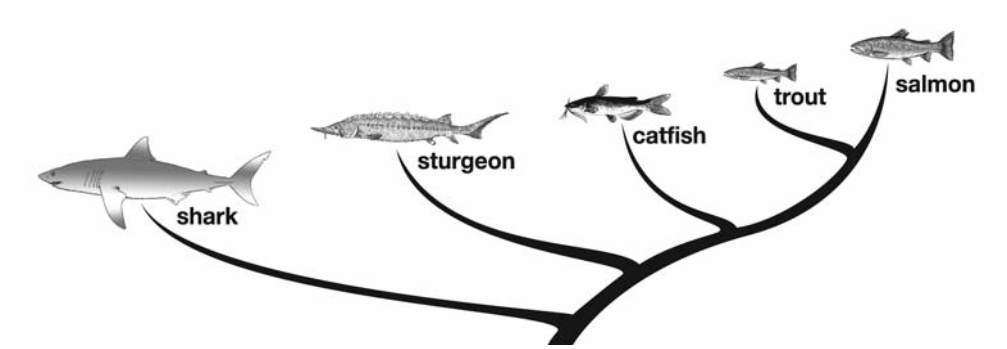


Biotechnology Explorer™

Comparative Proteomics Kit I: Protein Profiler Module

Catalog Number 166-2700EDU

explorer.bio-rad.com

Note: Kit contains temperature-sensitive reagents. Open immediately and see individual components for storage temperature.

Please visit explorer.bio-rad.com to access our selection of language translations for Biotechnology Explorer kit curricula.

BIO-RAD

Dear Educator:

Does molecular evidence support the theory of evolution?

The Human Genome Project has completed the task of sequencing all human genes. Far from closing the book, this body of work opened up a whole new field.

Proteomics asks the question: What do our genes do? Genes encode proteins that determine an organism's form, function and phenotype–the raw material of natural selection. Proteomics is the study of the structure, function, and interaction of proteins with each other and with their environment.

The Protein Profiler moves beyond DNA and allows students to employ protein electrophoresis, the most widely used technique in life science research, to study protein structure and function. Students learn to use SDS-PAGE to generate protein profiles from the muscles of both distantly and closely related species of fish. From their results they compare the different species profiles to test the hypothesis that protein profiles can be indicators of evolutionary relatedness.

This kit allows your students to explore evolution at the molecular level within the context of the central molecular framework of biology:

DNA > RNA > Protein > Trait = Phenotype

Changes in proteins can reflect changes in the gene pool. Muscle consists mainly of actin and myosin, but muscle tissue comprises numerous other proteins. Actin and myosin are the major muscle proteins essential for locomotion and survival in all animals. As such, the structures and functions of actin and myosin have remained relatively stable "or conserved" in all animals over evolutionary time. However, other muscle proteins exhibit considerable variation even among closely related species. Detectable variations between organisms' protein profiles reflect physiological adaptations to different environments, but they originate as random DNA mutations. Such mutation events, if favorable, persist through the natural selection process and contribute to the evolution of species–with new specialized functions.

Mutation > Variation > Specialization > Speciation = Evolution

This is an open-ended inquiry-based kit. Students make predictions about their results in pre-lab activities using internet databases and published phylogenetic information. They generate novel results and apply their findings directly to the problem of solving evolutionary relationships by constructing cladograms (phylogenetic trees). From their gel data, they build up a tree and assign each organism a branch. Students can decide whether their results support their predictions.

The kit guides students through the thought processes involved in a laboratory-based scientific investigation. Students are asked: Can molecular evidence support the theory of evolution? Why or why not? What explanations can you suggest?

This curriculum was developed in collaboration with Dr. Kristi DeCourcy of the Fralin Biotechnology Center at Virginia Tech. We'd like to thank Dr. DeCourcy for her invaluable guidance and contribution to this curriculum.

Biotechnology Explorer Team
Bio-Rad Laboratories
Biotechnology_Explorer@bio-rad.com
www.explorer.bio-rad.com

Create context. Reinforce learning. Stay current.
New scientific discoveries and technologies create more content for you to teach, but not more time. Biotechnology Explorer kits help you teach more effectively by integrating multiple core content subjects into a single lab. Connect concepts with techniques and put them into context with real-world scenarios.

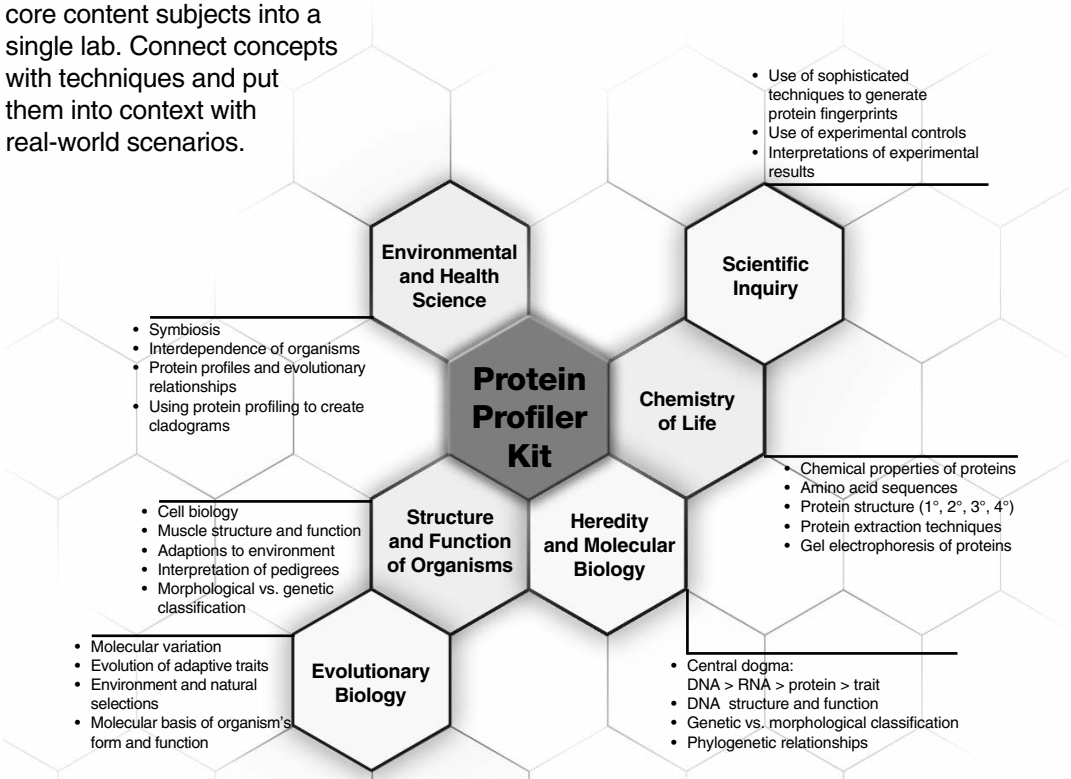


Table of Contents

Kit Summary

Overview

- In this investigation, students use SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel electrophoresis) to separate and analyze the protein profiles of the muscle tissue of different fish species. By comparing the protein profiles of different fish species, they test the hypothesis that protein profiles are indicators of genetic and evolutionary relatedness.
- This kit allows students to investigate evidence for biological evolution. Using protein electrophoresis, students compare the muscle proteomes of different fish species, create cladograms (phylogenetic trees) from their own results, and determine evolutionary relatedness between the species analyzed.
- This kit is multidisciplinary. It integrates scientific experimentation, number crunching (math), graphing, and computer-based bioinformatics.
- This kit addresses proteomics as the new frontier of molecular biology and its importance in the context of understanding the structure and function of the human genome and the genomes of other organisms. In this lab we focus on comparing the proteomes of muscle cells and the differences that exist in these proteomes between closely and distantly related species of fish.
- This is an open-ended inquiry-based kit. Students will obtain different and sometimes novel results and apply their results directly to the problem of solving evolutionary relationships. By constructing cladograms based on their scientific results, students decide whether their results support or refute their own predictions and published phylogenetic data.
- This kit can be performed using polyacrylamide gel electrophoresis as well as agarose gel electrophoresis. The latter is only recommended for those without the proper equipment to perform SDS-PAGE. This is explained in full detail in Appendix A.
- Computer-based informatics activities have been developed for this lab that allow students to understand the power and use of databases and how they are used in scientific research. A pre-lab computer activity incorporates use of an online Fish Base Database to make predictions about the similarities in muscle protein profiles between fish species and some of the factors that can affect evolutionary outcomes. A post-lab activity allows students to use the NIH's National Center for Biotechnology Information's BLAST database to derive a cladogram based on downloaded amino acid sequence information.
- The comparative proteomics kit I: protein profiler module (catalog #166-2700EDU) was developed in conjunction with the comparative proteomics kit II: western blot module (catalog #166-2800EDU). The protein profiler module can be used as a stand alone activity or together with the western blot module. The western blot module enables the specific identification of myosin light chain proteins. When planning to continue with the western blot module, please refer to the western blot module instruction manual for complete instructions for kits I and II on sample preparation, SDS-PAGE gel electrophoresis, electrophoretic transfer, and immunodetection. The western blot module requires electrophoresis using polyacrylamide gels.

Laboratory Time Line

Setting the Stage — Background

Pre-Lab Activity	**Proteomics, Evolution, and Classification of Fish**	1–2 days lecture and homework
Lesson 1	**Protein Extraction From Muscle**	45 min in lab
Lesson 2	**Load, Run, and Stain Gels**	45 min in lab
Lesson 3	**Destain and Dry Gels**	45 min in lab
Post-Lab Activity	**Analysis and Interpretation of Results**	1–2 days in lab and homework

Note: Lessons 1 and 2 can be completed in a single 90 min block period.

Lesson Extensions for Pre- and Post-Lab

Appendix A	Alternative protocol for protein electrophoresis using agarose gels and horizontal gel electrophoresis apparatus
Appendix B	Use databases to obtain real amino acid sequence data to create cladograms

Storage Instructions

Open kit immediately upon receipt and remove bag of temperature-sensitive components. Store these components at –20°C. Store all other reagents at room temperature.

Safety Precautions

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. DTT (dithiothreitol) is harmful when inhaled, swallowed, or in contact with skin. Please refer to the MSDS for more information on the safety assessment of the reagents in this kit. MSDS's are available from Bio-Rad at 1-800-424-6723 when calling in the U.S. or at www.bio-rad.com. Please consult your local environmental health and safety regulations for proper disposal.

Note: Students with known seafood allergies should avoid all contact with fish and/or shellfish samples.

Kit Inventory Checklist

This section lists the equipment and reagents necessary to conduct protein profiling in your classroom or teaching laboratory. We recommend that students be teamed up — two to four students per workstation.

Protein Profiler kits (catalog #166-2700EDU) are shipped at room temperature and contain consumable components to supply 8 student teams. Consumable components have unlimited shelf life. However, the Precision Plus Protein™ Kaleidoscope™ prestained standards and actin & myosin standards should be stored in the freezer (–20°C) within 4 weeks of receipt.

The number of gels, gel boxes, pipets and power supplies required will depend upon the number of workstations you choose to set up and must be ordered separately. Each Mini-PROTEAN® Tetra electrophoresis cell (protein gel box) is designed for one or two gels with one electrode assembly module. With the addition of a Companion Running Module, up to four gels can be run in the same protein gel box.

Tip: We recommend one 10-well gel be used per student team with each gel box running up to four gels at a time.

Kit Components	**Number/Kit**	**(✔)**
Prot/Elec™ pipet tips for gel loading	1 rack	❒
1.5 ml fliptop micro test tube	2 packs	❒
1.5 ml screwcap micro test tube	1 pack	❒
Disposable 1 ml pipets (DPTPs)	3 packs	❒
Laemmli sample buffer -– 30 ml, (catalog #161-0737EDU)	1 bottle	❒
Precision Plus Protein Kaleidoscope prestained standards, 50 µl	1 vial	❒
10x Tris-glycine-SDS electrophoresis buffer – 1 L, (catalog #161-0732EDU)	1 bottle	❒
Bio-Safe™ Coomassie stain for proteins – 200 ml	2 bottles	❒
Actin & myosin standard – 500 µg lyophilized, (catalog #166-0010EDU)	1 vial	❒
DTT — 0.3 g	1 vial	❒
Curriculum/instruction manual	1	❒
Required Accessories	**Number/Kit**	**(✔)**
Power supply, (catalog #164-5050EDU)	1–2	❒
2–20 µl adjustable-volume micropipet, (catalog #166-0506EDU)	8	❒
or		
10 µl fixed-volume pipet, (catalog #166-0512EDU) and 5 µl fixed-volume pipet, (catalog #166-0511EDU)	8	❒
Distilled water	1 gallon	❒
Fish samples – 1 g each per workstation		
See page 26 for recommended species.	5–8 types	❒
Scissors or knife to cut fish samples	1	❒
Water bath (catalog #166-0504EDU) or digital dry bath (catalog #166-0562EDU), ambient to 100°C,	1	❒

If using polyacrylamide gel electrophoresis:

Required Accessories	Number/Kit	(✔)
Vertical gel electrophoresis chambers, (catalog #165-8005EDU)	2–4*	❒
Mini-PROTEAN® TGX™ 4–20%, 10-well, (catalog #166-1093EDU) or Ready Gel® precast polyacrylamide gels, 15%, 10-well each (catalog #161-1103EDU)	8**	❒

*One Mini-PROTEAN Tetra cell (catalog #165-8005EDU) can run one to two gels with one electrode assembly or up to four gels with the addition of a Mini-PROTEAN Tetra companion running module (catalog #165-8038EDU).

**We recommend one gel per student team. Precast polyacrylamide gels have a maximum shelf life of 12 weeks and should be ordered shortly before using.

If using agarose gel electrophoresis:

(For details regarding the optional agarose gel electrophoresis protocol please refer to Appendix A.)

Required Accessories	Number/Kit	(✔)
Horizontal electrophoresis chambers with gel casting trays and combs, (catalog #166-4000EDU)	4–8	❒
Low-melt agarose, 25 g, (catalog #161-3113EDU)	1	❒
2–20 µl pipet tips, (catalog #223-9347EDU)	1	❒
Acetic acid	100 ml	❒
Reagent alcohol/ethanol	400 ml	❒
Gel staining trays, (catalog #166-0477EDU)	4	❒
Optional Accessories	**Number/Kit**	**(✔)**
Sample loading guides – one per Mini-PROTEAN Tetra cell, 10-well, (catalog #165-3146EDU)	4–8	❒
GelAir™ drying system, (catalog #165-1771EDU)	1	❒
Rocking platform, (catalog #166-0709EDU)	1	❒
Mini-PROTEAN Tetra companion running module, (catalog #166-8038EDU)	2	❒
Jellyfish foam floating racks, 8 racks, 12 microtube wells, (catalog #166-0479EDU)	8	❒
Green racks, set of 5 racks (catalog #166-0481EDU)	10 racks	❒

Refills Available Separately

Temperature-sensitive reagents bag: containing Precision Plus Protein Kaleidoscope prestained standards, actin & myosin standard, and DTT, (catalog #166-2701EDU)

Laemmli sample buffer, 30 ml, (catalog #161-0737EDU)

Precision Plus Protein Kaleidoscope prestained standards, 500 µl, (catalog #161-0375EDU)

10 x Tris-glycine-SDS electrophoresis buffer, 1 L, (catalog #161-0732EDU)

Bio-Safe Coomassie stain, 1L, (catalog #161-0786EDU)

Actin & myosin standard, 500 µg, lyophilized, (catalog #166-0010EDU)

DTT, 1 g, (catalog #161-0610EDU)

Syllabus Alignment

New national science education guidelines call for a movement away from traditional science teaching, which includes memorizing scientific facts and information, covering many subject areas, and concluding inquiries with the result of an experiment. Instead, teachers are encouraged to engage students in investigations over long periods of time, learning subject matter in the context of inquiry, and applying the results of experiments to scientific arguments and explanations.

This Protein Profiler kit and curriculum aligns with this approach. It provides a guided investigation in which students prepare common fish or other muscle protein extracts and use polyacrylamide gel electrophoresis to produce species-specific protein profiles, or fingerprints. From their own results students generate phylogenetic trees (cladograms) in order to infer evolutionary relationships between the species analyzed.

As an optional bioinformatic extension exercise students are also instructed to examine internet databases to construct phylogenic trees based on the actual variations in amino acid sequences between different species' muscle proteins.

Students who participate in this laboratory investigation are investigating a topical area of science in which there are few clear-cut answers. They use published articles and databases to gather information and make predictions. Students derive original data from their own electrophoresis gel results and use this data to evaluate their predictions.

The kit may be used to cover the following life science core content areas:

- Scientific inquiry: apply the scientific method and problem solving to investigate evidence regarding biological evolution
- The cell: cell structure and function
- Molecular basis of heredity: protein synthesis, protein structure & function, proteomics, and genomics
- Biological evolution: natural selection, phylogenetic relationships, cladograms, genetic mutation, and conservation of proteins
- Matter, energy, and organization in living systems: muscle structure and function, muscle proteins, conservation of organization across species
- Central dogma: relationship of DNA, RNA, and cellular interactions in the production of proteins

Instructor's Manual Background

Molecular biology has unlocked secrets of mystifying new diseases, given us the premier tools for defining biological identity, and created a pillar of data to support Darwin's theory of common descent. In short, molecular biology and its elegant techniques have revolutionized our understanding of life's origins and mechanisms.

Is it just genes that determine what proteins will be made? Current research in the field of proteomics suggests not. The following section is designed as a review of important background information for this laboratory investigation.

Proteomics

Proteomics is the study of proteins, particularly their structures and functions. This term was coined to make an analogy with genomics, and while it is often viewed as the "next step", proteomics is much more complicated than genomics. Most importantly, while the genome is a rather constant entity, the proteome differs from cell to cell and is constantly changing through its biochemical interactions with the genome and the environment. One organism will have radically different protein expression in different parts of its body, in different stages of its life cycle and in different environmental conditions.

The entirety of proteins in existence in an organism throughout its life cycle, or on a smaller scale the entirety of proteins found in a particular cell type under a particular type of stimulation, are referred to as the proteome of the organism or cell type, respectively.

With completion of a rough draft of the human genome, many researchers are now looking at how genes and proteins interact to form other proteins. The large increase in protein diversity is thought to be due to alternative splicing and posttranslational modification of proteins. This discrepancy implies that protein diversity cannot be fully characterized by gene expression analysis alone, making proteomics a useful tool for characterizing cells, tissues, and organisms of interest.

To catalog all human proteins and ascertain their functions and interactions presents a daunting challenge for scientists. An international collaboration to achieve these goals is being coordinated by the Human Proteome Organisation (HUPO).

Evolution

The term evolution probably brings to mind Charles Darwin and the theory of Natural Selection and common descent. The observation that every species on the planet, including humans, produces far more offspring in each generation than nature can support and that the pressure of so much excess population is a harsh but efficient test of the value of accidental variations in any species was the central observation that underlies Darwin's theory. As such, all species change gradually over time through natural selection. Each of these individuals is different — even among the same species. The environment selects organisms best suited to survive and reproduce based on those differences. Adaptations are the differences that make one organism more suited to the environment than another individual. These adaptations are phenotypic (physical) characteristics such as the variations in finch beaks that are determined by a genetic component. The genetic component is inherited from the parent in the form of genes.

The discovery of the chemical structure of DNA gave us an understanding of how the triplet code of nitrogen bases allows the synthesis of proteins (which is the phenotypic expression) and how phenotypic adaptations are the result of changes in the DNA code (mutations). However, current research in the field of proteomics is leading some scientists to question whether or not DNA is the final determining factor in the synthesis of proteins and thus the determining factor in evolution.

The central dogma of molecular biology (DNA → RNA → protein) has given us a comfortable explanation of how the information encoded by our DNA is translated and used to make an organism. It describes how a gene made of DNA is transcribed by messenger RNA and is then translated into a protein by transfer RNA in a complex series of events utilizing ribosomal RNA and amino acids. New discoveries about alternative roles for RNA, multiple forms of proteins being encoded by single genes in our cells, and changes to proteins after translation are changing this comfortable scenario and we are finding that things (as ever in biology) are not so simple. Although in essence the central dogma remains true, investigations into genomics and proteomics are revealing a complexity that many had never imagined.

In 1990, a massive research effort took place to sequence what was estimated to be the 100,000 genes that coded for each protein synthesized by humans (the human genome). This study, the Human Genome Project, took 13 years to complete (Jasny and Kennedy, 2001). When the study began, scientists estimated that there were over 100,000 human genes. Now, years after the genome has been sequenced, there is still no consensus on the actual number of human genes, but the current estimate is down to around 20,500 human genes (Clamp et al, 2007); this is only a few more genes than encodes the genome of a much simpler organism, C. *elegans*, a nematode worm that has around 19,000 genes.

So why are a similar number of genes required to make a worm and a person? Importantly, a human has a much larger total genome (3 billion base pairs) than a worm (100 million base pairs), suggesting that the total amount of DNA rather than the actual number of genes may be what gives rise to complexity. In addition, recent developments have shown it is quite common in complex organisms for a single gene to encode multiple proteins. Moreover, changing when, to what level and where a protein is expressed or changing a protein after it has been translated (posttranslational modification) can result in proteins with very different functions. This realization of the importance and diversity of proteins started a whole new field termed **proteomics**.

Proteomics was initially defined as the effort to catalog all the proteins expressed in all cells at all stages of development. That definition has now been expanded to include the study of protein functions, protein-protein interactions, cellular locations, expression levels, and

posttranslational modifications of all proteins within all cells and tissues at all stages of development. Thus, it is hypothesized that a large amount of the exogenous noncoding DNA in the human genome functions to highly regulate protein production, expression levels, posttranslational modifications, etc. and it is this regulation of our complex proteomes, rather than our genes, that makes us different from worms.

Researchers in the proteomics field have discovered a number of modification systems that allow one gene to code for many proteins and mechanisms that finely regulate the sub- and extracellular locations and expression levels of proteins. These include alternative splicing of exons, use of different promoters, posttranscriptional modification, translational frameshifting and posttranslational modification. Let's examine some of these systems.

Posttranscriptional Modifications

• RNA Editing

A newly discovered form of posttransciptional modification is RNA editing. Higher eukaryotes can change the sequence of their messenger RNAs (mRNAs) by substituting bases in their primary mRNA transcripts at specific positions, while lower eukaryotes insert and delete specific bases. These types of changes to the codons of mRNA can change the amino acid sequence of a protein, create a new open reading frame where one did not originally exist, or introduce a new stop codon to create a truncated protein. These changes can be regulated by the location of their expression, developmentally and by the organism's environment, and the resulting proteins can have very different functions. For example, fasting rats produce 50% less of an edited form of apolipoprotein B (a protein involved in cholesterol metabolism) than rats on a high carbohydrate diet (Maas and Rich, 2000). This happens when a cytidine is substituted with uridine. These base substitutions happen at specific locations, indicating they are involved in the regulation of metabolism.

