EDVOTEK®

The Biotechnology Education Company ®

Revised and Updated

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EDVO-Kit #

# Diversity of Fish Proteins

**Storage:**
Some components require refrigerator storage.
See page 3 for details.

## EXPERIMENT OBJECTIVES:

The objective of this experiment is to determine relationships between three species of fish based on their protein profiles.



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Material Safety Data Sheets can be found on our website: www.edvotek.com

# Experiment Components

There is enough of each sample for six (6) groups sharing three polyacrylamide gels.

Store fish proteins (components A-D) in the freezer.

| | | Storage |
|---|---|---|
| A | Standard Protein Markers | Freezer with desiccant |
| B | Perch Proteins | Freezer with desiccant |
| C | Salmon Proteins | Freezer with desiccant |
| D | Walleye Proteins | Freezer with desiccant |
| • | Tris-Glycine-SDS buffer (10X) | Room Temperature |
| • | Protein InstaStain® | Room Temperature |
| • | Practice Gel Loading Solution | |
| • | Transfer Pipets | |

LyphoProtein™ samples are protein samples which are denatured, lyophilized and ready for electrophoresis after rehydration and heating.

**None of the components have been prepared from human sources.**

# Requirements

- MV10 or MV20 vertical electrophoresis apparatus
- D.C. power supply
- Three 12% precast SDS polyacrylamide gels (Cat. #651 or #652)
- Micropipet and tips (Cat. #638 Fine Tip Micropipet Tips)
- 500 ml graduated cylinder
- Hot plate or burner
- Methanol (150 ml)
- Distilled or deionized water
- Beakers
- Glacial acetic acid (80 ml)
- Glass staining tray (optional)
- Aluminum foil or microtest tube holder
- Scissors
- Plastic wrap
- Spatula or gel spacer
- 500 gram weight
- White light box
- Small plastic tray or weigh boat
- Photodocumentation system (optional)

## Background Information

### DIVERSITY OF FISH PROTEINS

This experiment examines protein content of perch, salmon, and walleye. The mixture of proteins from the three fish are separated on denaturing SDS-polyacrylamide gels. The protein bands are prestained and the protein patterns are compared. Before introducing basic procedures for the analysis of structure and molecular weight of proteins, a brief background information will be provided on fish.

There are 20,000 species of bony fish worldwide with every possible size and shape. They are called "bony" because their skeletons are calcified. Fish have specialized mouths and great maneuverability. Bony fish exploit most marine and freshwater habitat on earth. The fish that are part of this experiment belong to the family of bony fish.

Perch are fresh water fish known for being very flavorful. There are hundreds of species of perch and few are more than four inches long with an average weight of eight ounces or less. These fish are swift bottom feeders. The yellow perch, *Perca flavescen,* is abundant in the Eastern and Midwestern United States. Their yellowish color and their seven, characteristic vertical black bars give this fish it's distinctive appearance. The European yellow perch closely resembles the American yellow perch and for a long time they were thought to be the same species. Perch are versatile feeders, taking crustaceans, aquatic insect larvae, worms, small clams and snails and anything else that is convenient, including the eggs of other fish.

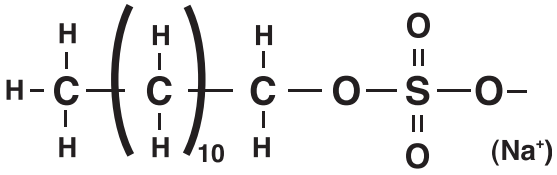



Figure 1 - The chemical structure of sodium dodecyl sulfate (SDS).

The yellow pike-perch, also known as walleyed perch or walleye, *Stizostedion vitreum,* is the largest member of the perch family averaging about two pounds in weight. They prefer cold waters where they inhabit the deep waters and lakes. The natural color of this fish is olive or yellowish, but their color varies considerably depending on where they live. The sides of walleye are mottled with black or brown with indefinite markings and a black or brown marking on the last rays of the first dorsal fin.

