Biotechnology Explorer™

Protein Fingerprinting

Instruction Manual

Catalog Number
166-0100EDU

explorer.bio-rad.com

Components of this kit ship in separate containers. Store the protein standards in the freezer within 4 weeks of receipt.

BIO-RAD

There's Nothing Fishy about Teaching Evolution!
Introduction to the Protein Fingerprinting Curriculum and Kit

In 1859 Charles Darwin published his famous work on evolution, The Origin of Species. In it he wrote:

> "Whatever the cause may be of each slight difference in the offspring from their parents, and a cause for each must exist – it is the steady accumulation, through natural selection, of such differences by which the innumerable beings on the face of this earth are enabled to struggle with each other and the best adapted to survive."

Since Darwin's time, much has been learned about the genetic basis of evolution. This laboratory activity moves beyond DNA and focuses on what it produces: the proteins that encode form, function, and traits of organisms. Students learn to use one of the key tools of molecular biology research—protein electrophoresis—to visualize and analyze the molecular "fingerprints" of protein content in fish muscle tissue. Since proteins are encoded by specific DNA sequences, protein fingerprints can reflect genetic similarities as well as differences among organisms. This curriculum engages students to think critically about the molecular basis for such similarities and differences, the connection between genes and functions, and the implications of evolutionary relationships among species, from a perspective deeper than outward body forms. The protein electrophoresis technique gives students a powerful tool for generating their own molecular data to test hypotheses about the relatedness of sample species.

This curriculum is an overview of the molecular processes underlying evolution, suitable for students with a basic knowledge of molecular biology. The students produce data to examine the theory of evolution in the context of the central framework of molecular biology, DNA→RNA→Protein→Trait. Gradual and accumulated changes in DNA, which translate to modified or novel proteins and traits, are acted upon by natural selection. These processes create diversity in living forms, and preserve and perpetuate those that are successful in their environments. This is the modern version of Darwin's theory of evolution, a central and unifying concept in biology today.

This manual is organized into two main sections—the Teacher's Guide and the Student Manual. The Teacher's Guide contains three sections: an overview of the molecular basis of evolution background about the techniques being introduced, and a day-by-day guide to the students' lab procedures. There is much information to work with, so the teacher should decide on the level of complexity to present. The Student Manual presents background information, experimental objectives, and detailed laboratory procedures. A list of sources for further information is given at the end of the manual.

Teaching Strategy – Guided, Inquiry-Based Investigation

The intent of this curriculum is to guide students through the thought processes of a laboratory-based scientific investigation. The central question that ties together all aspects of this investigation is: Do biomolecules reflect evolutionary relationships? In their analyses, students compare similarities and differences in their samples' protein profiles against an evolutionary tree and are asked: Are the molecular data consistent with the given tree? If not, what explanations might there be? What is the connection between proteins, and the criteria that were used to draw the given tree? These questions help the students to interpret their laboratory data, to use them to test hypotheses, and to lead them to integrate the morphological and molecular evidence for evolution.

Students who engage in Biotechnology Explorer activities develop a positive sense of their ability to apply real research tools to relevant questions and hypotheses. Thought-provoking questions embedded in the Student Manual are designed to maximize students' engagement in the laboratory. Student involvement in this process results in an increased understanding of the value of approaching a scientific challenge in an organized and logical fashion.

We strive continually to improve our curricula and products and welcome your stories and ideas!

Ron Mardigian
Dr. Patti Taranto
Bio-Rad Laboratories
ron_mardigian@bio-rad.com
patti_taranto@bio-rad.com
1-800-424-6723

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Introduction and Overview for the Teacher

This laboratory centers on the theory and practice of correlating molecular characteristics with evolutionary relationships among species. In this protein fingerprinting activity your students will move beyond DNA to explore proteins as an indicator of evolution, and use the technique of electrophoresis to compare protein profiles of closely and distantly related species of fish. Molecular biology has given us strong evidence that all of the diverse forms of life evolved from a common ancestor and also provides a plausible mechanism for the emergence of new traits. You and your students will be amazed at what you discover!

Intended Audience

It is our goal to provide biotechnology curricula and activities that integrate seamlessly into existing high school and college biology curricula, textbooks, and lab activities. This protein fingerprinting activity can stand alone, or complement the lessons and activities in our DNA fingerprinting, PCR-DNA amplification, and bacterial transformation kits. All Biotechnology Explorer kits and curricula are designed to deepen student understanding of concepts, techniques, and issues current in molecular biology and biotechnology.

Curricular Synergy, Fit, and Related Topics

- Molecular biology
- Biotechnology
- Biology
- Genetics
- Evolution

Recommended Student Background

Prior to this activity, your students should have some familiarity with the following concepts. Please read through these sections and cover these concepts with your students before beginning the laboratory. Answers to the questions and considerations posed throughout the student laboratory activities are derived from the background material presented in the Teacher's Guide.

- Darwinian theory of evolution
- Phylogenetic relationships
- DNA's genetic instructions expressed as proteins
- Commonality of DNA chemical structure and code in all organisms
- DNA mutation: kinds, causes, and effects
- Protein synthesis: DNA→RNA→Protein
- Protein structure: primary, secondary, tertiary and quaternary
- Protein functions: enzymatic and structural
- Major proteins in muscle
- Electrophoresis: basic theory and SDS-PAGE
- Microscale units of measure
- Use of micropipets

Expected Outcomes

Following this laboratory your students should be able to:

- Explain how polyacrylamide gel electrophoresis gives information about proteins
- Describe how DNA mutations might lead to new traits over time
- Interpret protein profiles as molecular evidence for evolution
- Compare phylogenetic relationships with protein gel data
- Apply the framework of molecular biology to understand evolution: DNA→RNA→Protein→Trait
- Propose a molecular mechanism to explain evolution

Activity Timeline

The three laboratory sessions are designed to be carried out in consecutive 50 minute periods. We recommend at least one day be added in advance to set the stage for this laboratory and at least one day be added at the end for discussion and student presentations of their results and interpretations.

Laboratory Day 1	Sample preparation – muscle protein extraction
Laboratory Day 2	Electrophoresis – load, run and stain polyacrylamide gels
Laboratory Day 3	Gel preservation; analyze and interpret results

Safety Issues

Precast polyacrylamide gels are safe and nontoxic. However, as standard practice, gloves, laboratory coats and goggles should always be worn by students throughout any laboratory activity.

Electrophoresis combines liquid and electricity and, therefore, presents a potential electric shock hazard. Proper use of equipment prevents this risk, but students must be made aware of the possibility and taught to handle equipment as instructed.

Students with known or suspected allergies should avoid all contact with shellfish samples.

The curriculum for this activity was developed in collaboration with:

Dr. Kristi Decourcy
Fralin Biotechnology Center
Virginia Tech, Blacksburg VA

Protein Fingerprinting Laboratory – Kit Inventory and Supplies Checklist:

This section lists the equipment and reagents necessary to conduct protein fingerprinting in your classroom or teaching laboratory. We recommend that students be teamed up—two to four students per workstation. The complete Biotechnology Explorer protein fingerprinting kit (Catalog # 166-0100EDU) contains consumable components and will supply 24 student teams. Many components have unlimited shelf life. The Kaleidoscope™ prestained standards and actin and myosin standards are shipped at room temperature and should be stored in the freezer (-20°C) within 4 weeks of receipt.

The number of gels, gel boxes, pipets and power supplies needed will depend upon the number of workstations you choose to set up and should be ordered individually. Each Mini Protean 3 electrophoresis module (gel box) is designed to run one or two gels.

TIP We recommend one 10-well gel be used per student workstation with each gel box running two gels.

Complete Protein Fingerprinting Kit Complete Contents, 166-0100EDU

Prot/Elect pipet tips for gel loading—rack of 200	❑	Refill, 5 racks/box	223-9917EDU	❑
1.5 ml EZ fliptop micro test tube—120, 4 bags of 30	❑	Refill, 500/box	223-9480EDU	❑
1.5 ml screwcap micro test tube—200, 4 bags of 50	❑	Refill, 500/box	224-0100EDU	❑
Disposable 1 ml pipets (DPTPs)—30, 3 bags of 10	❑	Refill, 500/box	223-9522EDU	❑
Laemmli sample buffer—30ml	❑	Refill, 30 ml	161-0737EDU	❑
Kaleidoscope prestained standards—500 µl	❑	Refill, 500 µl	161-0324EDU	❑
10X Tris-glycine-SDS electrophoresis buffer—1 L	❑	Refill, 1 L	161-0732EDU	❑
Bio-Safe™ Coomassie Stain for proteins—1 L	❑	Refill, 1 L	161-0786EDU	❑
Actin and myosin standard—500 µg lyophilized	❑	Refill, 500 µg/vial	166-0010EDU	❑
Gel staining trays—24	❑			
Floating microtube racks—24	❑			

Note: Contents shipped in separate containers.

Required Laboratory Equipment, Gels, Water, and Samples:

* Ready Gel® precast polyacrylamide gels, 15%, 10-well, each one per workstation	161-1103EDU	❑
Mini-PROTEAN 3 electrophoresis module (gel box)—runs one or two gels	165-3302EDU	❑
Power supply (200 volt constant)		
PowerPac Junior™ 100 V and 200 V constant—runs two gel boxes at once	165-5048EDU	❑
or PowerPac™ 300 variable: 0~300 V, 0~400 mA—runs four gel boxes at once	165-5050EDU	❑
2–20 µl micropipet per workstation	166-0506EDU	❑
or 10 µl fixed-volume pipet	166-0512EDU	❑
Distilled or deionized water—1 gallon	grocery store	❑
**Fish samples (5–8 types)—1 gm each per workstation	store, river or lake	❑
Scissors or knife to cut fish samples		❑

*** Precast polyacrylamide gels have a shelf life of 12 weeks and should be ordered shortly before using.**

** Fish specimens to be analyzed

Recommended (optional) Accessories:

Sample loading guides – for 10 well comb	each	165-3146EDU	❑
GelAir™ cellophane support, 50 sheets		165-1779EDU	❑
GelAir drying frames (2 frames, 16 clamps up to 6 gels per frame)		165-1775EDU	❑
GelAir assembly table		165-1776EDU	❑
GelAir Dryer–gel drying oven (holds 4 drying racks)		165-1777EDU	❑
Water bath, ambient to 100°C		166-0504EDU	❑
Gloves			❑
Safety glasses			❑

Protein Electrophoresis System – Hardware to Set Up Two Complete Student Workstations

1 - Mini-PROTEAN 3 electrophoresis module (workstation = 1 gel; 2 gels/box)

1 – Sample loading guides for 10-well gels

1 - PowerPac Junior power supply

2 - Micropipets, 2–20 µl

This laboratory exercise focuses on comparisons of different fish species, all of which should be available from your grocery store, ocean, river, lake or stream. Please refer to the teacher preparation section on pages 12–15 for recommended varieties. Other aquatic organisms, such as shrimp, mussels, crab, and imitation crab also work well with this laboratory protocol, and their use can widen the discussions to include different phyla.

Background and Fundamentals for Students

We recommend that your lecture(s) for this activity cover the topics discussed below. These concepts enrich student understanding of this laboratory.

The basis of this investigation: Students will use polyacrylamide gel electrophoresis to compare protein profiles derived from muscle tissue of different fish. Since proteins are specified by DNA sequences, as genetic (DNA) changes occur and pass from generation to generation, so do protein composition and organismal form change over time. To some degree, genetic similarities and differences can be detected and inferred from such electrophoretic protein profiles.

The hypothesis for this activity: The closer that two species are placed on an evolutionary tree based on morphological characters, the more similar their protein fingerprints will be.

The Molecular Basis of Evolution

Genetic evolution can be defined as the change in a gene pool (collective DNA of a species) over time. Alterations in DNA, called mutations, can alter proteins so that they function differently. These changes can lead to novel traits and generate diversity – individual differences among members of a population. Occasionally, a genetic mutation will alter a protein's structure to confer a functional advantage, enhancing an organism's survival in a particular niche, environment, or predator-prey relationship. Thus, genetic diversity makes evolution possible, since natural selection favors some individuals and not others, and this leads to changes in the composition of the gene pool over time. DNA provides both for the continuity of traits from one generation to the next and accounts for the variation that can lead to differences within a species – and even to entirely new species. As Francis Crick, one of the discoverer's of the structure of DNA, said of his and James Watson's finding:

> "We have discovered the secret of life."
> Francis Crick, Eagle Pub, Cambridge, 1953

The scientific theory of **genetic evolution** is based on the assumption that all life evolved from a common single-celled ancestor, through modification and diversification of earlier lineages into the forms that exist today. This theory is strongly supported by the discovery that a great deal of DNA sequence similarity exists among the genes of all modern-day organisms. For example, scientists were astounded to discover that the same family of genes (Hox genes) controls the embryonic development of animals as diverse as fruit flies, zebrafish, and humans.

> "At the level of embryology we are glorified flies."
> Matt Ridley, Genome, 1999

The high levels of gene sequence similarity among diverse organisms can only be due to common ancestry, or **homology.** (This term is also used in reference to anatomical structures with a common derivation, such as primate hands, bat wings, and whale flippers.) Complex proteins in higher organisms have evolved by the shuffling and alteration of functional protein domains that originated in ancient life forms. Families of ion channels, gene activators, gene replicators, muscle proteins, and other functions are found in organisms everywhere, including those deep in the sea, underneath rocks, and even flying above us in the sky.

Human evolutionists have long argued over which primate is more closely related to us, the chimpanzee or the gorilla. With modern techniques for analyzing DNA, chimpanzees have been shown to share 98.4% of their DNA with humans, slightly more than either does with the gorilla (King and Wilson 1975).

Traditional classifications of organisms, including kingdom, phylum, class, order, etc., have been based primarily on morphological characteristics—traits that can be seen with the unaided eye or microscope. Molecular biology allows us to reassess these classifications with a deeper knowledge of the common genetic constitution of all organisms. During this laboratory activity your students will explore evolution at its very roots—by looking at the genetically determined molecules that give organisms their traits.

The Molecular Framework of Biology
DNA→RNA→PROTEIN→Trait

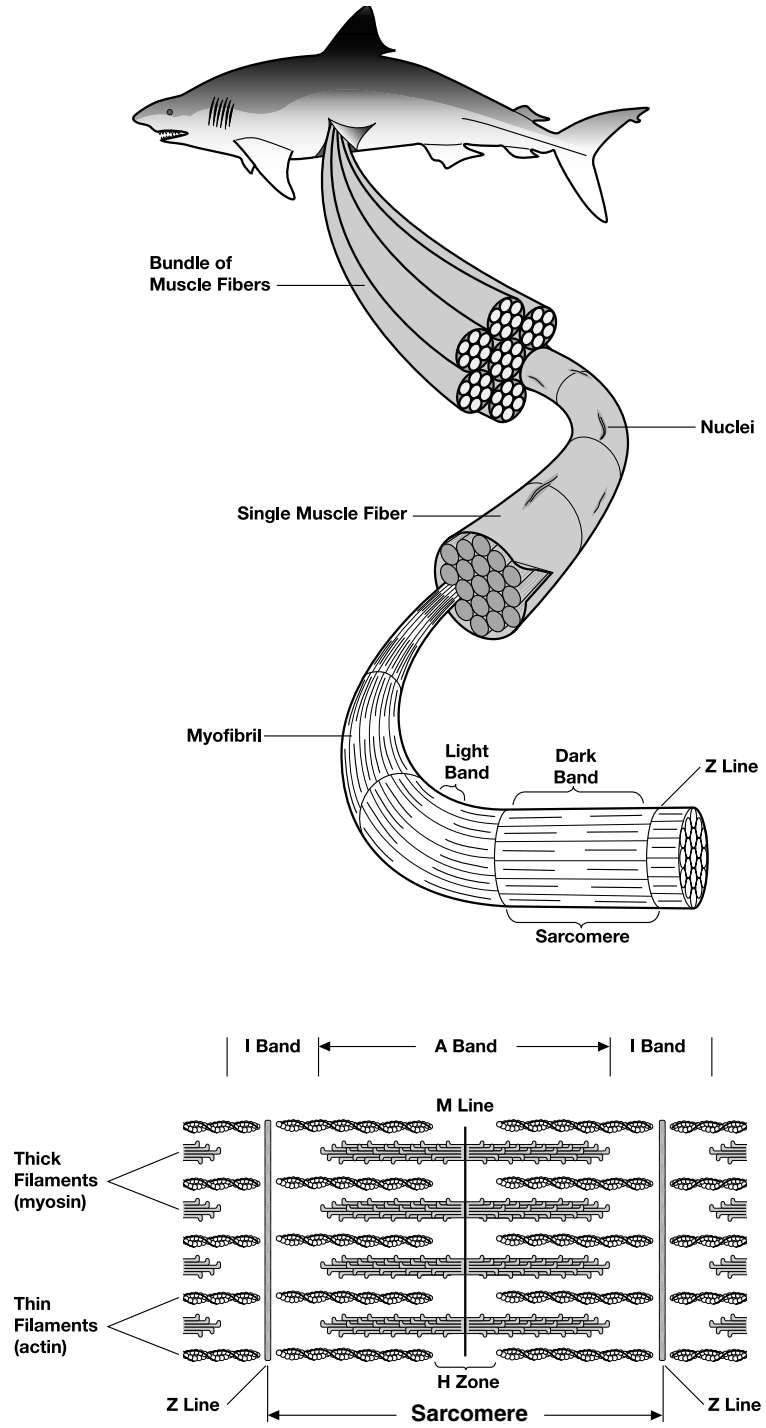
DNA has been a primary focus of research recently, but the ultimate function of DNA is to specify what proteins are made. In fact, recent studies of the genome sequences of humans and other organisms have revealed that the number of proteins expressed by a species contributes more to its complexity than does the number of genes (Jasny and Kennedy 2001, International Human Genome Consortium 2001). Protein molecules perform a stunning variety of functions, from catalyzing chemical reactions to forming the structures of our body. Universally, every living cell requires specific enzymes for the thousands of chemical reactions that maintain life. Each enzyme is a protein with a unique sequence of amino acids, which is specified by a gene with a unique DNA sequence.

Proteins also form structural units such as cartilage and muscle tissue. For example, the contraction of muscle results from the interaction of two main proteins, actin and myosin. Like enzymes, muscle proteins such as actin and myosin are specified by genes with unique DNA sequences. Both actin and myosin and the unique structures into which they are organized are conserved among all animals, no matter how diverse. This conservation implies that the encoding DNA sequences have changed little through evolutionary time.

Just as similarities of structures indicate a common descent, variations and differences indicate divergence on the evolutionary tree. In this laboratory, students generate muscle protein fingerprints from several different species of fish and use them to infer evolutionary relationships. Before giving some background on fish evolution, we will provide more detail about the types of proteins found in muscle tissue.