RNA editing is not limited to messenger RNA. In a number of yeast and higher eukaryotes, transfer RNA is also edited at the wobble positions of its anticodons (Maas and Rich, 2000).

• Alternative Splicing

Messenger RNA (mRNA) of higher eukaryotes has two types of sequence segments — introns and exons. Introns are portions of the code that are removed or edited from the sequence to be transcribed into protein. The remaining base segments, exons, move to the ribosome for translation. Exons can be differentially included or excluded in a process called alternative splicing to produce different mature mRNAs, which in turn generate distinct proteins. This process can be used at different stages in development or simply when a cell is signaled to alter its protein production.

• mRNA Synthesis and Degradation

Since the level of mRNA within a cell in part determines the level of protein expression, the rate at which mRNA is synthesized and the rate at which mRNA is degraded are important factors in protein expression levels. Some mRNAs are inherently more unstable than others and there are active processes within cells that regulate mRNA's degradation as well as its synthesis. Modifications to mRNAs can also change their stability and thus their levels of protein expression. For example, capping an mRNA with a 7-methyl guanosine nucleotide and the presence of a polyadenosine tail increases its stability. As such, the regulation of mRNA by capping and polyadenylation increase protein expression.

Posttranslational Modifications

• Proteolytic Cleavage

Most proteins undergo cleavage after translation: the simplest form of this is the removal of the initiation methionine. In addition, many enzymes are regulated by proteolytic cleavage and are either activated or inactivated (or both) by cleavage of specific domains within the protein. Examples of this are the procollagenases that digest extracellular matrix collagens only after the removal of their 'pro' domain. Protein cleavage results in different proteins of different sizes derived from the same gene.

• Protein Degradation

Similar to mRNA synthesis and degradation, proteins are actively regulated by degradation mechanisms. Proteins have specific half-lives and these can be regulated by posttranslational modifications. In one mechanism, that won its elucidators the 2004 Nobel Prize in Chemistry, proteins are tagged with ubiquitin proteins and degraded in highly regulated cellular degradation machinery known as proteasomes. By regulating the degradation of its proteins, a cell can quickly change the levels, as well as the presence or absence of a protein in response to outside factors.

• Protein-Protein Interaction

Many proteins exist in complexes and the presence or absence of other proteins from these complexes can determine their function. Thus proteins are dependent on other proteins for their activity. Although a specific protein may be present in abundance, if its binding partner is in short supply, then the protein will have little, if any, activity.

• Carbohydrate Modification (Glycosylation)

Many proteins, especially those associated with the plasma membrane and those that are secreted, are covalently bound to carbohydrates – usually sugars. Carbohydrate modification drastically changes the way proteins behave and interact with other proteins or structures and act as targeting molecules to direct proteins to specific locations within the organism or cell. Lymphocytes (white blood cells) have carbohydrate groups on their outer membranes which are vital in determining how lymphocytes infiltrate sites of infection. Carbohydrate modifications can result in proteins with different molecular weights and different physio-chemical properties derived from the same gene.

• Phosphorylation

Many proteins are regulated by phosphorylation. Most phosphorylation reactions change the activity of enzymes, often by causing conformational changes within the protein. This is the case with myosin light chain 2 or myosin regulatory light chain, which contracts smooth muscle when phosphorylated. There is a special class of enzymes that phosphorylate proteins called kinases, and their counterparts, which dephosphorylate proteins are called phosphatases. Many proteins are phosphorylated on multiple sites, and each phosphorylation site has a specific role in the function or activity level of the protein. Thus a protein may exist in many different states of activity depending on which sites have been phosphorylated. Other forms of posttranslational modification include methylation, sulfation, prenylation and acetylation.

Based on this new research, proteomic researchers can feasibly argue that it becomes increasingly important to examine differences in the proteins being expressed in different species just as it is important to examine differences in DNA code. Phenotypic diversity is

achieved with little cost. Life shows amazing economy when one gene can encode many proteins and these proteins can be subsequently modified to suit a particular environment. Imagine the flexibility of an organism with an extensive RNA editing system!

We previously thought protein sequence could only be changed at the level of DNA mutations, which were rare and occurred randomly, an adaptation mechanism inherent with high risk. We are now beginning to understand that there are indirect forces, in addition to gene mutation, that can drive evolution. Modifying an RNA editing system to change a protein complement would be far less risky than irreversibly changing the genes encoding proteins themselves since edits are optional and reversible. Thus an organism would have much more flexibility to adapt to new and different environments. Mounting research suggests that the number of RNA editing systems is great and that the similarity in these systems may be of evolutionary significance.

In the Comparative Proteomics Kit I: Protein Profiler Module we focus on comparing the proteomes of muscle cells and the differences in these proteomes between distantly and closely related species. The differences found can then be used to determine, or infer, evolutionary relationships between different species. The following section reviews important information about muscle proteins.

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 muscle cells are the myofibrils that are bundled into muscle fibers. Each myofibril consists of a linear series of contractile units called sarcomeres.

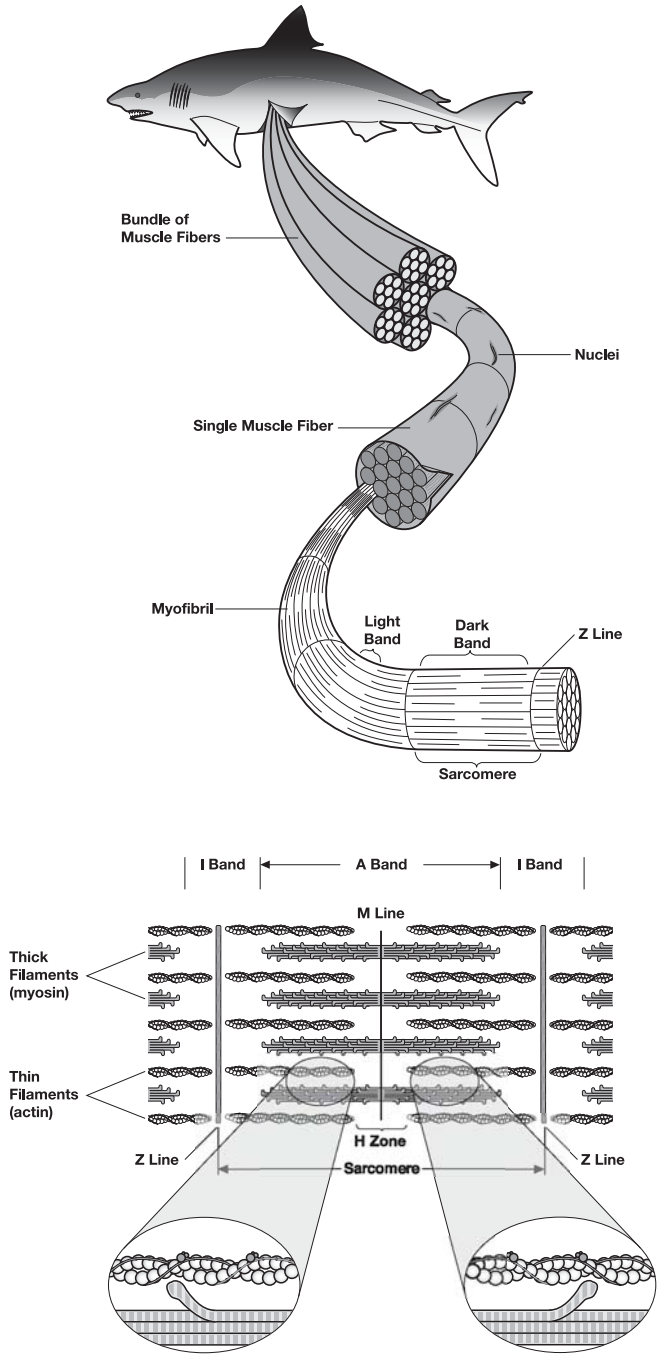


Fig. 1. Telescopic view of muscle structure: Thick myosin filaments and thin actin form myofibrils, which are bundled together to make muscle fibers. (Figure modifed from Campbell 1996 with permission.)

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.

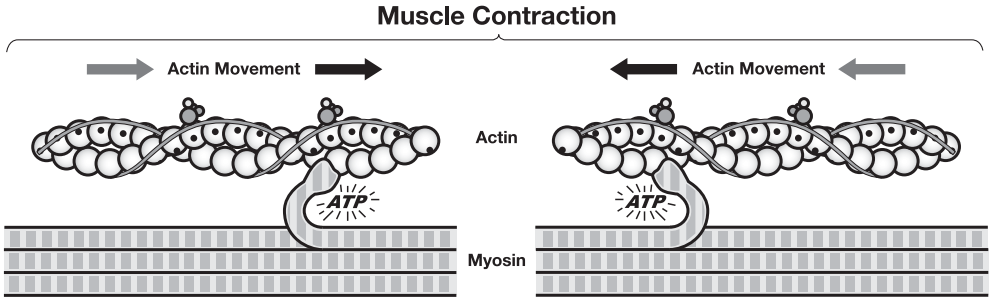


Fig. 2. Hydrolysis of ATP causes myosin and actin filaments to slide past one another, shortening the sarcomere and contracting the muscle. (Figure modified from Campbell 1996 with permission.)

Other Muscle Proteins

Numerous proteins besides actin and myosin are also required for muscle contraction (refer to the 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 (specialization) that are adaptive to particular niches, environments, or physiological stresses.

Table 1. Characterized muscle proteins, in order of decreasing size, adapted from Alberts et al. 2002.

Protein	MW (in kD)	Function
titin	3,000	centers myosin in sarcomere
dystrophin	400	anchoring to plasma membrane
filamin	270	crosslinks 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	15–25	slide actin filaments
troponin (I)	19	regulates contraction
troponin (C)	17	regulates contraction
thymosin	5	sequesters actin monomers

Evolutionary Trees

Phylogenetic trees can be based on many different types of data, some trees are constructed using a single type of data and some trees use multiple types of data. Comparing the morphology of organisms, including sizes, shapes and developmental structures of both living organisms and fossils is the traditional way evolutionary trees were constructed. Today, similarities and differences in protein and DNA sequences are also being used. Although both methods are valuable and often complement each other, they may not always agree as some shared morphological characteristics, although similar in structure and function, may have evolved independently.

An evolutionary tree shows the evolutionary lineages of different species over relative time. The following evolutionary tree is not precise or drawn to scale but is intended only as a reference for selecting a diverse mix of fish samples that may be available in your region.

The data used to construct the evolutionary tree below was obtained from the cladograms on the tree of life web page from the University of Arizona (www.tolweb.org). (Please note that the field of phylogenetics is ever changing and different methods used to construct a phylogenetic tree often result in differences between trees, hence the data on the tree of life web page may not concur exactly with "textbook" evolutionary trees.)

Evolution and Classification of Fishes

Most fish are contained within the superclass Gnathostoma (jawed vertebrates), which also includes all tetrapods. Only hagfish and lampreys are outside this group. These two fish types are sometimes classed together as Agnatha, but can also be separated into Hyperotreti and Hyperoartia. Hyperotreti (hagfish) are craniates (animals with skulls), but not vertebrates because they have no backbone, while Hyperoartia (lamprey) are very primitive vertebrates, that do not have a jaw. The Gnathostoma fishes are divided into the classes Chondrichthyes (cartilaginous fishes) and Osteichthyes (bony fishes). The Chondrichthyes include the sharks and rays, and the Osteichthyes, include all other modern fishes and all tetrapods (amphibians, birds, and mammals). Below are brief descriptions of some of the major fish groups, in order from most ancient to most recently diverged.

Hyperotreti (e.g., hagfish) are eel-like, jawless fishes that have a skull, but no backbone with parasitic and scavenging lifestyles. They are very primitive and may approach the condition of the common ancestor to all craniates.

Hyperoartia (e.g., lamprey) are eel-like, jawless fishes that are primitive vertebrates. They are identified by a single nostril and a sucker-like mouth with which they attach to fishes and rocks.

Chondrichthyes (e.g., shark, ray, skate & sawfish) have a cartilaginous rather than bony skeletons that reflects a more evolutionarily ancestral state. Their skin is thick and without true scales, and they do not have swim bladders or lungs.

Osteichthyes (e.g., coelancanth, tuna, & haddock). 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:

- Lobe-finned fish, Sarcopterygians, which contain the living fossil, the coelacanth, and the tetrapods (amphibians, reptiles, mammals, birds, and dinosaurs)
- Ray-finned fish, Actinopterygians, which contains most other fish

Sarcopterygians (e.g., lungfish and coelacanth) also include modern amphibians, reptiles, birds, and mammals. Coelacanth were thought to have become extinct at about the same time as the dinosaurs, until a live specimen was found in 1938. They form an important evolutionary link between fish and four-legged land animals.

Actinopterygian (e.g., gar, sturgeon, mackerel & anglerfish) is the subclass encompassing most modern ray-finned fish including the chondrostei, semionotiformes and teleosts.

- **Chondrostei (e.g., sturgeon)** are considered relic bony fishes. They lack scales on most of the body, have a cartilaginous skeleton, and have developed a shark-like, heterocercal tail and a rostrum extending past the mouth.
- **Semionotiformes (e.g., gar)** are also ancient fish, they have bony scales and a mainly cartilaginous skeleton.

Teleosts (e.g., herring, carp & pufferfish) comprise the remainder of the bony fishes. These include Clupeomorpha (e.g., herring, sardine & anchovy), Ostariophysi (e.g., carp, catfish, minnow, piranha & electric eel), Salmoniformes (e.g., salmon, trout & smelt), Esociformes (e.g., pike), and the diverse group, Acanthomorphia (e.g., tuna, cod & pufferfish).

Acanthomorphia (e.g., pollock, bass & sole) comprises two main superorders, Paracanthopterygians (e.g., cod, pollock & anglerfish) and Acanthopterygians. The Acanthopterygians include the Perciformes (e.g., the scombridae (e.g., swordfish, mackerel & tuna) and the serranidae (e.g., bass, snapper & grouper)), the Pleuronectiformes (e.g., flat fish, flounders & sole) and the Tetradontiformes (e.g., pufferfish).

Based on this new information, what questions can students ask about how muscle varies from species to species? Do muscle proteins vary between species in a manner related to their evolutionary history? Can we make predictions about how the muscles of two species are similar based on the environment in which they live? Can we use SDS-PAGE as a qualitative measure to see if our predictions are correct? Since actin and myosin are present in all samples can we use the presence or absence of other muscle proteins to infer relationships? How can we use variations in amino acid sequences to construct phylogenic trees and to infer evolutionary relationships? Can we use information from bioinformatic databases to construct phylogenetic trees? Can we examine proteins from different tissues to study protein expression?

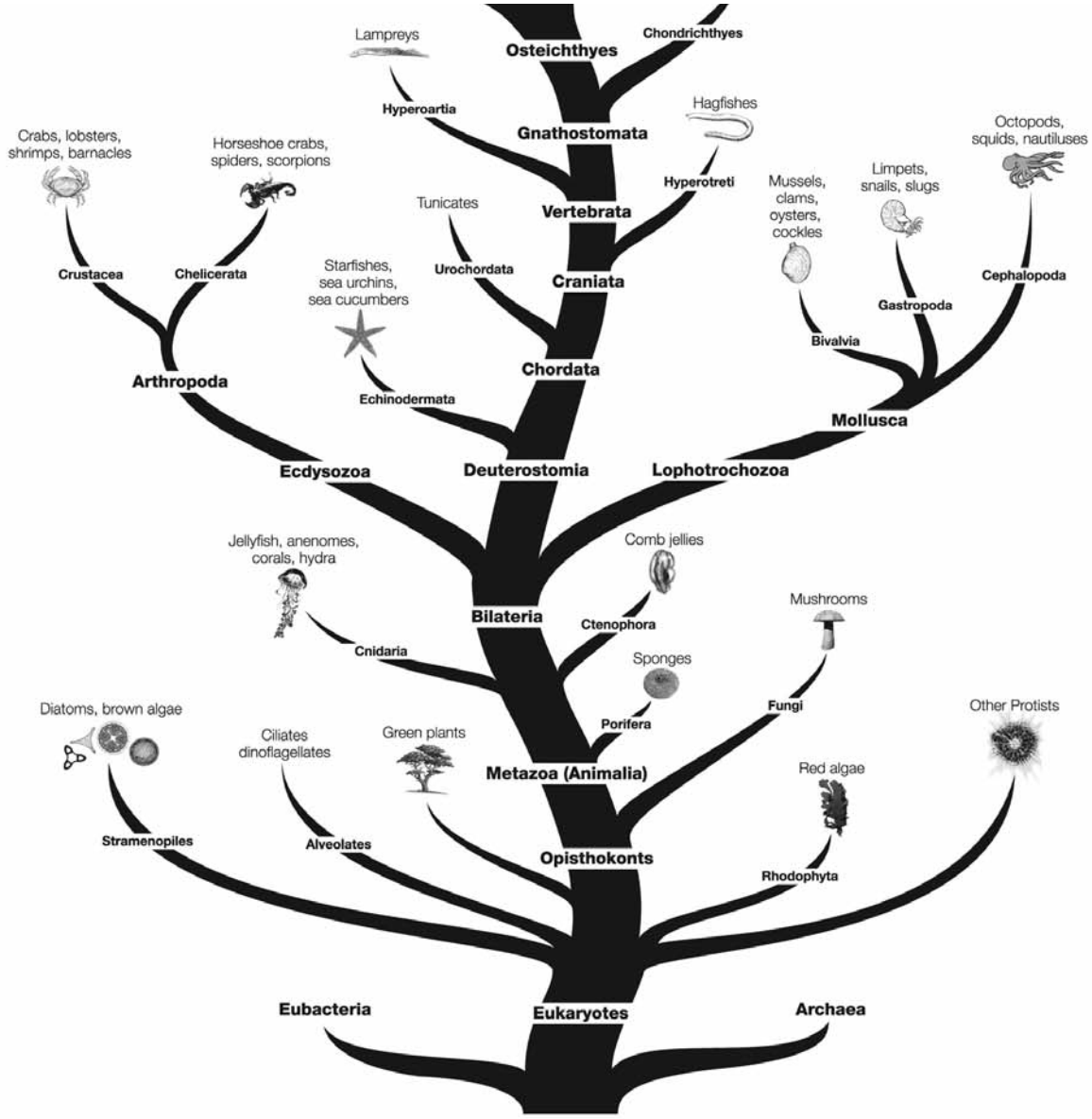


Fig. 3. Evolutionary tree showing the relationships of eukaryotes. (Figure adapted from the tree of life web page from the University of Arizona (www.tolweb.org).)

Swordfishes, mackerels, tunas
Snappers, perches, seabasses, groupers
Scombroidei
Percoidei
Perciformes
Flat fishes, flounders, soles
Pleuronectiformes
Pikes
Puffers, triggerfishes
Tetraodontiformes
Acanthopterygii
Smelts
Salmons, trouts
Osmeroidei
Salmonoidei
Esociformes
Protacanthoptrygii
Herrings, sardines, anchovies
Clupeomorpha
Sturgeons
Chondrostei
Sharks, rays, sawfishes
Actinopterygii
Gars
Semionotiformes
Teleostei
Carps, minnows, piranhas, catfishes, electric eels
Osteoglossomorpha
Acanthomorpha
Paracanthopterygii
Trout-perches, cavefishes
Percopsiformes
Cods, haddocks, pollacks
Gadiformes
Anglerfishes, goosefishes
Lophiiformes
Mammals
Mammalia
Coelacanths
Coelacanthimorpha
Sarcopterygii
Amphibians
Amphibia
Aminiota
Reptilia
Tortoises, turtles, terrapins
Testudines
Diapsida
Lizards, snakes
Lepidosauromorpha
Archosauria
Crocodiles, alligators
Crocodylomorpha
Birds
Aves
Dinosaurs
Dinosauria

An Introduction to SDS-PAGE

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.

SDS-PAGE (Sodium dodecyl sulfate – polyacrylamide gel electrophoresis) is a form of electrophoresis that treats samples with SDS to denature proteins.

This technique is widely used in molecular biology research to examine proteins to answer a variety of questions. For example:

- How many 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?

Proteins are usually separated using polyacrylamide gels rather than agarose gels, which are used to separate DNA. This is because most proteins are much smaller than DNA fragments and polyacrylamide gels have pore sizes similar to the sizes of proteins. The gel matrix formed by polyacrylamide is much tighter than agarose and able to resolve much smaller molecules. However, polyacrylamide gels are often used to separate very small DNA fragments (<500 bp) for DNA sequencing or PCR analysis, while agarose gels are sometimes used to separate very large proteins. In Appendix A, a protocol to run fish samples on agarose gels is included. While agarose gels never resolve bands as tightly as polyacrylamide gels, this protocol will separate the larger muscle protein of the fish samples.

Polyacrylamide gel electrophoresis (PAGE) uses two phases of polyacrylamide. An upper stacking gel typically of 4% acrylamide and a lower resolving gel of a higher percentage of acrylamide, (this lab uses 15%) (see Figure 4). This is called a discontinuous system and results in all of the proteins in a sample separating, or resolving, at the same time (Laemmli, 1970). 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 gel simultaneously. However, the low percentage of the stacking gel allows the proteins to migrate rapidly and be compressed 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 resolving gel and begin to be separated according to their molecular weights.

A tight band of proteins is formed by establishing two ion fronts that act to sandwich the protein bands between them. To establish the ion fronts, the SDS-PAGE running buffer is made with Tris and glycine (pH 8.3), while the Ready Gel polyacrylamide gel is made with Tris-HCl buffer (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.

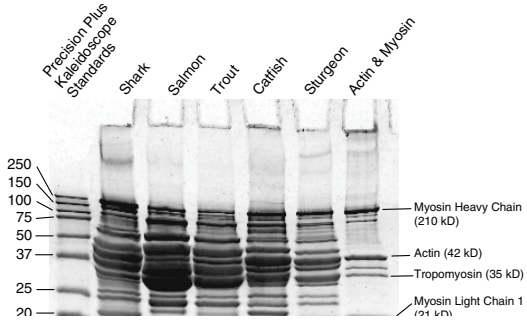


Fig. 4. A typical SDS-polyacrylamide gel.

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. 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 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. The comb is removed after polymerization is complete to create wells for sample loading. Note: Although powdered or liquid unpolymerized acrylamide monomers are neurotoxins, the precast recommended with 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 strongly recommended.