Salmon are highly carnivorous, feeding on live animals. Members of the family of *Salmonidae* are the best known and are important of all fish. Members of this group include trouts, salmon, white fishes and draylings. Salmon and their relatives are primitive fishes with fossil relatives that date for more than 100 million years. Salmon lack spines in the fins. Their requirement for oxygen is high and they are closely tied to the sea. The Atlantic salmon, *Salmo salar,* are bred in fresh rivers and spend their lives in salt water. After salmon enter salt water, they usually remain there until the urge to spawn drives them back to their original waters. Spawning occurs in American rivers in October and November. Salmon spend long periods of time in salt water prior to returning to fresh water to spawn. Most large salmon enter fresh water after about two years in salt water. In fact, it is thought that the exceptionally large fish, "maidens", spend a longer number of years in salt water prior to entering fresh water for the first time. However, the return to fresh water is not directly correlated to the age of fish since immature fish known as "grilse" sometimes accompany adults on their spawning migration.

## Background Information

### PROTEIN CHEMISTRY

Proteins are a highly diversified class of biomolecules. Differences in their chemical properties, such as charge, organic functional groups, shape, size, and solubility enable them to perform many biological functions. These functions include enzyme catalysis, metabolic regulation, transport of small molecules, gene regulation, immunological defense and cell structure.

The amino acid sequence variations provide a virtually unlimited set of polypeptides. A protein can have a net negative or a net positive charge, depending on its amino acid composition and the pH. At certain values of pH, the molecule can be electrically neutral overall, i.e. negative and positive charges are balanced. In such a case, the protein is isoelectric. In the presence of an electrical field, a protein with a net charge will migrate towards the electrode of opposite charge.

Proteins exhibit many different three-dimensional shapes and folding patterns which are determined by their amino acid sequence and intracellular processing. The precise three-dimensional configuration of a protein is critical to its function. Proteins have spherical, elliptical or rod-like shapes. The molecular weight is a function of the number and type of amino acids in the polypeptide chain. Proteins can consist of a single polypeptide or several polypeptides specifically associated with each other. Proteins that are in their biologically active forms are referred to as native proteins.

The physical-chemical properties of proteins affect the way they migrate during gel electrophoresis. Gels used in electrophoresis (e.g. agarose, polyacrylamide) consist of microscopic pores of a defined size range that act as a molecular sieve. Only molecules with net charge will migrate through the gel when it is in an electric field. Small molecules pass through the pores more easily than large ones. Molecules having more charge than others of the same shape and size will migrate faster. Molecules of the same mass and charge can have different shapes. Those with a more compact shape, like a sphere, will migrate through the gel more rapidly than those with an elongated shape, like a rod. In summary, the size and charge affect electrophoretic migration rates.

Sodium dodecyl sulfate (SDS) is a detergent which consists of a hydrocarbon chain bonded to a highly negatively charged sulfate group as shown in **Figure 1**. SDS binds strongly to most proteins and causes them to unfold to a random, rod-like chain. No covalent bonds are broken in this process. Therefore, the amino acid composition and sequence remain the same. Since its specific three-dimensional shape is abolished, the protein no longer possesses biological activity. Proteins that have lost their specific folding patterns and biological activity but have their polypeptide chains intact are called denatured. Proteins which contain several polypeptide chains that are associated only by non-covalent forces will be dissociated by SDS into separate, denatured polypeptide chains. Proteins can also contain covalent cross-links known as disulfide bonds. These bonds are formed between two cysteine amino acid residues that can be located in the same or different polypeptide chains. High concentrations of reducing agents, such as 2-mercaptoethanol, can break disulfide bonds. This allows SDS dissociate and denature proteins. Proteins that retain their disulfide links bind less SDS, causing anomalous migration.

## Background Information

In most cases, SDS binds to proteins in a constant ratio of 1.4 grams of SDS per gram of protein. On average, the number of bound SDS molecules is half the number of amino acid residues in the polypeptide. The negative charge due to SDS is much more than the negative and positive charges of the amino acid residues. The large quantity of bound SDS efficiently masks the intrinsic charges in proteins. Consequently, SDS denatured proteins are net negative and since the binding of the detergent is proportional to the mass of the protein, the charge to mass ratio is constant. The shape of SDS denatured proteins are rod-like. The size of the rod-like chains is the only gross physical difference between SDS denatured proteins. The larger the molecular weight of the protein the longer the rod-like chain. The pores in the gel distinguish these size differences. During SDS electrophoresis, proteins migrate through the gel toward the positive electrode at a rate that is inversely proportional to their molecular weight. In other words, the smaller the protein, the faster it migrates.