Muscle Proteins

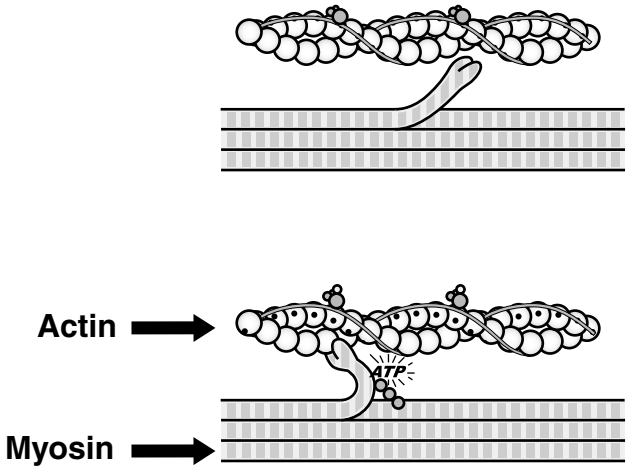
Our most familiar daily movements, from walking to simply breathing, are driven by the interactions between specialized proteins in our muscle fibers. The basic contractile elements of the muscle cells are the myofibrils that are bundled into muscle fibers. Each myofibril consists of a linear series of contractile units called sarcomeres.



(Figure modified from Campbell 1996 with permission)

Figure 1. Telescopic view of muscle structure: Thick myosin filaments and thin actin filaments form myofibrils, which are bundled together to make muscle fibers.

Sarcomeres are precisely arranged assemblies of actin and myosin protein filaments. Thin filaments of actin are aligned with thick filaments of myosin in a parallel and partly overlapping manner. The sarcomere shortens when myosin hydrolyzes ATP to slide along the actin filament, pulling the ends of the sarcomere towards each other. The combined contraction of many sarcomeres along a muscle fiber causes contraction of the entire muscle. It is important to note that, although actin and myosin are the major components, other proteins are also found in muscle tissue.



(Figure modified from Campbell 1996 with permission)

Figure 2. Hydrolysis of ATP causes myosin and actin filaments to slide past one another, shortening the sarcomere and contracting the muscle.

Other Muscle Proteins

Numerous proteins besides actin and myosin are also required for muscle contraction (see table below). While actin and myosin are highly conserved across all animal species, other muscle proteins show more variability. These variations in an organism's muscle proteins may reflect refinements of muscle function and performance that are adaptive to particular niches, environments, or physiological stresses.

Characterized muscle proteins, in order of decreasing size, adapted from Alberts et al. 1994.

Protein	MW (in kD)	Function
titin	3000	centers myosin in sarcomere
dystrophin	400	anchoring to plasma membrane
filamin	270	cross-links actin filaments into a gel
myosin heavy chain	210	slides actin filaments
spectrin	265	attaches filaments to plasma membrane
M1/M2	190	myosin breakdown product
M3	150	myosin breakdown product
C protein	140	myosin breakdown product
nebulin	107	regulates actin assembly
α-actinin	100	bundles actin filaments
gelsolin	90	fragments actin filaments
fimbrin	68	bundles actin filaments
actin	42	forms filaments
tropomyosin	35	strengthens actin filaments
troponin (T)	30	regulates contraction
myosin light chains	24, 17, 15	slide actin filaments
troponin (I)	19	regulates contraction
troponin (C)	17	regulates contraction
thymosin	5	sequesters actin monomers

In this protein fingerprinting laboratory, actin and myosin will be present in all species but the presence of other proteins will be more variable. Students will compare the variable proteins of the different fish samples to assess evolutionary relatedness among species. Once students have carried out this analysis, they can compare their results with evolutionary trees based on morphological data.

Next, we will outline fish evolution before covering how protein fingerprinting works.

Evolution and Classification of Fishes

To help with the selection of fish for the laboratory and to aid interpretation of the molecular data, students need to have some understanding of fish evolution. The evolutionary charts below will aid you in selecting appropriate varieties. The idea is to compare both closely related and distantly related fish. We recommend that you, or your students, research additional information on the evolutionary histories of fishes, using the Internet and biology and zoology books. A list of useful references is included in the Further Reading list at the end of this manual.

Evolutionary trees

An evolutionary tree shows the evolutionary lineages of different species over time. The following evolutionary trees are not precise or drawn to scale but are intended as a reference for selecting a diverse mix of fish samples that may be available in your region. Figure 3 shows the relationship among a number of animal phyla.

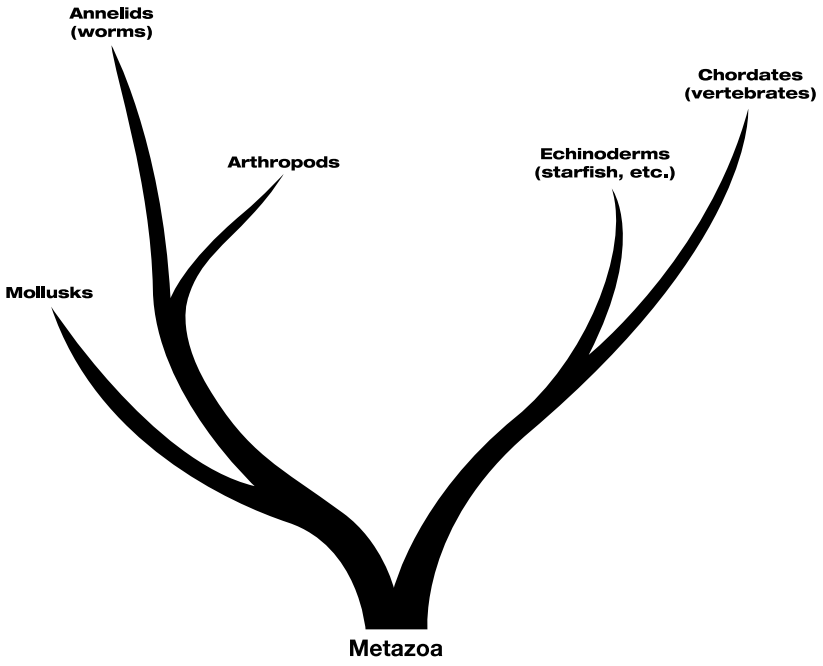


Figure 3. Evolutionary tree showing the relationships among the vertebrates (chordates, including all fish), mollusks, worms, echinoderms, and arthropods.

The modern fishes are divided into the classes Chondrichthyes (cartilaginous fishes) and Osteichthyes (bony fishes), with the lampreys and hagfishes in the separate class Agnatha (jawless vertebrates) that diverged earlier from the ancestral fishes. The Chondrichthyes include the sharks and rays, and the Osteichthyes, include all other modern fishes. Below are brief descriptions of some of the major fish groups, in order from most ancient to most recently diverged.

Agnatha. Lampreys and hagfishes are eel-like, jawless fishes with parasitic and scavenging lifestyles. They do not have scales or paired fins. They evolved from the earliest vertebrate ancestor before the jawed fishes appeared and diversified. The fossil record for Agnatha stretches back nearly 500 million years.

Chondrichthyes. Sharks, rays, and skates belong to this class. Their cartilaginous rather than bony skeletons reflect a more evolutionarily ancestral state. Their skin is thick and without true scales, and they do not have swim bladders or lungs.

Osteichthyes. The bony fishes are the most diverse class of fish. The class is characterized by having bony skeletons, true scales, paired fins, and movable rays in their fins and tail. Osteichthyes are divided into two subclasses, the lobe-finned fish (Sarcopterygians) and the ray-finned fish (Actinopterygians).

Sarcopterygians are the fish most closely related to modern amphibians, reptiles, birds, and mammals, and this subclass includes the lungfish and coelacanth. Coelacanth were thought to have become extinct at about the same time as the dinosaurs, until a live specimen was found in 1938 (Glausiusz 1999)!

Most modern fish are members of the ray-finned, Actinopterygian subclass.

Sturgeons, bowfins, and gars are considered relic bony fishes. There are about 50 species of these relic fish extant around the world today, although the fossil record shows there were many more in the past.

Teleosts. The remainder of the bony fishes are in the subdivision **Teleostei,** which includes 42 orders and over 23,000 species. The orders that include **herrings** and **anchovies** and the **eels** are considered specialized offshoots of the teleosts and comprise about 6% of the subdivision.

Other subdivisions of Teleostei:

Superorder Ostariophysi
- Order Cypriniformes (minnows, carps)
- Order Siluriformes (catfish)

Superorder Protacanthopterygii
- Order Esociformes (pikes)
- Order Osmeriformes (smelts)
- Order Salmoniformes (salmon, trout, whitefish)

Superorder Paracanthopterygii
- Order Gadiformes (cod, hakes, pollock)

Superorder Acanthopterygii
- Order Percoidei (perches, snook, basses)
- Order Pleuronectiformes (flounders, soles)
- Order Perciformes (mackerel, tuna, swordfish)

Which Fishes Should You Select for this Laboratory?

The aim is to choose fish specimens that will provide striking and distinct results for your students to analyze. Figure 4 is a more detailed fish evolutionary tree, with common names to help you identify examples in the grocery store. We recommend that you select some closely related fish from the same branch of the tree, such as salmon and trout, as well as distantly related fish from different branches, such as shark and tuna.

Note: These evolutionary trees were drawn to represent relationships among fish species. They are not meant to be strictly accurate.

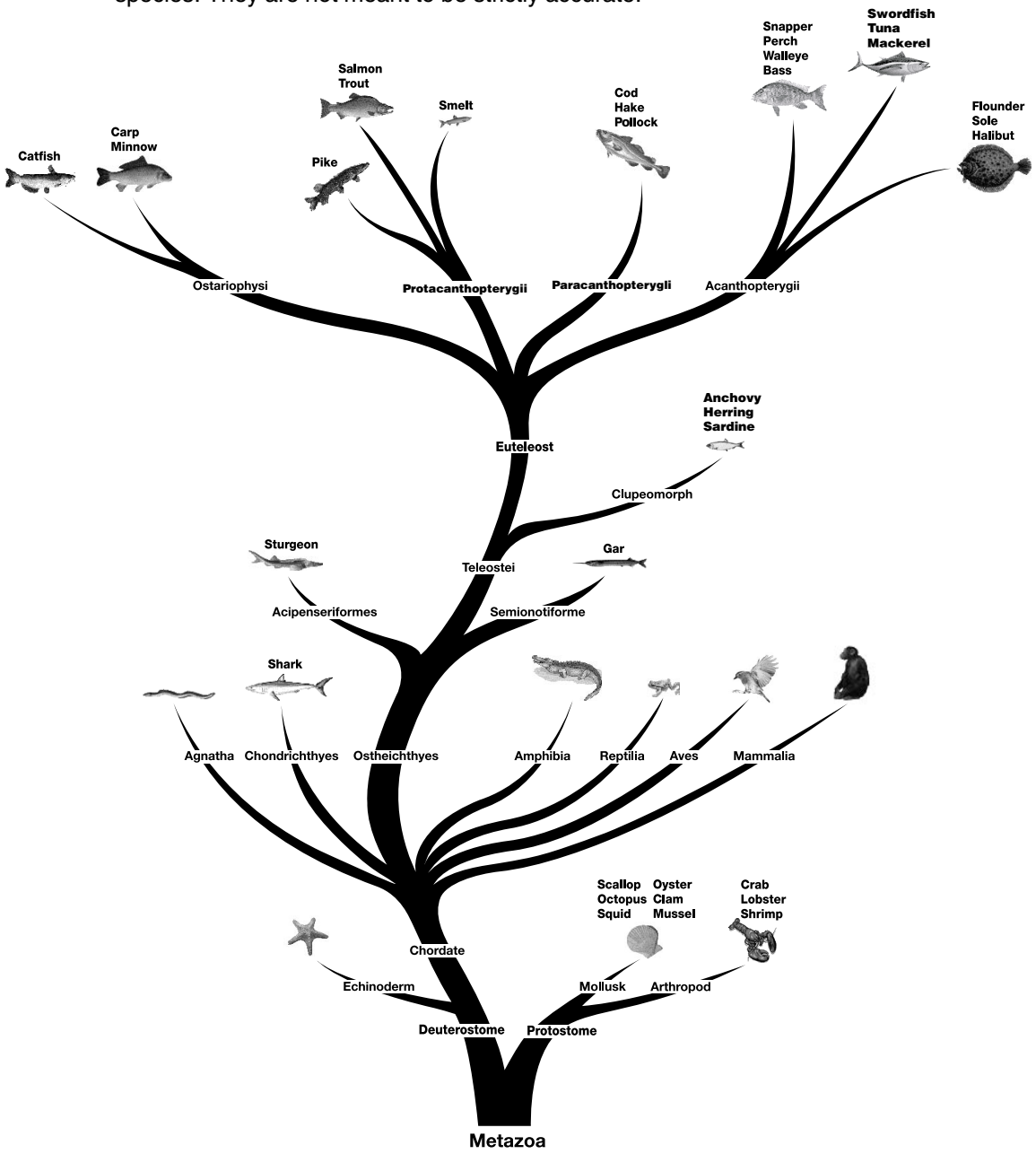


Figure 4. Representative fish evolutionary tree showing the relationships among fish species, based on Ichthyology Web resources (www.biology.ualberta.ca/jackson.hp/IWR/index.php) and the Tree of Life (phylogeny.arizona.edu).

Other aquatic organisms that could be used in this experiment include mollusks (*e.g.*, scallops, octopus, clams, oysters) and arthropods (*e.g.*, crab, shrimp, lobster, crayfish). These organisms are evolutionarily distant from fish so they will provide interesting comparative data on broader evolutionary relationships.

TIP When purchasing fish, keep in mind that you only need less than a gram of each sample. At the fresh fish counter at your grocery store, you can probably get small samples for minimal or no charge. Frozen fish works just as well as fresh. Be sure to keep track of which fish is which!

Armed with a selection of closely related and distantly related samples, the next step is to learn about the method of protein fingerprinting.

Overview of Protein Electrophoresis

These concepts are critical to the students' understanding of this laboratory activity.

- Proteins are charged molecules whose sequences are determined by DNA.
- Polyacrylamide gels are used to separate small molecules, such as proteins, whereas agarose gels are used to separate larger molecules, such as DNA.
- The percentage of polyacrylamide gel to be used depends on the size range of the proteins of interest. The higher the percentage, the denser the matrix. As a result, gels of higher percentages are used to separate smaller proteins, and gels of lower percentages are used to separate larger proteins.
- Proteins are separated according to their size when they are subjected to SDS-PAGE, a form of polyacrylamide gel electrophoresis (PAGE) that incorporates the detergent sodium dodecyl sulfate (SDS). If proteins of known molecular weights, called standards, are also run on the gel, their migrations can be used to estimate the molecular weights of unknown proteins.
- SDS-PAGE protein samples are pretreated by heating them in the presence of the negatively charged detergent SDS, so that proteins and protein complexes are disrupted, or denatured, and all proteins acquire a uniform charge-to-mass ratio. Proteins then will migrate at rates dependent only on their molecular weights, without their native 3-dimensional shapes or charges being factors.

Some relevant history …

Protein fingerprinting was first carried out in 1956 to show that the genetic disease sickle-cell anemia is caused by a change to a single amino acid of the hemoglobin protein (Ingram 1956). The gel electrophoresis techniques that you are using, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), was developed by U.K. Laemmli, whose 1970 Nature paper has the highest number of citations of any scientific paper (Laemmli 1970). SDS-PAGE is still the predominant method used in vertical gel electrophoresis of proteins.

General Principles of Protein Electrophoresis and SDS-PAGE

Electrophoresis ("to carry with electricity") is **the migration of charged molecules in an electric field toward the electrode with the opposite charge**. This technique is widely used in molecular biology research to examine proteins to answer a variety of questions. For example:

- **What proteins are in my sample?**
- **What are the molecular weights of the proteins?**
- **What differences are there in the proteins from different sources?**
- **How pure is my protein of interest?**
- **How much protein do I have?**

Laemmli developed his system of polyacrylamide gel electrophoresis with two gel phases, so that all of the proteins in a gel begin separating, or resolving, at the same time. Since sample volumes can vary from lane to lane, forming vertically narrow or broad bands in the wells, all of the proteins in a sample do not enter the stacking gel zone simultaneously. However, the low percentage (4%) of the stacking gel allows the proteins to migrate rapidly and accumulate at the edge of the denser resolving gel, regardless of their sizes. The samples of mixed proteins are thus concentrated into uniformly thin bands in each lane, before they move into the denser (5-20%) resolving gel and begin to separate according to their weights.

There is no obvious visual border between the stacking and resolving zones of the Ready Gel, but if you watch your samples immediately after turning on the power supply, you will see the protein samples being focused into a narrow band at the interface. The prestained Kaleidoscope markers first stack into a tight band, and then the individual prestained proteins become distinct as the proteins begin to separate according to their molecular weights.

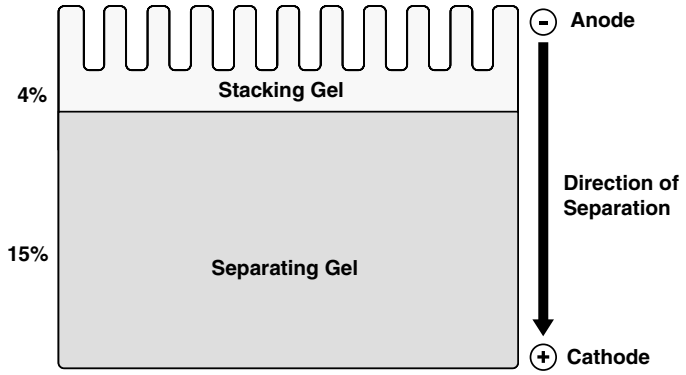


Figure 5. Bio-Rad's Ready Gel precast gels are very thin polyacrylamide gels sandwiched between clear plates. Each gel has two separate zones, the stacking gel and the separating gel, which is also known as the resolving gel. In vertical gel electrophoresis, samples are loaded into wells at the top of the stacking gel, and the proteins move downward toward the positively charged electrode.

Why Are We Using Polyacrylamide, Not Agarose,Gels to Analyze Proteins?

The gel matrix formed by polyacrylamide is much tighter and able to resolve much smaller molecules than agarose gels. Polyacrylamide gels have pore sizes similar to the sizes of proteins. DNA molecules are orders of magnitude larger than proteins, and for nucleic acids, agarose is usually the preferred gel medium. However, when

separating very small fragments of DNA, for example during DNA sequencing, polyacrylamide is the gel matrix of choice.

The Chemistry and Physics behind Electrophoresis

The size of biomolecules is expressed in daltons, a measure of molecular weight. One dalton equals the mass of a hydrogen atom, which weighs 1.66 x 10^{-24} gram. Most proteins have masses on the order of thousands of daltons, so the term kilodalton (kD) is used for protein molecular weights. Proteins range in size from several kilodaltons to thousands of kilodaltons. In contrast, the nucleic acids we study are often larger than 1000 base pairs, or 1 kilobase (kb), and each kilobase pair has a mass of approximately 660 kD. For example, when cloning DNA, a 2 kb fragment of DNA can be inserted into a plasmid vector of 3 kb, giving a total plasmid length of 5 kb. The mass of this 5 kb plasmid would be approximately 3.3 million daltons or 3,300 kD, much larger than the average protein!

