matrix to ensure there are no clumps. **INCUBATE** for 30-60 minutes, stirring occasionally to fully rehydrate.
5. **ALIQUOT** 6 mL for each of the 6 groups.
   NOTE: The prepared Molecular Sieve Matrix can be used immediately or stored covered at 4°C for up to 72 hours.

Cell Extracts Containing GFP and BFP

6. **THAW** the frozen Control Cell Extract containing GFP (A) and BFP (B) at room temperature and immediately **PLACE** on ice.
7. **LABEL** 6 tubes "GFP extract". **ALIQUOT** 220 µL of the GFP extract into the tubes. **PLACE** immediately back on ice.
8. **LABEL** 6 tubes "BFP extract". **ALIQUOT** 220 µL of the BFP extract into the tubes. **PLACE** immediately back on ice.

MODULE II

Reconstitution of Lyophilized Protein Molecular Weight Standards and Protein Solutions

Once rehydrated, the tube of Protein Molecular Weight Standards (J) contains enough material for loading 6 wells.

1. **ADD** 130 µL of distilled or deionized water to the tube of Standard Protein Markers (D) and **ALLOW** the material to hydrate for several minutes. **VORTEX** or mix vigorously.
2. **ALIQUOT** the Standard Protein Markers for each pair of student groups, or students can share the rehydrated sample stock tube. The volume of sample to load per well is 20 µL.
3. **STORE** any unused portion of reconstituted sample at -20°C.

continued

FOR MODULE II
Each Group Requires:

- Protein Standard markers (shared)
- 1 tube Glycerol
- 4 Microcentrifuge tubes (2 snap cap, 2 screw cap)
- 1 tube Protein Denaturing Solution (50 µL)

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Pre Lab Preparations, continued

MODULE II, CONTINUED

4. **ALIQUOT** 75 µL of Protein Denaturing Solution (F).
5. **ALIQUOT** 75 µL 50% Glycerol Solution (G).
6. Make sure each group also has a beaker, foil, and hot plate.

Preparation of the Electrophoresis Buffer

Prepare the electrophoresis buffer by adding and mixing 1 part Tris-Glycine-SDS electrophoresis buffer (10x) to 9 parts distilled water.

The approximate volume of 1X electrophoresis buffer required for EDVOTEK® Protein Vertical Electrophoresis units are listed in Table B, below. The buffer should just cover the back plate of the gel cassette.

Electrophoresis Time and Voltage

Your time requirements will dictate the voltage and the length of time for your samples to separate by electrophoresis. Approximate recommended times are listed in Table A, below.

Table A Time and Voltage Guidelines

	Recommended Time	
Volts	Minimum	Optimal
100	70 min.	90 min.
125	50 min.	60 min.
150	40 min.	50 min.

Table B Tris-Glycine-SDS Electrophoresis Buffer

EDVOTEK Model #	Conc. (10x) Buffer	+	Distilled Water	=	Total Volume
MV10	58 mL		522 mL		580 mL
MV20	95 mL		855 mL		950 mL

Preparation for Staining Gels

1. **PREPARE** a stock solution of white vinegar and ethanol* by combining 400 mL white vinegar with 200 mL ethanol. Gently **MIX**. **LABEL** as "Staining/Destaining Solution".
2. **ADD** 125 mL of the Staining/Destaining Solution to the bottle of FlashBlue™ Protein Stain. Shake briefly to **MIX**.
3. **STORE** both solutions at room temperature until needed.
4. Student groups will **SHARE**: 30 mL FlashBlue™ Protein Stain, 140 mL Staining/Destaining Solution, water, a staining tray, and plastic wrap.