The Chemistry and Physics Behind Electrophoresis

In contrast to DNA, which is quantified in terms of its length, (i.e., the number of base pairs), proteins are quantified in terms of their molecular weights relative to a hydrogen atom, in Daltons. This is because DNA is composed of only 4 nucleotides, which are in roughly equal proportions and are roughly the same molecular weight. Proteins on the other hand are composed of 20 amino acids with molecular weights from 89 to 204 Daltons (the average is 110) and whose peptide chains vary considerably in percentage amino acid composition. One Dalton equals the mass of a hydrogen atom, which is 1.66×10^{-24} grams. Most proteins have masses on the order of thousands of Daltons, so the term kilodalton (kD) is used for protein molecular masses. Proteins range in size from several kilodaltons to thousands of kilodaltons, but most fall between the range of 10 kD and 220 kD. In comparison, the nucleic acids we study are often larger than 1,000 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 Da 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 (Figure 5). The intrinsic charges of proteins are obscured by placing a strongly anionic (negatively charged) detergent, sodium dodecyl sulfate (SDS), in both the sample buffer and the gel running buffer. SDS binds to and coats the proteins and also keeps them denatured as relatively linear chains. In this form, proteins migrate in a polyacrylamide gel as if they have equivalent negative charge densities, and mass becomes the main variable affecting the migration rate of each protein (note: posttranslational modifications such as glycosylation can also affect protein migration). This technique is called SDS-polyacrylamide gel electrophoresis or **SDS-PAGE**.

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** = denatured 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

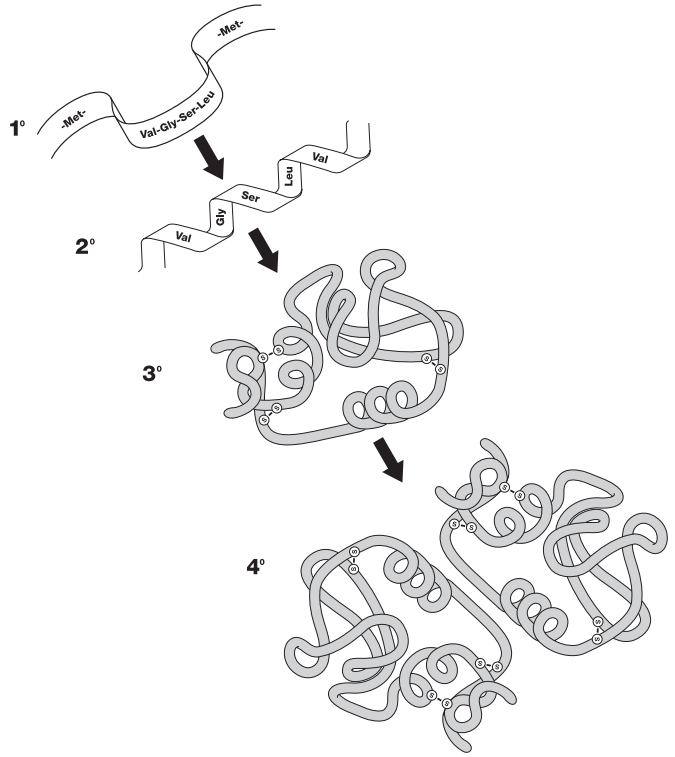


Fig. 5. Secondary (2°), tertiary (3°), and quaternary (4°) protein structure must be distrupted, or denatured, to accurately separate proteins by size.

A reducing agent, such as β-mercaptoethanol (BME) or dithiothreitol (DTT), is sometimes added to samples to ensure complete breakage of disulfide bonds. 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 (see Figures 6 and 7). These molecules snake through the gel at rates proportional to their molecular masses.

Tip: Both BME and DTT are potentially hazardous and produce an unpleasant smell. The protein banding patterns of fish muscle tissues are not greatly affected by the exclusion of reducing agents, but the addition of a reducing agent to the Laemmli sample buffer does reduce background bands. DTT is included in this kit and may be used at the discretion of the instructor for the protein profiler module. If the comparative proteomics kit II: western blot module will be used subsequent to the protein profiler module, it is necessary that the reducing agent be included in the Laemmli sample buffer.

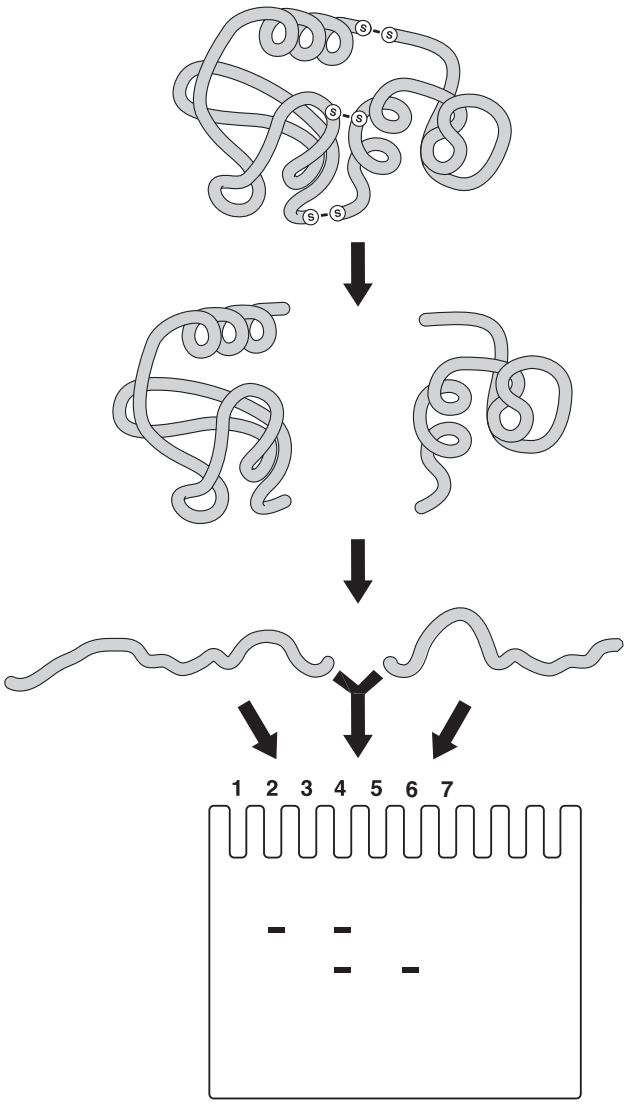


Fig. 6. A quaternary protein complex denatured with reducing agents, heat, and SDS, can be separated into individual proteins and resolved by size using SDS-PAGE.

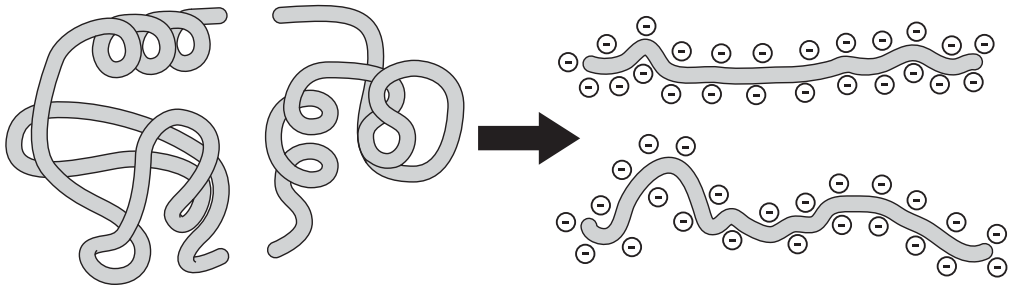


Fig. 7. The combination of heat and the detergent SDS denatures proteins for SDS-PAGE analysis.

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 stained 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 standard provided in this kit helps to identify these proteins. The Precision Plus Protein Kaleidoscope prestained protein standards are used to determine the molecular masses of the unknown proteins and to help monitor the progress of the run.

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 or due to post-translational modifications. 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.

The comparative proteomics kit II: western blot module (#166-2800EDU) allows students to specifically identify myosin light chains in gels using immunodetection and to possibly discover differences in the molecular weights of previously uninvestigated fish species. In western blotting, after polyacrylamide gels are run, all the proteins are transferred to a nitrocellulose membrane by passing an electric current through the gel (or using capillary action) and the negatively charged proteins migrate from the gel and bind to the membrane. Then an antibody that specifically binds to myosin light chains is added and incubated with the membrane. A second antibody that specifically binds the first or primary antibody is linked to a color-producing enzyme and incubated with the membrane. A substrate is then added that reacts with the enzyme-linked secondary antibody and color develops on the membrane only at the specific position of the bound myosin light chains. Thus, this technique provides certainty about the position of the myosin light chains and allows precise determination of molecular weight by constructing a standard curve from the migration distances of the known molecular weights for each of the proteins contained in the Precision Plus Protein Kaleidoscope Prestained protein standards (also present on the membrane).

Background on the Reagents Used in This Kit

- **Molecular weight markers**. Precision Plus Protein Kaleidoscope prestained protein standards, also referred to as molecular weight markers, are made from proteins that have been genetically engineered to be specific molecular weights and then bound to dye molecules so that they are visible on the gel or a western blot. Since they are known molecular weights, the molecular weight markers can be used to generate a standard curve. Plotting the distance each migrates against its known molecular weight produces a linear curve that can be used to calculate the molecular weights of the unknown proteins in your muscle samples. Precision Plus Protein Kaleidoscope prestained protein standards should be stored at –20°C, thawed at room temperature, and heated to 37°C for 5 min before use to dissolve any precipitated SDS. Unlike the fish samples and the actin & myosin standard, the Precision Plus Protein Kaleidoscope prestained protein standards do not require heating to 95°C prior to electrophoresis, although heating will not adversely affect them. Load 5 µl of Precision Plus Protein Kaleidoscope prestained protein standards per gel.
- **Laemmli sample buffer** is used to solubilize the proteins in the fish samples. It 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.
- **Actin & myosin standard**. The actin & myosin standard included in the kit is a reference to help identify the major, conserved muscle proteins and to serve as a positive control for gel analysis. A control sample containing rabbit actin and myosin proteins is provided in a lyophilized form, and like the fish samples, after rehydration with Laemmli buffer, the actin and myosin sample must be heated for 5 minutes at 95°C before loading. The actin & myosin standard consists of myofibrils (see Figure 1) that have been isolated from rabbit skeletal muscle. Actin, myosin heavy chain, three myosin light chains, and tropomyosin will be visible after the gel has been destained. The intense 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. Biologically active myosin has a quaternary structure with both heavy and light chains, which when denatured separate in the gel. The myosin light chains (15-25 kD) vary in molecular weight between fish species and can be investigated using the comparative proteomics kit II: western blot module (#166-2800EDU).
- **DTT**. A reducing agent, such as dithiothreitol (DTT) or β-mercaptoethanol (BME), is sometimes added to Laemmli buffer to ensure complete breakage of disulfide bonds. Both DTT and BME are potentially hazardous and produce an unpleasant smell. The protein banding patterns of fish muscle tissues are not greatly affected by the exclusion of reducing agents, but the addition of a reducing agent to the Laemmli sample buffer does reduce background bands. DTT is included in this kit and may be used at the discretion of the instructor for the protein profiler module. If the comparative proteomics kit II: western blot module will be used subsequent to the protein profiler module, it is highly recommended that the reducing agent be included in the Laemmli sample buffer.

- **TGX or Ready Gel precast gels**. Bio-Rad's precast gels are very thin polyacrylamide gels sandwiched between clear plates. Each gel has two separate zones, the stacking gel and the resolving gel. The tape at the bottom of the gel and the comb must be removed before use to allow the electrical current to run from the negative electrode in the inner chamber through the top of the gel out of the bottom of the gel to the positive electrode in the outer chamber. 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. We recommend you use 4-20% Tris-HCl TGX gels (catalog #456-1093EDU) or 15% Tris-HCl Ready Gels (catalog #161-1103EDU) for this laboratory. The high percentage of acrylamide resolves small molecular weight proteins better than larger ones and differences between the protein profiles of fish species are well demonstrated in this small molecular weight range. TGX gels have a maximum shelft life of 12 months from the date of manufacture, and Ready Gel precast gels have a maximum shelf life of 12 weeks. Both types must be stored at 4°C (and never frozen) until the time of use.

  More information about Bio-Rad's precast gels is available in bulletins 2144, 5874, 5932, and LIT188, which can be requested online or from Bio-Rad's technical support.

- **Electrophoresis running (1x TGS) buffer**. Tris-glycine-SDS (TGS) running buffer contains Tris to buffer the pH, glycine to provide ions to transmit current, and SDS to maintain the denaturation of the proteins in the gel. Distilled or deionized water should be used to prepare the 1x buffer because impurities in tap water can adversely alter the conductivity of the buffer.

- **Bio-Safe Coomassie protein stain**. Bio-Safe Coomassie is based on a colloidal suspension system. Once the colloidal dye particles are near the proteins in the gel, the dye is removed from the colloids by the nearby proteins due to the relative high affinity of the dye for proteins. Gels can be transferred directly into Bio-Safe Coomassie stain after electrophoresis is complete. However, if time permits, the resolution of the bands will be improved if the gels are washed 3 times with deionized water for 5 min 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. The optimal gel staining time is 1 hour with gentle shaking, but gels can be left in stain overnight with only minor diffusion of low molecular weight proteins. Depending on how long the gels have been in stain, the destaining of the gel can also act as a development reaction, with protein bands becoming much more intense as destaining occurs. To destain, rinse the stained gel with several changes of a large volume of deionized water and complete by destaining overnight in water.

Practice Using Adjustable-Volume Micropipets (Optional)

We recommend that you familiarize your students with proper pipetting techniques prior to Lesson 2. Have your students learn how to transfer different volumes of a solution from one tube into another with a micropipet. Students may practice using either Laemmli sample buffer or food coloring mixed with either a dense saturated sugar or glycerol solution. A simple and less expensive alternative to using adjustable micropipets for this lab are 5 µl and 10 µl fixed-volume pipets.

Here is a quick summary on how to use adjustable volume micropipets:

1. Look at the micropipet to determine the volume range.
2. Twist the dial on the micropipet to set the desired volume.
3. Attach a clean pipet tip.

4. Press the micropipet plunger to the **first** (soft) stop.
5. Insert the pipet tip into the solution to be transferred.
6. **Slowly** release the plunger to retrieve the liquid.
7. Insert the pipet tip into the desired tube.
8. Press the plunger past the first stop to the **second** (hard) stop to transfer the liquid. Make sure to keep the plunger pressed when lifting the pipet tip out of the tube.
9. Eject the pipet tip.

Pre-Lab Activity

Using Computer Databases to Predict What Factors Affect Fish Muscle Protein Profiles

In the pre-lab computer activity described below, students will use an Internet database (www.FishBase.net) that contains information on the biology of most of the world's fish species. This exercise is intended to focus the students' thoughts onto what factors may cause similarities in muscle protein profiles, to become familiar with the fish that they will be investigating, and finally to make predictions as to the results of their electrophoresis experiment. In addition, they will learn about the power of databases, how to search databases, and the role of databases in scientific research. Alternatively, the phylogenetic tree provided may be used to develop basic understanding of fish evolution and for making predictions about the relatedness of the various fish species investigated.

FishBase was developed at the International Center for Living Aquatic Resources Management (ICLARM) in collaboration with the Food and Agriculture Organization (FAO) of the United Nations and many other partners and has been funded mainly through sequential grants from the European Commission. More information can be found at FishBase.net. The data provided by FishBase is intended for use by scientists, policymakers, teachers, students, and enthusiasts.

Students will obtain information from FishBase on the fish species that they will be using for the laboratory. They will then make educated guesses on which pieces of information from the database will help them make predictions on what proteins are expressed in the muscles of their different fish species. They will justify how they think this information can help them predict which fish will express a similar array of proteins and which fish species will have contrasting muscle protein expression. Lastly they will make predictions on which two fish will have the most and least similar protein profiles obtained from SDS-PAGE in the lab and then test these predictions in the subsequent laboratories.

In order to form a hypothesis, and make predictions about the relatedness of the fish species they will examine, students should have some understanding of fish evolution. The evolutionary tree will aid them in the process and help you in selecting appropriate varieties of fish for this lab. Following the analysis and interpretation of their electrophoresis results, students create a cladogram from their own results. Students then compare their cladograms to a variety of published data including the phylogenetic tree provided and decide if their own molecular evidence supports or refutes their own predictions.

As an alternative to the computer-based pre-lab activity described above, students may use just the evolutionary tree provided to make predictions about the relatedness of the fish species they will examine in this lab. Following the analysis and interpretation of their electrophoresis results, they will create a cladogram from their own results and compare their cladograms with their predictions. This is provided as the Alternative Pre-Lab Activity in the student manual.

Which Fish Should You Choose for This Activity?

Although environmental and fish lifestyle factors may influence protein expression in fish muscle, the biggest predictor of similarity of fish muscle protein expression is the closeness of the evolutionary relationship of the fish. Thus, you should obtain some fish that will have similar profiles, such as fish from the same family (for example salmon and trout are both in the family Salmonidae), and some fish that have contrasting profiles such as fish from different classes or orders (for example, salmon are in the class Actinopterygii, while sharks are in the class Chondrichthyes).

Other aquatic organisms that may be analyzed 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 (and each other) so they will provide interesting comparative data on broader evolutionary relationships.

The evolutionary tree provided should give you an idea of evolutionary relatedness of common fish available in the grocery store.

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!

Materials required: Internet access, Student Fish Data Sheets provided in the Student Manual (five per student, if they will investigate five fish species)

Time required: One class period (45 minutes) and homework time

Start the lesson by asking students to name several types of environments in which fish might live. Answers may include freshwater, saltwater, brackish water, still water, light currents, high velocity currents, deep water, shallow water, etc. Ask whether these factors would influence how fish move in their environment and whether these factors would influence what proteins are present in the muscles of different fish. Then ask what other factors may influence what proteins are present (or expressed) in the muscles of different fish. Answers may include fish shape, fish evolution, fish diet, etc.

Next write the common names of the five fish you have selected on the board. Remind students these are common names. Many different species can be called by the same common name. Provide as much information as possible to students, such as yellow fin or blue fin tuna instead of just tuna. If possible also inform them of the country of origin. Explain to students that they are going to use an online fish biology database to gather as much information about the fish as possible.

Students will fill in the fields on the Student Fish Data Sheets. Students will then answer the focus questions to predict which factors will most influence the protein profiles of fish muscles and to predict which fish will have the most and least similar protein profiles as determined by these chosen factors.

Note: You or your students may find discrepancies between different information sources on the exact classification of fish with regard to which class, subclass, superorder, etc. they belong. The Animal Diversity Web from the University of Michigan, Museum of Zoology (http://animaldiversity.ummz.umich.edu) is useful to obtain breakdowns of the classification of fish species.

Instructor's Advance Preparation
Lesson 1: Protein Extraction From Muscle

In this lesson, students prepare protein extracts from their fish muscle samples. Tiny pieces of fish are added to Laemmli sample buffer, the tubes flicked to release proteins, the sample buffer decanted into screw cap tubes and then heated to 95°C. This lesson can be immediately followed by lesson 2 if time permits. The use of DTT is optional for this laboratory exercise*.

If you are planning to go on to perform the western blot module (comparative proteomics kit II) we recommend you use the Advance Preparation Guide in the western blot module manual since there are slight differences in the protocols.

Preparation Overview

- Read through introductory material on the previous pages (2 hours)
- Inventory kit and accessories (20 minutes)
- Obtain 5 or more fish samples of different species and 2 liters of distilled water — trip to grocery store as needed. To make this a blind study, students will assign a letter (e.g., A–E) to each fish sample to be investigated
- Aliquot student reagents (0.5–1 hour)
- Set up student and common workstations (30 minutes)

Prepare student reagents

Fish	Cut each fish sample into eight roughly 0.5–1 cm square chunks, place on card or plastic and label A–E for a blind study.
Laemmli sample buffer with DTT (optional)*	Add all the DTT (0.3 g) to the 30 ml bottle of Laemmli sample buffer and mix well. Final concentration is 70 mM DTT. Store at –20°C.
Aliquot Laemmli sample buffer	Aliquot 1.5 ml of Laemmli sample buffer into eight 1.5 ml flip-top microtubes and label SB. If no DTT is added, store at room temperature, if DTT is added, store at –20°C.

***Use of DTT** – Dithriothreitol (DTT) is a reducing agent and disrupts disulfide bonds between peptide chains. Its use is optional for the Protein Profiler laboratory exercise (but is necessary if you are using the western blot module in the comparative proteomics kit II). Inclusion of DTT in the Laemmli sample buffer will result in sharper, cleaner banding patterns on your stained polyacrylamide gels since it disrupts nonspecific protein-protein interactions within the protein extracts. However, DTT is potentially harmful and you may not want to introduce this chemical into your classroom. Importantly, your gels will be perfectly clear and interpretable without its use.

Student Workstations

Materials Needed for Each Workstation	Quantity
1.5 ml fliptop microtubes	5
1.5 ml screwcap microtubes	5
1 ml disposable plastic transfer pipet (DPTP)	1
Fish samples, labeled	5 species
Marking pen	1
Laemmli sample buffer	1.5 ml
Knife or scissors to cut fish samples	1

Common Workstation

Materials Required	Quantity
Water bath set to 95°C	1

Lesson 2: Electrophoresis – Gel Loading, Running, and Staining

In this lesson, students separate the proteins in their fish muscle extracts using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then stain their gels to visualize the proteins. If you do not have the vertical electrophoresis equipment to run polyacrylamide gels, an alternative protocol to run agarose gels using horizontal electrophoresis equipment is included in Appendix A.

Preparation Overview

- Prepare reagents and other items for student (10 minutes)
- Aliquot student reagents (15 minutes)
- Set up student and common workstations (20 minutes)

Required Materials for Reagent Preparation (for eight workstations)	**Quantity**
Actin and myosin standard, 500 µg, lyophilized	1 vial
Precision Plus Protein Kaleidoscope prestained protein standards, 50 µl	1 vial
1–20 µl adjustable-volume micropipet and tips (or 5 µl and 10 µl fixed-volume pipet)	1
10x Tris-glycine-SDS (TGS) running buffer	200–400 ml
Distilled or deionized water	2–4 L
1.5 ml screwcap microtubes	9
1.5 ml fliptop microtubes	8
Laemmli sample buffer, 500 µl	1 vial

Prepare Reagents

Rehydrate Actin and Myosin Standard. Add 500 µl of Laemmli sample buffer to the vial of actin and myosin standard and incubate at room temperature for 20–30 minutes. Transfer the rehydrated actin and myosin sample to an "AM" labeled screwcap tube and heat for 5 minutes at 95°C. Store at –20°C.

Tris-Glycine-SDS (TGS) running buffer. One Mini-PROTEAN Tetra cell with two gels requires 700 ml of 1x TGS running buffer. One Mini-PROTEAN Tetra cell using the companion running module to run four gels requires 1.1 L of 1x TGS running buffer. To make 3 L of 1x TGS running buffer, mix 300 ml of 10x TGS with 2,700 ml of distilled water. Store at room temperature.