A molecule's electrical charge and its mass affect its mobility through a gel during electrophoresis. The ratio of charge to mass is called **charge density**. Since every protein is made of a unique combination of amino acids, each of which may have a positive, negative, or neutral charge, the net charge of each protein is naturally different. The inherent charges of proteins must be removed as a factor affecting migration in order for polyacrylamide electrophoresis to be effective as a method of protein molecular weight determination.

The intrinsic charges of proteins are obscured by placing a strongly anionic (negatively charged) detergent, SDS, in both the sample buffer and the gel running buffer. SDS binds to and coats the proteins and also keeps them denatured as linear chains. In this form, proteins migrate in a polyacrylamide gel as if they have equivalent negative charge densities, and mass becomes the only variable affecting the migration rate of each protein. This technique is called **SDS-PAGE**.

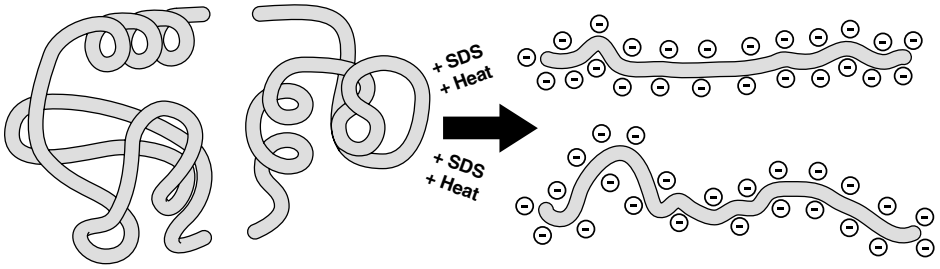


Figure 6. The structure of a protein complex is disrupted when SDS binds to proteins in the presence of heat. Individual negatively charged proteins can be separated by size on an SDS-PAGE gel.

Polyacrylamide Acts As a Molecular Sieve

The degree of sieving within a gel can be controlled by adjusting the polyacrylamide concentration. Higher concentrations of polyacrylamide resolve smaller molecular weight ranges. For example, a 5% polyacrylamide gel separates large proteins of 100 to 300 kD, while an 18% polyacrylamide gel is better for separating smaller proteins in the range of 5 to 30 kD.

For this activity we recommend using 15% polyacrylamide gels, which provide excellent separation for proteins in the range of 10 to 100 kD. Our attention will be focused on variations among the smaller proteins, in the range of 15 to 50 kD, since it is easier to discern differences among these proteins. Smaller proteins migrate further through the gel and are better resolved than proteins of high molecular weights.

Running a Polyacrylamide Gel

To run a polyacrylamide gel, a Ready Gel cassette is prepared according to the instructions on page 33, and the well-forming comb is removed. The prepared cassette is locked into a vertical electrophoresis apparatus and the running buffer is added to the upper and lower buffer chambers. Samples, controls, and molecular weight markers are loaded into the preformed wells of the gel. A lid is placed on the electrophoresis box, and leads are plugged into a power supply. A current is applied at constant voltage, bubbles rise from the electrodes, and the blue dye and proteins in the samples begin to enter the gel.

Sample Preparation – Disrupting Protein Structure

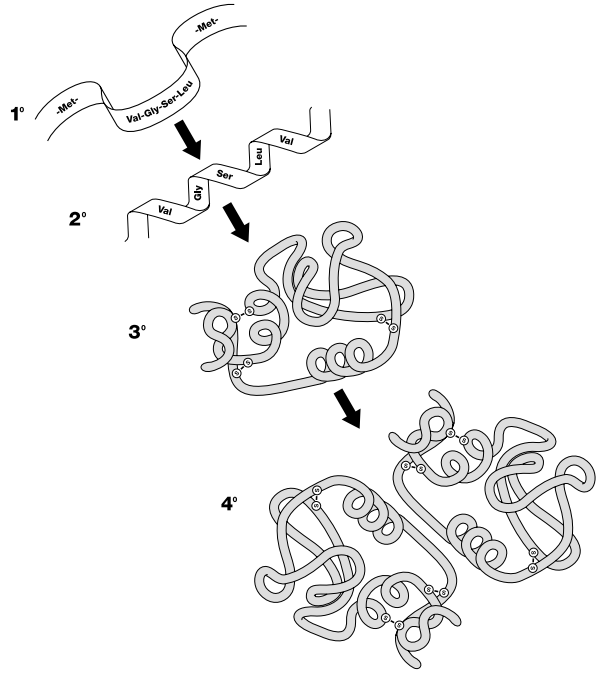


Figure 7. Secondary (2°), tertiary (3°), and quaternary (4°) protein structure must be disrupted, or denatured, to separate proteins by size.

To effectively determine the molecular weights of proteins, the secondary (2°), tertiary (3°), and quaternary (4°) structures of the protein complexes within a protein extract are disrupted prior to electrophoresis. This process of structural disruption is called **denaturation.**

- **Primary structure** = linear chain of amino acids
- **Secondary structure** = domains of repeating structures, such as β-pleated sheets and α-helices
- **Tertiary structure** = 3-dimensional shape of a folded polypeptide, maintained by disulfide bonds, electrostatic interactions, hydrophobic effects
- **Quaternary structure** = several polypeptide chains associated together to form a functional protein

Secondary, tertiary, and quaternary structures are disrupted by the combination of heat and SDS. A reducing agent, such as β-mercaptoethanol (BME) or dithiothreitol (DTT), may be added to ensure complete breakage of disulfide bonds. These three factors – heat, ionic detergent, and reducing agent – completely disrupt the 2°, 3°, and 4° structures of proteins and protein complexes, resulting in linear chains of amino acids. These molecules snake through the gel at rates proportional to their molecular masses.

TIP Both BME and DTT are potentially hazardous and smell awful. Fortunately, neither is required for this activity. The protein banding patterns of fish muscle tissues are not visibly affected by the inclusion of reducing agents in the Laemmli sample buffer. The Laemmli sample buffer that comes with the kit is ready to use.

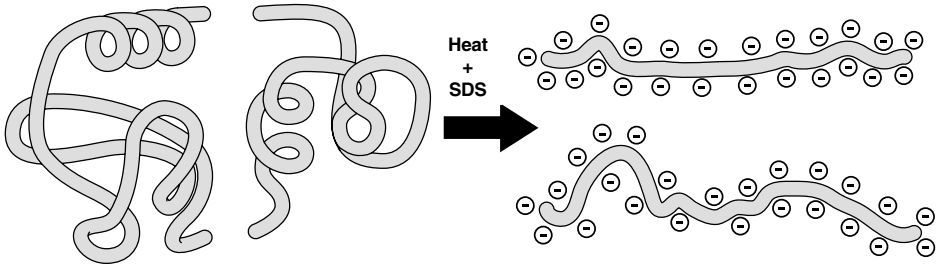


Figure 8. The combination of heat and the detergent SDS denatures proteins for SDS-PAGE analysis.

Fish samples are denatured for SDS-PAGE by heating them in Laemmli sample buffer, which contains SDS.

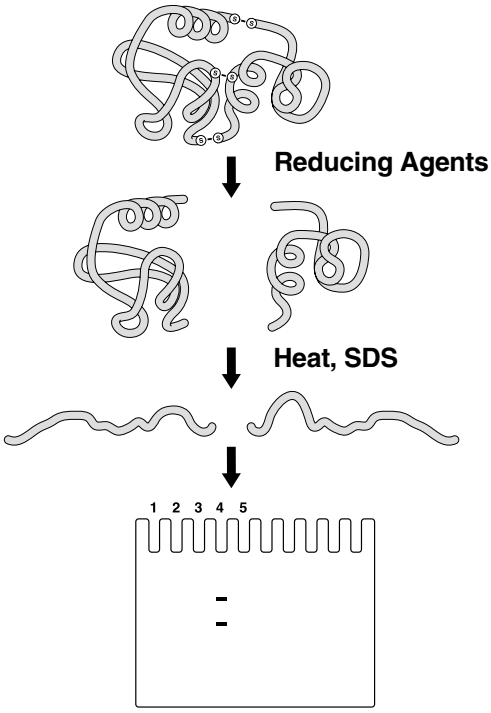


Figure 9. A quaternary protein complex denatured with reducing agents, heat, and SDS, can be separated into individual proteins and resolved by size using SDS-PAGE.

Visualizing the Proteins

After electrophoresis is complete, the Ready Gel is stained so that blue-colored protein bands appear against a clear background. For a record of the experiment, the stained gel can be sandwiched between two sheets of cellophane to dry, or sealed in a plastic bag and kept hydrated. Alternatively, the wet gel can be photographed or photocopied for a permanent record.

Molecular Weight Standards

Electrophoresis protein standards, or molecular weight markers, consist of a mixture of proteins of known molecular weight. They are available in a number of protein size ranges. The markers to be used should correspond to the sizes of the proteins of interest.

Molecular weight standards are available either prestained or unstained. Unstained markers are not visible until the gel is stained with a protein stain, such as Bio-Safe™ Coomassie stain. The prestained Kaleidoscope standards included in this kit are visible as they run on the gel. The dyes bound to the Kaleidoscope marker proteins affect the migrations of the proteins, and the actual sizes of the dyed molecules differ slightly from batch to batch. Please refer to the size chart that comes with each vial of Kaleidoscope prestained standards for the calibrated molecular weights of each of the dyed proteins.

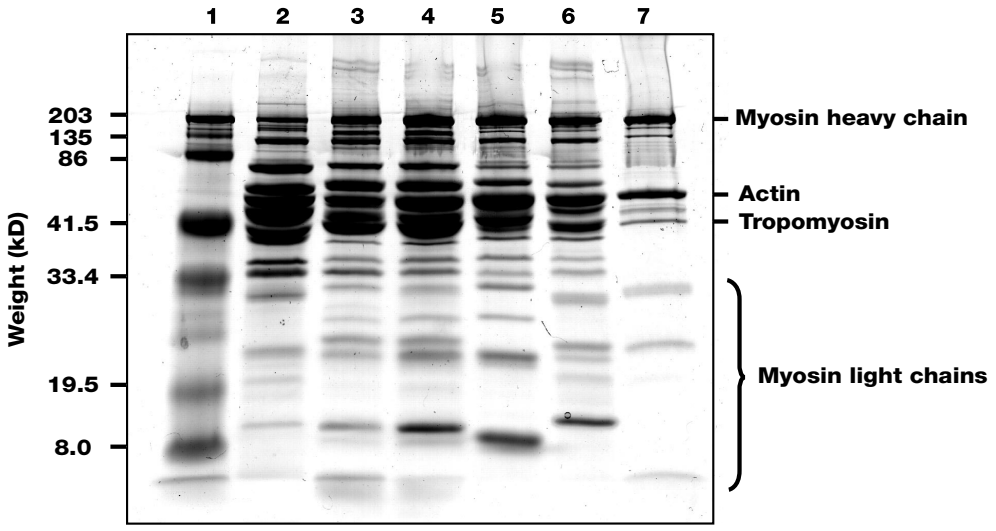
Actin and Myosin Standards

The actin and myosin standard is included in the kit as a reference to help identify the major, conserved muscle proteins and to serve as a positive control for gel analysis. This protein sample consists of myofibrils (see Figure 1) that have been isolated from rabbit skeletal muscle. Actin, myosin heavy and light chains, and tropomyosin will be visible after the gel has been destained. This sample contains sufficient protein to be seen clearly when the gel is stained and destained according to kit instructions. If the only proteins visible on the destained gel are the Kaleidoscope prestained standards, there was a probelm with gel loading, staining, or destaining.

The heavy bands at 210 kD and 43 kD, present in all of the muscle samples, are myosin heavy chain and actin, respectively. Their presence in all samples signifies that all fish have actin and myosin as a primary component of muscle and that both proteins have been conserved throughout evolution. Myosin itself is composed of several large polypeptides (heavy and light chains) that also are separated on the gels.

Analyzing Student Electrophoresis Results

To make meaningful inferences about the evolutionary relationships among the sampled fish species, students must analyze and compare the protein banding patterns. A typical set of results from SDS-PAGE of fish muscle proteins is shown here:



Lane: 1. Kaleidoscope standards
2. Shark
3. Salmon
4. Trout
5. Catfish
6. Sturgeon
7. Actin and myosin standard

Figure 10. 15% polyacrylamide Ready Gel electrophoresed at 200 V for 30 minutes, stained with Bio-Safe Coomassie stain and destained in water.

Obvious similarities and differences between protein fingerprints are easily spotted. Comparing the banding patterns of salmon (lane 3) and trout (lane 4), it is clear that these are very similar. For instance, they share a common band at approximately 70 kD, as well as a pair of bands at 20 kD. This similarity is consistent with the proposed fish evolutionary tree (Figure 4), as salmon and trout are both on the same branch. In contrast, a comparison of salmon (lane 3) with catfish (lane 5) reveals significant differences in their patterns. Only one of the approximately 25 kD bands found in the salmon is present in the catfish.

Constructing a Standard Curve

For a more precise description of the protein fingerprints, the bands can be analyzed quantitatively. Just as with agarose gel electrophoresis of DNA fragments, a standard curve of molecular weights is made by plotting the migration distances (x-axis) of the proteins in a set of standards versus their known molecular weights (y-axis) on a semi-log graph. The molecular weights of unknown proteins can then be derived by measuring their migration distances and finding the corresponding molecular weights on the standard curve.

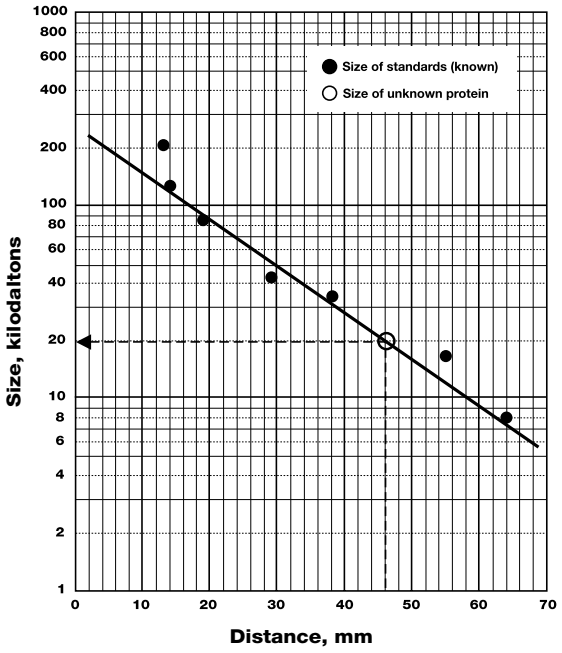


Figure 11. The distance migrated by each prestained protein was plotted against its size on semi-log graph paper to generate a standard curve for the

Another useful determination that can be made from a protein gel is relative protein abundance. Different proteins within a sample are present in different quantities, and the same protein may be present at different levels in different samples. A thick, broad, darkly stained band indicates an abundance of molecules in that band, compared to a faint or narrow band. Using this information, you can assess the abundance of different proteins within a sample. If equal amounts of total protein are loaded in different sample lanes, the abundance of a particular protein can be compared among different samples.

Identifying Proteins in Polyacrylamide Gel

It is not possible to definitively identify unknown proteins in an SDS-PAGE gel without additional confirming information. In an experiment like this one, each protein extract contains a complex mixture of proteins. The different proteins appear as distinct blue-stained bands on the finished gel. From the positions and intensities of these bands, we can determine the size and relative abundance of the proteins, but we can only make educated guesses about the identity of each protein, based on available references. For example, since the samples are all from muscle tissue, you may correctly assume that there would be large quantities of the predominant muscle proteins such as actin and myosin. The actin and myosin sample provided in this kit allows you to confirm the identity of these two proteins in your samples.

Even when the molecular weight of a protein is known, and used as a criterion for identification, there are two possible sources of error. First, bands that migrate almost identically on a gel may actually be different proteins of very similar sizes. Second, proteins of very similar composition, function, and evolutionary origin may be different in molecular weight, because of variations acquired as they evolved. Definitive identification of a protein requires mass spectrometry, sequencing, or immunodetection. Immunodetection methods, such as western blotting, use antibodies that specifically recognize the proteins of interest. Such antibodies can provide positive identification when bands cannot be identified by molecular weight alone.

This is the end of the section dealing with the theory behind protein fingerprinting. Before turning to the detailed laboratory protocol, we provide ideas on how to present this laboratory in the classroom and suggest some questions to test students' theoretical understanding.

Ideas on How to Present This Activity in the Classroom

Evolutionary approach: The fish muscle samples your students will run on their gels represent actual products of evolution. Before your students begin any laboratory activities, have them look at the evolutionary tree to see the relationships among the samples you have chosen. More information and ideas about the different fish families you might use can be found on the following pages. Have your students hypothesize where they might expect differences in biochemical profiles among the samples. For example, species closely placed on the tree would be expected to show more protein similarities than more distantly placed species. This approach gets students to think about how evolutionary biologists have reconstructed the course of evolution and lineages of species—traditionally through morphological characters but currently through molecular data. After the gels are run, see if the students' hypotheses were correct.

There will be a lot of similarity among the sample protein profiles, simply because they are from the same type of tissue. Nevertheless, there will be clear differences among samples—see our gel example on page 21. You can see that trout and salmon have very similar patterns, while the shark, sturgeon, and catfish patterns are distinct from each other as well as from trout and salmon. Other aquatic organisms, such as shrimp, mussels, clams, and crab, can also be incorporated into this laboratory protocol for a broader examination of the evolutionary tree.

Protein approach: You can take advantage of the fact that all of the samples are from muscle tissue, and hence the most abundant proteins in each sample should be associated with muscle function. This approach, like the evolutionary approach, can be used to guide the students to think about the correlation between tissue- and organ-level features, such as muscle, and the proteins of which they are composed.

Have students construct a standard curve using the Kaleidoscope prestained standard and/or actin and myosin standard provided in this kit. Then have them derive the molecular weights of the predominant "unknown" bands in their samples. Do any of these molecular weights correspond to the sizes of known muscle proteins? Are any proteins more abundant in one fish compared to another? Or are there proteins present in "true fish" that are absent in "shellfish"? Freshwater versus saltwater fishes? Cold water versus warm water fishes? How about differences between sedentary and more active fish? Even if many of the bands in their samples cannot be identified with names and functions, students can be encouraged to think about what types of proteins might be found in fish of different environments and lifestyles. For example, flounder is a bottom-dweller and relatively inactive, whereas mackerel is a fast-moving ocean fish. What kinds of protein functions might be associated with such lifestyles? Species of fish that live in very cold waters are known to possess anti-freeze proteins (Davies and Hew 1990). Proteins are what make cells, tissues, and organs able to perform and adapt in such diverse ways!

Food identification approach: See if students can figure out the composition of imitation crab by comparison to other samples such as real crab, shrimp, crayfish, scallops, and true fishes. Since imitation crab is usually made from a whitefish such as pollock, you should be able to find something similar. Which samples most closely resemble the true crab sample? You might predict that arthropods such as lobster, crayfish, or shrimp would show more similar banding patterns to real crab than would fish species. Protein and DNA profiling are used in the seafood industry for this very purpose—to detect adulteration or mislabeling of processed seafood products (Piñeiro et al. 1999).