FOR STAINING GELS
Two Groups will SHARE:

- 30 mL FlashBlue™ Protein Stain
- 140 mL Staining/Destaining Solution
- Staining tray and plastic wrap

**White vinegar, sometimes called distilled or spirit vinegar, is an easy to find cooking and cleaning vinegar with an acetic acid concentration between 5-8% and a pH ~2.6. Ethanol is a common lab supply which is available at various concentrations. Our FlashBlue™ Protein Stain has been designed to work with a wide range of white vinegars. However, we do recommend using 95% Ethanol or higher.*

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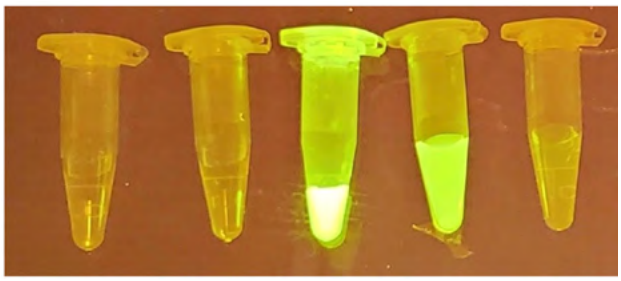
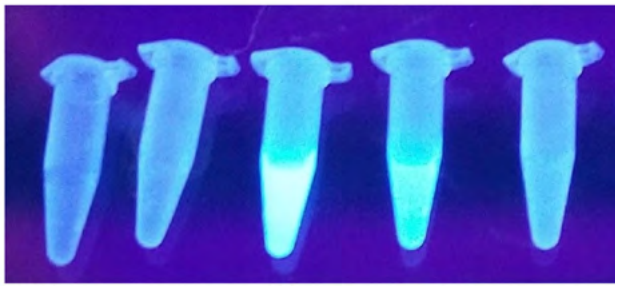
Experiment Results and Analysis

MODULE I

GFP Fractions



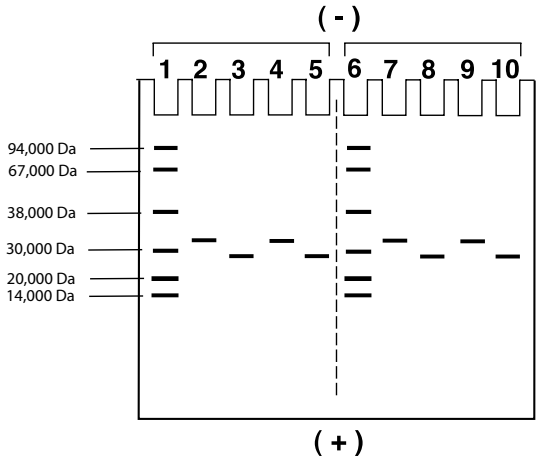
BFP Fractions

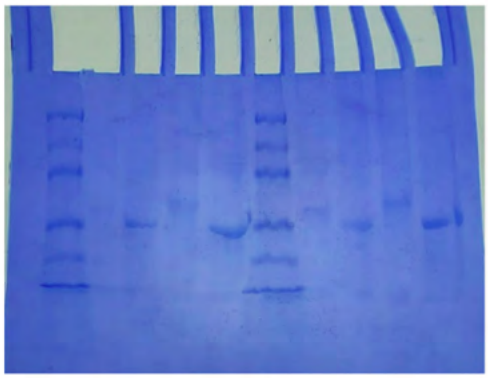


Results can vary. In this example, tubes #3 and #4 contained GFP and tubes #8 and #9 contained BFP.

MODULE II

The molecular weights of GFP and BFP are ~27 kDa and ~29 kDa. Lanes 3, 5, 8, and 10 show denatured proteins that have lost their tertiary structure. The proteins in lanes 2, 4, 7, and 9 have maintained their native structure and should still fluoresce.





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Please refer to the kit insert for the Answers to Study Questions