Tip: you may want to prepare 1–2 L of extra 1x TGS buffer in case your gel boxes leak after assembly. If you do have a leak, the outer chamber of the gel box can be filled to above the inner small plates, to equalize the buffer levels in both reservoirs. This requires approximately 1,200 ml of 1x TGS buffer per gel box and is a more convenient fix than reassembling the apparatus mid-lesson.

Aliquot Student Reagents

Actin & myosin standard	Label eight 1.5 ml screw-cap tubes "AM" on their sides. Aliquot 12.5 µl of rehydrated and preheated actin & myosin standard into each. Store at –20°C.
Precision Plus Protein Kaleidoscope prestained protein standard	Label eight 1.5 ml flip-top tubes "Stds." Aliquot 6 µl of standards into each tube. Store at –20°C.

Student Workstations

Materials Needed for Each Workstation	Quantity
Fish protein extracts from Lesson 1	5 species
Actin & myosin standard, 12.5 µl	1 vial
Precision Plus Protein Kaleidoscope prestained protein standard, 6 µl	1 vial
4-20% Mini-PROTEAN TGX or 15% 10-well Ready Gel precast gel	1
1–20 µl adjustable-volume micropipet (or 5 µl and 10 µl fixed-volume pipet)	1
Prot/Elec pipet tips for gel loading	7 tips
Mini-PROTEAN Tetra cell electrophoresis module (gel box)	1 per 2 gels
1x Tris-glycine-SDS (TGS) running buffer	700 ml per gel box
Power supply (200 V constant) to be shared between workstations	1
Sample loading guide for 10-well comb (optional)	1 per gel box
Buffer dam (required only if running 1 gel/box)	1
Staining trays	1 per 2 gels
Bio-Safe Coomassie stain for proteins	50 ml per 2 gels

Common Workstation

Materials Required	Quantity
Water bath set at 95°C	1
Water for gel destaining (tap water is fine)	

Lesson 3: Dry Gels

In this lesson, students dry their gels between cellophance sheets to keep a record of their gels. Alternatively, gels can be scanned, photographed, or photocopied.

If you are planning to go on to perform the western blot module (comparative proteomics kit II) we recommend you use the advanced preparation guide in the western blot module manual since there are slight differences in the protocols. If you are going to perform a western blot, you can choose to stain the gel or not depending on your teaching goals; refer to the western blot manual for details.

Preparation Overview

- Set up student and common workstations (20 minutes)

Please note, the materials for drying gels are not provided in the kit; refer to the recommended optional accessories section on p. 4 for ordering information.

Student Workstations

Materials Needed for each Workstation	Quantity
Container of tap water to wet cellophane	1
GelAir cellophane support sheets	2
Square plastic container and 2 rubber bands (if not using GelAir drying frame)	1
Ruler & graph paper if performing band analysis	

Common Workstation

Materials Required	Quantity
GelAir drying frames	1
GelAir assembly table (optional)	1
GelAir dryer (optional)	1

Notes on Preserving Gels and Gel Analysis

The information from gels can be preserved in many ways. The wet gels themselves can be scanned or photocopied (taking care both not to tear the delicate gel and not to damage electrical equipment with wet gels). Alternatively, the gels can be dried between two sheets of cellophane (a cellophane sandwich). In contrast to delicate wet gels, dried gels are quite tough, which makes analysis much easier. In addition, they last for years, can be much more easily scanned and photocopied, and also give your students a physical record of their experiment.

The GelAir gel drying assembly table and drying frames allow you to very easily put together a cellophane sandwich with 4 gels per sandwich. Additionally, if you have the GelAir drying oven, you can dry the gels in 2–3 hours.

The alternative to using the GelAir apparatus is to make your own cellophane sandwiches using plastic tubs and rubber bands. This method is more cumbersome, and without the gel drying oven, gels take 1–2 days to dry; however, this method gives perfectly good results.

With regards to gel analysis, in the laboratory, scientists often use specialized scanning equipment to analyze gels, such as the GS-800™ densitometer, with specialized software such as Quantity One® 1-D analysis software which scans wet gels and determines the size, position, and intensity of every band on the gel. Your students will mimic the work performed by computers by performing post-lab analysis of their gel.

Post-Lab Activity

Analyzing and Interpreting Student Results

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

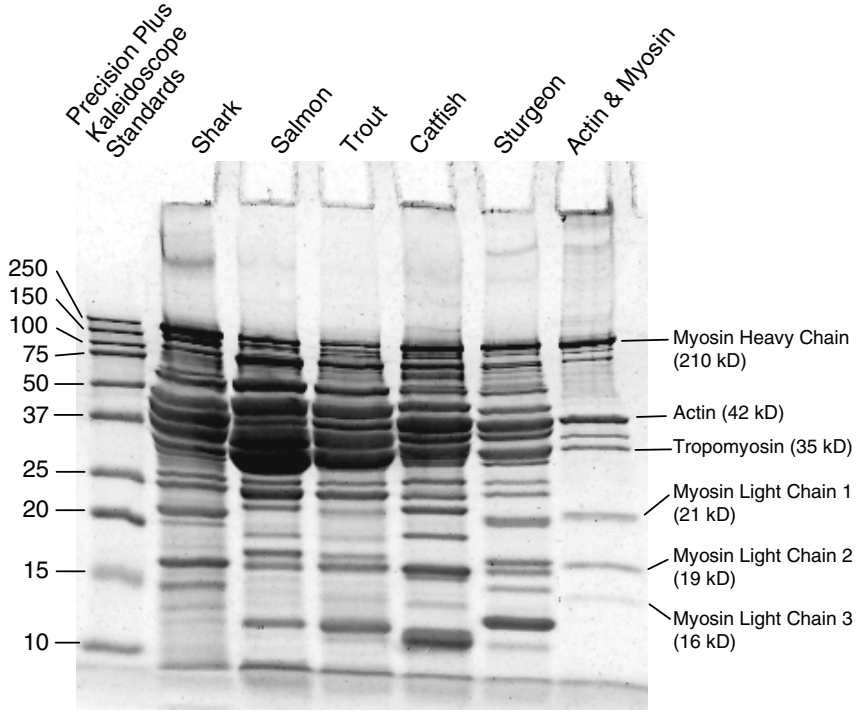


Fig. 8. 15% Ready Gel polyacrylamide gel electrophoresed at 200 V for 30 minutes, stained with Bio-Safe Coomassie stain, and destained in water.

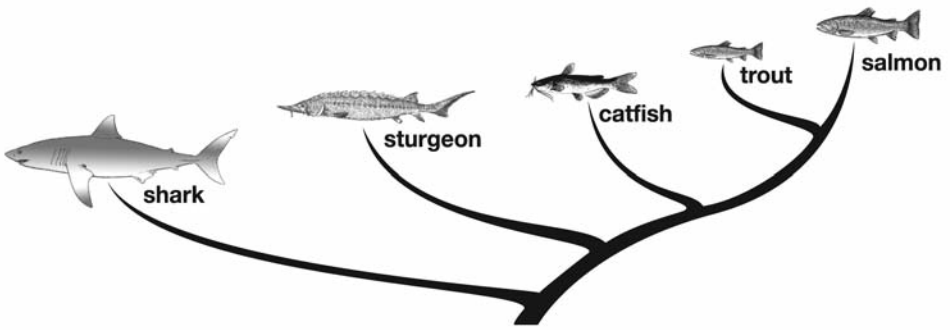
Obvious similarities and differences between protein fingerprints are easily spotted. Comparing the banding patterns of salmon and trout, it becomes clear that these are very similar. For instance, they share a common band at approximately 18 kD, as well as a pair of bands at 16 kD. This similarity is consistent with the provided fish evolutionary tree, as salmon and trout are both on the same branch. In contrast, a comparison of salmon with catfish reveals significant differences in their patterns. Only one of the bands of approximately 16 kD found in salmon is present in catfish.

In the post-lab activity below, students construct a cladogram based on their own gel analysis.

Detailed Gel Analysis

This section is the "so what?" part of the activity. Students engage in a multidisciplinary approach to problem-solving to determine whether their electrophoresis data support or refute the original predictions they made during the pre-lab activity. Students create a fish family tree, or cladogram, from scratch, using their own gel results. Students determine whether their cladogram matches the evolutionary relatedness of the fish species determined by traditional morphological analysis in the evolutionary tree provided.

Each protein band that a fish has in common with another fish is considered a shared characteristic. A fish family tree, or cladogram, can be constructed based on protein bands that the fish have in common. Cladistic analysis assumes that when two organisms share a common characteristic, they also share a common ancestor with that same characteristic. This lineage can be represented on a cladogram as a node with two branches representing the descendent organisms. In this exercise your students will identify shared characteristics by listing all the different proteins in each fish sample, determining which proteins (characteristics) are shared between the different fish species in a character matrix, and constructing a cladogram based on their own electrophoresis data.



Creating a Cladogram

Each protein band that a fish has in common with another fish is considered a shared characteristic. A protein can be identified by its molecular weight or by the distance the protein migrated though the gel. Because a cladogram may be created simply from the distance each protein band migrated through the gel, it is not strictly necessary to construct a standard curve to determine the molecular weights of the proteins in each sample. However, as a lesson in graphing, you may choose to instruct your students to create a standard curve using the protein standards they ran on their gels to determine the actual size of the protein bands in each sample.

Procedure: Generate a standard curve to calculate protein sizes

The different protein bands in the gel can be defined by their different molecular masses. Indeed many proteins are named for their molecular weights. For example p53, a protein implicated in tumor progression is 53 kD in size. To determine the molecular masses of the proteins, a standard curve is created plotting the known molecular masses of the proteins in the Precision Plus Protein Kaleidoscope prestained standards against the distance they have migrated down the gel from the base of the well.

A 15% polyacrylamide gel is designed to separate small proteins- proteins less than 40 kD. The gel analysis will concentrate on this size range. Note: If a different percentage acrylamide gel or an agarose gel has been run, analyze the section of the gel that has the best separation.

1. As shown in the figure below, draw a line between the 37 and 25 kD bands of the prestained standards. The gel analysis will be restricted to the proteins below this line.

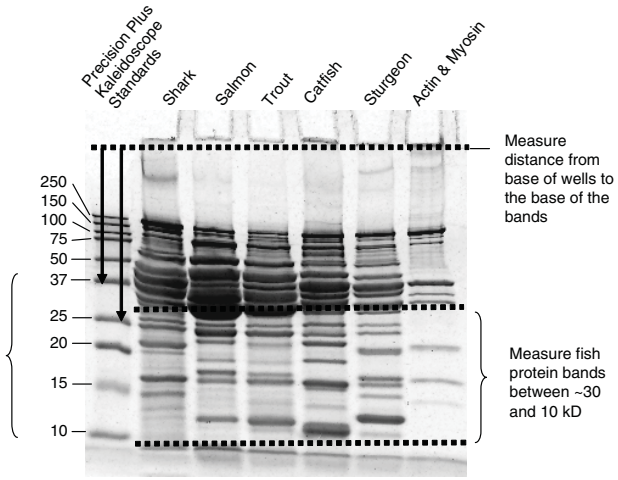


Fig. 9. Image of fish muscle proteins separated by SDS-PAGE and stained with Bio-Safe Coomassie stain. Lines illustrate measurement of bands for constructing the standard curve.

2. To create the standard curve students measure and record in the table below the distances the five sub 40 kD protein bands of the prestained standard have migrated from the base of the well i.e. measure the 37, 25, 20, 15 and 10 kD bands. Accuracy to 0.5 mm is required (see example below).

Precision Plus Protein Kaleidoscope Prestained Standards Molecular Mass (kDa)	Distance Migrated (mm)
37	23
25	30
20	35
15	42
10	51.5

3. On the graph paper provided, students plot the distances migrated in mm on the x-axis against the molecular masses of the prestained protein bands in kD on the y-axis as a scatter plot and draw a line of best fit through the points. On semi-logarithmic graph paper with the molecular mass of the proteins on a logarithmic scale the data should result in a linear (straight line) curve.

Define the characteristics (proteins) of the different fish

4. For each fish sample that has been analyzed, students determine the molecular masses of the proteins below the 25-37 kD line by measuring the distance each band has migrated from the base of its well and using the standard curve to determine the molecular mass.

Alternatively, students could use graphing software to generate the standard curve, make a line of best fit (or trend line) through the points and formulate an equation to calculate the mass of the unknown proteins on the gel.

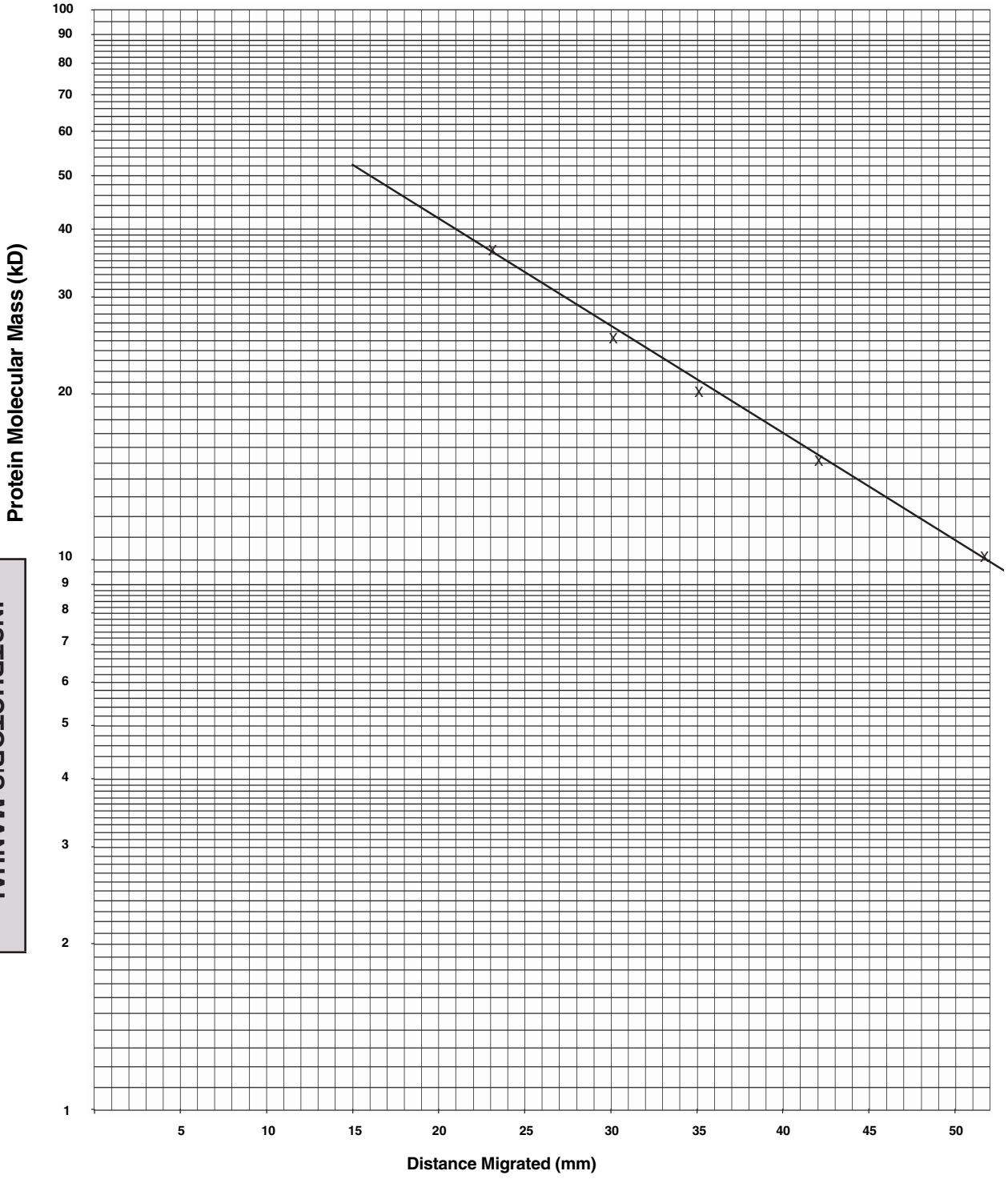


Fig. 10. Example of a standard curve drawn on two-cycle semi-logarithmic graph paper. The curve is constructed by plotting the protein molecular mass against the distance migrated. Example shows a protein that migrated 29 mm having a molecular mass of 29 kD.

5. Students then enter this data into a table with the molecular masses of the proteins for each fish (see example below).

Shark	
Distance Migrated (mm)	Molecular Mass (kD)
25	32.5
26.5	31
29	28.6
36	21.7
36.5	21.2
39	18.8
44	13.9
52	6

Determine which fish have each characteristic (protein)

6. Students record their gel data by filling out the table as shown below. There should be a row for every measurable band size and/or distance migrated and a column for each type of fish on their gel. Then students put an X in each cell of the table where the fish has that size band and put the total count of protein bands in the analyzed section at the bottom of each column (see example below).

Characteristic

Distance migrated (mm)	Protein Molecular Mass (kD)	Shark	Salmon	Trout	Catfish	Sturgeon
25	32.5	X				
26	31.5		X	X	X	X
26.5	31.0	X				
27.5	30.0		X	X	X	X
28.5	29.1					
29	28.6	X	X	X	X	
30	27.6			X		X
30.5	27.1					X
32	25.6		X	X	X	
33	24.7					X
34.5	23.2		X	X		
35.5	22.2					X
36	21.7	X				
36.5	21.2	X	X	X	X	
37	20.7					X
37.5	20.2		X	X		
38	19.7				X	
38.5	19.3				X	
39	18.8	X				X
39.5	18.3					X
40.5	17.3		X	X		
41	16.8				X	
41.5	16.3					
42	15.8		X	X		X
43	14.8					
44	13.9	X				X
45	12.9		X	X		
46	11.9				X	
46.5	11.4			X		
47	10.9					X
47.5	10.4				X	
51.5	6.5			X		
52	6.0	X				
	COUNT	**8**	**10**	**13**	**10**	**12**

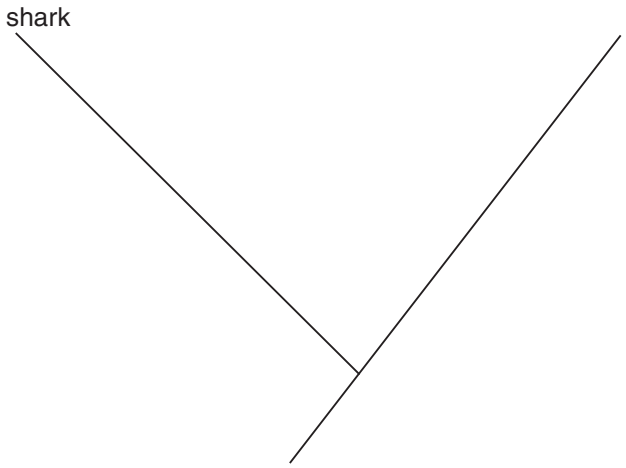
7. Find the number of characteritics shared by each of the fish. Students then create one final table that will be the basis for constructing their cladograms. In this table both the row and column headings are the types of fish analyzed. For each fish students separately compare the number of bands in common with every other fish in their gel and put those numbers into the table so that each fish is individually compared with every other fish. In this example salmon and shark have just two bands in common, while trout and salmon have ten bands in common.

	Shark	**Salmon**	**Trout**	**Catfish**	**Sturgeon**
Shark	8	2	2	2	2
Salmon		10	10	5	3
Trout			13	5	4
Catfish				10	2
Sturgeon					12

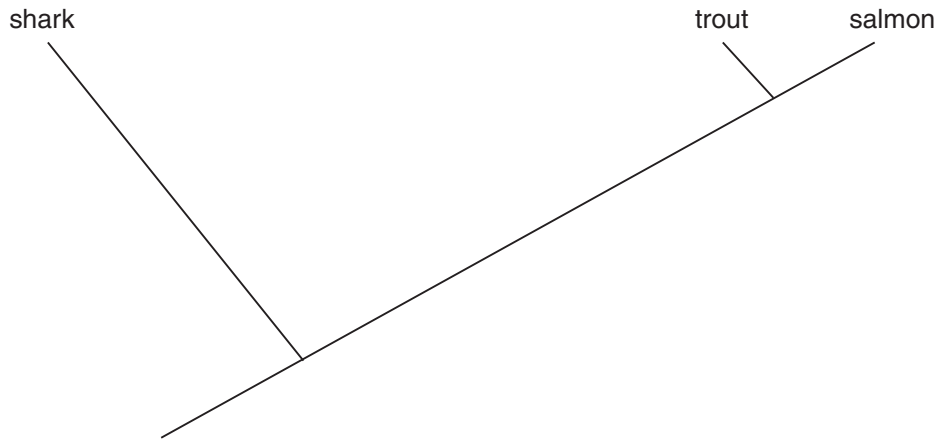
8. Now your students are ready to construct their cladograms.

Construct the cladogram

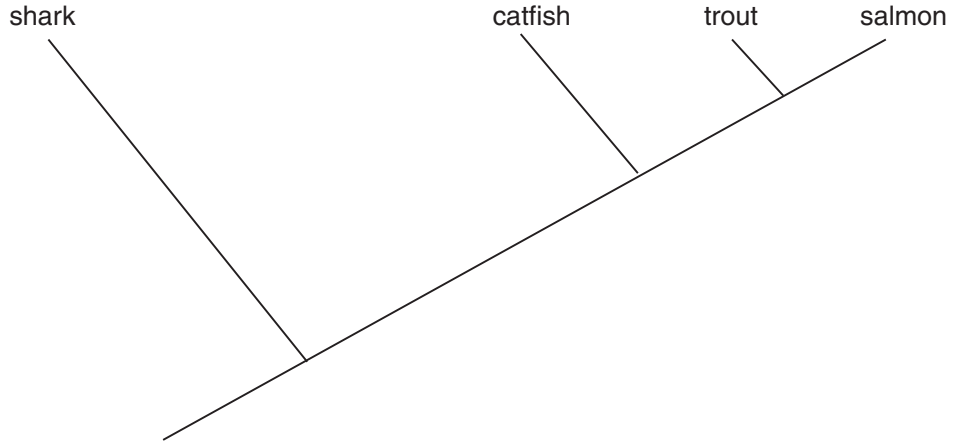
A. First, draw a diagonal line across the page from left to right in the upward direction. This line will form the "trunk" of the cladogram. Identify the fish with the least bands in common with any of the other fish (in this example it is shark, which has only 2 bands). Draw a side branch at 90 degrees near the bottom of the trunk and label the end with the fish's name, in this case, shark. This fish is the outlier because it is the least similar to any of the others. The node where the shark's side branch forks from the trunk represents an ancestor that is common to all the fish in this analysis.



B. Next, find the two fish with the most bands in common (in this example it is salmon and trout which have 10 bands in common). Draw a side branch off the line near the top to form a fork and label the 2 ends of the fork with the fishes names, in this case, salmon and trout (it doesn't matter which branch gets which label). The node represents a common ancestor of salmon and trout that had all those proteins.