Why Fish?

This laboratory exercise focuses on comparisons of fish species because fish represent a diverse and ancient group of vertebrates that has adapted to a range of niches. In addition, a wide variety of specimens is readily available from grocery stores or local waters.

Why Muscle Tissue?

Fish muscle is easy to prepare for electrophoresis because, in general, it is low in fat and tough connective tissue. This allows the proteins to be easily extracted, and produces reliable results in three simple steps.

TIP Tissues obtained from insects, warm-blooded animals, and plants exhibit a wide range of textures and may contain high levels of chitin, lipids, and cellulose, respectively. These factors affect protein solubility and may yield poor results.

Extension Activities

1. Have the students use the Kaleidoscope prestained protein standards, and/or the actin and myosin controls, to create a standard curve. Then have them estimate the molecular weights of several unknown proteins in their fish samples. Use the estimated molecular weight to predict the number of amino acids within a protein, using 110 daltons as the average weight of an amino acid. For example: actin has a molecular weight of 43 kD (43,000 daltons), so you would predict that it contains 391 amino acids.
2. Using similar logic, you may also have students estimate the number of base pairs within the gene that encodes this 43 kD protein. Given that each amino acid is encoded by a triplet of nucleotides, and the average weight of a base pair is 660 daltons, you can approximate the molecular weight of the gene that specifies the protein. This activity reminds students of the relationship between DNA and proteins.
3. Discuss any differences between values obtained by students and the known values. α-actin of carp is actually composed of 377 amino acids and encoded by 1134 base pairs.

Focus Questions

Q: What information would you need to accurately calculate a protein's actual molecular weight?

A: The actual amino acid (or DNA) sequence, not just the number of amino acids is needed to calculate accurately a protein's actual molecular weight.

Q: Why might the weight you've determined from a gel differ from the actual molecular weight?

A: Any molecular weight determinations made from a gel are limited by the precision of your measurement, which may not be very accurate. The estimated values for high molecular weight proteins will be less accurate than for smaller proteins because the scale of their migration is compacted; i.e., bands separate more distinctly the farther they travel down the gel. Actin (43 kD) and the myosin heavy chain (205 kD) provide a visible, instructive example of this discrepancy. Some

proteins are modified by phosphates or carbohydrates, which causes them to migrate differently than expected. Finally, the standard curve is not strictly linear, even when plotted on semi-log graph paper. The log function, however, changes slowly enough that the straight line gives reasonable molecular weight estimates.

Teacher's Laboratory Guide

This section presents an overview of each laboratory day. Provided for each laboratory day is the lesson flow, advance preparation, student workstation setup, and techniques and concepts to highlight.

Advance Preparation for Student Laboratory Days 1 through 3

Suggested Laboratory Schedule for the Students

The laboratory activities are designed to be carried out in three consecutive 50-minute laboratory periods, or in two 2-hour laboratory periods, plus one period each for pre-laboratory and post-laboratory discussions. The detailed laboratory protocol and Laboratory Quick Guide for each period can be found in the sections below and in the Student Manual.

Implementation Timeline

2–3 days	Pre-laboratory	• Background lecture
		• Student considerations/focus questions
50 minutes	Laboratory day 1	• Obtain muscle samples from a variety of fish species
		• Extract and denature sample proteins
		• Student considerations/focus questions – page 58
50 minutes	Laboratory day 2	• Polyacrylamide gel electrophoresis (PAGE)
		• Stain gels, destain overnight
		• Student considerations/focus questions – page 62
50 minutes	Laboratory day 3	• Analyze protein fingerprints
		• Construct standard curves, determine molecular weights of proteins
		• Infer evolutionary relationships from students' data, compare to evolutionary trees
		• Student considerations/focus questions
50 minutes	Data analysis	• Discussion and/or student presentations

Laboratory Day 1 - Protein Extraction and Sample Preparation

Teacher's Advance Preparation for Laboratory Day 1

Preparation Overview		Time required
Step 1	Read through introductory material on the previous pages	2–3 hours
Step 2	Obtain fish samples and distilled water—trip to grocery store	As needed
Step 3	Set up student and teacher workstations	20 minutes

Laboratory Checklist (✔)

Student workstations. The following materials, supplies, and equipment should be present at each student workstation prior to beginning the laboratory activities. The materials in this kit are designed to supply 24 student laboratory stations.

Teacher's workstation. The following materials, supplies, and equipment should be present at a common location that can be accessed by all student groups. It is up to the teacher's discretion whether students should access common buffer solutions and equipment, or whether the teacher should aliquot solutions into microtubes.

Student Workstations		(✔)
1.5 ml EZ Micro™ test tube	6 each	❑
1.5 ml screw-cap micro test tube	7 each	❑
Disposable 1 ml pipets (DPTPs)	1 each	❑
Fish samples (5 types)	~1 gm each	❑
Indelible marking pen, fine point	1	❑
Laemmli sample buffer	1.5 ml	❑
Knife or scissors to cut fish samples	1	❑

Teacher's (Common Workstation)		(✔)
Water bath or hot plate set to 95°C	1	❑
Laemmli sample buffer—30 ml	1	❑
*Actin and myosin standard—rehydrated	1 vial	❑
*Kaleidoscope prestained standards—500 µl	1 vial	❑

* Kaleidoscope prestained standards and actin and myosin standards may be prepared along with fish samples during laboratory day 1, or the teacher may choose to prepare these two reagents in advance of laboratory day 2.

Lesson Points to Highlight – Laboratory Day 1

Selecting Fish Specimens

This laboratory focuses on comparing different fish species that are available in your area (in stores or in the natural environment). Please refer to the phylogenetic charts on page 14 to select appropriate varieties for the experiment. Choose a variety of different fish species, including representatives from as many branches as possible. The idea is to look at both closely related (same branch) and distantly related fish (different branches) for comparison. In our example on page 21 we used salmon, trout, catfish, sturgeon, and shark. Protein banding patterns for different fish groups should reveal similarities and differences that can be compared to the arrangement of the evolutionary tree on page 14. Other aquatic organisms, such as shrimp, mussels, clams, crab, and imitation crab can be included in this activity to analyze a broader section of the evolutionary tree.

When purchasing your fish, keep in mind that you only need a very small amount of each sample. Be sure to keep track of which fish is which!

Safety Precautions

No toxic chemicals are used in this laboratory. However, good laboratory practice should be followed while carrying out all aspects of any laboratory procedure. We recommend that students wear gloves and safety glasses while handling fish samples, polyacrylamide gels, protein stain, and the other reagents used in this exercise. Gloves not only protect students from exposure to the reagents, such as the blue protein stain, but also protect the samples and gels from unwanted contamination from your students' hands.

Be sure that students wash their hands and benchtops after working with fish!

Note: Students with known allergies should avoid all contact with shellfish samples.

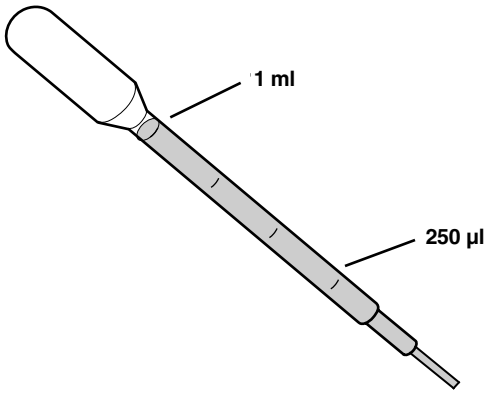


Figure 12. The disposable transfer pipet is marked at 250 µl intervals.

Use of the plastic disposable pipets

Before beginning the laboratory sessions, point out to the students the graduations on the pipet. The 250 µl mark will be used as a basic unit of measurement during this laboratory.

Use of the Micropipets

Explain the 2-stop action of the adjustable, digital micropipet. Micropipets are accurate and expensive, so emphasize that they should be handled with care. Also explain the importance of using fresh tips to prevent cross-contamination and the appropriate disposal of used tips. The 10 µl fixed-volume micropipet offers a less expensive and easier-to-use alternative if desired.

Reagents Used on Laboratory Day 1

Laemmli sample buffer is used to solubilize the proteins in the fish muscle samples. Each bottle contains 30 ml and is a mixture of Tris buffer, the anionic detergent SDS, electrophoresis tracking dye (Bromophenol Blue), and glycerol. Glycerol increases the density of the samples so that they sink into the wells as they are loaded.

TIP Addition of β-mercaptoethanol (BME) is not recommended for this laboratory protocol. Do not add BME to the sample buffer. Follow the simple instructions to prepare the samples for electrophoresis.

Molecular weight markers. Kaleidoscope prestained standards, also referred to as molecular weight markers, come in a 1.5 ml microcentrifuge tube that contains 500 µl of standards in solution. One tube contains enough marker for up to 40 gels. Store this tube in the freezer until ready to use. Thaw the Kaleidoscope standards at room temperature and heat the tube briefly at 37°C to dissolve any precipitated SDS before removing any aliquots. Unlike the fish and actin and myosin standard, the Kaleidoscope standards do not require heating to 95°C prior to electrophoresis, although heating will not adversely affect the Kaleidoscope standards.

You may choose to provide a 12.5 µl aliquot of the Kaleidoscope standards, labeled KS, to each student workstation during the laboratory day 1, or you may choose to dispense a 12.5 µl aliquot to each student workstation as the students load their gels during laboratory day 2.

Actin and myosin standard. A control sample containing actin and myosin proteins is provided in a lyophilized form that is stable at room temperature. Store these proteins, along with the Kaleidoscope standards, in the freezer for long term safe-keeping. To rehydrate the sample, add 500 µl of Bio-Rad Laemmli sample buffer to the vial and incubate it at room temperature for 5 minutes. Transfer the rehydrated actin and myosin sample to a labeled screwcap tube using a disposable plastic transfer pipet. Like the fish protein samples, the actin and myosin sample must be heated for 5 minutes at 95°C before loading on gels.

You may choose to provide a 12.5 µl aliquot of the rehydrated actin and myosin standard in a screwcap tube, labeled AM, for each student workstation to heat during laboratory day 1, or you can complete preparation of the actin and myosin standard yourself and dispense 12.5 µl to each student workstation just before the students load their gels during laboratory day 2.

Student Laboratory Protocol for Day 1

Extract and Denature Fish Proteins

For each fish to be analyzed:

1. Use an indelible pen to label one 1.5 ml flip-top microtube with the name of each fish sample being prepared for electrophoresis. Also label a screwcap microtube for each fish sample.

2. Add 250 µl of Laemmli sample buffer to each labeled 1.5 ml flip-top tube.

3. Obtain a piece of each fish muscle sample to be analyzed and cut it to approximately 0.25 x 0.25 x 0.25 cm^3 (); avoid taking any skin, fat, or bones. Transfer the fish sample into the appropriately labeled flip-top microtube and close the lid.

4. Gently flick the microtube 15 times with your finger to agitate the tissue in the sample buffer.

5. Incubate samples for 5 minutes at room temperature to extract and solubilize the proteins.

6. Carefully pour the sample buffer containing the extracted proteins, but not the solid fish piece, into a labeled 1.5 ml screwcap tube.

   Note: It's not necessary to transfer all of the fluid to the screwcap tube. Only a small volume (<20 µl) is actually needed for gel loading.

7. Heat the fish samples and the actin and myosin standard (AM), if your teacher has provided it, for 5 minutes at 95°C to denature the proteins in preparation for electrophoresis.

8. You may store all the samples at room temperature if they are to be loaded onto gels within 3–4 hours or store them at –20°C for up to several weeks.

Laboratory day 2 – Electrophoresis: Gel Loading, Running, and Staining

Teacher's Advance Preparation

Preparation Overview		Time required
Step 1	Review section on electrophoresis on pages 15–20	30 minutes
Step 2	Prepare reagents and other items for student workstations	15 minutes
Step 3	Set up student and teacher workstations	20 minutes

Laboratory Checklist

Student Workstations - Daily Checklist		(✔)
Fish protein extracts prepared on laboratory day 1	5 each	❑
Prot/Elec pipet tips for gel loading	7 tips	❑
Mini-PROTEAN 3 electrophoresis module (gel box) – runs one or two gels	1	❑
Buffer dam (if running only 1 gel/box)	0.5–1	
Power supply (200 V constant)	1	❑
1–20 µl micropipet	1	❑
Ready Gel precast gel, 15%—10 wells	1	❑
Sample Loading Guide - for 10 well comb	1	❑
Thin metal weighing spatula	1	❑
*Actin and myosin standard, aliquotted	1 vial	❑
*Kaleidoscope prestained standards, aliquotted	1 vial	❑

Teacher's (Common) Workstation (✔)		
1x Tris-glycine-SDS (TGS) Electrophoresis	~350 ml/station	❑
Bio-Safe Coomassie stain for proteins	40 ml/staining tray	❑
Staining trays		❑
Water bath		❑
Water (tap water works fine for gel destaining)	As needed	❑

***Kaleidoscope prestained standards and the actin and myosin standard may be prepared along with fish samples during laboratory day 1, or the teacher may choose to prepare these two reagents in advance of laboratory day 2. See advance preparation steps for laboratory day 1 for detailed instructions.**

Reagents and Materials Used on Laboratory Day 2

Fish protein extracts. If samples have been frozen since laboratory day 1, simply thaw them at room temperature, heat them briefly (1 minute at 37°C or 30 seconds at 95°C) to dissolve the SDS detergent, then keep the samples at room temperature until you are ready to load them.

Ready Gel precast gels. Gels should be stored in a refrigerator until the time of use. **Do not** freeze the gels.

To set up the gels for the laboratory, cut the gel packages open over a sink or container, drain out the excess buffer, and throw away the filter paper and plastic wrap. Remove the comb from between the plates by pushing it upward gently with your fingertips. Use a razor blade to cut along the black line on the tape at the bottom front of the gel cassette, and peel off the strip of plastic covering the bottom front of the gel, as indicated on the gel cassette. Make sure the entire section of tape is removed completely, to allow the full length of the bottom of the gel to be exposed to electric current. For best results, use a transfer pipette and 1x TGS Running Buffer to rinse any debris out of the wells.

Electrophoresis running (1x TGS) buffer. Tris-glycine-SDS (TGS) running buffer comes in a 10x concentrated solution (10x TGS). To prepare a 1x working concentration, dilute 1 part of the 10x TGS concentrate with 9 parts distilled or deionized water. Distilled or deionized water is used to prepare the 1x buffer because impurities in tap water can increase the conductivity of the buffer. Approximately 350 ml of 1x TGS electrophoresis buffer is required for each gel box.

Bio-Safe Coomassie protein stain. The stain is ready to use. Gels can be transferred directly into Bio-Safe Coomassie stain after electrophoresis is complete. If time permits, the gels can be rinsed 3 times with water before staining, as directed on the Bio-Safe Coomassie stain label. Bio-Safe Coomassie protein stain is not reusable and should be disposed according to local disposal regulations (see label for details).

Optimal gel staining time is 1 hour while shaking gently, but gels can be left in stain for as long as 8 hours. Overnight staining causes diffusion of low molecular weight proteins and should be avoided. To destain, rinse the stained gel with several changes of a large volume of water and destain overnight in water.

Laboratory Protocol for Day 2

Step 1: Prepare samples, electrophoresis gels and gel boxes

Note: Reheat frozen samples at 80–95°C for 2–5 minutes to redissolve any precipitated detergent.

Set Up Electrophoresis Gels and Boxes

Note: Teachers may choose to assemble the gel boxes up to one hour prior to laboratory.

1. Make sure the comb and the tape along the bottom of the Ready Gel cassette have been removed. If two gels are to be run in one electrophoresis box, place a Ready Gel cassette on each side of the electrode assembly, with the short plates facing the inside of the assembly. If you are running only one gel in the box, place a Ready Gel cassette on one side of the electrode assembly and a buffer dam on the other side (see figure on page 42). Be sure to place the side of the buffer dam that says "BUFFER DAM" toward the electrode assembly.

2. Open the gates (cams) on the front of the clamping frame. Hold the two Ready Gel cassettes, or the one Ready Gel and buffer dam, against the electrode assembly and slide the electrode assembly into the clamping frame.

3. Press down on the outer edge of the electrode assembly, not the gels, while closing the cams of the clamping frame to ensure a seal on the bottom edge of each cassette.

4. Place the assembled clamping frame containing the gel(s) into the gel box tank. Fill the upper buffer chamber, the space between the two gels, with ~150 ml 1x TGS electrophoresis buffer so the buffer level is above the inner short plates. Check for leaks. If the assembly is leaking, remove the assembled clamping frame, pour off the buffer, reopen the cams, and push down on the electrode assembly again while closing the cams before filling again with buffer.*

5. Pour ~200 ml of 1x TGS electrophoresis buffer into the lower buffer chamber, or tank. Double-check the buffer fill level within the upper buffer chamber.

*Note: If leakage of the upper buffer cannot be corrected by reassembling the clamping frame in Step 4, the lower chamber can be filled to above the inner small plates, to equalize the buffer levels in both reservoirs. This requires approximately 900 ml of 1x TGS electrophoresis buffer.

Step 2: Load and run electrophoresis gels

Place a yellow sample loading guide on the top of the electrode assembly. The guide will direct the pipet tip to the correct position for loading each sample in a well.

Assign samples to wells, loading samples in middle of the gel, where separation is best with the standards on each side. For example, for 5 fish samples on a 10 well gel, you may choose to follow this guide:

Lane	Volume	Sample
1	empty	None
2	empty	None
3	10 µl	Kaleidoscope prestained standard
4	10 µl	Fish sample 1
5	10 µl	Fish sample 2
6	10 µl	Fish sample 3
7	10 µl	Fish sample 4
8	10 µl	Fish sample 5
9	10 µl	Actin and myosin standard
10	empty	None

To load each sample, use a thin, gel loading micropipet tip to withdraw 10 µl of each protein sample from its tube and gently transfer it into the designated gel well. After loading all samples, remove the sample loading guide, place the lid on the tank, and insert the leads into the power supply, matching red to red and black to black.

Run gels for 30 minutes at a constant voltage of 200 V.

Step 3: Stain gels

Wear gloves when disassembling and staining the gels to avoid contaminating the gels with human proteins, as well as to avoid staining your skin. When the gels are finished running, discard the buffer from the inner chamber, release the cams, and remove the gel cassettes from the assembly. Lay each gel cassette flat on the bench with the short plate facing up. Cut the tape along the sides of each gel cassette. Carefully pry apart the gel plates using your fingertips or a weighing spatula. The gel will adhere to one of the plates. Transfer the plate with the gel adhering to it to a staining tray containing Bio-Safe Coomassie stain. The staining liquid will cause the gel to detach from the plate. Alternatively, the gel can be gently lifted from the plate into the stain.

Note: If time permits, students' gels can be rinsed 3 times, 5 minutes each with gentle agitation in a large volume of water to remove SDS from the gels before staining. Residual SDS causes a temporary blue background of Bio-Safe Coomassie stain. This background disappears completely when gels are destained in a large volume of water overnight.

Stain the gels for 1 hour. For best results, gently agitate the tray during the staining period.