Appendix A

EDVOTEK® Troubleshooting Guide

PROBLEM:	CAUSE:	ANSWER:
Gel is not running properly.	Running buffer was not properly prepared.	Check buffer protocol, make fresh buffer.
	Wrong buffer used.	Check gel recipe, buffer must be compatible with the gel.
	Buffer volume is too low.	Buffer must fully cover the sample wells throughout the entire experiment.
	Gel is inserted in the wrong orientation.	Check with manufacturer for proper setup of the electrophoresis chamber.
	Malfunctioning electrophoresis chamber or power supply.	Consult with manufacturer of electrophoresis chamber or power supply.
	Tape at bottom of precast gel not removed.	Carefully remove tape before running the gel.
	Buffer volume is too low.	Buffer must fully cover the sample wells throughout the entire experiment.
	Electrodes not connected or polarity reversed.	Check electrode connections at the gel box and power supply.
Poor band resolution or separation.	Diffusion of samples before power was turned on.	Minimize time between loading samples and the start of electrophoresis.
	The gel is old or expired.	Make fresh gels or order new pre-cast gels.
	Wrong concentration of acrylamide gel.	The kit is designed for 12% acrylamide gels, other concentrations will affect results.
Smiling or frowing of bands.	Proteins have been overloaded.	EDVOTEK ® has optimized this kit to avoid overloading. Be sure to load the amount recommended by the protocol.
	Wrong buffer was used.	Check gel recipe, the buffer must be compatible with the gel.
	Incorrect voltage supplied to the gel.	Check the protocol for the recommended voltage (page 15).
No bands on gel/ smallest bands missing from gel.	Proteins ran off gel.	Use the appropriate length of time for the chosen voltage. Be sure to monitor the tracking dye while the gel is running. For best results, the tracking dye should run 8-9 cm and should not be allowed to run off the gel.
Proteins have accumulated in the wells of the gel.	Proteins have aggregated.	Ensure proteins have fully denatured; boil proteins for 5 min. and load while still warm.
Bands are smeary and distorted.	The gel has overheated.	Reduce voltage, check buffer concentration and dilute if necessary.
Bands are faint.	Proteins have diffused or faded.	Repeat staining using instructions on pages 16 and 17.
	Too little protein was loaded.	EDVOTEK ® has optimized this kit to avoid underloading. Be sure to load the amount recommended by the protocol.

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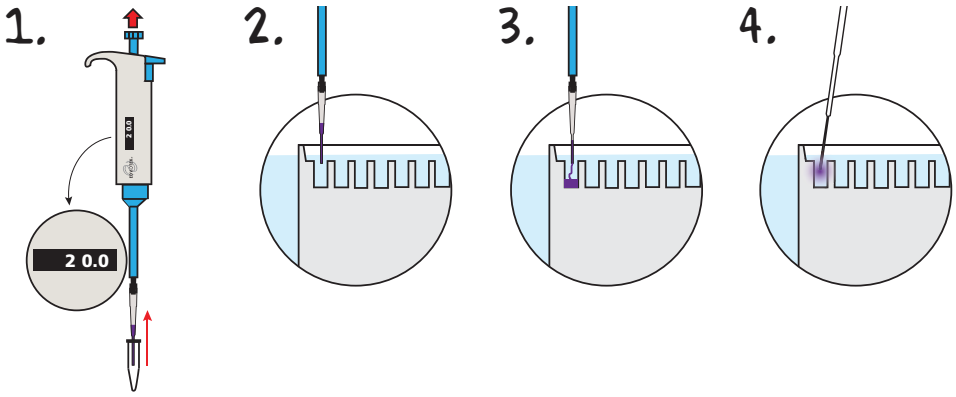
Appendix B
Practice Gel Loading

Prepare polyacrylamide gels and chambers beforehand using the instructions on page 14. Gel and chamber preparation and practice loading can be done prior to preparing the protein samples to provide more time flexibility.



Wear gloves and safety goggles

NOTE: EDVOTEK® Cat. #638, Fine Tip Micropipette Tips are recommended for loading samples into polyacrylamide gels. A regular microtip may damage the cassette and result in the loss of protein samples.

1. **PLACE** a fresh tip on the micropipette. **REMOVE** 20 µL of practice gel loading solution.

2. **PLACE** the lower portion of the pipette tip below the surface of the electrode buffer, directly over a sample well. The tip should be at an angle pointed towards the well. The tip should be partially against the back plate of the gel cassette, but the tip opening should be over the sample well. **Do not try to jam the pipette tip in between the plates of the gel cassette.**

3. **EJECT** all the sample by steadily pressing down on the plunger of the automatic pipette. Do not release the plunger before all the sample is ejected. Premature release of the plunger will cause buffer to mix with sample in the micropipette tip. Release the pipette plunger after the sample has been delivered and the pipette tip is out of the buffer.

4. **REMOVE** the practice gel loading solution from the sample wells. **FILL** a transfer pipet with buffer and **SQUIRT** a stream into the sample wells. This will displace the practice gel loading solution, which will be diluted into the buffer and will not interfere with the experiment. ***NOTE: Practice gel loading solution must be removed from the sample wells prior to sample loading.***

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