### DESCRIPTION OF PROTEIN SAMPLES

Standard Protein Markers are a mixture of proteins that give the following denatured molecular weights: 94,000; 67,000; 38,000; 30,000; 20,000 and 14,000 Da. The values have been rounded off for convenience in graphical analysis.

Protein samples have been denatured by SDS. Under the experimental conditions, proteins will have a mobility in the gel that is inversely proportional to the logarithm of their molecular weights. This assumes that proteins do not contain carbohydrate, lipid, or other biomolecules associated with them. Proteins of known molecular weights will be electrophoresed in parallel and used to estimate the molecular weights of the unknowns by graphical analysis. All protein samples contain buffer, SDS, a reducing agent for disulfide bonds, sucrose to create density greater than that of the electrode buffer, and the negatively charged tracking dye bromophenol blue. The tracking dye will migrate toward the positive (bottom) electrode, ahead of the smallest proteins.

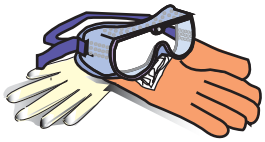
Since proteins are prestained, the protein bands will be visible during electrophoresis. The prestained proteins can be made more visible by staining the gel with Protein InstaStain®. Proteins are precipitated in the gel matrix by fixation. Fixation is necessary to prevent protein diffusion, which causes blurry bands and reduced intensity. Fixatives contain acetic acid and methanol. There are several staining methods for the visualization of proteins in the gel. Protein InstaStain® has been researched and developed by EDVOTEK®. It is a rapid and sensitive staining and destaining single step procedure that does not use corrosive chemicals.

## Experiment Overview

### EXPERIMENT OBJECTIVE:

The objective of this experiment is to determine relationships between three species of fish based on their protein profiles.

### LABORATORY SAFETY



Gloves and goggles should be worn routinely as good laboratory practice.

Un-polymerized acrylamide is a neurotoxin and should be handled with extreme caution in a fume hood. Gloves and goggles must be worn at all times. Use a pipet pump to measure polyacrylamide gel components. Polymerized acrylamide precast gels are safe but should still be handled with gloves.

## Protein Denaturation

The protein samples were shipped in lyophilized (freeze-dried) form and have been rehydrated by your instructor. These samples are denatured proteins which tend to form super-molecular aggregates and insoluble particulates. Heating disrupts aggregates of denatured proteins.

**NOTES:**

- ***If the protein samples (tubes A through D) have not been heated by your lab instructor, follow the heating procedure (Steps 1-2) to heat the samples.***
- ***If the protein samples have already been heated by your lab instructor, proceed with Electrophoresis of Proteins as outlines on page 9.***

**Quick Reference:**

The heating (Steps 1-2) disrupts aggregates of denatured proteins. Denatured proteins tend to form super-molecular aggregates and insoluble particulates.

1. Bring a beaker of water, covered with aluminum foil, to a boil. Remove from heat.
2. Make sure the sample tubes A through D are tightly capped and well labeled. The bottom of the tubes should be pushed through the foil and immersed in the boiling water for 5 minutes. The tubes should be kept suspended by the foil.
3. Proceed to loading the gel while the samples are still warm.

**NOTES:**

- ***Upon completion of loading the samples for electrophoresis, the unused portions of the protein samples can be frozen.***
- ***Remove the samples from the freezer and follow steps 1-3, above, to reheat and run the samples when using them at a later time.***

Experiment Procedure

## Electrophoresis of Proteins

### PREPARING THE POLYACRYLAMIDE GEL FOR ELECTROPHORESIS

**Precast Polyacrylamide Gels:**

Precast polyacrylamide gels will vary slightly in design. Procedures for their use will be similar.

1. Open the pouch containing the gel cassette with scissors. Remove the cassette and place it on the bench top with the front facing up.

   *Note: The front plate is smaller (shorter) than the back plate.*

2. Some cassettes will have tape at the bottom of the front plate. Remove all of the tape to expose the bottom of the gel to allow electrical contact.