Step 4: Destain gels

After gels have been stained for at least 1 hour, pour off the Bio-Safe Coomassie stain and dispose of it according to local disposal regulations. Destain the gels with a large volume of water, changing it several times if possible. Allow the gels to destain overnight in water, with gentle shaking for best results.

Note: Small proteins may diffuse if the gels are not destained with plenty of water.

After destaining, blue bands should be distinct on a clear background. Gels can be stored in water for up to one week before they are dried or photocopied.

Laboratory Day 3 - Gel Preservation

Teacher's Advance Preparation

Preparation Overview		Time Required
Step 1	Review section on electrophoresis on pages 15–20	30 minutes
Step 2	Set out and prepare equipment and supplies for student workstations	10 minutes
Step 3	Set up student workstations	20 minutes

Laboratory Checklist

Student Workstations		(✔)
Water (tap water)	1 L	❑
GelAir cellophane support sheets	2	❑
Square plastic container (if not using GelAir dryingframe) and rubber bands	1	❑

Teacher's (Common) Workstation		(✔)
GelAir drying frames	1	❑
GelAir assembly table (optional)	1	❑
GelAir dryer (optional)	1	❑

Laboratory Protocol for Day 3

1. Destained gels can be stored in water for up to one week before drying. Destained gels can be placed in gel drying solution for 30 minutes prior to drying to reduce the risk of gels cracking while drying.

2. Gels can be dried in GelAir drying frames, or by making GelAir cellophane sandwiches on square plastic containers.

Note: For best results, trim off the thick bottom edge of the gel before drying.

GelAir drying frame method:

TIP Use this quicker method if you have the GelAir assembly table and drying frames (Catalog # 165-1776EDU). Otherwise, use the slower GelAir cellophane sandwich and plastic container method.

1. Prewet 2 sheets of cellophane in a container of water for 15–20 seconds.

2. Place a plastic drying frame on the GelAir assembly table. Center one sheet of cellophane on the assembly table.

3. Carefully lay and position gels on the cellophane. Up to 6 gels can be accommodated on one drying frame. If there are bubbles between the gels and the cellophane, gently push them out with a gloved finger.

4. Flood the gels with water. Lay the second sheet of cellophane on top of the gels, trying not to trap any bubbles in the sandwich. If there are any bubbles, gently push them out with a gloved finger. Bubbles will cause cracks in the gel during drying!

5. Place the square metal frame on top of the cellophane sandwich. Secure the eight clamps onto the frame, two on each side. If you are not using a GelAir dryer oven, place the frames upright in a well-ventilated area for 12–36 hours.

   If you have a GelAir dryer, place up to four drying frames into the oven, turn the heater switch on and set the dial to 3 hours. The dryer will shut off automatically.

6. When the gels are completely dry, they will be flat. Remove the clamps and take the sandwich of gels and cellophane from the frame. With scissors, trim the excess cellophane surrounding each dried gel.

Alternatively, you may use the GelAir cellophane sandwich and plastic container method:

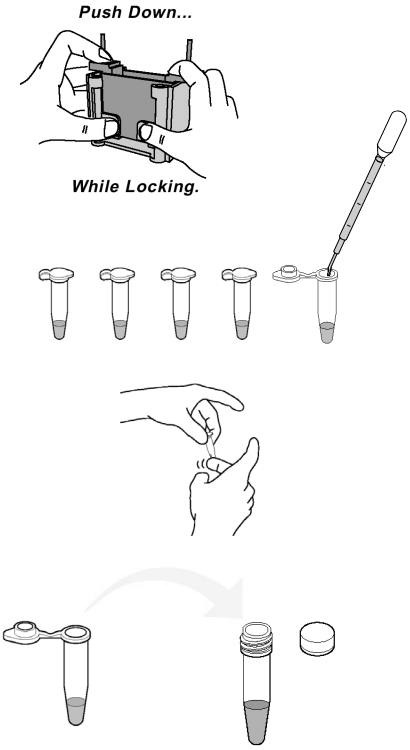
Wet two pieces of cellophane in a large volume of water. Place one sheet of cellophane over a plastic container. Pull the cellophane taut so that it makes a flat surface over the top of the container and use a rubber band to secure the sheet in place. Place a gel onto the cellophane. Remove any air bubbles that are under or around the gel. Flooding the surface of the cellophane around the gel with water will aid in the removal of bubbles. Place the second sheet of wetted cellophane over the gel, being careful not to trap any bubbles. Secure the second sheet of cellophane to the box with a second rubber band. Allow the gel to dry for several days in a well-ventilated area.

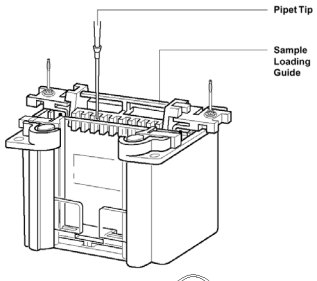
Protein Fingerprinting Kit – Quick Guide

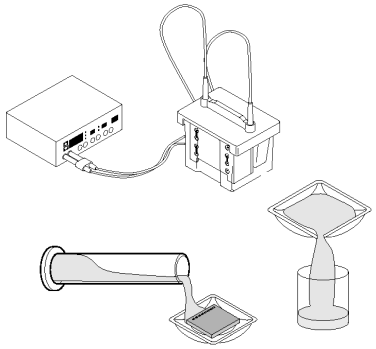
1. Setup Mini-PROTEAN 3 gel box and add 1x TGS electrophoresis buffer to the chamber.
2. Label one 1.5 ml **flip-top** micro tube for each of five fish samples. Also label one **screwcap** micro tube for each fish sample.
3. Add 250 µl of Bio-Rad Laemmli sample buffer to each labeled **flip-top** micro tube.
4. Cut a piece of each fish muscle about 0.25 x 0.25 x 0.25 cm^3 ( ) and transfer each piece into a labeled **flip-top** micro test tube. Close the lids.
5. Flick the microtubes 15 times to agitate the tissue in the sample buffer.
6. Incubate for 5 minutes at room temperature.
7. Carefully transfer the buffer by pouring from **each flip-top** microtube into a labeled **screwcap** microtube. Do not transfer the fish!
8. Obtain the Kaleidoscope prestained standards (KS) and the actin and myosin standard from your teacher.
9. Heat the fish samples and the actin and myosin standard (AM) in screwcap microtubes for 5 minutes at 95°C.
10. Load your gel:

Lane	Volume	Sample
1 & 2	empty	empty
3	10 µl	Kaleidoscope standards (KS)
4	10 µl	fish sample 1
5	10 µl	fish sample 2
6	10 µl	fish sample 3
7	10 µl	fish sample 4
8	10 µl	fish sample 5
9	10 µl	Actin and myosin standard (AM)
10	empty	empty

11. Electrophorese for 30 minutes at 200 V in 1x TGS electrophoresis buffer.
12. After electrophoresis, remove gel from cassette and transfer gel to a container with 40 ml Bio-Safe Coomassie Blue stain and stain gel for 1 hour, with gentle shaking for best results.
13. Discard stain and destain gels in a large volume of water for at least 30 minutes overnight, changing the water at least once. Blue-stained bands will be visible on a clear gel after destaining.
14. Dry gels using GelAir cellophane.

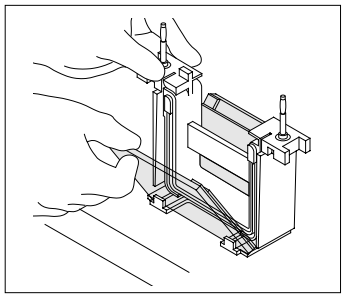




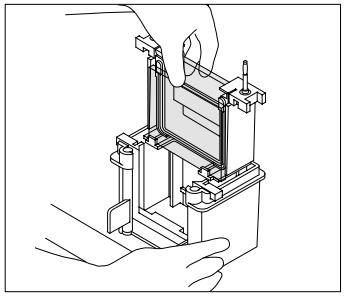


Mini-PROTEAN 3 Electrophoresis Module Assembly

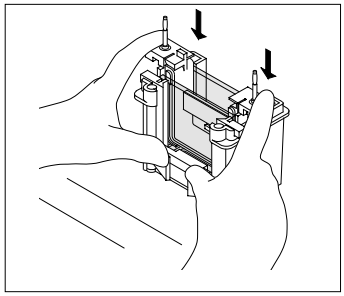
1. Prepare a Ready Gel cassette by cutting along the black line on the bottom of the cassette with a razor blade and pulling off the plastic strip, as indicated on gel cassette.



2. Remove the comb from the Ready Gel cassette.

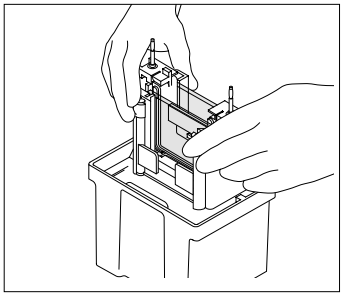


3. Place Ready Gel cassette into the electrode assembly with the short plate facing inward. Place a buffer dam or another Ready Gel cassette on the opposite side of the electrode assembly, with notch on buffer dam facing inward.



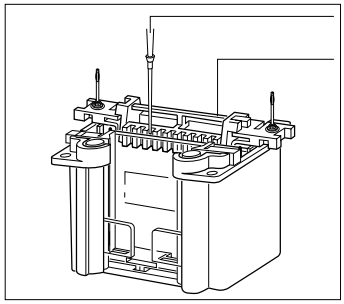
4. Slide gel cassette, buffer dam, and electrode assembly into the clamping frame

5. Press down the electrode assembly while closing the two cam levers of the clamping frame.



6. Lower the inner chamber into the mini tank.

7. Completely fill the inner chamber with 1x TGS electrophoresis buffer, making sure the buffer covers the short plate (~150 ml).



8. Fill mini tank with approximately 200 ml of 1x TGS electrophoresis buffer.

9. Place sample loading guide on top of the electrode assembly.

10. Load samples and run gel at 200 V for 30 minutes.

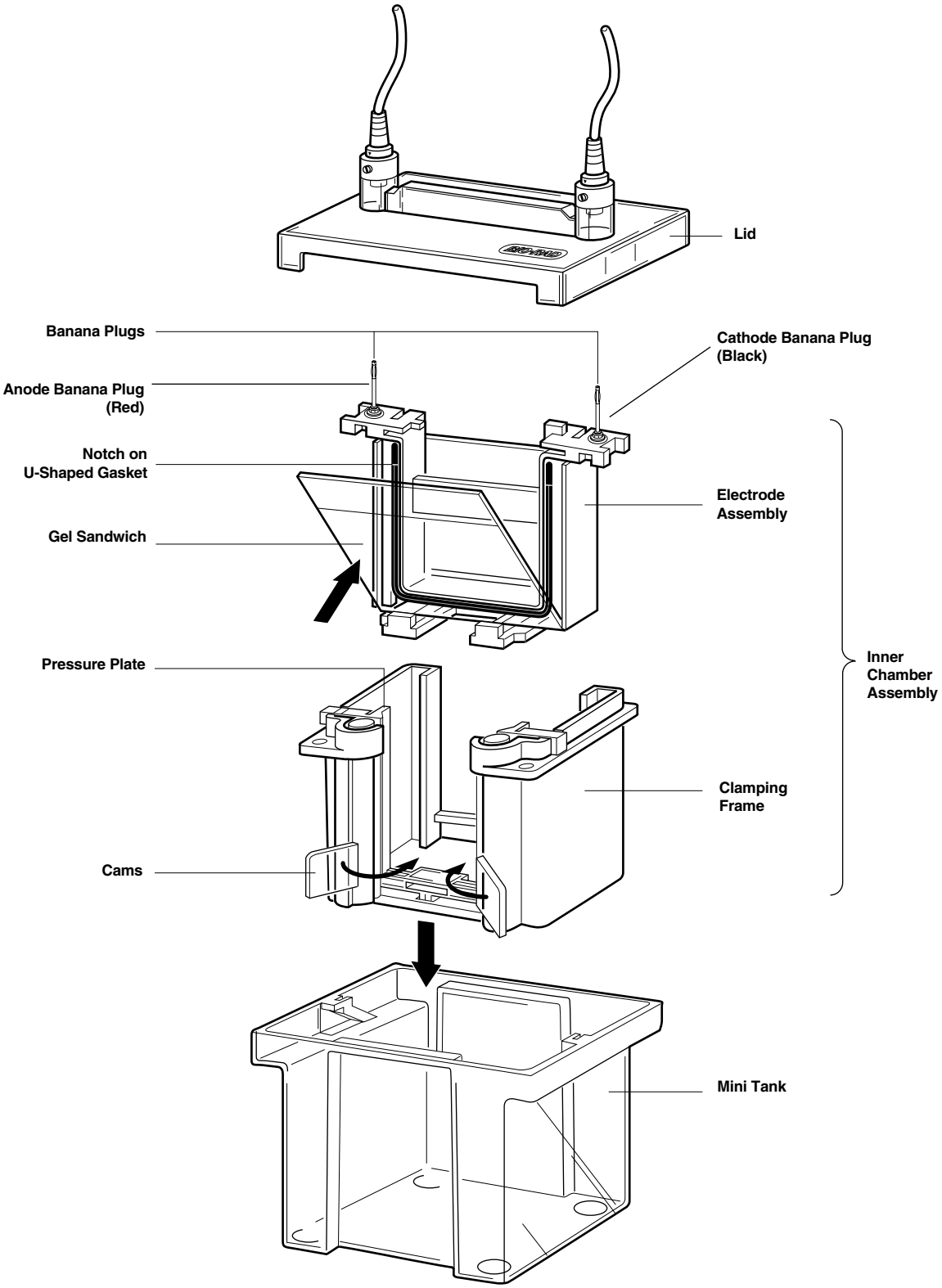


Figure 13. Assembling the Mini-PROTEAN 3 cell.

Student Manual

Student Pre-laboratory Background Considerations

Consideration 1 – Is There Molecular Evidence for Evolution?

Did you know that human genes are 98.4% identical to chimpanzee genes, but they're also like many of the genes in bacteria, mushrooms, and fish (King and Wilson 1975)? This remarkable similarity in genetic information among diverse species is our primary evidence that all organisms have the same ancient origin.

The Modern Theory of Evolution

Charles Darwin proposed the revolutionary idea that varieties of forms and species are derived from a common ancestor— "descent with modification." When a population of similar, related individuals acquires new characteristics over many generations, we refer to that process of modification over time as evolution. The millions of species living on earth today have one amazing, striking commonality: we all use essentially the same chemical language and mechanisms for metabolism and reproduction. Reliable fossil evidence for ancient bacteria, the earliest known cellular life, dates to at least 3.5 billion years ago. Since then, the expansion and modification of the gene pool of the original ancestral organisms, has resulted in the many millions of different species that exist on the planet today. Since DNA contains the information to make proteins, mutations or alterations in DNA can produce new proteins that function differently. These changes can lead to novel traits and diversity among related organisms. This genetic diversity makes evolution possible, since natural selection favors some individuals and not others, and so leads to changes in the composition of the gene pool over time. DNA both provides for the continuity of traits from one generation to the next and accounts for the variation that can lead to differences within a species—and even to entirely new species. As Francis Crick, one of the discoverers of the structure of DNA, put it:

> "We have discovered the secret of life."
> Francis Crick, Eagle Pub, Cambridge, 1953

Is There Proof?

Extensive studies have revealed that a great deal of DNA sequence similarity exists among the genes of all modern-day organisms. For example, scientists were astounded to discover that the same family of genes (Hox genes) controls the embryonic development of animals as diverse as fruit flies, zebrafish, and humans.

> "At the level of embryology we are glorified flies."
> Matt Ridley, Genome, 1999

The high levels of gene sequence similarity among diverse organisms can only be due to common ancestry, or **homology.** When the proteins of many diverse organisms are compared, they too show similarities, indicating that they have evolved by the shuffling and modification of simpler protein domains. Families of

related genes and proteins are found distributed wide and far – deep in the sea, underneath rocks, and flying above us in the sky.

So, the Question isn't Whether Evolution Happens but How It Happens?

Traditional classifications (kingdom, phylum, class, order, etc.) of organisms have been based primarily on morphological characteristics – traits that can be seen with the unaided eye or microscope. Charles Darwin's ideas about evolution were inspired by his observations of the large variety of beaks among finches. In 1859, he didn't even know about DNA. Nobody did – yet. However, he did have this revolutionary insight:

> "Whatever the cause may be of each slight difference in the offspring from their parents – and a cause for each must exist – it is the steady accumulation, through natural selection, of such differences...by which the innumerable beings on the face of this earth are enabled to struggle with each other and the best adapted to survive." (Darwin 1859)

A century later, scientists discovered the central framework of molecular biology: DNA→RNA→protein→trait, and came to understand the cause of these all-important differences. During this laboratory activity you will use a powerful technique of molecular biology to explore evolution by looking at organisms' individual differences and variations at the molecular level. Was Darwin right? See for yourself!

Consideration 2 - What Are Proteins?

The ultimate function of DNA is to specify what proteins are made. Proteins determine how an organism functions, what an organism can do, what it can eat, what it looks like, and where it can live. DNA gives instructions; proteins do the work. Each protein is composed of one or more folded chains of amino acids, each chain specified by a **gene** with a unique DNA sequence. Universally, every living cell requires proteins (enzymes) to carry out the thousands of chemical reactions that are needed to maintain life. The cell's structure is also an intricate and complex architecture of proteins and other molecules.

Within the complete genetic blueprints, or **genomes,** of organisms there may be a few thousand (e.g., in bacteria) to tens of thousands of genes (in complex eukaryotes) specifying individual proteins. A mutation that alters a gene's DNA sequence, even by a single nucleotide, may affect a protein's structure and thus the organism's ability to function – for better, or for worse.

Example – Sickle Cell Anemia

In sickle-cell anemia, a genetic disease, red blood cells contain an abnormal type of hemoglobin protein. This abnormal protein is the consequence of a single mutation in the DNA sequence that encodes hemoglobin. The mutation causes one amino acid, glutamic acid, to be replaced by a different amino acid, valine. This single substitution has profound effects on the three-dimensional shape and function of the hemoglobin protein, altering its ability to bind oxygen.

The Molecular Framework of Biology: DNA→RNA→Protein→Trait

DNA and RNA have informational roles in cells, whereas proteins and traits are the products of the information. In **transcription,** DNA sequences encoding individual proteins are copied to produce messenger RNA (mRNA). In **translation,** mRNA sequences are read by cellular machinery that synthesizes amino acid chains of precise order and content, as specified by the mRNA. These amino acid chains become the proteins that form structures and carry out the biochemical functions that we observe as traits.

The base units of mRNA are read in groups of three. Each triplet of bases, called a codon, specifies an amino acid, so the sequence of an mRNA molecule dictates the sequence of a chain of amino acids. Adaptor molecules called transfer RNA (tRNA) are the links between the mRNA and the amino acid sequences. tRNA molecules contact and "read" the mRNA codons, by supplying the appropriate amino acids to a growing protein chain. The coordination of mRNA and tRNA to synthesize amino acid chains takes place on intracellular structures called ribosomes.