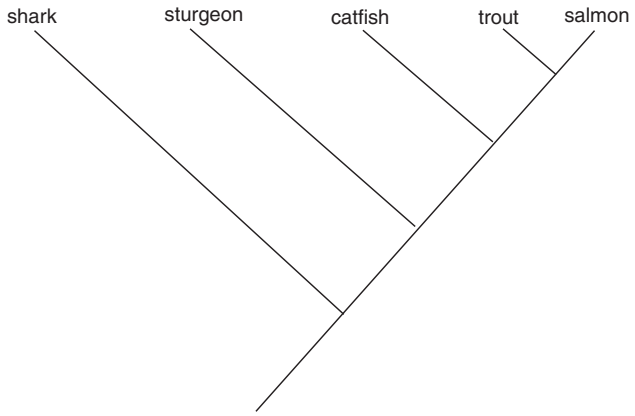


C. Now find the fish with the next most bands in common. In this example, catfish has five bands in common with salmon and trout, which indicates it is the same cladistic distance from salmon and trout (i.e. it is not more closely related to salmon over trout or vice versa). Draw a branch further down the trunk. This node represents an ancestor that is common to salmon, trout and catfish that had these same five proteins.

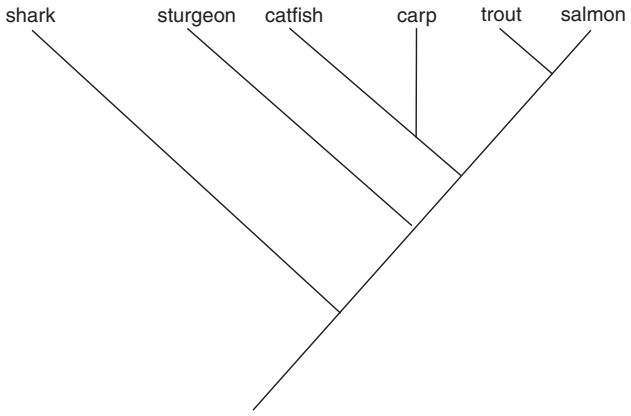


D. The last fish to add to the cladogram in this example is sturgeon, which shares four bands with trout, three bands with salmon, and only two bands with catfish and shark. This fish may seem trickier to place than the others because it shares more characteristics with salmon and trout than it does with catfish, but catfish shares more characteristics with salmon and trout than sturgeon does.

So, to place this fish you can ask, does sturgeon share the five proteins that the common ancestor of salmon, trout, and catfish had? Answer – no. Does sturgeon share more proteins with salmon, trout, and catfish than shark? Answer – yes. Therefore, sturgeon gets its own branch in between the catfish and shark branches to indicate that it has more shared characteristics with trout, salmon, and catfish than shark, but fewer shared characteristics with salmon and trout than catfish.



Note: It has not happened in this example, however, there may be a case in which two pairs of fish are more closely related to each other than to the other fish. For example a carp would likely have more bands in common with catfish, say 8 bands (while having 5 or less bands in common with the other fish). In this case, a sub-branch would be made of the catfish branch to indicate that catfish and carp have a shared ancestor that has more shared characteristics (in this case 8 proteins) than the shared ancestor of salmon, trout, catfish and carp represented by the node on the main trunk, which has only 5 protein bands in common.

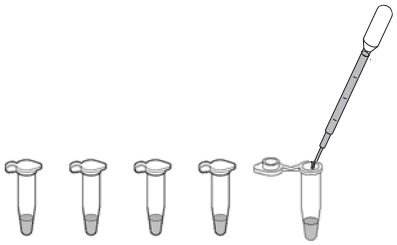


Compare cladogram to phylogenetic tree

9. Students then compare their cladograms with the phylogenetic tree provided and decide whether their molecular evidence supports or refutes published phylogenetic data.

Comparative Proteomics Kit I: Protein Profiler Module – Quick Guide

Lesson 1 Quick Guide



1 Label one 1.5 ml **fliptop** micro tube for each of five fish samples. Also label one **screwcap** micro tube for each fish sample.

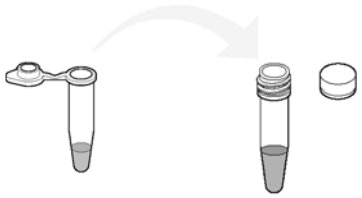
2. Add 250 µl of Bio-Rad Laemmli sample buffer to each labeled **fliptop** microtube.

3. 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 **fliptop** micro test tube. Close the lids.

4. Flick the microtubes 15 times to agitate the tissue in the sample buffer.

5. Incubate for 5 minutes at room temperature.

6. Carefully transfer the buffer by pouring from **each fliptop** microtube into a labeled **screwcap** microtube. Do not transfer the fish!



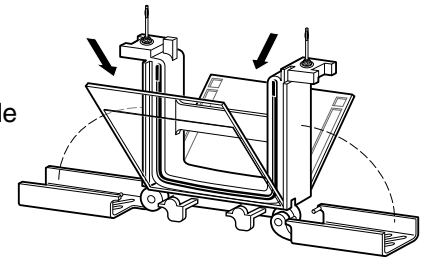
7. Heat the fish samples in screwcap microtubes for 5 minutes at 95°C.

Lesson 2 Quick Guide

1. Set up Mini-PROTEAN Tetra gel box.

2. Prepare a TGX or 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.

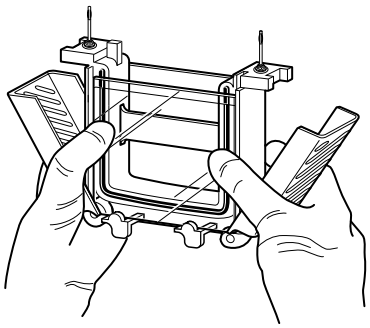
3. Remove the comb from the TGX or Ready Gel cassette.



4. Place TGX or Ready Gel cassette into the electrode assembly that has the banana plugs with the short plate facing inward. Place a buffer dam or another TGX or Ready Gel cassette on the opposite side of the electrode assembly, with notch on buffer dam facing inward.

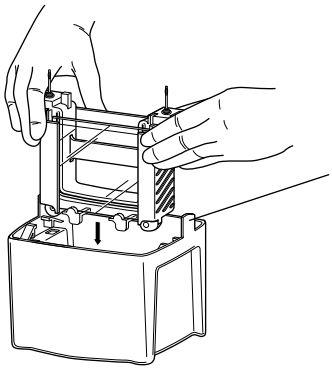
QUICK GUIDE

5. Push both gels towards each other, making sure that they are against the green gaskets that are built into the clamping frame; make certain that the short plates sit just below the notch at the top of the green gasket. Slide the green arms of the clamping frame over the gels, locking them into place.



6. Lower the electrode assembly with the gels in it into the mini tank on the side of the tank with the plastic tabs. Make sure that the red banana plug goes on the side of the tank with the red oval.

CAUTION: When running 1 or 2 gels only, DO NOT place the Companion Running Module in the tank. Doing so will cause excessive heat generation and prevent electrophoretic separation.



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 700 ml of 1x TGS electrophoresis buffer until the buffer reaches the 2 gels line on the tank.

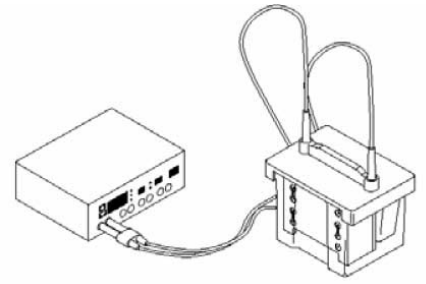
9. If using, place sample loading guide on top of the electrode assembly.

10. Heat fish samples and actin and myosin standard to 95°C for 2–5 min.

11. Load your gel:

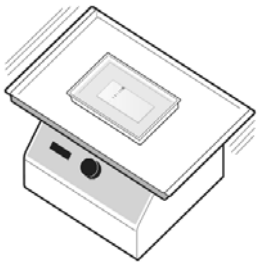
Lane	Volume	Sample
1 & 2	empty	empty
3	5 µl	Precision Plus Protein Kaleidoscope prestained standards (Stds)
4	*10 µl	fish sample 1
5	*10 µl	fish sample 2
6	*10 µl	fish sample 3
7	*10 µl	fish sample 4
8	*10 µl	fish sample 5
9	*10 µl	actin and mysin standard (AM)
10	empty	empty

*__Note__: If you are going on to perform the western blot module, load 5 µl of fish sample and actin and myosin standard.



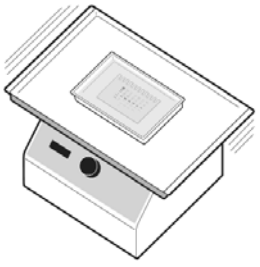
12. Electrophorese for 30 minutes at 200 V in 1x TGS electrophoresis buffer.

13. After electrophoresis, remove gel from cassette and place two gels in one staining tray. Rinse gels in tap water. If time allows rinse gels 3 times for 5 minutes.



14. Pour off water and add 50 ml of Bio-Safe Coomassie blue stain. Stain gels for at least 1 hour with gentle shaking for best results.

15. Discard stain and destain gels in a large volume of water overnight, changing the water at least once. Blue-stained bands will be visible on a clear gel after destaining.



Lesson 3 Quick Guide

1. Dry gels using GelAir cellophane.

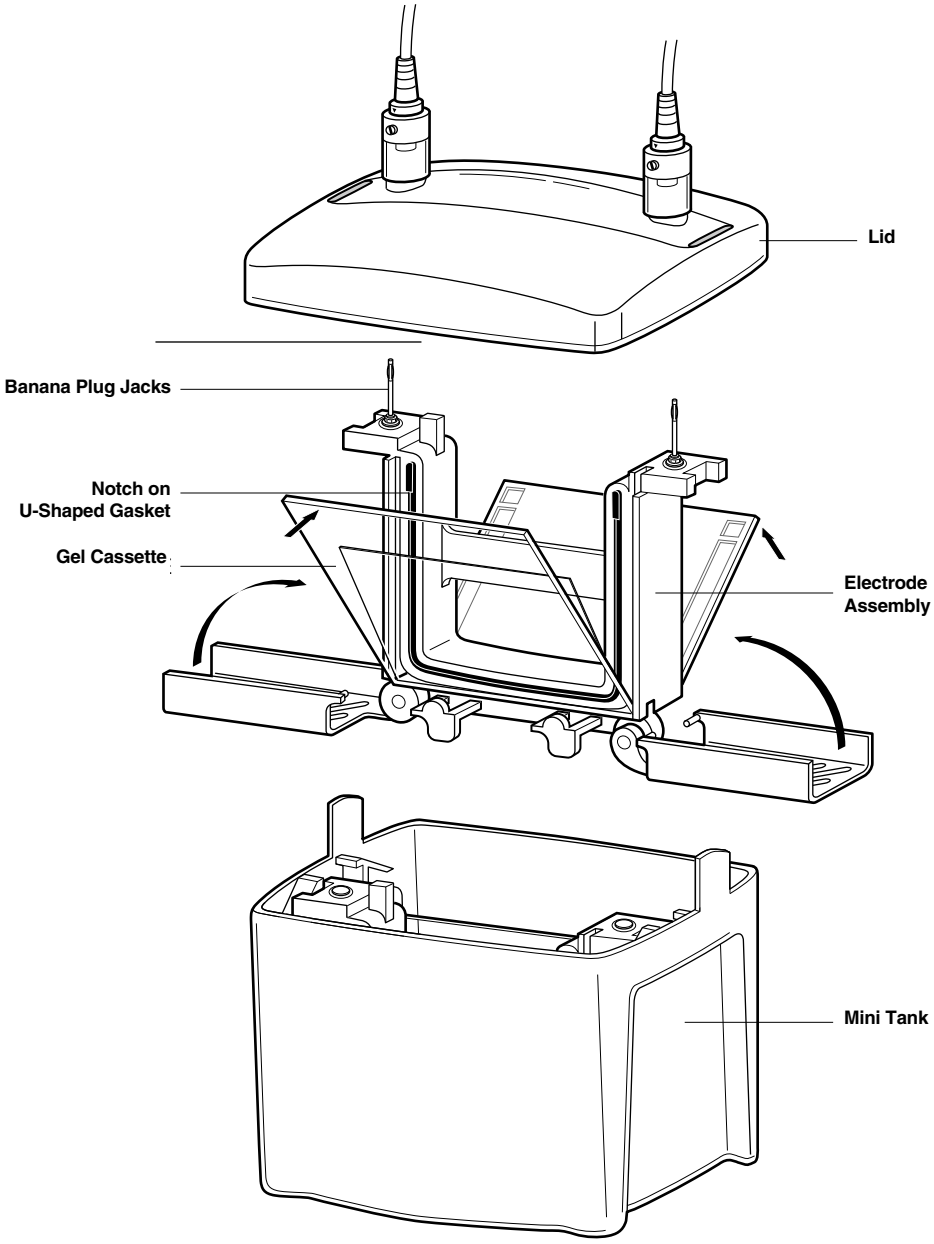


Fig. 11. Assembling the Mini-PROTEAN Tetra cell.

Student Manual

Does molecular evidence support or refute the theory of evolution? DNA gets a lot of attention, but proteins do all the work. Proteins determine an organism's form, function, and phenotype. As such, proteins determine the traits that are the raw material of natural selection and evolution.

In this lab you will use protein gel electrophoresis, the technique most widely used in biotechnology research, to examine muscle proteins from closely and distantly related fish species, and to identify similarities and differences in these organisms' protein profiles, or fingerprints.

Analogous in principle to DNA fingerprinting, protein profiles can also reveal genetic similarities or differences, and from such molecular data it is possible to infer relatedness. Each protein band that a fish has in common with another fish is considered a shared characteristic. A fish family tree, or cladogram, can be constructed based on protein bands that the fish have in common. Cladistic analysis assumes that when two organisms share a common characteristic, they also share a common ancestor with that same characteristic.

Muscle protein consists mainly of actin and myosin, but numerous other proteins also make up muscle tissue. While actin and myosin are highly conserved across all animal species, the other proteins are more diverse, varying even among closely related species.

During this laboratory-based scientific investigation you are asked: Can molecular data show similarities and differences among species? You will compare the similarities and differences in the protein profiles of various fish species, create a cladogram (family tree) from your own gel results, and compare your data to published evolutionary data. Then you will be asked: Do the data agree? Why or why not? What explanations can you suggest?

Molecular biology has unlocked secrets of mystifying new diseases, given us the premier tools for defining biological identity, and created a pillar of data to support Darwin's theory of common descent. In short, molecular biology and its elegant techniques have revolutionized our understanding of life's origins and mechanisms.

Is it just genes that determine what proteins will be made? Current research in the field of proteomics suggests not. The following section is designed as a review of important background information for this laboratory investigation.

Background

Proteomics

The central dogma of molecular biology of DNA♦RNA♦protein has given us a comfortable explanation of how the information encoded by our DNA is translated and used to make an organism. It describes how a gene made of DNA is transcribed by messenger RNA and then translated into a protein by transfer RNA in a complex series of events utilizing ribosomal RNA and amino acids. New discoveries about alternative roles for RNA, multiple forms of proteins being encoded by single genes in our cells, and changes to proteins after translation are changing this comfortable scenario and we are finding that things (as ever in biology) are not so simple. Although in essence the central dogma remains true, investigations into genomics and proteomics are revealing a complexity that we had never imagined.

In 1990, a massive research effort took place to sequence what was estimated to be the 100,000 genes that coded for each protein synthesized by humans (the human genome). This study, the Human Genome Project, took 13 years to complete. When the study began, scientists estimated that there were over 100,000 human genes. Now, years after the genome has been sequenced, there is still no consensus on the actual number of human genes, but the current estimate is down to around 22,000 human genes, this is only a few thousand more genes than encodes the genome of a much simpler organism, *C. elegans*, a nematode worm that has around 19,000 genes.

So why are a similar number of genes required to make a worm and a person? Importantly, a human has a much larger total genome (3 billion base pairs) than a worm (100 million base pairs) suggesting that the total amount of DNA rather than the actual number of genes may be what gives rise to complexity. In addition, recent developments have shown it is quite common in complex organisms for a single gene to encode multiple proteins. Moreover, changing when, to what level and where a protein is expressed, or changing a protein after it has been translated (posttranslational modification) can result in proteins with very different functions. This realization of the importance and diversity of proteins started a whole new field termed **proteomics**.

Proteomics is the study of proteins, particularly their structures and functions. This term was coined to make an analogy with genomics, and while it is often viewed as the "next step", proteomics is much more complicated than genomics. Most importantly, while the genome is a rather constant entity, the proteome differs from cell to cell and is constantly changing through its biochemical interactions with the genome and the environment. The entirety of proteins in existence in an organism throughout its life cycle, or on a smaller scale the entirety of proteins found in a particular cell type under a particular type of stimulation, are referred to as the *proteome* of the organism or cell type, respectively. One organism will have radically different protein expression in different parts of its body, in different stages of its life cycle, and in different environmental conditions.

Proteomics was initially defined as the effort to catalog all the proteins expressed in all cells at all stages of development. That definition has now been expanded to include the study of protein functions, protein-protein interactions, cellular locations, expression levels, and posttranslational modifications of all proteins within all cells and tissues at all stages of development. Thus, it is hypothesized that a large amount of the noncoding DNA in the human genome functions to highly regulate protein production, expression levels, posttranslational modification etc., and it is this regulation of our complex proteomes, rather than our genes, that makes us different from worms.

To catalog all human proteins and ascertain their functions and interactions presents a daunting challenge for scientists. An international collaboration to achieve these goals is being coordinated by the Human Proteome Organization (HUPO).

Research in the proteomic field has discovered a number of modification systems that allow one gene to code for many proteins and mechanisms that finely regulate the sub- and extracellular locations and expression levels of proteins. These include alternative splicing of exons, use of different promoters, posttranscriptional modification, translational frameshifting, posttranslational modification, and RNA editing.

Evolution

The term evolution probably brings to mind Charles Darwin and the Theory of Natural Selection. In short, this theory states that there are more organisms brought into the environment than can be supported by the environment. Each of these individuals are different – even among the same species. The environment selects organisms best suited to survive and reproduce based on those differences. Adaptations are the differences that make one organism more suited to the environment than another individual. These adaptations are phenotypic (physical) characteristics such as finch beaks that are determined by a genetic component. The genetic component is inherited from the parent in the form of genes.

Variations in an organism's proteins may reflect physiological adaptations to an ecological niche and environment, but they originate as chance DNA mutations. Such random mutation events, if favorable, persist through the natural selection process and contribute to the evolution of new species – with new specialized functions.

The discovery of the chemical structure of DNA by Watson, Crick, Wilkins, and Franklin and our understanding of how the triplet code of nitrogen bases leads to the synthesis of proteins (which is the phenotypic expression) convinced us that adaptations are the result of changes in the DNA code (mutations). However, current research in the field of proteomics is leading some scientists to question whether or not DNA is the final determining factor in the synthesis of proteins and thus the determining factor in evolution.

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.

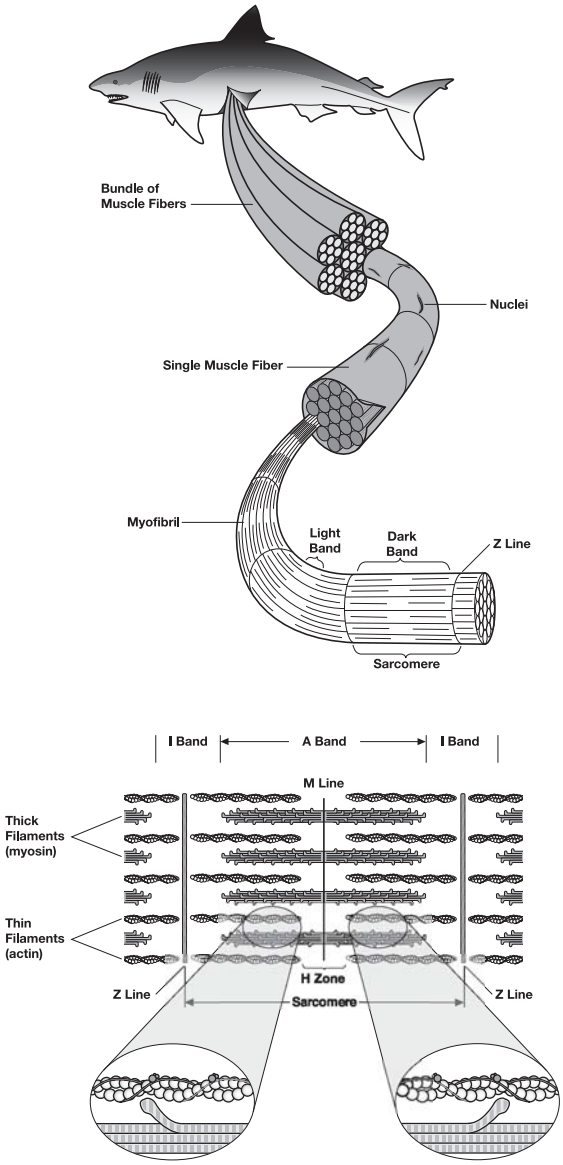


Fig. 12. Telescopic view of muscle structure: Thick myosin filaments and thin actin filaments form myofibrils, which are bundled together to make muscle fibers. (Figure modified from Campbell 1996 with permission.)

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.

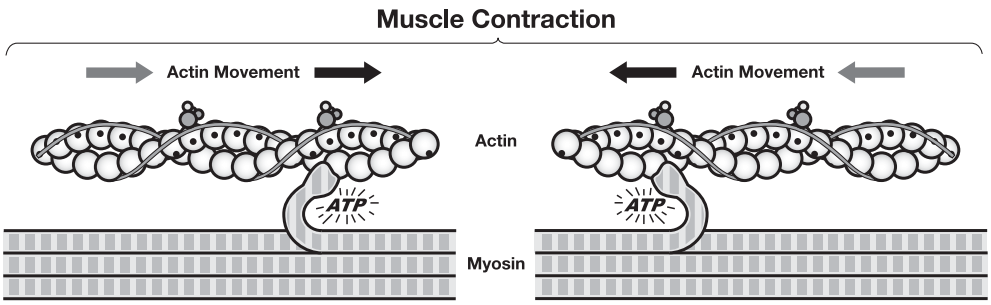


Fig. 13. Hydrolysis of ATP causes myosin and actin filaments to slide past one another, shortening the sarcomere and contracting the muscle. (Figure modified from Campbell 1996 with permission.)

Other Muscle Proteins

Numerous proteins besides actin and myosin are also required for muscle contraction (please refer to the 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.

Table 2. Characterized muscle proteins, in order of decreasing size, adapted from Alberts et al. (1994).