3. Insert the Gel Cassette into the electrophoresis chamber.

4. Remove the comb by placing your thumbs on the ridges and pushing (pressing) upwards, carefully and slowly.

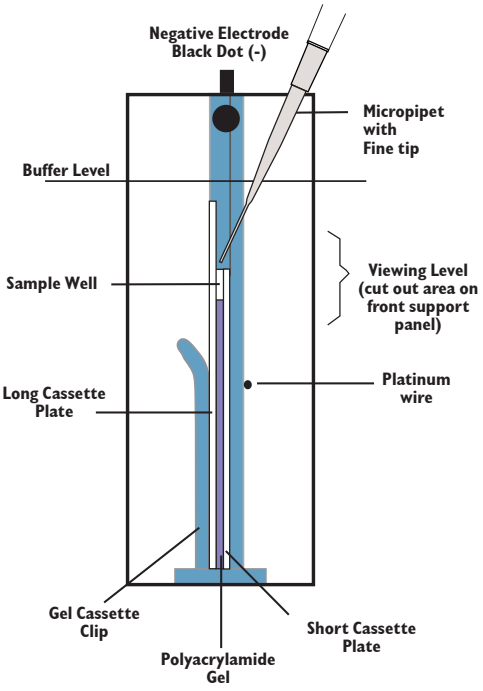
### PROPER ORIENTATION OF THE GEL IN THE ELECTROPHORESIS UNIT

1. Place the gel cassette in the electrophoresis unit in the proper orientation. Protein samples will not separate in the gel if the cassette is not oriented correctly. Follow the directions accompanying the specific apparatus.

2. Add the diluted buffer into the chamber. The sample wells and the back plate of the gel cassette should be submerged under buffer.

3. Rinse each well by squirting electrophoresis buffer into the wells using a transfer pipet.

   The gel is now ready for practice gel loading or sample loading.




The figure above shows a polyacrylamide gel cassette in the EDVOTEK® Vertical Electrophoresis Apparatus, Model #MV10.

Experiment Procedure

## Electrophoresis of Proteins




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

### PRACTICE GEL LOADING

**EDVOTEK® Cat. #638, Fine Tip Micropipet 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 fine tip on the micropipet. Aspirate 20 µl of practice gel loading solution.

2. Place the lower portion of the fine pipet tip between the two glass plates, 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, as illustrated in the figure on page 9.

   Do not try to jam the pipet tip in between the plates of the gel cassette.

4. Eject all the sample by steadily pressing down on the plunger of the automatic pipet.

   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 micropipet tip. Release the pipet plunger after the sample has been delivered and the pipet tip is out of the buffer.

5. Before loading protein samples for the actual experiment, the practice gel loading solution must be removed from the sample wells.

   Do this by filling a transfer pipet with buffer and squirting 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.

## Electrophoresis of Proteins

### LOADING PROTEIN SAMPLES

Change fine pipet tips between loading each sample. Make sure the wells are cleared of all practice loading solution by gently squirting electrophoresis buffer into the wells with a transfer pipet.

Groups 1 and 2 will share the first gel. Groups 3 and 4 will share the second gel. Groups 5 & 6 will share the third gel. Pipet the samples while still warm from boiling. Load the gel as follows.

| | |
|---|---|
| Lane 1 | 20 µl of Standard Protein Markers (A) |
| Lane 2 | 20 µl of perch soluble protein (B) |
| Lane 3 | 20 µl of salmon soluble protein (C) |
| Lane 4 | 20 µl of walleye soluble protein (D) |
| Lane 6 | 20 µl of Standard Protein Markers (A) |
| Lane 7 | 20 µl of perch soluble protein (B) |
| Lane 8 | 20 µl of salmon soluble protein (C) |
| Lane 9 | 20 µl of walleye soluble protein (D) |

### RUNNING THE GEL

1. After the samples are loaded, carefully snap the cover all the way down onto the electrode terminals. The black plug in the cover should be on the terminal with the black dot.

2. Insert the plug of the black wire into the black input of the power supply (negative input). Insert the plug of the red wire into the red input of the power supply (positive input).

3. Set the power supply at the required voltage and run the electrophoresis for the length of time as determined by your instructor. When the current is flowing, you should see bubbles forming on the electrodes. The sudsing is due to the SDS in the buffer.