During the process of transferring information from its original state in DNA to its functional form of a protein, the genetic code is read and interpreted several times. In the example below, the first three nucleotides in the β-globin gene are TAC. When mRNA is produced with sequences complementary to the DNA, its sequence is AUG, since U replaces T in RNA. The tRNA are in turn complementary to the mRNA, so the tRNA that reads the first codon has the anticodon UAC. The tRNA that contains the anticodon UAC and recognizes the mRNA codon AUG

carries a particular amino acid, methionine (Met). Thus the tRNA molecule adds methionine to the growing amino acid chain. In this way the information stored in DNA undergoes transcription by complementary copying of DNA to RNA and translation from nucleic acid sequence to amino acid sequence so that its function can be realized. Each gene is a DNA code specifying the linear order of amino acids that make up a protein.

The Transfer of Genetic Information: Transcription and Translation

β-globinA (β^A), wild type

DNA	TAC	CAC	GTG	GAG	TGA	GGA	CTC	CTC	TTG	AGA	CGG	CAA
mRNA	AUG	GUG	CAC	CUG	ACU	CCU	GAG	GAG	AAG	UCU	GCC	GUU
tRNA	UAC	CAC	GUG	GAC	UGA	GGA	CUC	CUC	UUC	AGA	CGG	CAA
amino acid	**Met**	**Val**	**His**	**Leu**	**Thr**	**Pro**	**Glu**	**Glu**	**Lys**	**Ser**	**Ala**	**Val**

β-globinA (β^S), sickle-cell mutant

DNA	TAC	CAC	GTG	GAG	TGA	GGA	CAC	CTC	TTG	AGA	CGG	CAA
mRNA	AUG	GUG	CAC	CUG	ACU	CCU	GUG	GAG	AAG	UCU	GCC	GUU
tRNA	UAC	CAC	GUG	GAC	UGA	GGA	CAC	CUC	UUC	AGA	CGG	CAA
amino acid	**Met**	**Val**	**His**	**Leu**	**Thr**	**Pro**	**Val**	**Glu**	**Lys**	**Ser**	**Ala**	**Val**

Figure 14. Transcription and translation of wild type and sickle cell mutant DNA.

Can you find the difference between the DNA sequences of the wild type and sickle cell mutant of the β-globinA gene? What is it? What is its effect on the respective amino acid sequences?

Would individuals with this altered form of hemoglobin continue to survive and reproduce? Could a mutant protein provide an advantage? How?

Muscle Proteins – Universal Soldiers

In addition to their vast array of functions as enzymes that assist biochemical reactions, proteins also have structural and mechanical functions. Muscle tissue consists of many different proteins with specialized functions, but the contraction of muscle results primarily from the interaction of two predominant proteins, actin and myosin. Actin and myosin form muscle fibers, the biochemical machinery that causes muscle to contract. These two proteins make up the structure and function of muscle that is common to all animals.

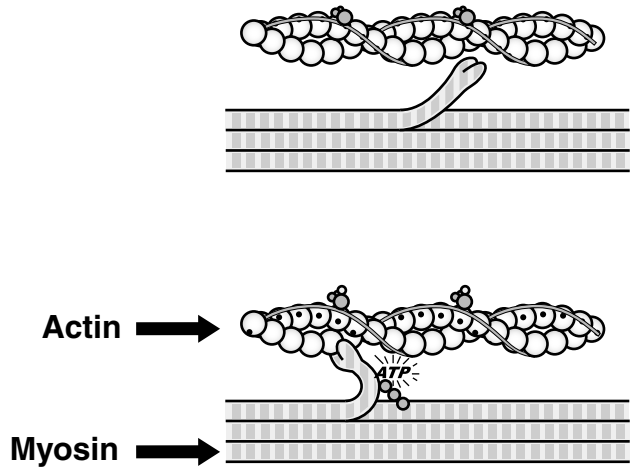
Characteristics of Actin and Myosin

Actin:

- 5% of total protein
- 20% of vertebrate muscle mass
- 377 amino acids = 42 kD
- Forms thin filaments

Myosin:

- 2 heavy subunits (220 kD)
- 4 light subunits (15-24 kD)
- Breaks down ATP for muscle contraction
- Forms thick filaments



(Figure modified from Campbell 1996 with permission)

Figure 15. Hydrolysis of ATP causes myosin and actin filaments to slide past one another, shortening the sarcomere and contracting the muscle.

The Evolution of New Functions

The genes for actin and myosin are members of gene families that encode proteins that enable movement. Other proteins associated with muscle have known, unknown, or speculated functions and may vary in their occurrence among different species. The variations in an organism's proteins are the results of random DNA mutations within the encoding genes, which have occurred over thousands to millions of years. Each mutation may result in some kind of change in a protein. The changes can be beneficial, neutral, or detrimental to an organism's ability to survive and reproduce. As Darwin said, only the "best adapted" survive natural selection. What he meant was that a trait persists if it brings about a competitive advantage, or gives an organism an ability to succeed in a challenging environment. In many cases, such advantages are related to new varieties of proteins. May the best protein win!

Focus Questions

1. **Besides actin and myosin, how many other proteins make up a functional muscle?**

2. **Can you name 10?**

3. **What does each protein do?**

Consideration 3 – How Do You See a Protein Molecule?

Gel electrophoresis is a powerful tool for separating and visualizing the individual proteins in complex samples like muscle tissue. The two types of molecules most often analyzed by electrophoresis are nucleic acids, including DNA, and proteins. Electrophoresis not only lets you determine how many distinct types of molecules are in the sample, it can also tell you their sizes. The size of a molecule, along with other information, can be a clue to its identity.

In this investigation, you will use high-resolution polyacrylamide gel electrophoresis to display the various proteins in the muscle tissue of different fish and to perform molecular weight determinations. By the displays of the protein assortments in the different fish, you can test the hypothesis that proteins are indicators of genetic and, therefore, evolutionary relatedness.

In this laboratory you will use a combination of a detergent and heat to extract and denature (disrupt) the proteins in several fish muscle samples. The detergent, SDS, coats dissolved proteins and polypeptides with negative charges. The SDS-coated proteins then all move toward the positive electrode, but at different rates depending on their sizes.

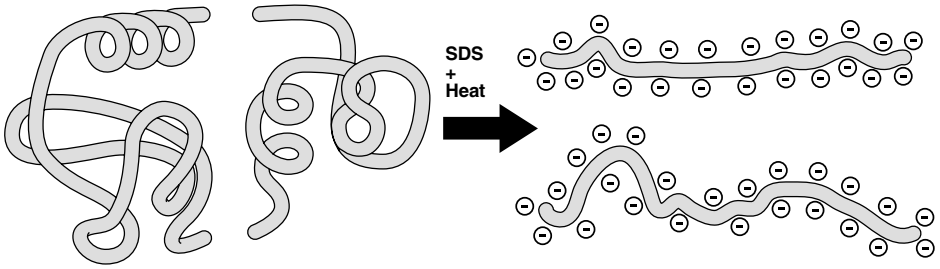


Figure 16. The combination of heat and the detergent SDS denatures proteins for SDS-PAGE analysis.

When the functional proteins (on the left) are coated with SDS and heated, they lose their three-dimensional structure and take on a net negative charge (on the right). Bigger polypeptides are coated with more molecules of SDS, so the ratio of a protein's molecular weight to its charge is approximately the same for all proteins. This means that size (molecular weight) becomes the determinant of mobility through the gel.

Many large proteins are made up of smaller protein subunits. These polypeptides are held together by bonds between sulfur atoms in the amino acids. Certain chemical treatments break these disulfide bridges and release the separate polypeptide units. Thus, one functional, native protein can give rise to several smaller polypeptides. Myosin, for example, is a complex of 2 heavy protein chains and 4 light chains. The light chains are of two different sizes, so a purified myosin sample will form 3 separate bands when treated appropriately prior to electrophoresis.

Consideration 4 - What Can Electrophoresis Tell Us about Proteins?

Electrophoresis is the migration of charged molecules in an electric field toward the electrode with the opposite charge. This technique is widely used in molecular biology to analyze mixtures containing proteins or nucleic acids like DNA and RNA.

Charged molecules would move toward an oppositely charged electrode even if the electrophoresis were performed in a liquid solution alone. However, with so little resistance to their movement, the molecules would all travel very fast, essentially at the same rate. Electrophoresis is commonly performed by placing a sample in a gel matrix of either polyacrylamide or agarose. The gel matrix acts as a molecular sieve, such that smaller molecules move through it more quickly than larger molecules. The degree of sieving can be controlled by selecting the appropriate gel substance, agarose or polyacrylamide, and by changing the concentration of the gel matrix.

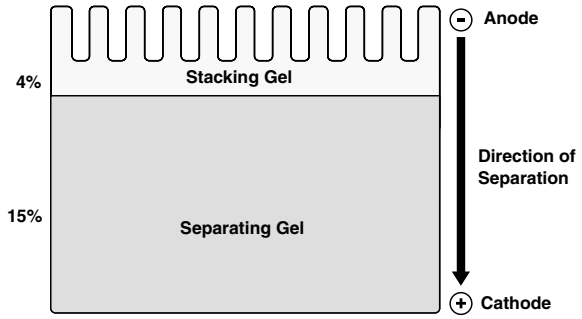


Figure 17. A simplified diagram of a polyacrylamide gel. The glass plates and supports that hold the gel upright are not shown. Mixtures of proteins are loaded into the "wells" at the top, and when the electric current is turned on, the proteins migrate through the gel, toward the bottom.

Since molecules migrate through the gel at different rates, they separate, which allows us to ask a variety of questions:

- **How many different proteins are there in my sample?**
- **How big are these proteins?**
- **What similarities or differences are there between different samples?**

Determining the Sizes of Proteins via Electrophoresis

One of the predominant uses of polyacrylamide gel electrophoresis is to determine the molecular weight of a protein.

Untreated, or **native**, proteins will migrate in a gel at rates based on both their electrical charges and their masses. If we equalize the charge-to-mass ratios (charge densities) of all protein molecules, mass becomes the only factor determining the migration rate of each protein. This is accomplished by treating the proteins with the ionic detergent SDS, which is present in both the gel running buffer and the sample loading buffer. This technique is called SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

A polyacrylamide gel is positioned in a buffer-filled chamber between two electrodes, treated protein samples are placed in wells at the top of the gel, and the electrodes are connected to a power supply that generates a voltage gradient across the gel. The negatively charged, SDS-coated proteins then move downward through the gel toward the positive electrode.

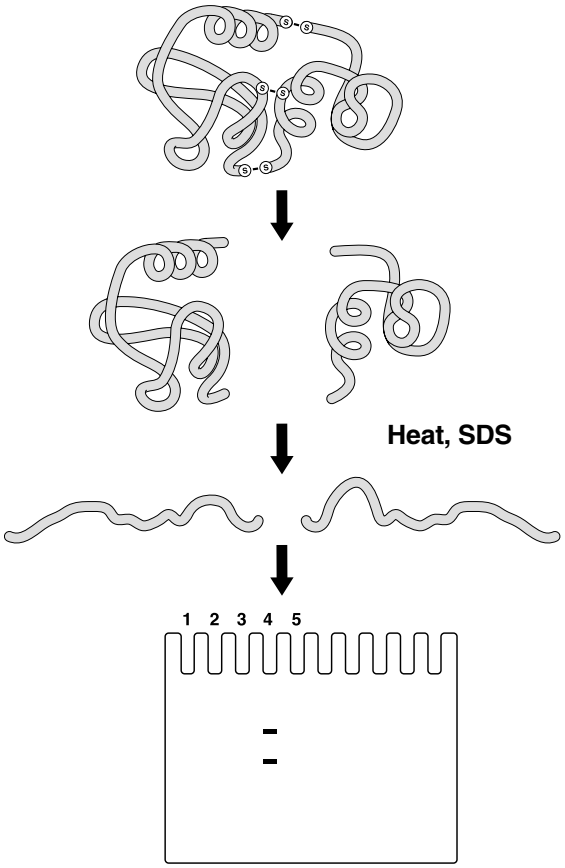


Figure 18. As soon as the electric current is applied, the SDS-coated proteins begin their race toward the positive electrode. The smaller proteins can move through the gel more quickly than the larger ones, so over time, the proteins will be separated according to their sizes.

Protein size is measured in **daltons,** a measure of molecular weight. One dalton is defined as the mass of a hydrogen atom, which is 1.66×10^{-24} gram. Most proteins have masses on the order of thousands of daltons, so the term **kilodalton** (kD) is often used to describe protein molecular weight. Given that the average weight of an amino acid is 110 daltons, the number of amino acids in a protein can be approximated from its molecular weight.

- Average amino acid = 110 daltons
- Approximate molecular weight of protein =
  number of amino acids x 110 daltons/amino acid

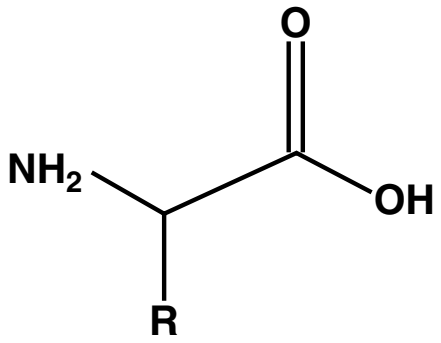


Figure 19. Chemical structure of an amino acid.

Proteins in your samples are not visible while the gel is running, unless they are prestained with covalently attached dyes, like the Kaleidoscope standards. If the electric current is left on for too long, the proteins will run off the gel at the bottom. To avoid this, a blue tracking dye is mixed with the protein samples. This blue dye is negatively charged and is also drawn toward the positive electrode. Since the dye molecules are smaller than the proteins expected in most samples, they move more quickly through the gel. When the dye approaches the bottom of the gel, it is time to turn off the power.

After turning off the electric current and removing the gel from the glass plates that hold it in place, the gel is placed in a stain. The stain used in this technique was originally developed to dye wool, which, like your own hair, is composed of protein. This stain binds specifically to proteins and not to other macromolecules like DNA or lipid. After destaining, distinct blue bands appear on the gel, each band representing on the order of 10^{12} molecules of a particular protein that have migrated to that position. The larger the amount of protein, the more intense the blue staining.

Consideration 5 – What Can Proteins Tell Me about Evolution?

The banding patterns on your gel reveal information about the protein composition of muscle from your fish samples. Proteins are synthesized according to the genes of an organism's DNA. Since closely related organisms share similar DNA sequences, the proteins encoded by their DNA should be very similar as well, and similar protein compositions should be reflected in banding patterns. In this investigation you can test this hypothesis of correlation between protein profiles and evolutionary relatedness.

1. Why do SDS-coated proteins move when placed in an electrical field?

2. On the gel diagram below, imagine loading lanes 1, 2, and 3 with different samples.

   Lane 1 is loaded with a mixture of 4 proteins composed of 100 amino acids, 175 amino acids, 250 amino acids, and 300 amino acids.

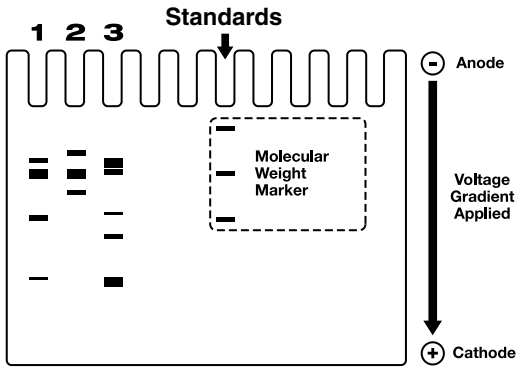
   Lane 2 is loaded with a mixture of 3 proteins composed of 200 amino acids, 250 amino acids, and 350 amino acids.

   Lane 3 is loaded with a mixture of 5 proteins composed of 100 amino acids, 150 amino acids, 175 amino acids, 250 amino acids, 300 amino acids.

   Given that the average weight of amino acids is 110 daltons, calculate the molecular weight of each protein and identify each on the gel below.

3. If each lane represents proteins from a different animal species, which two species would you say are the most closely related? Why?

4. If two proteins have the same molecular weight, are they necessarily the same protein?



Consideration 6 - Are Proteins a Window to DNA?

Each of the bands in the protein fingerprint is due to a different protein. But how much can we infer about DNA sequences from protein alone?

As we have already stated, a gene is a code specifying the fundamental composition of a protein. The sequence of "letters" in DNA is transcribed and translated into a sequence of amino acids, and the chain of amino acids takes on a three-dimensional structure to become a functional protein. In the translation step, triplets of RNA bases correspond to individual amino acids. With a DNA/RNA "alphabet" of 4 letters, there are 64 possible triplets, yet cells make their proteins from only 20 amino acids. Because of this excess coding capacity, most amino acids are encoded redundantly, by more than one codon. Leucine, for example, can be coded on mRNA by UUA, UUG, CUU, CUC, CUA, and CUG. This redundancy leads to a code that is degenerate, which cannot be decoded accurately in reverse.

An amino acid sequence can always be derived unambiguously from a DNA or RNA sequence, but we cannot derive an unambiguous RNA or DNA sequence from an amino acid sequence. Because of the similarities of the redundant sets of codons, an RNA or DNA sequence can be partially reconstructed from a given amino acid sequence. Identical polypeptides derived from two species are likely, therefore, to reflect highly similar, but not identical, genes encoding them.

The advantage of the degenerate code is that it allows organisms to tolerate some mutations. For example, if an mRNA contains the codon CUA, for leucine, the third position of the codon could mutate to any other letter (G, C, or U) and not affect the amino acid at that position in the polypeptide. In this case, the first position could also be changed to U without any effect on the polypeptide.

Large regions of an organism's genome and portions of individual genes, called introns, do not get transcribed and translated into protein. Furthermore, within a specific tissue such as muscle, only a fraction of all of the genes in the genome are expressed. Proteins thus reflect only a small subset of an organism's total genomic composition. In spite of this limitation, protein profiles are a simple and effective means of indirectly detecting variation in DNA sequences.

Focus Questions:

1. How many mRNAs are produced by each gene?

2. What function, if any, do you think introns may serve?

3. Why can't the DNA sequence encoding a protein be determined unambiguously from the sequence of amino acids?

Let's review...

1. **Traits** are the result of:

    - Structural and functional proteins
    - Enzymes that are made of protein

2. **DNA** codes for proteins that confer traits
   DNA→RNA→Protein→Trait

3. **Changes in DNA produce:**

    - alterations in the structures of existing proteins, or new proteins
    - novel traits arising from the altered or new proteins
    - positive, negative, or neutral effects from those traits

4. **Genetic diversity provides a pool for natural selection→evolution**

    - Molecular studies show a great deal of similarity between DNA sequences of present-day organisms, indicating a common ancestry.
    - Over time small changes add up and can eventually produce new species.
    - Natural selection eliminates detrimental traits but preserves beneficial traits within a given environment.

5. Look at the evolutionary tree below.

Different branches of related organisms separated at different evolutionary times. The further apart species are on the tree, the less related they are. Mollusks and arthropods diverged from one another before the emergence of chordates, animals with backbones, very early in evolutionary time. These animals are only distantly related to fish, birds, reptiles, mammals, and amphibians, which are more closely related to each other.