Protein	MW (in kD)	Function
titin	3,000	centers myosin in sarcomere
dystrophin	400	anchoring to plasma membrane
filamin	270	crosslinks 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	15–25	slide actin filaments
troponin (I)	19	regulates contraction
troponin (C)	17	regulates contraction
thymosin	5	sequesters actin monomers

Pre-Lab Activity: Using Computer Databases to Predict What Factors Affect Fish Muscle Profiles

Your teacher has assigned you 5 different species of fish. You will fill in a Fish Data Sheet for each fish. You will then use the information you have gathered to make predictions on how the muscle proteins of these fish will be similar or different.

Go to http://www.fishbase.net

You should see a screen very similar to this:

First follow the example below to become familiar with how the database works.

Example – Rainbow Trout

Type "rainbow trout" into the Common Name field on the FishBase home page.

This search term returns at least 22 different types of fish called rainbow trout. All species are the same. Click on the species *Oncorhynchus mykiss* from the USA. Use the web page this link takes you to in order to fill in the Fish Data Sheet. Note: some information, such as swim type, may need to be reached by following an additional link on the page.

Common Name: Rainbow trout

Scientific Name: *Oncorhynchus mykiss*

Taxonomic Classification:

Family: Salmonidae (Salmonids)

Order: Salmoniformes (Salmons)

Class: Actinopterygii (Ray-finned fishes)

Size: Max weight 25.4 kg

Environment: benthopelagic; anadromous freshwater; brackish; marine; depth range 0–200 m

Biology: Survive better in lakes than streams. Needs fast flowing well oxygenated waters for spawning. Can adapt to sea water if necessary.

Swim Type: Moves body and caudal fin

Definitions of unfamiliar terms: Benthopelagic – feeds on bottom, midwaters, and near surface. Hovers near bottom.

Anadromous – ascend rivers to spawn.

Now it is time to investigate your own fish species. Enter the common name of one of the species of fish you will be investigating into the FishBase search field. Then click on the scientific name link and use the information it brings up to fill in the fields of your Fish Data Sheet. If you think an additional factor may be important in predicting what proteins are expressed in fish muscles, feel free to add it to your data sheet in the additional factors field, making sure you specify that factor and fill it in for each fish species. Fill in a Fish Data Sheet for each fish species you will be investigating. If you find terms you are unfamiliar with, find out what they mean and write definitions in the field for definitions of unfamiliar terms. **Hint**: performing an Internet search with the unfamiliar term and then writing "definition" after it (for example. "benthopelagic definition") usually brings up dictionary definitions of terms.

Once your Fish Data Sheets are filled in, use the information you have gathered to answer the questions below.

Focus Questions

1. Which of the fields you have filled in on your Fish Data Sheets (taxonomic classification, environment, biology, swim type and additional factors) do you think will help you most in predicting which fish have the most similar muscle protein profiles?

2. For each field you have chosen, state why you think these fields will help you to make these predictions, and for those fields you haven't chosen, state why you don't think these will help.

3. Using the fields you have stated above to help you predict which fish will have similar muscle protein profiles, predict which two fish will have the most similar protein profiles. Describe how you have come to this prediction.

4. Similarly, predict which two fish will have the most contrasting protein profiles, i.e., the fewest proteins in common. Describe how you have come to this prediction.

After the lab is complete, remember to come back and see whether your predictions were correct.

Fish Data Sheet

Common Name:

Scientific Name:

Taxonomic Classification:

- Family
- Order
- Class

Size:

Environment:

Biology:

Swim Type:

Additional Factors:

Definitions of unfamiliar terms:

Alternative Pre-Lab Activity

Evolution and Classification of Fish

An evolutionary tree shows the evolutionary lineages of different species over relative time. Evolutionary trees, (also called cladograms), can be based on many different types of data. Some trees are constructed using a single type of data and some trees use multiple types of data. The traditional way of constructing evolutionary trees was to look at the physical morphology of organisms, including sizes, shapes and developmental structures of both living organisms and fossils. Today, similarities and differences in protein and DNA sequences are being used. Both methods are valuable and often complement each other but they may not always agree. Can you propose why this may be so?

Use the evolutionary tree below to make predictions about the relatedness of the fish species you will examine in this lab. Following the analysis and interpretation of your electrophoresis results, you will create a cladogram from your own results and compare your cladogram with your predictions. Will your lab data support or refute your own predictions? Why, or why not?

For this activity, and in order to generate meaningful data, it is useful to compare both closely related and distantly related fish. In addition to using the tree below to make your predictions, we recommend that you research additional information on the evolutionary histories of fishes, using the Internet, and biology and zoology books.

The data used to construct the evolutionary tree below was obtained from the cladograms on the tree of life web page from the University of Arizona (www.tolweb.org). (Please note that the field of phylogenetics is ever changing and different methods used to construct a phylogenetic tree often result in differences between trees, hence the data on the tree of life web page may not concur exactly with "textbook" evolutionary trees.)

Most fish are contained within the superclass Gnathostoma (jawed vertebrates), which also includes all tetrapods. Only hagfish and lampreys are outside this group. These two fish types are sometimes classed together as Agnatha, but can also be separated into Hyperotreti and Hyperoartia. Hyperotreti (hagfish) are craniates (animals with skulls), but not vertebrates because they have no backbone, while Hyperoartia (lamprey) are very primitive vertebrates, but do not have a jaw. The Gnathostoma fishes are divided into the classes Chondrichthyes (cartilaginous fishes) and Osteichthyes (bony fishes). The Chondrichthyes include the sharks and rays, and the Osteichthyes, include all other modern fishes and all tetrapods (amphibians, birds, and mammals). Below are brief descriptions of some of the major fish groups, in order from most ancient to most recently diverged.

Hyperotreti (e.g., hagfish) are eel-like, jawless fishes that have a skull, but no backbone with parasitic and scavenging lifestyles. They are very primitive and may approach the condition of the common ancestor to all craniates.

Hyperoartia (e.g., lamprey) are eel-like, jawless fishes that are primitive vertebrates. They are identified by a single nostril and a sucker-like mouth with which they attach to fishes and rocks.

Chondrichthyes (e.g., shark, ray, skate, & sawfish) have a cartilaginous rather than bony skeletons that reflects a more evolutionarily ancestral state. Their skin is thick and without true scales, and they do not have swim bladders or lungs.

Osteichthyes (e.g., coelancanth, tuna & haddock). 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 which contains the living fossil, the coelacanth, and the tetrapods (amphibians, reptiles, mammals, birds and dinosaurs)
- The ray-finned fish, Actinopterygians, which contains most other fish

Sarcopterygians (e.g., lungfish and coelacanth) also include modern amphibians, reptiles, birds, and mammals. Coelacanth were thought to have become extinct at about the same time as the dinosaurs, until a live specimen was found in 1938. They form an important evolutionary link between fish and four-legged land animals.

Actinopterygian (e.g., gar, sturgeon, mackerel & anglerfish) is the subclass encompassing most modern ray-finned fish including the chondrostei, semionotiformes, and teleosts.

- **Chondrostei (e.g., sturgeon)** are considered relic bony fishes. They lack scales on most of the body, have a cartilaginous skeleton, and have developed a shark-like, heterocercal tail and a rostrum extending past the mouth.
- **Semionotiformes (e.g., gar)** are also ancient fish, they have bony scales and a mainly cartilaginous skeleton.

Teleosts (e.g., herring, carp & pufferfish) comprise the remainder of the bony fishes. These include Clupeomorpha (e.g., herring, sardine & anchovy), Ostariophysi (e.g., carp, catfish, minnow, piranha & electric eel), Salmoniformes (e.g., salmon, trout & smelt), Esociformes (e.g., pike), and the diverse group, Acanthomorphia (e.g., tuna, cod & pufferfish).

Acanthomorphia (e.g. pollock, bass & sole) comprises two main superorders, Paracanthopterygians (e.g., cod, pollock & anglerfish) and Acanthopterygians. The Acanthopterygians include the Perciformes (e.g., the scombridae (e.g., swordfish, mackerel & tuna) and the serranidae (e.g., bass, snapper & grouper)), the Pleuronectiformes (e.g., flat fish, flounders & sole) and the Tetradontiformes (e.g., pufferfish).

Pre-Lab Focus Questions

1. Name three proteins found in muscle

   i. _____

   ii. _____

   iii. _____

2. Make a prediction of which two of your fish species will have the most similar protein profiles and why. You may want to use a database on fish (http://www.fishbase.net) to find more information on the different fish you are analyzing.

3. Give a prediction of which two of your fish species will have the least similar protein profiles and why.

4. Predict how the other fish in the study will compare by drawing an evolutionary tree and describing your reasoning.

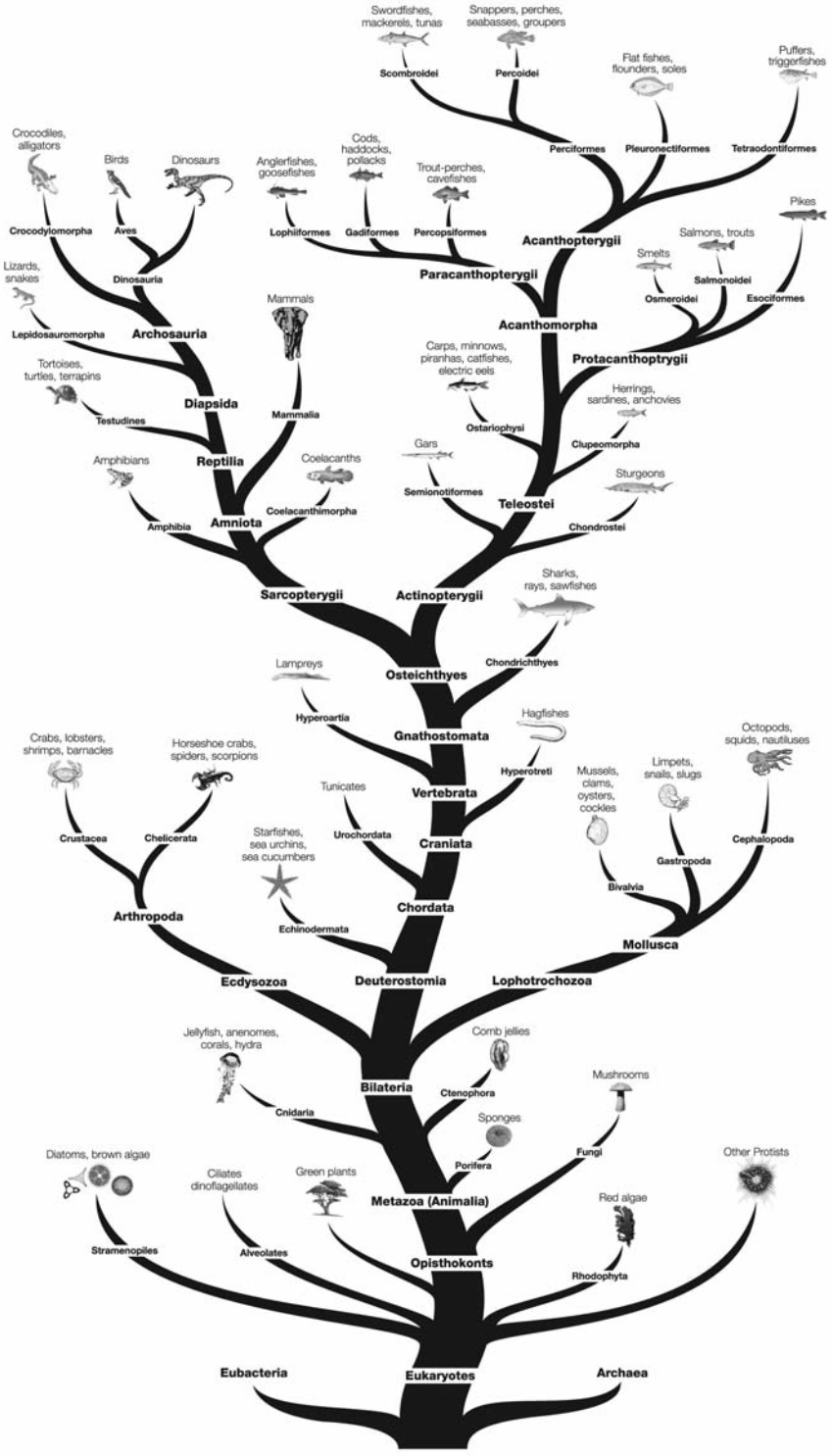


Fig. 14. Evolutionary tree showing the relationships of eukaryotes. (Figure adapted from the tree of life web page from the University of Arizona (www.tolweb.org).)

STUDENT MANUAL
ALTERNATE PRE-LAB
ACTIVITY

Lesson 1: Introduction to Protein Electrophoresis and SDS-PAGE

How Can We Study Proteins Found in Muscle?

Polyacrylamide gel electrophoresis (PAGE) can be used to separate small molecules such as proteins. Understanding protein structure is important to understanding how we can use PAGE for protein analysis.

Proteins are made of smaller units (monomers) called amino acids. There are 20 common amino acids. The sequence and interaction between these different amino acids determine the function of the protein they form. Amino acids are joined together by peptide bonds to form polypeptide chains. Chains of amino acids constitute a protein. In turn these chains may interact with other polypeptides to form multi-subunit proteins.

Amino acids can be combined in many different sequences. The sequence of the amino acids in the chain is referred to as the primary protein structure. All amino acids have the same basic structural component (Figure 15).

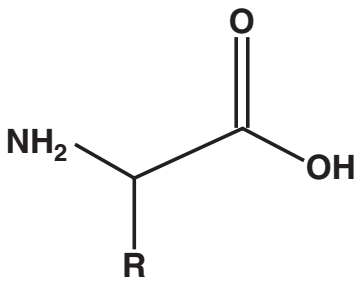


Fig. 15. Chemical structure of an amino acid.

The "R" group may be charged or uncharged, or may be a long side chain. Thus, each amino acid has different properties and can interact with other amino acids in the chain. Hydrogen bonding between these side chains, primarily between the C=O and the N-H groups, causes the protein to bend and fold to form helices, pleated sheets, reverse turns, and non-ordered arrangements. Disulfide bonds between methionines can also bend and loop the amino acid chain. This is considered the secondary structure of the protein.

The tertiary structure of the protein is determined by the interaction of the hydrophilic and hydrophobic side chains with the aqueous environment. The hydrophobic regions aggregate to the center of the molecule. The hydrophilic regions orient themselves toward the exterior. These ordered bends and folds make the protein compact. Examples of tertiary protein structure are structural and globular proteins.

The quaternary structure of proteins is achieved from the interaction of polypeptide chains with others. Multiple polypeptides can combine to form complex structures such as the muscle protein myosin, or the blood protein hemoglobin, which are both composed of four polypeptide chains. These complex proteins are often held together by disulfide bonds between cysteines. In fact, PAGE analysis was first carried out in 1956 to show the genetic disease sickle cell anemia is caused by a change to a single amino acid of the hemoglobin protein (Ingram 1956).

Prior to electrophoresis, the proteins are treated with the detergent sodium dodecyl sulfate (SDS) and heated. SDS and heat denatures (destroys) the protein tertiary and quaternary structures, so that the proteins become less three dimensional and more linear. SDS also gives the protein an overall negative charge with a strength that is relative to the length of its polypeptide chain, allowing the mixture of proteins to be separated according to size.

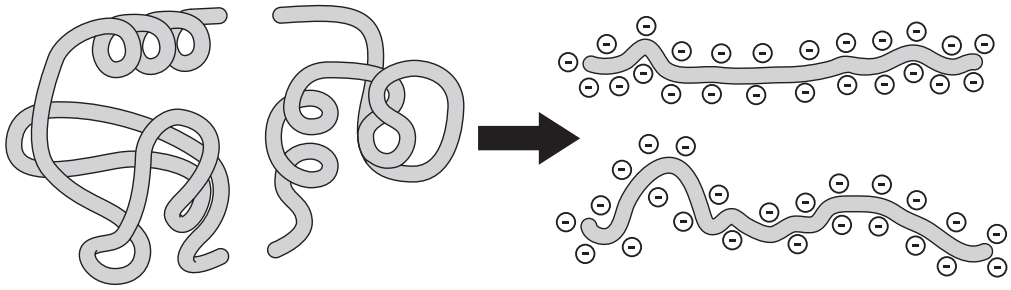


Fig. 16. The combination of heat and the detergent SDS denatures proteins for SDS-PAGE analysis.

The proteins, in their SDS-containing Laemmli sample buffer, are separated on a gel with a matrix that acts to sieve the proteins by size upon addition of an electric current. A polyacrylamide gel is positioned in a buffer-filled chamber between two electrodes, 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 SDS-coated, negatively charged proteins migrate through the gel away from the negatively charged anode toward the cathode, with the larger proteins moving more slowly than the smaller proteins. This technique was developed by U.K. Laemmli, whose 1970 Nature paper has received the highest number of citations of any scientific paper. SDS-PAGE is still the predominant method used in vertical gel electrophoresis of proteins.

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 mass. 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

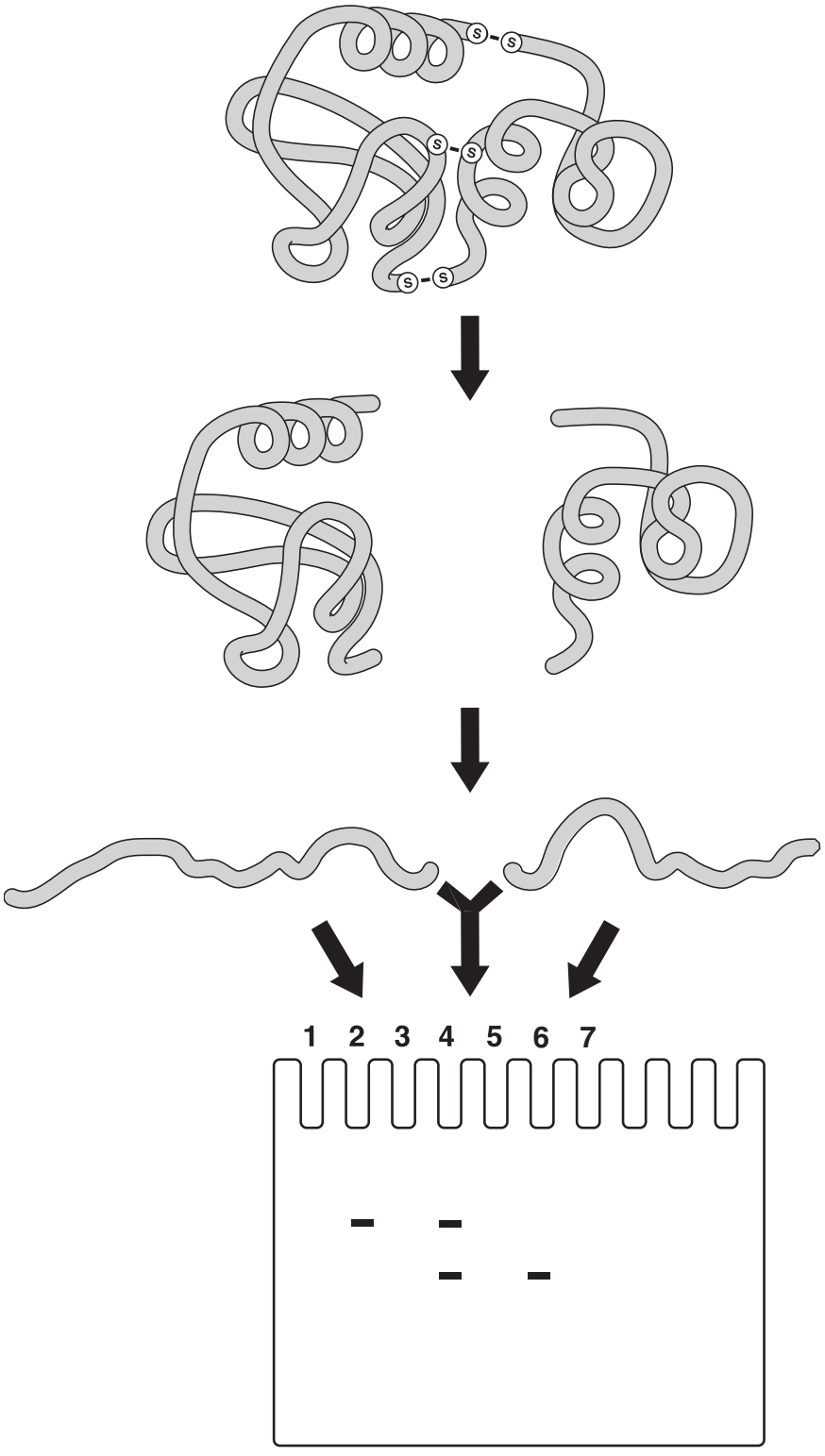


Fig. 17. A quaternary protein complex denatured with reducing agents, heat, and SDS, can be separated into individual proteins and resolved by size using SDS-PAGE.

In this investigation, you will use SDS-PAGE to separate and analyze the protein profiles of the muscle tissue of different fish. By comparing the protein profiles of different fish species you can test the hypothesis that protein profiles are indicators of genetic and evolutionary relatedness.

Visualizing your proteins

Proteins in your samples are not visible while the gel is running. The only visible proteins will be those in the Precision Plus Protein Kaleidoscope standard that have been prestained with covalently attached dyes. You should see these proteins resolve into multicolored bands that move down the gel as power is run through the gel. If the electric current is left on for too long, the proteins will run off the bottom of the gel. To guard against this and to show you the progress of your gel if you did not have the standards, a blue tracking dye is mixed with the Laemmli sample buffer used to prepare your 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 ahead of the proteins in the gel.

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 such as DNA or lipids. 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.

Lesson 1: Protein Extraction From Muscle

Your first task is to extract proteins from muscle tissue, unfold and denature them, and give each protein an overall negative charge using Laemmli sample buffer, mechanical forces, and heat. In this lab you will add tiny pieces of muscle to Laemmli sample buffer and manually disrupt the tissue by flicking the tubes. This will release muscle specific proteins from the cells, unfold them, and add an overall negative charge to each protein. You will then pour off the extract and heat the extracted proteins to 95°C, which will complete their denaturation.