4. After the electrophoresis is finished, turn off power, unplug the unit, disconnect the leads and remove the cover.

**Time and Voltage**

| Volts | Recommended Time | |
|---|---|---|
| | Minimum | Optimal |
| 125 | 60 min | 75 min |

## Staining the Gel

Wear gloves
and safety goggles

### STAINING WITH PROTEIN INSTASTAIN® IN ONE EASY STEP

EDVOTEK features a state-of-the-art, proprietary stain for DNA or Protein gels called InstaStain®. Protein Polyacrylamide gels can be stained with Protein InstaStain® cards in one easy step. Staining is rapid, sensitive and Polyacrylamide gels are ready for visualization in 1-3 hours.

InstaStain® Blue and InstaStain® Ethidium Bromide are also available from EDVOTEK for staining of DNA gels.

1. After electrophoresis, turn off the power and remove the gel cassette from the gel electrophoresis apparatus.
2. To remove the gel from the cassette, lay the cassette down and carefully remove the front plate by placing a coin or a spatula in the slot at the top edge, near the sample wells, and twist to separate the two plates of the cassette.
3. Gently lift the front plate away from the larger back plate. In most cases, the gel will stay on the back plate. If the gel partially sticks to the front plate, let it fall onto the back plate.
4. Pour approximately 100 ml of fixative solution in a small tray.
5. Transfer the back plate of the cassette (with the gel) into the tray containing the fixative solution. Wet gloved fingers with fixative solution and gently nudge the gel off the back plate and remove the plate, leaving the gel submerged in the fixative solution.
6. Gently float a sheet of Protein InstaStain® card with the stain side (blue) facing in the liquid. Remove the Protein InstaStain® card after 30 minutes.
7. Cover the staining tray with saran wrap to prevent evaporation.
8. Gently agitate on a rocking platform for 1-3 hours or overnight.
9. After staining, Protein bands will appear medium to dark blue against a light background* and will be ready for excellent photographic results.

* *Destaining is usually not required but can be carried out if the gel background is too dark. Gels can be destained in several changes of fresh destaining solution until the appearance and contrast of the protein bands against the background improves.*

**NOTE:**

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

**Fixative and Destaining Solution for each gel (100ml)**

| | |
|---|---|
| 50 ml | Methanol |
| 10 ml | Glacial Acetic Acid |
| 40 ml | Distilled Water |

Experiment Procedure

## Staining the Gel

### Storing the Gel

Once satisfactory result is achieved, the gel can be stored in distilled or deionized water.

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.

## Study Questions

1. A purified, preparation of a fish protein was submitted to native polyacrylamide gel electrophoresis. Three major bands were observed after staining. The same preparation of protein was denatured and submitted to SDS-polyacrylamide gel electrophoresis. More than three bands were observed after staining. Explain these results.

2. Can an estimate of the native molecular weight of a protein be determined from DNA sequences of structural genes? (HINT: DNA is transcribed and translated. An average amino acid is estimated to be 100 daltons in molecular weight.)

3. Why are varying intensities in protein bands obtained upon separating a mixture of proteins by denaturing SDS-polyacrylamide gel analysis and after staining with Protein InstaStain®?

4. Would there be a pattern variation if a native protein is denatured with SDS in the presence and absence of a reducing agent, such as β-mercaptoethanol?

Experiment Procedure

# 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). One group should load samples in lanes 1-4 and the other group should load samples in lanes 6-9. Enough buffer is included for three (3) vertical electrophoresis units (Model MV10 or equivalent). Additional electrophoresis buffer is required for more than three units.

**Note: Polyacrylamide gels are not included. You may choose to purchase precast gels (Cat. #s 651 or 652).**

The experimental procedures consist of three major parts:
1) separation of proteins on polyacrylamide gels,
2) optimal staining of protein bands,
3) identifying major protein bands in various extracts.

The staining of protein bands can be conducted using Protein InstaStain®, a new state-of-the-art method of staining. Protein InstaStain® is a proprietary staining method available exclusively from EDVOTEK®.

## APPROXIMATE TIME REQUIREMENTS FOR PRE-LAB AND EXPERIMENTAL PROCEDURES

1. Pre-lab preparations will require approximately 20 minutes on the day of the lab.

2. Students will require approximately 15 minutes to heat samples and load the gel. Practice gel loading may require an additional 15 minutes if performed the same day of the lab.