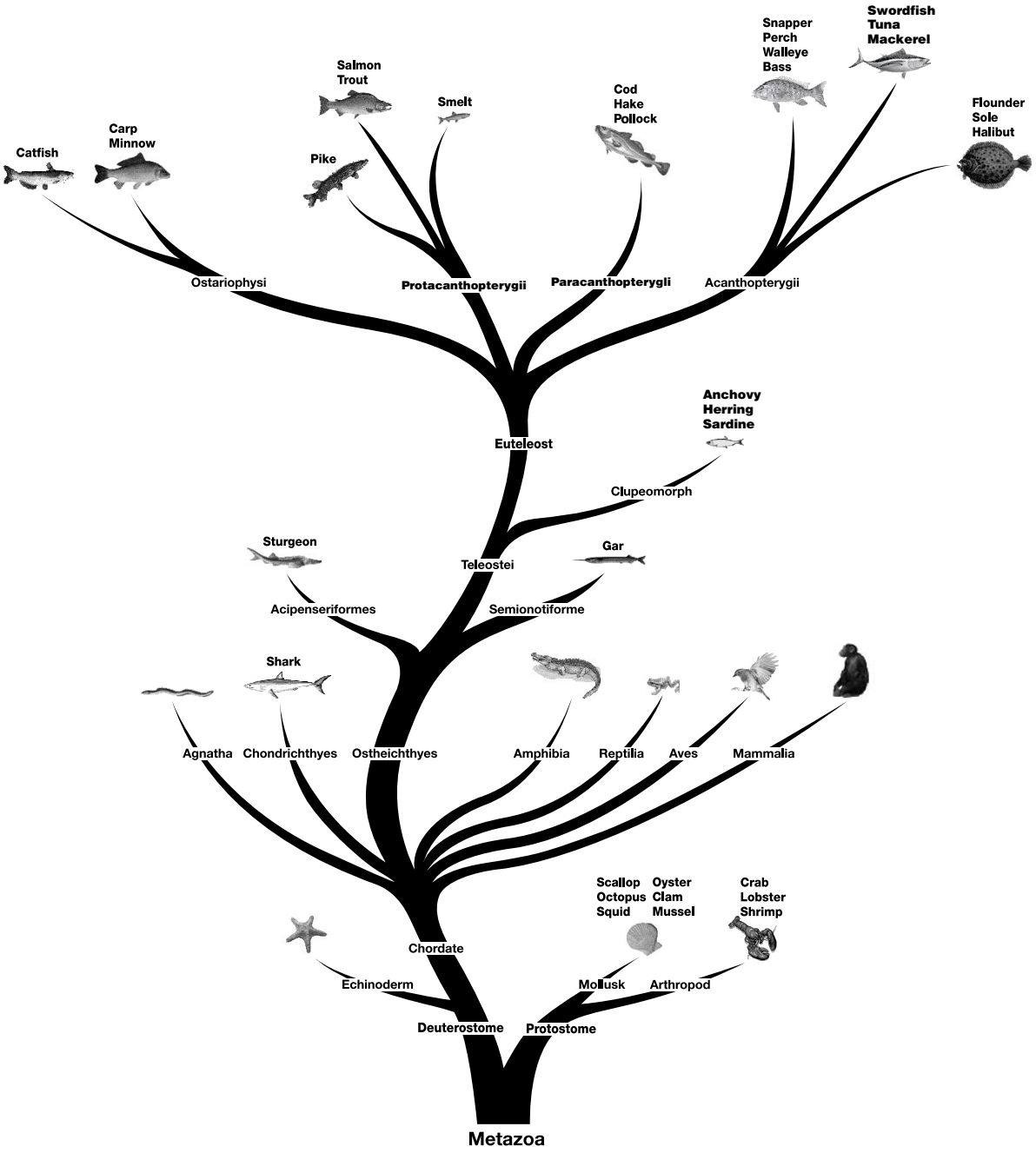


Figure 20. Fish evolutionary tree based on Ichthylogy Web Resources and the Tree of Life.

Evolutionary Tree of Fishes

1. List the names of the fishes whose muscle proteins you will be investigating.

2. According to the fish phylogenetic tree, which of your fish are most closely related to each other? Which fish are most distantly related to each other?

3. Which samples would you expect to share the most DNA sequence similarity? The least DNA sequence similarity?

4. Which samples would you expect to share the most protein banding similarity? The least protein banding similarity?

Now that we've considered the objective of the activity and your expectations of the outcome, you are now ready to proceed with the extraction and examination of the muscle proteins.

Laboratory Day 1

What Is the Question?

Scientific investigations are conducted to gather data to answer a specific question. For example, the question relevant to this investigation is:

Do Biomolecules Reflect Evolutionary Relationships?

In this investigation, you will use high-resolution polyacrylamide gel electrophoresis to compare the proteins in the muscle tissues of different fish. You will generate protein profiles, or fingerprints, for the specimens and examine them for similarities and differences among the different species. You will then draw conclusions from your data about the relatedness of the species and compare them to evolutionary classifications.

Laboratory Day 1 - Sample Preparation: Muscle Protein Extraction

Purpose of this laboratory:

Prepare protein extracts of muscle tissue from fish samples

Procedure Overview:

1. Extract proteins from tissue
2. Denature proteins

Step 1 Read through introductory material on the previous pages
Step 2 Check your workstation for the following items
Step 3 Go

Laboratory Checklist (✔)

Check that all supplies and equipment are at your student workstation and the common workstation.

Student Workstations	Daily checklist	(✔)
1.5 ml fliptop micro test tube	5 each	❑
1.5 ml screwcap micro test tube	7 each	❑
Disposable 1 ml pipet (DPTP s)	1 each	❑
Fish samples (5–8 types)	1 gm each	❑
Indelible marking pen, fine point	1	❑
Laemmli sample buffer	1.25 ml	❑

Teacher's (Common) Workstation		(✔)
Water bath or hot plate set to 95°C	1	❑
Laemmli sample buffer – 30 ml	stock bottle	❑
Kaleidoscope prestained standards	1 vial	❑
Actin and myosin standard	1 vial	❑

Laboratory Day 1

Step 1: Sample Preparation – Muscle Protein Extraction

Procedure

1. Label (with indelible pen) 1.5 ml flip-top microtubes with the names of the fish samples to be analyzed. There should be one labeled tube for each fish sample being prepared for electrophoresis.

2. Add 250 µl of Laemmli Sample Buffer to each labeled tube.

3. For each sample, obtain a piece of fish muscle (avoid skin, fat, and bones) approximately 0.25 x 0.25 x 0.25 cm^3 (), and transfer it to the appropriately labeled microtube. Close the lid.

4. Gently flick the microtube 15 times with your finger to agitate the tissue in the sample buffer.

5. Incubate the samples for 5 minutes at room temperature to extract and solubilize the proteins.

6. Pour the buffer containing the extracted proteins, but not the solid fish piece, to a labeled 1.5 ml **screwcap** tube. Note: It's not necessary to transfer all of the fluid to the screwcap tube, since only a small volume (<20 µl) is actually needed for gel loading.

7. Obtain aliquots of the Kaleidoscope (KS) and actin and myosin (AM) standards from your teacher.

8. Heat the fish samples and the actin and myosin (AM) sample in their screwcap tubes for 5 minutes at 95°C to denature the proteins in preparation for electrophoresis.

9. Store the samples at room temperature if they are to be loaded onto gels within 3–4 hours, or store them at –20°C for up to several weeks.

Focus Questions:

1. What molecules might be present in the sample dissolved in the Laemmli sample buffer?

2. How have the molecules changed as a result of treatments you have performed?

3. Why was it necessary to change the molecules in this way?

Laboratory Day 2

Electrophoresis

So far you have extracted, denatured, and given the proteins from fish muscle tissue a negative charge. Now they can be separated according to their molecular weights using gel electrophoresis.

Electrophoresis: gel loading, running, and staining

Purpose of this laboratory:

Generate profiles for various fish species via electrophoresis of extracted protein samples.

Procedure Overview:

1. Reheat fish and actin and myosin standard controls
2. Set up electrophoresis gel boxes
3. Load and run gels
4. Stain gels to visualize protein bands

Step 1 Review previous section about electrophoresis
Step 2 Check your workstation for materials and supplies
Step 3 Go

Laboratory Checklist (✔)

Student Workstation		**(✔)**
Fish protein extracts prepared on laboratory day 1	5 each	❑
Prot/Elec pipet tips for gel loading	7 tips	❑
Mini-PROTEAN 3 electrophoresis module (gel box – runs one or two gels)	1	❑
Buffer dam (if running only one gel/box)		❑
Power supply (200 V constant)	1	❑
2–20 µl micropipet	1	❑
Ready Gel precast gel, 15% – 10 wells	1	❑
Sample loading guides – for 10 well comb	1	❑
Thin metal weighing spatula	1	❑

Teacher's (Common) Workstation		**(✔)**
**Actin and myosin standard sample, rehydrated	1 vial	❑
Kaleidoscope prestained standards	1 vial	❑
1X Tris-glycine-SDS (TGS) electrophoresis buffer	As needed	❑
Bio-Safe Coomassie stain for proteins	As needed	❑
Staining trays		❑

**You may already have aliquots at student stations

Laboratory Day 2

Step 1: Prepare Samples, Electrophoresis Gels and Gel Boxes

Note: Teachers may have already assembled the gel boxes. If not, follow these instructions.

1. Reheat frozen samples at 80–95°C for 2–5 minutes to redissolve any precipitated detergent.

2. Make sure the comb and the tape along the bottom of the Ready Gel cassette have been removed. If two gels are to be run in one electrophoresis box, place a Ready Gel cassette on each side of the electrode assembly, with the **short** plates facing the inside of the assembly. If you are running only one gel in the box, place a Ready Gel cassette on one side of the electrode assembly and a buffer dam on the other side. Be sure to place the side of the buffer dam that says "BUFFER DAM" toward the electrode assembly.

3. Open the gates (cams) on the front of the clamping frame. Hold the two Ready Gel cassettes, or one Ready Gel cassette and buffer dam, against the electrode assembly and slide the electrode assembly into the clamping frame.

4. Press down on the outer edge of the electrode assembly, not the gels, while closing the cams of the clamping frame to ensure a seal on the bottom edge of each cassette.

5. Place the assembled clamping frame containing the gel(s) into the gel box tank. Fill the upper buffer chamber, the space between the two gels, with ~150 ml 1X TGS electrophoresis buffer, so the buffer level is **above** the inner short plates. Check for leaks. If the assembly is leaking, remove the assembled clamping frame, pour off the buffer, reopen the cams, and push down on the electrode assembly again while closing the cams.

6. Pour ~200 ml of 1X TGS electrophoresis buffer into the lower buffer chamber, or tank. Double-check the buffer fill level within the upper buffer chamber.

Note: If leakage of the upper buffer cannot be corrected by reassembling the clamping frame in Step 4, the outer chamber can be filled to above the inner small plates, to equalize the buffer levels in both reservoirs. This requires approximately 900 ml of 1X TGS electrophoresis buffer.

Laboratory Day 2

Step 2: Load and run gels

Place a yellow sample loading guide on the top of the electrode assembly. The guide will direct the pipet tip to the correct position for loading each sample in a well.

Assign samples to wells, loading samples in middle of the gel, where separation is best with the standards on each side. For example, for 5 fish samples on a 10 well gel, you may choose to follow this guide:

Lane	Volume	Sample
1	empty	None
2	empty	None
3	10 µl	Kaleidoscope prestained standard (KS)
4	10 µl	Fish sample 1
5	10 µl	Fish sample 2
6	10 µl	Fish sample 3
7	10 µl	Fish sample 4
8	10 µl	Fish sample 5
9	10 µl	Actin and myosin standard (AM)
10	empty	None

To load each sample, use a thin, gel loading micropipet tip to withdraw 10 µl of each protein sample from its tube and gently transfer it into the designated gel well. After loading all samples, remove the sample loading guide, place the lid on the tank, and insert the leads into the power supply, matching red to red and black to black. Set the voltage to 200 V and run the gels for 30 minutes.

Record your samples here:

Lane	Sample
1	empty
2	empty
3	Kaleidoscope prestained standard
4	_____
5	_____
6	_____
7	_____
8	_____
9	Actin and myosin standard
10	empty

Laboratory Day 2

Step 3: Stain and Visualize the Proteins

Gel staining:

1. When gels are finished running, turn off the power supply and disconnect the leads. Remove the lid and lift out the electrode assembly and clamping frame.

2. Pour out the running buffer from the electrode assembly. Open the cams and remove the gel cassettes.

3. To keep the gel free of contamination from your fingertips, wear gloves to handle the gels from this point on. Lay a gel cassette flat on the bench with the short plate facing up. Cut the tape along the sides of the gel cassette. Carefully pry apart the gel plates, using a spatula or your fingertips. The gel will usually adhere to one of the plates. Transfer the plate with the gel adhering to it to a tray containing Bio-Safe Coomassie Blue stain, allowing the liquid to detach the gel from the plate. The gel may also be lifted directly (and gently!) from the plate and placed into the stain.

4. Allow the gels to stain for 1 hour, with shaking if available.

5. Your teacher will discard the stain and replace it with a large volume of water to destain the gel overnight.

Focus Questions

1. What happened to the proteins in your loaded samples when you turned on the electric current?

2. Why were you able to see the different colored Kaleidoscope standard proteins while the gel ran?

3. What must happen to make the proteins in your samples become visible?

Laboratory Day 3

Gel Preservation

Purpose of this laboratory:

Examine data and make a permanent record

Procedure Overview:

1. Initial examination of protein profiles from fish samples
2. Set up of gels for drying

Step 1 Review Considerations 4 and 5 for interpreting electrophoresis gels
Step 2 Check your workstation for materials and supplies
Step 3 Go

Laboratory Checklist (✔)

Student Workstations		**(✔)**
Water (tap water)	1 liter	❑
GelAir cellophane support	2	❑
Plastic container (if not using GelAir drying frame)	1	❑
Rubber bands	2	❑
Teacher's (Common) Workstation		
GelAir drying frames	1	❑
GelAir dryer (optional)	1	❑

1. Examine your gels. Blue protein bands should be visible on the clear, destained gel. You may want to make a photocopy of your gel so that your data are available to analyze sooner.

   Your teacher will tell you if you will be drying your gel with GelAir drying frames, or by the plastic container method. Follow the instructions below accordingly.

   Note: For best results, trim off the thick bottom edge of the gel before drying.

GelAir drying frame method:

Prewet 2 sheets of cellophane in a container of water for 15–20 seconds.

2. Place a plastic drying frame on the GelAir assembly table. Center one sheet of cellophane on the assembly table.

3. Carefully lay your gel on the cellophane, positioning it to accommodate other gels (up to 6 total). If there are bubbles between the gel and the cellophane, gently push them out with your gloved finger.

4. Flood the gels with water and lay the second sheet of cellophane on top of them, trying not to trap any bubbles in the sandwich. If there are any bubbles, gently push them out with a gloved finger. Bubbles will cause cracks in the gel during drying!

5. Place the square metal frame on top of the cellophane sandwich. Secure the eight clamps onto the frame, two on each side. If you are not using a GelAir Dryer oven, place the frames upright in a well-ventilated area for 12–36 hours.

   If you have a GelAir dryer, place up to four drying frames into the oven, turn the heater switch on and set the dial to 3 hours. The dryer will shut off automatically.

6. When the gels are completely dry, they will be flat. Remove the clamps and take the gel/cellophane sandwich from the frame. Trim the excess cellophane surrounding your dried gel with scissors.

Alternatively, you may use the GelAir cellophane sandwich and plastic container method:

Wet two pieces of cellophane in a large volume of water, around 500 ml. Place one sheet of cellophane over a plastic container. Pull the cellophane taut so that it makes a flat surface over the top of the container, and use a rubber band to secure the sheet in place. Place a gel onto the cellophane. Remove any air bubbles that are under or around the gel. Flooding the surface of the cellophane around the gel with water will aid in the removal of bubbles. Place the second sheet of wetted cellophane over the gel, being careful not to trap any bubbles. Secure the second sheet of cellophane to the box with a second rubber band. Allow gel to dry for several days in a well-ventilated area.

Focus Questions: What Is the Significance of Your Data? What Do the Bands Reveal?

You have completed a procedure that displays a profile of protein composition in the muscle tissue of different fish. Since proteins are a reflection of an organism's DNA, variation in these composition profiles reflects genetic, that is, DNA sequence, variation. Evolutionary relationships among species are inferred from the degrees of genetic (DNA) similarity among them. The protein fingerprints you've generated, indirectly representing the genetic compositions of your chosen fish species, are molecular-level indicators of evolutionary relationships. The conclusions you draw from your own data can be compared to evolutionary relationships based on traditional morphological criteria.

Interpreting the bands collectively: Qualitative comparisons of protein profiles

1. Is there any variation among the protein profiles of your samples?

2. How would you distinguish the protein profiles of different species from each other?

3. What are possible explanations for this variation?

4. Which samples are most alike?

Tape your gel or a photocopy of it in the box. The bands are the data upon which your analysis will be based.

Post Laboratory - Quantitative Analysis of Data

The most similar species:

Now that you've had a chance to make a general comparison of the similarities and differences of protein banding patterns among the fish samples you've analyzed, a more quatitative analysis of your data may further support or refute your hypotheses.

1. Before you conducted the investigation, which two fish species did you list as being most related?

   _____ and _____

2. Of all the muscle proteins that you found in these two species, how many are present in both species?_____

3. What is the total number of different kinds of proteins that you were able to detect on your gel, in these two species? _____

4. Of the total number of proteins in this pool, how many are found in common to both species listed in question 1 above?

   $$\frac{\text{(number of proteins in question 2)}}{\text{(number of proteins in question 3)}} \times 100 = \_\_\_\_\_ \%$$

The least similar species:

1. Prior to starting the laboratory, which two fish species did you indicate to be least related? _____ and _____

2. Of all the muscle proteins that you found in these two species, how many are present in both species?_____

3. What is the total number of different kinds proteins that you were able to detect on your gels, in these two species? _____

4. What percent of the muscle proteins were common to these least similar species? _____%

5. Do your data support the relationships indicated by the phylogenetic tree?

Interpretation of Individual Bands

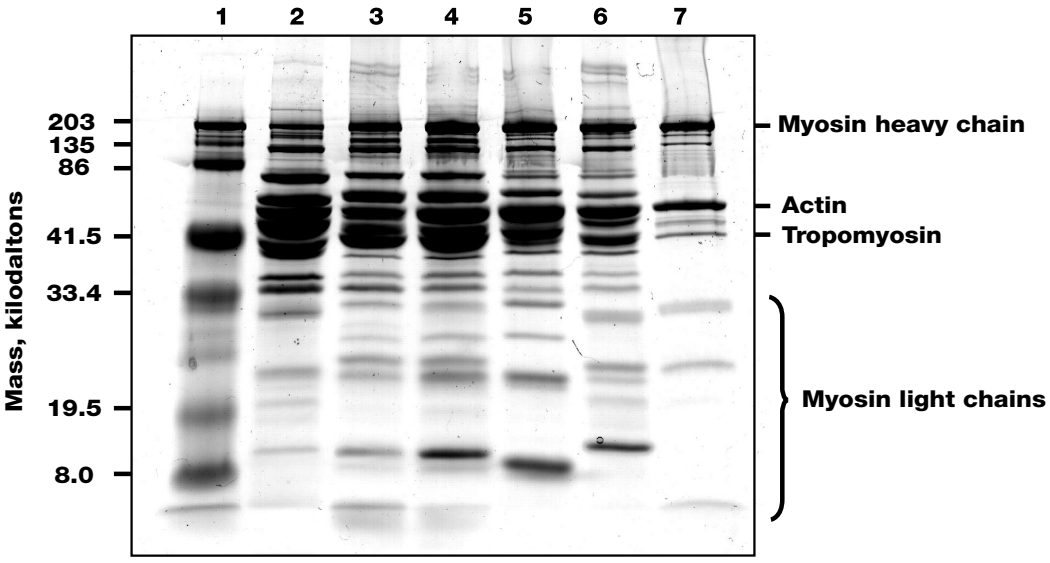
1. What do the relative positions of the bands on the gel indicate about the proteins in the bands?