Student Workstations

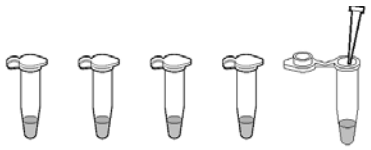
Material	Quantity
1.5 ml fliptop microtubes	5
1.5 ml screwcap microtubes	5
1 ml transfer pipet	1
Fish samples, labeled 1–5	5 species
Marking pen	1
Laemmli sample buffer	1.5 ml
Knife or scissors to cut fish samples	1

Common Workstation

Material	Quantity
Water bath set to 95°C	1

Procedure

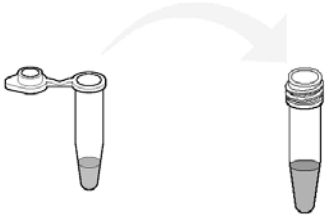
1. To make this a blind study, assign a letter (e.g., A–E) to each fish sample to be investigated. Keep a record of which fish got which number and hide their true identities until after the analysis is complete.
2. Label 1.5 ml fliptop microtubes with the number of the fish species to be analyzed. There should be one labeled tube for each fish sample being prepared for electrophoresis.
3. Add 250 µl of Laemmli sample buffer to each labeled tube.



4. 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.

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

6. Incubate the samples for 5 min at room temperature to extract and solubilize the proteins.
7. 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.



8. Heat your fish samples in their screwcap tubes for 5 min 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 hr, or store them at –20°C for up to several weeks.

Lesson 1 Focus Questions

1. Why did you add Laemmli sample buffer to your fish samples?

2. What was the purpose of heating the samples?

3. How are the proteins extracted from the fish samples?

4. Have all the proteins been extracted from the fish slice or are some still left after the extraction? How could you test your hypothesis?

Lesson 2: Electrophoresis: Gel Loading, Running, and Staining

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, which will generate profiles for various fish species

Student Workstations

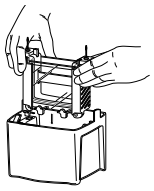
Material	Quantity
Fish protein extracts from lesson one	5 species
Actin & myosin standard, 12.5 ul	1 vial
Precision Plus Protein Kaleidoscope prestained standards, 6 µl	1 vial
4-20% Mini-PROTEAN TGX or 15% 10-well, Ready Gel precast gel	1 vial
1–20 µl adjustable-volume micropipet	1
Prot/Elec pipet tips for gel loading	7 tips
Mini-PROTEAN Tetra cell electrophoresis module	1 per 2 gels
1x Tris-glycine-SDS (TGS) running buffer	700 ml per gel box
Power supply (200 V constant) to be shared between workstations	1
Sample loading guide – for 10-well comb (optional)	1 per gel box
Buffer dam (only required if running 1 gel/box)	1
Staining trays	1 per 2 gels
Bio-Safe Coomassie stain for proteins	50 ml per 2 gels

Common Workstation

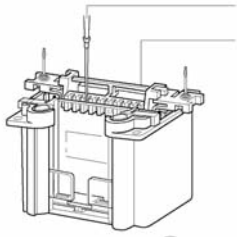
Material	Quantity
Water bath set at 95°C	1
Water for gel destaining (tap water is fine)	

Procedure

1. Reheat frozen fish samples and actin and myosin standard at 95°C for 2–5 minutes to redissolve any precipitated detergent. Note: If you have prepared your fish samples in this lesson, there is no need to reheat them.
2. Assemble gel boxes. Use the pictorial guide found in the Quick Guide to insert your TGX or Ready Gel polyacrylamide gels into the vertical electrophoresis module if your instructor has not preassembled them.
3. Double-check that the buffer in the inner buffer chamber is well above the top of the smaller plate. If it is not, you may have a leak; consult with your instructor.
   Note: If you do have a leak, the outer chamber of the gel box can be filled to above the inner small plates, to equalize the buffer levels in both reservoirs.



4. If available, place a yellow sample loading guide on the top of the electrode assembly. The guide will direct your pipet tip to the correct position for loading each sample in a well.

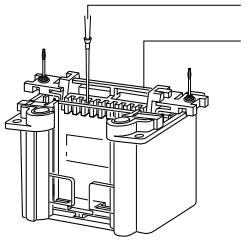


5. Record in which well of your gel you will load which of your samples in the table below:

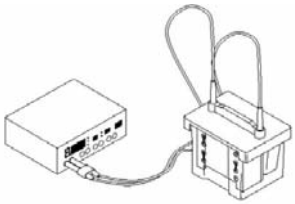
Well	Volume	Sample Name
1	empty	none
2	empty	none
3	5 µl Stds	Precision Plus Protein Kaleidoscope prestained standard (Stds)
4	*10 µl sample A	_____
5	*10 µl sample B	_____
6	*10 µl sample C	_____
7	*10 µl sample D	_____
8	*10 µl sample E	_____
9	*10 µl AM	actin & myosin standard (AM)
10	empty	none

*Note: If you are going on to perform the western blot module load 5 µl of each fish sample and the actin & myosin standard. This will prevent overloading the lanes.

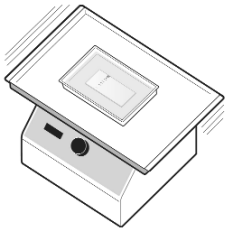
6. Load **5 µl** of Precision Plus Protein Kaleidoscope prestained standard gently into well # 3 using a thin gel loading tip. **Note**: The fine barrel of the gel loading tips means liquid is slower to go into the tip than normal tips. You must therefore release the plunger of the micropipet very slowly, otherwise you will not pipet the correct volume.



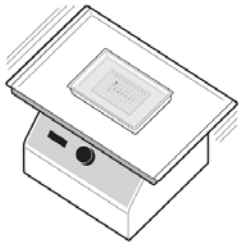
7. Using a fresh tip each time, load **10 µl** of each of your protein samples gently into the wells designated in your table above. Note: If you are going on to perform the western blot module, load 5 µl of each protein sample.
8. Using a fresh tip, load **10 µl** of the actin & myosin standard gently into well # 9. Note: If you are going to perform the western blot module load 5 µl of the actin & myosin standard.
9. After loading all samples, remove the yellow sample loading guide (if used), 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. Watch for the separation of the standard.



10. 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.
11. Pour out the running buffer from the electrode assembly. Open the cams and remove the gel cassettes.
12. Now it's time to stain the proteins in your gel. Lay your gel cassette flat on the bench with the short plate facing up. Carefully pry apart the gel plates, using the gel opening key. The gel will usually adhere to one of the plates. Transfer the plate with the gel adhering to it to a tray containing tap water 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 water. If there is sufficient time, rinse the gel 3 times with tap water for 5 minutes by carefully pouring out the water and replacing it. Rinsing the gel will improve the intensity of the protein bands.
13. Carefully pour out the water and replace with 50 ml of Bio-Safe Coomassie stain per 2 gels.



14. Allow the gels to stain for at least 1 hour, with shaking if available. Gels maybe stained overnight but seal the container to reduce evaporation.
15. After at least 1 hour discard the stain and replace it with a large volume of water to destain the gel overnight with rocking action if available. Change water 2–3 times if possible. Bands will become visible after a few hours of destaining.



Lesson 2 Focus Questions

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

2. What is the purpose of the actin & myosin standards and the Precision Plus Protein Kaleidoscope prestained standard?

3. Which proteins will migrate farthest? Why?

4. What is the purpose of the stain?

Lesson 3: Dry Gels

You will now dry your gel to make a permanent record.

Student Workstations

Material	Quantity
Container of tap water to wet cellophane	1
GelAir cellophane support sheets	2
Square plastic container and 2 rubber bands (if not using GelAir drying frame)	1
Ruler & graph paper if performing band analysis	

Common Workstation

Material	Quantity
GelAir drying frames (optional)	1
GelAir assembly table (optional)	1
GelAir dryer (optional)	1

- Examine your gel. Blue protein bands should be visible on the clear, destained gel. You may want to make a photocopy of your gel so that you can perform the detailed analysis sooner. Your instructor 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.
- Answer the focus questions on qualitative comparisons of protein profiles before drying your gels.

GelAir drying frame method:

1. Precast polyacrylamide gels must have the ridge at the bottom of the gel removed by chopping them off (not slicing) using a plastic card, e.g., an I.D. card or ruler.
2. Prewet 2 sheets of cellophane in a container of water for 15–20 seconds.
3. Place a plastic drying frame on the GelAir assembly table. Center one sheet of cellophane on the assembly table.
4. 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.
5. 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 bubbles, gently push them out with a gloved finger. Bubbles will cause cracks in the gel during drying!
6. 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.

7. 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.
8. In contrast to Fast Blast™ stain for DNA staining, Bio-Safe Coomassie protein stain is not light sensitive.

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

1. Wet two pieces of cellophane in a large volume of water, around 500 ml.
2. 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.
3. 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.
4. Place the second sheet of wetted cellophane over the gel, being careful not to trap any bubbles.
5. 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. Cutting the bottom out of the plastic container can speed up drying.
6. In contrast to Fast Blast™ Stain for DNA staining, Bio-Safe Coomassie protein stain is not light sensitive.

Lesson 3 Focus Questions

1. Which two fish have the most similar protein profiles?

2. Which two fish have the least similar protein profiles?

3. Give an explanation for why you think the protein profiles of some fish species share more bands than other fish species.

4. Did your predictions from your Pre-Lab Activity turn out to be true or not? If not, why do you think that was?

Post-Lab Activity

Analysis and Interpretation of Results

Detailed Gel Analysis

Does molecular evidence support or refute the theory of evolution?

Does your molecular evidence support or refute your predictions?

Create a cladogram using your results to find out.

From your gel you can create a cladogram based on proteins that the fish have in common. You can then determine whether your cladogram supports your predictions and/or matches the evolutionary relatedness of the fish species determined by morphological analysis in the evolutionary tree provided. Each protein band that a fish has in common with another fish is a shared characteristic. Cladistic analysis assumes that when two organisms share a characteristic, they had a common ancestor that had that characteristic, and this can be represented as a node on a cladogram with two branches coming from that node representing the descendent organisms.

In this exercise you will define the shared characteristics (i.e., make a list of all the different proteins in fish muscle), find which proteins (characteristics) are shared between fish, and construct a cladogram based on your data.

Procedures

Generate a standard curve to calculate protein sizes

The different protein bands in your gel can be defined by their different molecular masses. Indeed many proteins are named for their molecular weights. For example p53, a protein implicated in tumor progression is 53 kD in size. To determine the molecular masses of the proteins, a standard curve is created plotting the known molecular masses of the proteins in the Precision Plus Protein Kaleidoscope prestained standards against the distance they have migrated down the gel from the base of the well.

A 15% polyacrylamide gel is designed to separate small proteins- proteins less than 40 kD. Your gel analysis will concentrate on this size range. Note: If a different percentage acrylamide gel or an agarose gel has been run, analyze the section of the gel that has the best separation.

1. As shown in the figure below draw a line between the 37 and 25 kD bands of the prestained standards. Your gel analysis will be restricted to the proteins below this line.

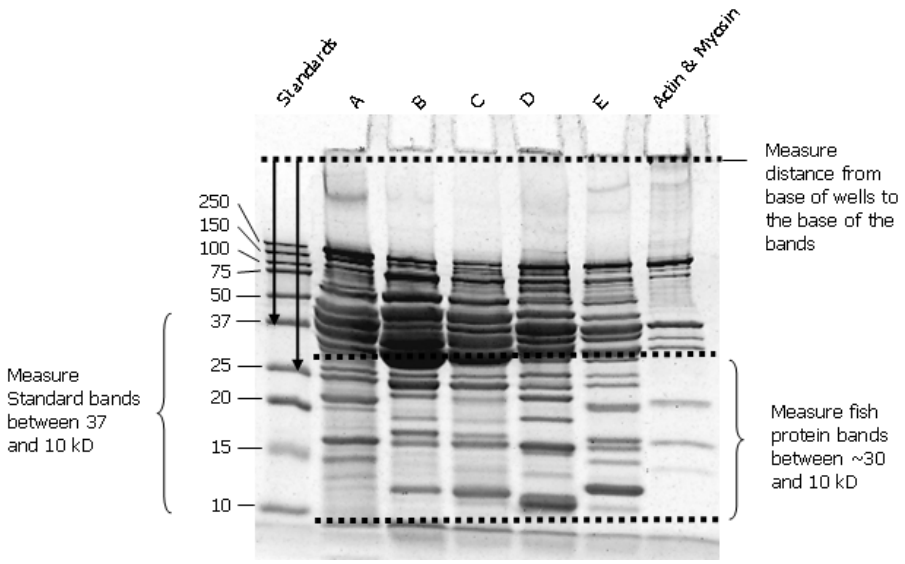


Fig. 18. Image of fish muscle proteins separated by SDS-PAGE and stained with Bio-Safe Coomassie stain. Lines illustrate measurement of bands for constructing the standard curve.

2. To create the standard curve measure and record in the table below the distances the five sub 40 kD protein bands of the prestained standard have migrated from the base of the well i.e. measure the 37, 25, 20, 15 and 10 kD bands. Accuracy to 0.5 mm is required.

Precision Plus Protein Kaleidoscope Prestained Standards Molecular Weight (kD)	Distance Migrated (mm)
37	
25	
20	
15	
10	

3. On the graph paper provided, plot the distances migrated in mm on the x-axis against the molecular masses of the prestained protein bands in kD on the y-axis as a scatter plot. Draw a line of best fit through the points. On semi-logarithmic graph paper with the molecular mass of the proteins on a logarithmic scale the data should result in a linear (straight line) curve.

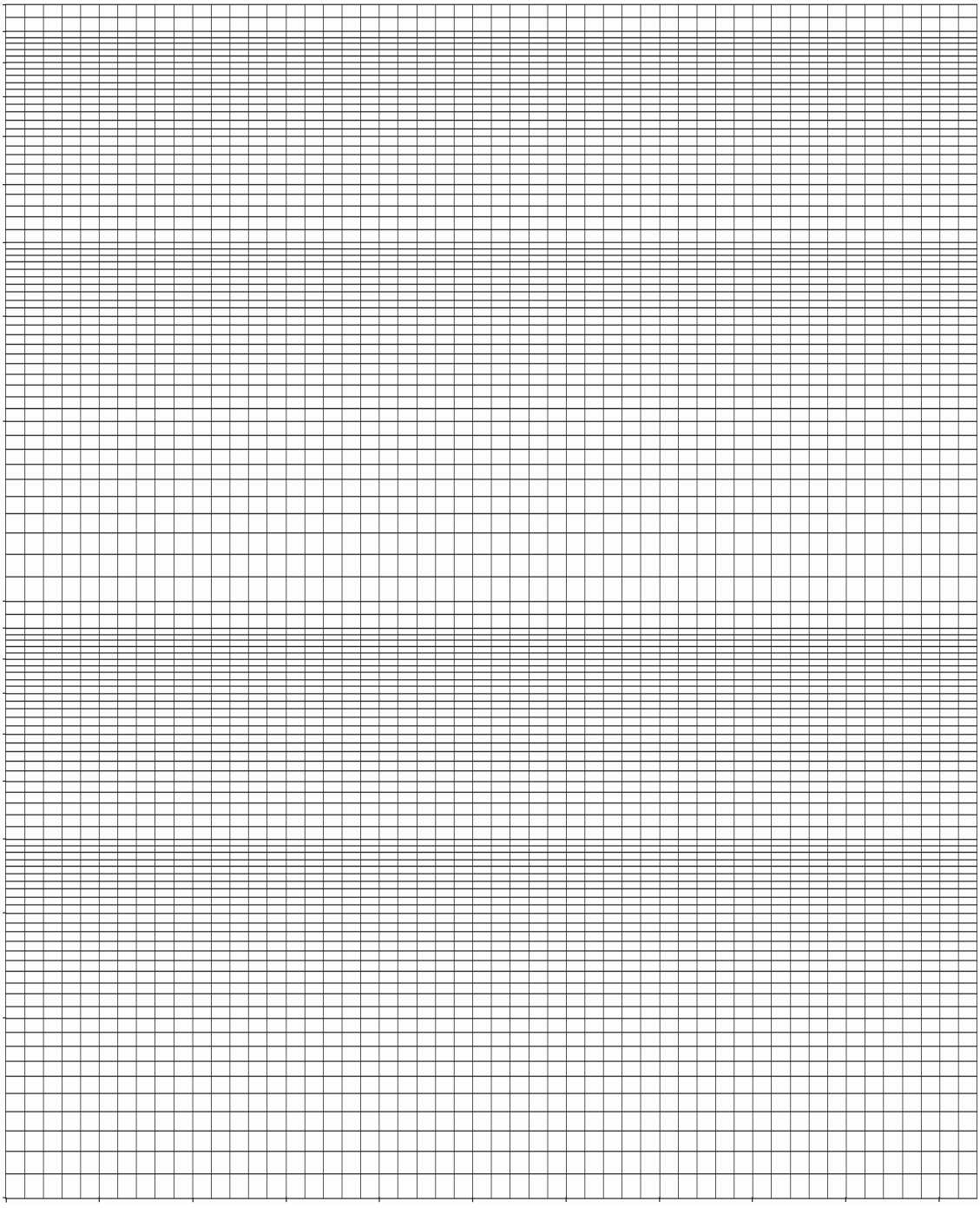


Fig. 19. Two-cycle semi-logarithmic graph paper to construct curve of the protein molecular mass against the distance migrated.

Define the characteristics (proteins) of the different fish

4. For each fish sample that has been analyzed, determine the molecular masses of the proteins below the 25-37 kD line. Measure the distance each band has migrated from the base of its well. Find that distance on the x-axis of the standard curve. Draw a line up from the x-axis to the curve. Read across to the y-axis to determine the molecular mass.

Alternatively, use graphing software to generate the standard curve. Make a line of best fit (or trend line) through the points and formulate an equation to calculate the mass of the unknown proteins on the gel.

5. Enter this data into a table with the molecular masses of the proteins for each fish (see example below).

Fish Species A	
Distance Migrated (mm)	Molecular Mass (kD)
25	32.5
26.5	31
29	28.6
36	21.7
36.5	21.2
39	18.8
44	13.9
52	6

Determine which fish have each characteristic (protein)

6. Make a table with a row for every band size you have recorded for all your fish samples and a column for each type of fish on your gel. Then make a mark in each cell of the table where the fish has that size band (see example below).

Characteristic

Distance Migrated (mm)	Protein Molecular Mass (kDa)	Species A	Species B	Species C	Species D	Species E
25	32.5	X				
26	31.5		X	X	X	X
26.5	31.0	X				
27.5	30.0		X	X	X	X
28.5	29.1					
29	28.6	X	X	X	X	
30	27.6			X		X
30.5	27.1					X
32	25.6		X	X	X	
33	24.7					X
34.5	23.2		X	X		
35.5	22.2					X
36	21.7	X				
36.5	21.2	X	X	X	X	
37	20.7					X
37.5	20.2		X	X		
38	19.7				X	
38.5	19.3				X	
39	18.8	X				X
39.5	18.3					X
40.5	17.3		X	X		
41	16.8				X	
41.5	16.3					
42	15.8		X	X		X
43	14.8					
44	13.9	X				X
45	12.9		X	X		
46	11.9				X	
46.5	11.4			X		
47	10.9					X
47.5	10.4				X	
51.5	6.5			X		
52	6.0	X				
	COUNT	**8**	**10**	**13**	**10**	**12**

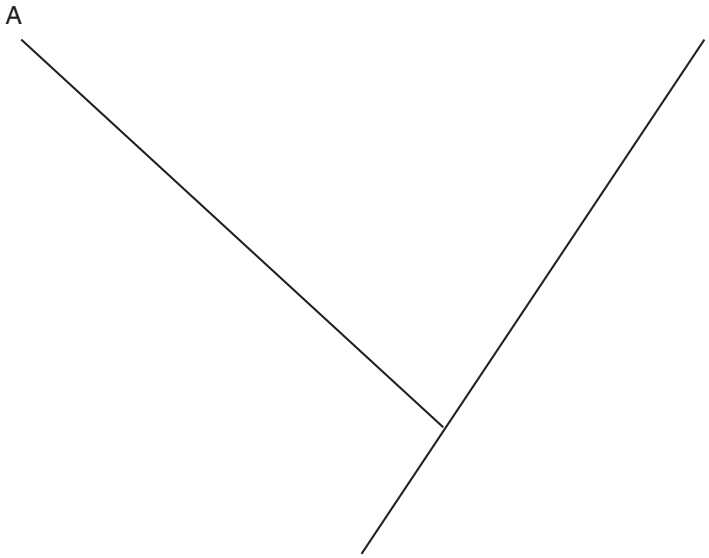
Find the number of characteristics shared by each of the fish

7. In the table below both the row and column headings are the types of fish. From the table above, separately compare the number of bands (X's) in common with every other fish sample from your gel and put those numbers into the table below, such that each fish is individually compared with every other fish. In this example, species A and B have just 2 bands in common while species B and C have 10 bands in common. Your table will be the basis for drawing your cladogram.

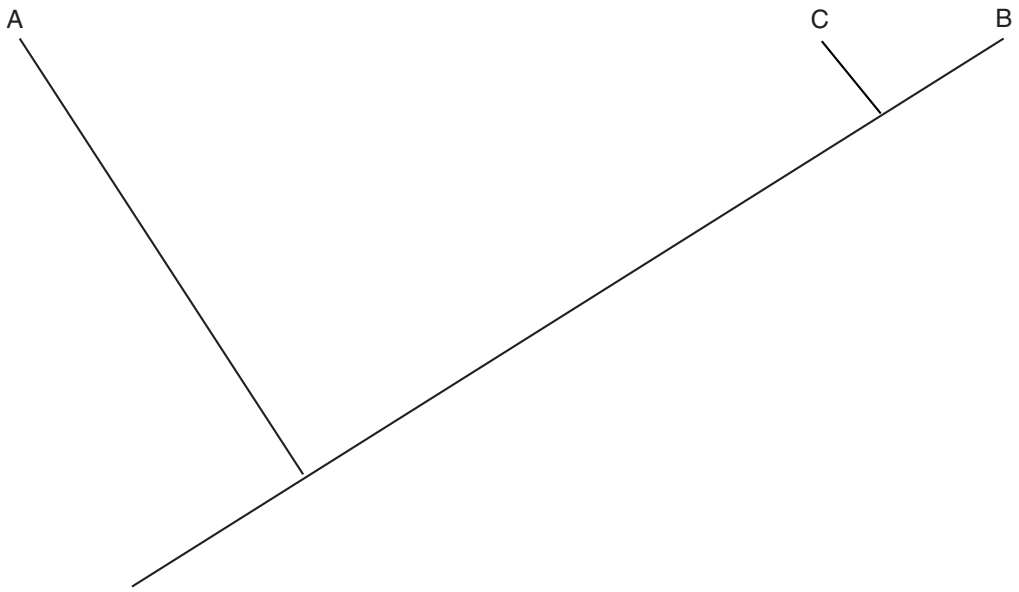
	Species A	**Species B**	**Species C**	**Species D**	**Species E**
Species A	8	2	2	2	2
Species B		10	10	5	3
Species C			13	5	4
Species D					2
Species E					12

Construct your cladogram

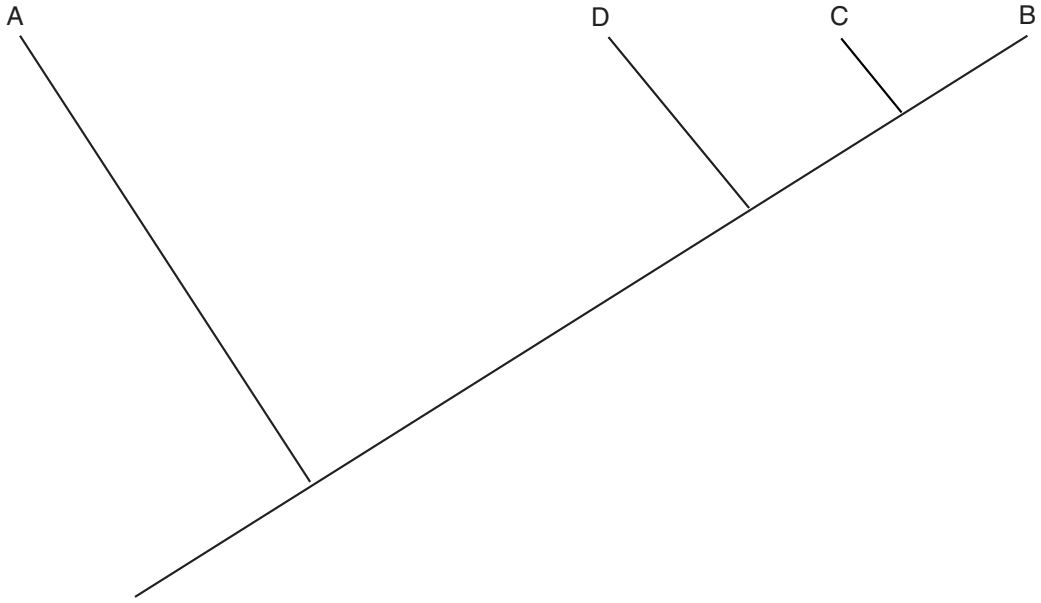
8. Now you are ready to construct your cladogram. First draw a line to form the trunk of your cladogram. Find the fish with the least bands in common. In the example above it is species A, which has only 2 bands in common with any of the other fish. Then draw a side branch off the line near the bottom of the trunk and label that branch with the fish's name, in this case, species A. This fish is the outlier, i.e., it is the least similar to any of the others. The node (where the side branch meets the trunk) represents an ancestor that is common to all the fish in this analysis.