3. Electrophoresis will require approximately 1 to 1.5 hours, depending on the voltage.

## PRACTICE GEL LOADING

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.

## PreLab Preparations

### RECONSTITUTION OF LYOPHILIZED FISH PROTEINS (LYPHOPROTEINS™)

Each tube contains enough material for loading 6 wells.

1. Add 125 µl of distilled or deionized water to each tube (A-D) and allow the samples to hydrate for several minutes. Vortex or mix vigorously.

2. Bring a beaker of water, covered with aluminum foil, to a boil. Remove from heat.

3. Make sure the sample tubes A through D are tightly capped and well labeled. The bottom of the tubes should be pushed through the foil and immersed in the boiling water for 5 minutes. The tubes should be kept suspended by the foil.

4. Samples can be aliquoted for each of the 6 student groups, or students can share the rehydrated sample stock tubes. **Have students load samples onto the polyacrylamide gel while the samples are still warm to avoid aggregation.** The volume of sample to load per well is 20 µl.

5. Store any unused portion of reconstituted sample at -20°C and repeat steps 2 and 3 when using samples at a later time.

### PREPARING ELECTROPHORESIS BUFFER

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

The approximate volume of 1x electrophoresis buffer required for EDVOTEK Protein Vertical Electrophoresis units are listed in the table below. The buffer should just cover the back plate of the gel cassette.

**Tris-Glycine-SDS Electrophoresis (Chamber) Buffer**

| EDVOTEK Model # | Concentrated Buffer (10x) | + | Distilled Water | = | Total Volume |
|---|---|---|---|---|---|
| MV10 | 58 ml | | 522 ml | | 580 ml |
| MV20 | 95 ml | | 855 ml | | 950 ml |

## Pre-Lab Preparations

### 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 the table at right.

**Time and Voltage**

| Volts | Recommended Time | |
|---|---|---|
| | Minimum | Optimal |
| 125 | 60 min | 75 min |

### PREPARING STAINING AND DESTAINING SOLUTIONS

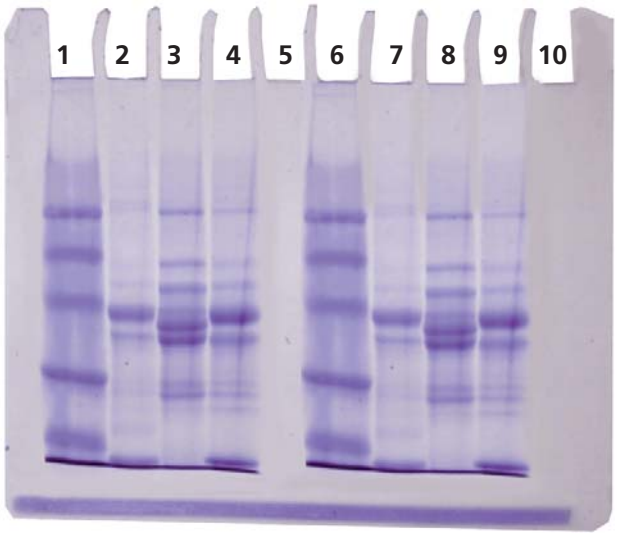
***The stock solution is used for staining and destaining with Protein InstaStain®***

1. **Solution for staining with Protein InstaStain®**
   - Prepare a stock solution of Methanol and Glacial Acetic Acid by combining 180 ml Methanol, 140 ml Distilled water, and 40 ml Glacial Acetic Acid.
   - Staining of Protein Gel(s) is optional.
2. **Destaining Solution**
   - Use the stock solution of Methanol, Glacial Acetic acid and distilled water (in Step 1) to destain the gel(s).

## Idealized Schematic of Results

The figure below is an idealized schematic showing relative positions of the protein bands. Actual results will yield broader bands of varying intensities. The idealized schematic shows the relative positions of the bands, but are not depicted to scale.

| Lane | Sample | Protein |
|---|---|---|
| 1, 6 | A | Standard Protein Markers |
| 2, 7 | B | Walleye soluble protein |
| 3, 8 | C | Salmon soluble protein |
| 4, 9 | D | Perch soluble protein |



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