2. Are all of the bands of equal thickness?

3. How would you explain the observation that some proteins form thin bands while others form thick bands?

Determining the Molecular Weights of Proteins

Look at the sample gel below. Proteins of known sizes were run along with 5 different fish muscle extracts, which are complex mixtures of unknown proteins. By comparing the migration distance of an unknown protein to that of a series of known molecular weight standards, the size of the unknown protein can be estimated. While it is not possible to determine the exact identity of a particular protein using this technique alone, a good estimate of the protein's molecular weight is obtained.



Lane: 1. Kaleidoscope standards
2. Shark
3. Salmon
4. Trout
5. Catfish
6. Sturgeon
7. Actin and myosin standard

Figure 21. Protein standards and fish extracts were run on a 15% polyacrylamide Ready Gel at 200 V for 30 minutes, stained with Bio-Safe Coomassie stain, and destained in water.

Construct Your Own Standard Curve

To determine the molecular weights of unknown proteins, construct a standard curve by plotting the molecular weights of known protein markers on the y-axis against the migration distances of those markers on the x-axis. Representing the molecular weights on a logarithmic scale results in a linear (straight) standard curve, as seen in the illustration below.

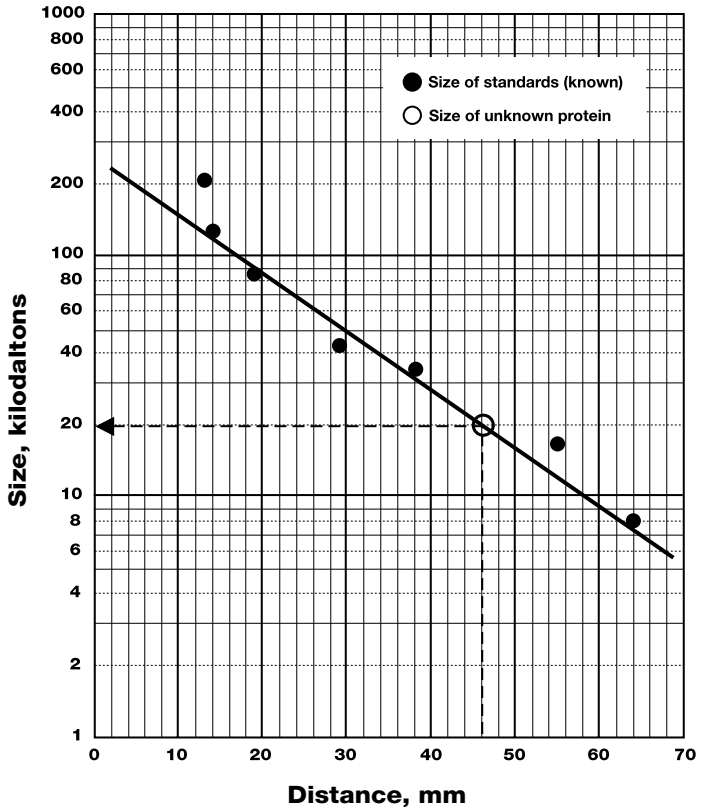
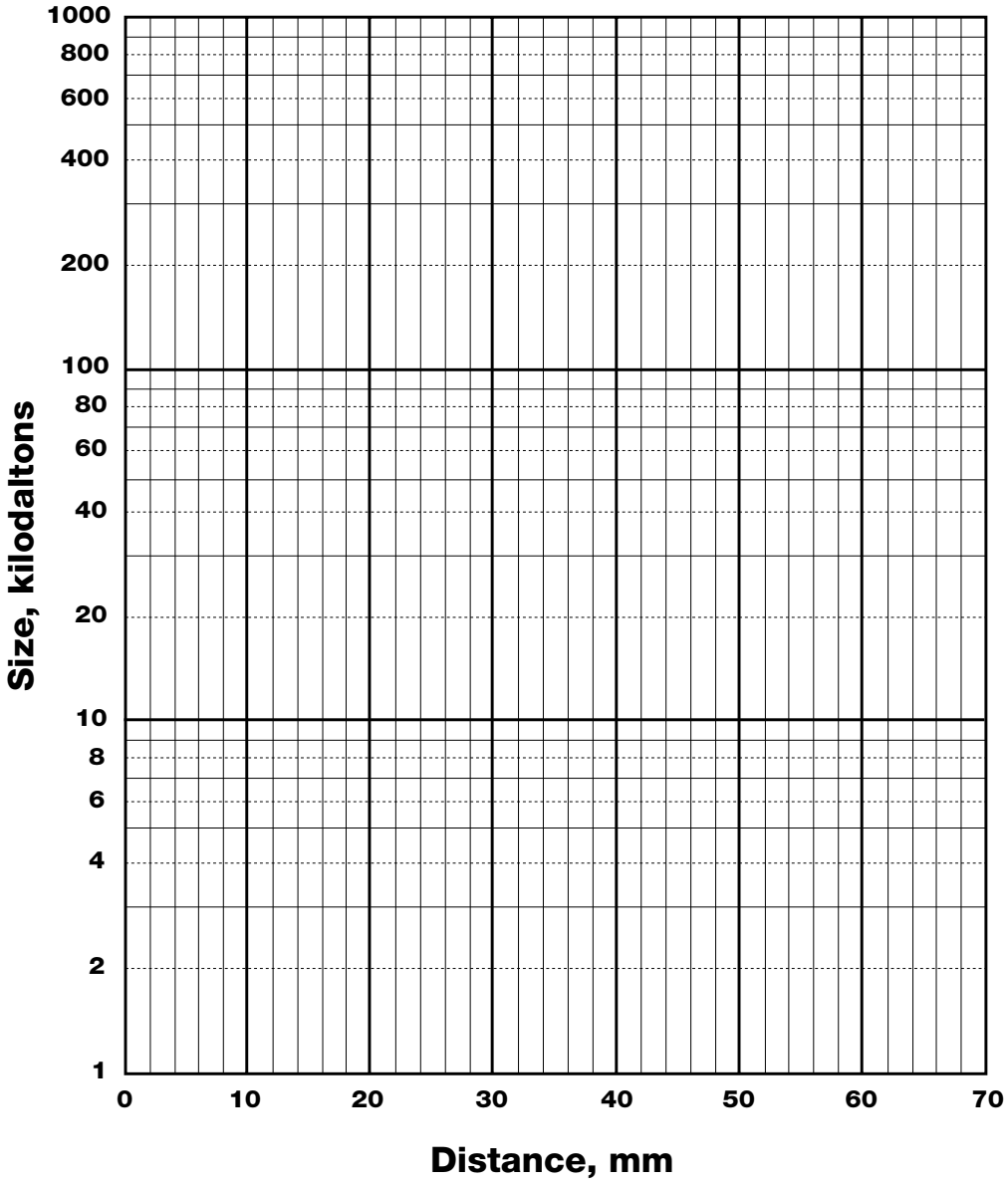


Figure 22. The distance migrated by each prestained protein was plotted against its size on semi-log graph paper to generate a standard curve for the gel.

From the standard curve, the molecular weight of an unknown protein can be estimated. First, measure the migration distance of a protein band from the bottom of the well to the lower edge of the band. Find the value for this migration distance on the x-axis of the standard curve and draw a vertical line up to the standard curve. Then draw a horizontal line from that point on the curve to the y-axis. The point where this line intersects the y-axis will give the value of the protein's apparent molecular weight.

Use the semi-log paper provided here:

Semi-Log Graph Paper



1. Construct a standard curve for your gel, using the Kaleidoscope standards' migration distances and their known molecular weights.

2. Now use the standard curve to determine the molecular weights of actin and myosin in your control lane (your teacher will tell you which bands in the control sample are actin and myosin)

   Actin: _____ Myosin: _____

3. In your own fish extracts, are there protein bands that migrated to identical positions as actin and myosin in the control lane?

4. After determining the apparent molecular weights of actin and myosin from the gel, predict the number of amino acids in actin, using an average molecular weight of 110 daltons per amino acid.

5. Predict the number of DNA base pairs in the gene that encodes actin. DNA base pairs have an average molecular weight of 660 daltons.

6. Compare answers obtained by the class for questions 1–5. Are there differences? What information would you need to determine actual molecular weights, and numbers of amino acids and DNA base pairs?

7. Why might the apparent weights you have determined from your gel differ from the true molecular weights of actin and myosin?

8. Would you expect the molecular weight approximations that you have determined from your gel and standard curve to be closer to true values for larger or smaller proteins? Why?

9. Describe the relative concentrations of actin and myosin in your fish samples.

10. Actin and myosin are proteins found in muscle tissue of all animals. Based on your data, what can you say about these two proteins in the fish you have investigated? What might you find if you looked at actin and myosin in other animals?

The following is a list of the proteins common to all animal muscle, adapted from Alberts et al. 1994.

Protein	MW (in kD)	Function
titin	3000	centers myosin in sarcomere
dystrophin	400	anchoring to plasma membrane
filamin	270	cross-links actin filaments into gel
myosin heavy chain	210	slides actin filaments
spectrin	265	attaches actin filaments to plasma membrane
M1/M2	190	myosin breakdown product
M3	150	myosin breakdown product
C protein	140	myosin breakdown product
nebulin	107	regulates actin assembly
α-actinin	100	bundles actin filaments
gelsolin	90	fragments actin filaments
fimbrin	68	bundles actin filaments
actin	42	forms filaments
tropomyosin	35	strengthens actin filaments
troponin (T)	30	regulates contraction
myosin light chains	24, 17, 15	slides actin filaments
troponin (I)	19	regulates contraction
troponin (C)	17	regulates contraction
thymosin	5	actin monomers

11. Based on these molecular weights provided, can you guess the identities of any of the proteins in your gel?

12. Can you definitively assign the identity of a protein based on its molecular weight? Why or why not?

13. Do all of your samples have similar band profiles? How can you explain the variation in the muscle protein profiles of different fish species?

14. Describe the relative concentrations of some of the proteins in your samples.

15. Given what you know about the fish species you selected for this study, *e.g.*, their habitats, lifestyles, and presumed evolutionary relationships, what might be the functions of some of the proteins you can't identify? Use your imagination.

Conclusions

1. You have isolated, separated, and compared profiles of muscle proteins from a number of different fish species. You have also correlated the similarities of these protein profiles with the species' presumed phylogenetic relationships. What do protein profiles tell us about DNA similarities among species? Be as explicit as possible.

2. What implications might these kinds of molecular data have in relation to the theory of evolution?

3. Do your data correlate with the arrangement of branches of the evolutionary tree?

4. What new questions or investigations might be stimulated by your findings in this activity?

5. Suppose someone tells you they don't understand the evidence for the theory of evolution, since no one has been able to witness the process of species evolving through time. How would you use your experimental data to respond to their statement?

Glossary

Actin	major muscle protein organized into thin filaments
Anode	positive electrode
BME	β-mercaptoethanol
Cathode	negative electrode
Charge density	ratio of charge to mass of a protein
Codon	a set of three nucleotides (DNA bases) that code for an amino acid
Dalton (Da)	unit of molecular weight equal to the mass of a hydrogen atom, 1.66×10^{-24} gm
Denature	to disrupt a protein's 3-dimensional structure
Disulfide bond	S-S bond between amino acids in a polypeptide chain; contributes to tertiary and quaternary structure of proteins
Exon	region of a gene that is translated into amino acids (compare to intron)
Fingerprint	distinct pattern of bands on a protein gel, useful as an identifying characteristic of a sample or species
Gene	a defined region of DNA that encodes information for the synthesis of a single polypeptide
Genome	the entire complement of genes in an organism
Homology	similarity between genes of different species due to common ancestry
Intron	region of a gene that is not translated into amino acids (compare to **exon**)
Kilodalton (kD)	1000 daltons
mRNA	message derived from a gene, with information to make one polypeptide
Myosin	major muscle protein organized into thick filaments
Native	the natural structure of a protein or protein complex, as found within the organism
PAGE	polyacrylamide gel electrophoresis

Phylogeny	the evolutionary relationship of species based on lineage and history of descent
Protein	a functional assembly of one or more polypeptides, made of sequences of amino acids
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis; a form of electrophoresis that treats samples with SDS to denature proteins
Transcription	production of mRNA from DNA genetic information
Translation	production of a protein from messenger RNA (mRNA)
tRNA	transfer RNA that acts as adaptor molecule between mRNA and an amino acid

Appendix: SDS-PAGE Chemistry

Why This Technique Works So Well...

Polyacrylamide gels used for SDS-PAGE have two features that make them ideal for high-resolution protein separation. Unlike agarose gels, different buffers are used to cast and run the SDS-PAGE gel. In addition, the gel itself is made up of two separate zones, the **stacking** and the **separating** gels. This system was developed to make sure that all the proteins loaded into each sample well start being separated at the same time. Since vertical electrophoresis samples are loaded into wells at the top of the gel in 5–25 µl, all the proteins in the sample cannot enter the gel simultaneously. To eliminate this problem, proteins are concentrated, or stacked, into a tight band before they enter the part of the gel where they will be separated, or resolved, according to their mass.

A tight band of proteins is formed by establishing two ion fronts that sandwich the proteins between them. To establish the ion fronts, the SDS-PAGE running buffer is made with Tris and glycine at pH 8.3, while the Ready Gel polyacrylamide gel is made with Tris-HCl buffer at pH 8.8. Since chloride ions migrate more rapidly than glycine ions in an electric field, and proteins have intermediate mobility, the proteins become trapped in a narrow band between the two ion fronts when electrophoresis is begun.

The protein stacking takes place in the short stacking gel, which contains the sample wells. The stacking gel has a total polyacrylamide concentration of 4%, in contrast to the separating zone of the gel, which can have a concentration of polyacrylamide of 5–20% (Figures 5 and 17). The low percentage stacking gel allows the proteins to migrate uniformly, regardless of their size. When the tightly stacked proteins reach the higher concentration of the resolving gel, the proteins begin to separate based on their mass. The percentage of polyacrylamide used in the resolving gel can be selected based on the sizes of the proteins being studied, but the concentration of the stacking gel remains the same.

There is no obvious visual border between the stacking and resolving gel zones of the Ready Gel precast gel, but if you watch your samples immediately after turning on the power supply, you will see the protein samples being focused into a narrow band. The Kaleidoscope prestained standards will stack into a tight band first, and then the individual prestained proteins will become visible as the electrophoresis separates the proteins according to their mass.

Casting Polyacrylamide Gels

Unlike agarose gels, which are cast by melting agarose and then allowing it to solidify, polyacrylamide gels require a chemical reaction to cause polymerization of two acrylamide monomers. To cast a polyacrylamide gel, a reaction initiator, ammonium persulfate (APS), and catalyst, tetramethylethylenediamine (TEMED), are added to a solution containing the desired concentrations of acrylamide and bis-acrylamide monomers in a Tris buffer. The solution is quickly poured between glass plates that are separated by a narrow spacer. To cast a gel with a resolving and stacking gel of different polyacrylamide concentrations, a high-concentration resolving gel is poured first and the low concentration stacking gel is poured on top of it. A sample comb is inserted into the unpolymerized stacking gel solution, and the comb is removed to create wells for sample loading.

Note: Although powdered or liquid unpolymerized acrylamide monomers are neurotoxins, the precast ReadyGels included in this kit are already polymerized and are safe to use in your classroom. As always, proper laboratory safety precautions, such as wearing gloves and protective eyewear, are recommended.

References:

Campbell N, Biology, 4th edition, Benjamin/Cummings Publishing Company, Inc., Menlo Park (1996)

Davies PL and Hew CL, Biochemistry of Fish Antifreeze Proteins, FASEB J 4, 2460-2468 (1990)

Glausiusz J, The Old Fish of the Sea, Discover 20, 49 (1999)

Ingram VM, A Specific Chemical Difference between Globins of Normal and Sickle-Cell Anemia Hemoglobin, Nature 178, 792-4 (1956)

International Human Genome Sequencing Consortium, Initial Sequencing and Analysis of the Human Genome, Nature 409, 860-921 (2001)

Jasny BR and Kennedy D, The Human Genome, Science 291, 1153 (2001)

King MC and Wilson AC, Evolution at two levels in humans and chimpanzees, Science 188, 107-16 (1975)

Laemmli UK, Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4, Nature 227, 680-685 (1970)

Piñeiro C et al., Development of a Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Reference Method for the Analysis and Identification of Fish Species in Raw and Heat-Processed Samples: A Collaborative Study, Electrophoresis 20, 1425-1432 (1999)

Ridley M, Genome, Fourth Estate Limited, London, (1999)

Further Reading:

Books and journal articles

Alberts et al., Molecular Biology of the Cell, 3rd edition, Garland Publishing, Inc., New York (1994)

Attenborough D, Life on Earth: A Natural History, BBC/Collins, London (1979)

Darwin C, The Origin of Species, John Murray, London (1859)

Dawkins R, The Selfish Gene, Oxford University Press, Oxford (1976)

Gould SJ, Wonderful Life, Penguin, London (1991)

Jones S, The Language of the Genes, HarperCollins, London (1993)

Long LA, The Rise of Fishes, John Hopkins University Press, Baltimore (1995)

Nelson LS, Fishes of the World, 3rd edition, John Wiley and Sons, New York (1994)

Raff RA, The Shape of Life: Genes, Development and the Evolution of Animal Form, University of Chicago Press, Chicago (1996)

Weiner J, The Beak of the Finch, Vintage Books, New York (1994)

Wolpert L, The Triumph of the Embryo, Oxford University Press, Oxford (1991)

Web pages

Ichthyology Web Resources, Taxon pages
www.biology.ualberta.ca/jackson.hp/IWR/index.php

Tree of Life, University of Arizona
phylogeny.arizona.edu/tree/life.html

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Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***Bio-Rad Laboratories Main Office*** *2000 Alfred Nobel Drive, Hercules, CA 94547, Ph. (510) 741-1000, Fx. (510) 741-5800*
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