Now, find the two fish with the most bands in common (in this example it is species B and C, which have 10 bands in common). Draw a side branch off the trunk near the top and label the two ends with the fishes' names, in this case, species B and species C (it doesn't matter which branch gets which label). The node represents a common ancestor of species B and species C that had all the same characteristics (proteins).



Now, identify those fish species with the next most bands in common. In this example, species D has five bands in common with species B and species C, which indicates species D is the same cladistic distance from B and C (i.e. species D is not more closely related to either B or C). Draw a branch further down the trunk. This node represents an ancestor that is common to species B, C, and D that had these 5 characteristic proteins.

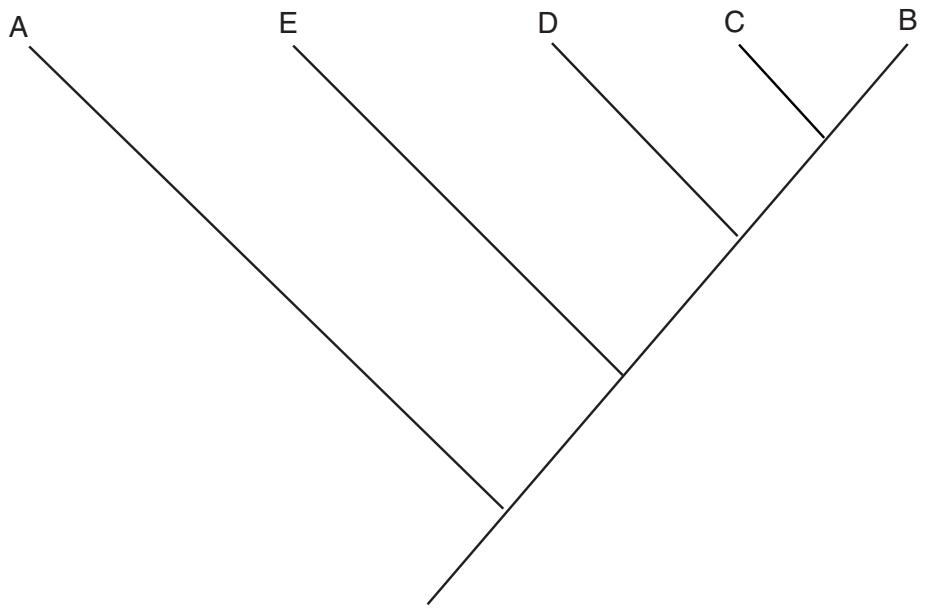


The last fish to add to the cladogram in this example is species E, which shares four bands with species C, three bands with species B, and only two bands with species A and D. This fish may seem trickier to place than the others because it shares more characteristics with species B and C than it does with D, but D shares more characteristics with B and C than E does. So, to place this fish you might ask:

Does species E share the five proteins that the common ancestor of species B, C, and D had? Answer (no).

Does species E share more proteins with B, C, and D than A? Answer (yes).

Therefore, species E gets its own branch in between the D and A branches to indicate that it has more shared characteristics with B, C, and D than A, but fewer shared characteristics with B and C than D.



Now compare your cladogram with your original predictions. Write your deductions below.

Appendix A: Protein Electrophoresis Using Agarose Gels and Horizontal Gel Electrophoresis Apparatus

The principles of protein separation by electrophoresis can be taught using agarose gels and horizontal electrophoresis apparatus that is typically used for separating DNA. Proteins are rarely separated on agarose in the real world because proteins are much smaller than DNA (compare the number of carbons in a base pair to an amino acid). Polyacrylamide has a tighter matrix than agarose which, in addition to the discontinuous system used in SDS-PAGE (see Lesson 1: Introduction to Protein Electrophoresis SDS-PAGE), is much better at resolving most proteins into distinct bands. However, agarose gels are used in scientific laboratories to separate large proteins, for example serum proteins from blood. The following protocol describes a method to separate fish muscle proteins using agarose gels and horizontal gel electrophoresis apparatus. The same experimental principles apply to this method as SDS-PAGE, such as the SDS conveying a net negative charge and the denaturation of proteins using heat. The main difference is that the gel is not a discontinuous system like the polyacrylamide gels.

An example of the results is shown in Figure 18 below. You can see that the bands are not as distinct as with polyacrylamide gels, and that the larger proteins separate better with more distinct bands than the smaller ones, which diffuse in the more porous agarose.

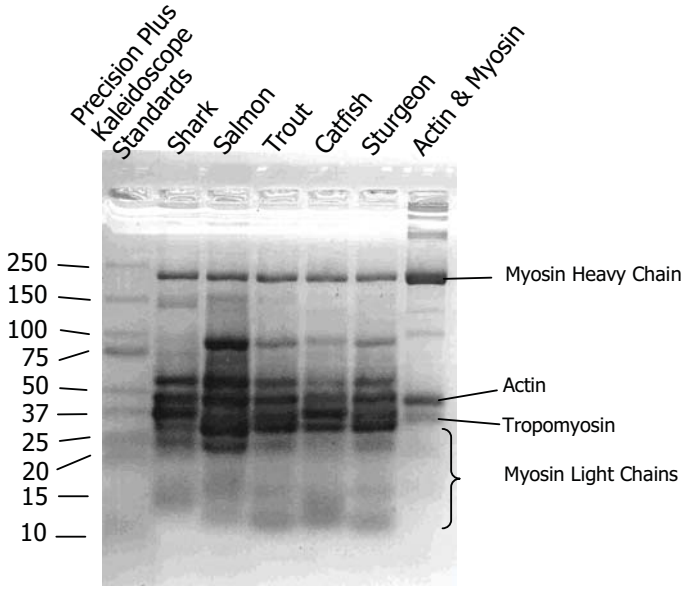


Fig. 20. 4% agarose gel electrophoresed at 100 V for 45 minutes, fixed, stained with Bio-Safe Coomassie stain, and destained in water.

It is important to note that both the type of agarose and the running buffer are different from what is usually used to separate DNA. **Regular agarose will not work with this protocol**. The agarose used to make these gels is high-percentage PCR low-melt agarose and must be purchased separately from the kit (Certified PCR low-melt agarose, 25 g, catalog #161-3113). This special type of agarose is superior to regular molecular biology agarose at separating very small molecules. Low-melt agarose is different from regular agarose because once dissolved and formed into gels, it melts at around 65°C and will remain molten to around 34°C. Therefore, if the gels are prepared in a hot climate, it may be necessary to put the gels in the refrigerator to set. **Tip**: Thinner agarose gels (e.g., 3–5 mm) will resolve proteins better than thick gels, so pour your gels carefully with minimal agarose. The running buffer and the buffer

used to dissolve the agarose is 1x Tris-glycine-SDS (TGS) buffer. This is the same running buffer used for polyacrylamide gels.

It is important to prepare the agarose gels by the method described below using a hot water bath, and not in the microwave or on a hot plate as is often done when preparing DNA agarose gels. SDS is a detergent, and boiling the TGS-agarose in a microwave or hot plate will result in a boiling foam that will not form good gels and is a safety hazard.

The protocol for performing protein electrophoresis using agarose gels differs from that used for polyacrylamide gels described in lesson 2 by a few points: extra electrophoresis time; a fixation step, a longer staining step and extra destaining. Note: an extra day may be required to complete the lab when using this protocol.

- The gels are electrophoresed for 45 minutes at 100 V. The extra electrophoresis time is necessary to see good separation of protein bands. Note: Increasing the voltage to reduce the running time is not recommended since this may increase the temperature of your gel running buffer, which could melt the low-melt agarose gels.
- After the gels have been run, the proteins need to be fixed into the agarose, otherwise they will diffuse and the protein bands will become very fuzzy. The fixative is 10% acetic acid and 40% ethanol. The fixation causes the proteins to aggregate and partially precipitate. This makes them larger and so they diffuse less in the porous agarose matrix. 60% vinegar can replace acetic acid, but will not work quite as well. Gels should be fixed for 1 hour to overnight.
- The gels are stained for 2 hours to overnight, this extra time is required because the gels are much thicker than polyacrylamide gels.
- The water used to destain the gels should be changed at least 3 times to remove the stain from the agarose.

Post-lab activities: The activities to determine protein sizes and the relatedness of the fish species described in the Post-Lab Activity can be performed from agarose gel data. However, the analysis should concentrate on the larger proteins rather than the smaller proteins. The 250 kD to 25 kD molecular mass markers should be used to generate a standard curve, rather than the 37 to 10 kD. Your students may have difficulty deciding which point on the protein band to measure to, because the protein bands can be quite fat in the agarose gels. So long as they are consistent, they can either measure to the base of each band or to the middle of each band.

Note: The western blot protocol does not work using agarose gels. This protocol requires polyacrylamide gels to properly resolve the myosin light chain protein band.

Instructor's Advance Preparation Guide
Lesson 2: Agarose Electrophoresis – Agarose Gel Loading, Running, Fixing and Staining

Preparation Overview

These reagents may be carried out 1 to 2 days ahead of time by the instructor or done during class by individual student teams.

- Prepare 1x TGS & agarose gels (3–4 hours)
- Prepare and aliquot other reagents for students (20 minutes)
- Set up student and common workstations (20 minutes)

Required Materials for Reagent Preparation (for eight workstations)	Quantity
10x Tris-glycine-SDS (TGS) running buffer	300 ml
Distilled/deionized water	3.5 L
Certified PCR low-melt agarose	12 g
Waterbath set to 95°C	1
Gel casting trays	4–8
Gel combs	8
Lab tape (optional)	1 roll
Acetic acid	100 ml
Reagent alcohol/ethanol (95–100%)	400 ml
Laemmli sample buffer, 500 µl	1 vial
Actin & myosin standard, 500 µg, lyophilized	1 vial
Precision Plus Protein Kaleidoscope prestained standards, 50 µl	1 vial
1–20 µl adjustable-volume micropipet & tips	1
1.5 ml screwcap microtubes	9
1.5 ml fliptop microtubes	8

Prepare Reagents

Prepare 1x Tris/glycine/SDS (TGS) gel running buffer (time required,10 minutes). The electrophoresis buffer is provided as a 10x concentrated solution. 1x TGS buffer is needed to make the agarose gel and is also required for each electrophoresis chamber. 3 liters of 1x TGS buffer will be sufficient to run 8 electrophoresis chambers and pour 8 agarose gels. To make 3 L of 1x TGS from a 10x TGS concentrate, add 300 ml of concentrate to 2.97 L of distilled water. Store at room temperature.

Prepare 4% TGS PCR low-melt agarose solution (time required, 2–4 h). For eight 4% agarose 7 x 7 cm gels add 12 g of PCR low-melt agarose to 300 ml of 1x TGS gel running buffer in a suitable container such as a 1 liter Erlenmeyer flask, and swirl to suspend the agarose powder in the buffer. Place the flask into a water bath set to 95–100°C. Leave to melt with occasional swirling for 1–2 hours. You will know when the agarose is ready because all the tiny bubbles will have dissipated. Once all the bubbles are gone, either let the agarose cool to around 60°C for a short while before pouring the gels yourself, or reduce the temperature of the water bath to 60°C and have students pour their own gels when ready to do so. If the agarose sets, it can be repeatedly remelted in a 65°C water bath.

It is important to prepare the agarose gels by the method described above using a hot water bath, and not in the microwave or on a hot plate as is often done when preparing DNA agarose gels. SDS is a detergent, and boiling the TGS-agarose in a microwave or hot plate will result in a boiling foam that will not form good gels and is a safety hazard.

Note: if you require fewer gels, recalculate the amount of agarose you will need: 7 x 7 cm gels and 7 x 10 cm gels require 20–30 ml and 30–40 ml of agarose solution per gel, respectively. A 4% agarose solution requires 4 g of PCR low-melt agarose per 100 ml of TGS gel running buffer.

Cast 4% PCR low-melt agarose gels (time required-10 minutes). Using the Mini-Sub® cell GT system, gels can be cast directly in the gel box by using the casting gates with the gel tray. If casting gates are unavailable, use the taping method for casting gels, as outlined below. Other methods are detailed in the Sub-Cell GT instruction manual. 7 x 7 cm gel trays allow a single gel to be cast. 7 x 10 cm gel trays allow a double stacked gel to be cast, i.e., a gel with two rows of wells that can be loaded with the samples of two student teams. These longer gels do not fit the casting gates and need to be taped. Once formed gels should be stored covered in 1x TGS gel running buffer at room temperature.

Step 1: Seal the ends of the gel tray securely with strips of standard laboratory tape. Press the tape firmly to the edges of the gel tray to form a fluid-tight seal.

Step 2: Level the gel tray on a leveling table or workbench using the leveling bubble provided with the instrument.

Step 4: Cool the agarose to at least 60°C before pouring (a water bath is useful for this step).

Step 5: While the agarose is cooling to 60°C, place the comb into the appropriate slot of the gel tray. Gel combs should be placed within 3/4" of an inch of the end of the gel casting tray (not in the middle of the gel, unless 2 gel combs are used for double stacked gels).

Step 6: Pour 25–40 ml of molten agarose into the tray to a depth of 0.3–0.5 cm. Thinner gels will resolve proteins better than thick gels.

Step 7: Allow the gel to solidify at room temperature for 20 to 30 minutes – it will appear cloudy and translucent when ready to use. If you are preparing the gels in a particularly hot climate, you may need to put the gels in the refrigerator to set, since low-melt agarose gels may not set at around 30°C or above. However, do not store the gels in the refrigerator, since the SDS will precipitate out of the gels.

Step 8: Carefully remove the comb from the solidified gel. Remove the tape from the edges of the gel tray. Once the gels have set, do not allow them to dry out or they will crack. Store gels at room temperature covered with 1x TGS gel running buffer.

Step 9: To run the gels, place the tray into the leveled horizontal electrophoresis chamber so that the sample wells are at the cathode (black) end of the base. Protein samples will migrate towards the anode (red) end of the base during electrophoresis.

Step 10: Fill the electrophoresis chamber with 1x TGS running buffer to about 2 mm above the surface of the gel.

Step 11: Load gel in manner directed in student manual.

Step 12: Run gel at 100 V for 45 minutes; be careful not to let the blue dye front to migrate off the gel. If double stacked gels are used, take care to not let the blue dye front migrate from the top gel into the bottom gel.

Prepare Gel Fixative (time required 10 minutes). A solution of 40% ethanol and 10% acetic acid will be used to fix the proteins into the agarose gel prior to staining the gel. If acetic acid is not available, a solution of 40% ethanol and 60% vinegar can be used (the proteins bands may not be as distinct as with 10% acetic acid). Store at room temperature.

	Water	Acetic Acid	Clear Vinegar (Any Variety)	95-100% Ethanol (Reagent Alcohol)
Ideal fix	500 ml	100 ml	-	400 ml
Less ideal fix	-	-	600 ml	400 ml

Rehydrate actin and myosin standard. Add 500 µl of Laemmli sample buffer to the vial of actin and myosin standard and incubate at room temperature for 20–30 minutes. Transfer the rehydrated actin and myosin sample to a labeled screwcap tube and heat for 5 minutes at 95°C. Store at –20°C.

Aliquot Student Reagents:

Actin and myosin standard	Label eight 1.5 ml screwcap tubes AM on their sides. Aliquot 25 µl of rehydrated and preheated actin and myosin standard into each. Store at –20°C.
Precision Plus Protein Kaleidoscope prestained standards	Label eight 1.5 ml fliptop tubes Stds. Aliquot 6 µl of standards into each tube. Store at –20°C.

Student Workstations

Materials Required	Quantity
Fish protein extracts from lesson one	5 species
Actin and myosin standard, 25 µl	1 vial
Precision Plus Protein Kaleidoscope prestained standard, 6 µl	1 vial
4% TGS PCR low-melt agarose gel	1
1–20 µl adjustable-volume micropipet	1
Pipet tips for gel loading	7 tips
Horizontal electrophoresis module (gel box)	1
1x Tris-glycine-SDS (TGS) running buffer	250–300 ml per gel box
Power supply (100 V constant) to be shared between workstations	1
Staining trays	1 per 2 gels
Gel fixative solution	100 ml
Bio-Safe Coomassie stain for proteins	50 ml per 2 gels

Common Workstation

Materials Required	Quantity
Water bath set at 95°C	1
Rocker	1
Water for gel destaining (tap water is fine)	

Student Protocol
Lesson 2: Agarose Electrophoresis – Agarose Gel Loading, Running, Fixing, and Staining

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, which will generate profiles for various fish species

Student Workstations

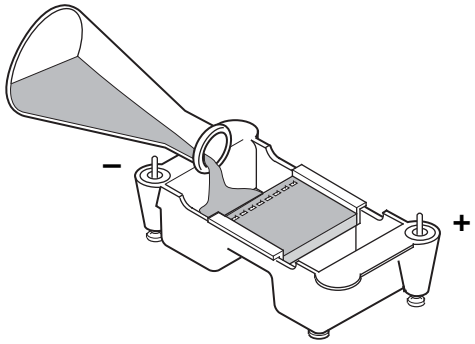
Materials Required	Quantity
Fish protein extracts from lesson one	5 species
Actin & myosin standard, 25 ul	1 vial
Precision Plus Protein Kaleidoscope prestained standards, 6 µl	1 vial
4% TGS PCR low-melt agarose gel	1
1–20 µl adjustable-volume micropipet	1
Pipet tips for gel loading	7 tips
Horizontal electrophoresis module (gel box)	1
1x Tris-glycine-SDS (TGS) running buffer	275 ml per gel box
Power supply (100 V constant) to be shared between workstations	1
Staining trays	1 per 2 gels
Gel fixative solution	100 ml
Bio-Safe Coomassie stain for proteins	100 ml

Common Workstation

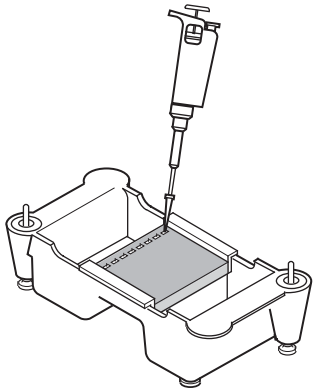
Materials Required	Quantity
Water bath set at 95°C	1
Rocker	1
Water for gel destaining (tap water is fine)	

Protocol

1. Obtain a TGS low-melt agarose gel from your teacher, or, if your teacher instructs you to do so, pour your own gel.
2. Place the casting tray, with the solidified gel in it, onto the central platform in the gel box. The wells should be at the negative (cathode) end of the box where the black electrical lead is connected. Very carefully remove the comb from the gel by pulling it straight up.
3. Pour about 275 ml of TGS gel running buffer into the electrophoresis chamber. Pour enough buffer into the box until it just covers the wells of the gel by 1–2 mm.



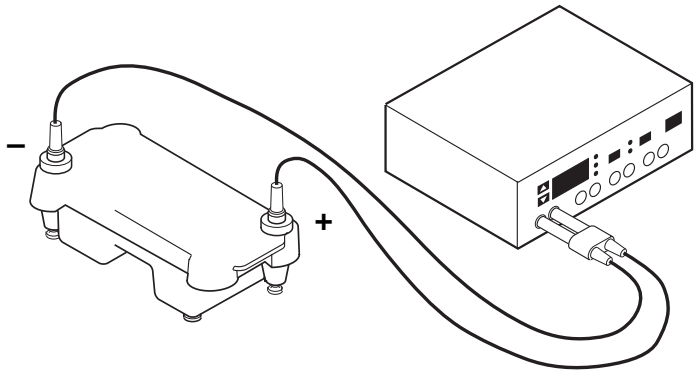
4. Obtain your 5 muscle extracts from the previous lesson. Heat to 95°C for 2–5 minutes to redissolve any precipitated detergent. Note: If you have prepared your muscle extracts today, there is no need to reheat the samples.
5. Using a fresh pipet tip and either an adjustable micropipet set to 5 µl, or a fixed-volume 5 µl micropipet, load 5 µl of Precision Plus Protein Kaleidoscope prestained standards to lane 1 of your TGS agarose gel. **Important: use a fresh tip each time**.



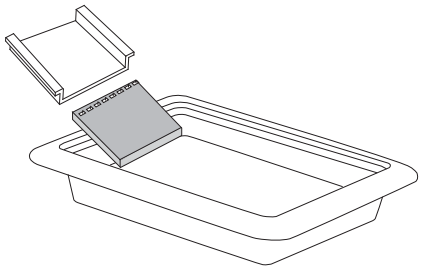
6. Using the table below as a guide, load 20 µl of each of your protein samples and the Actin & Myosin standard into your gel in the order indicated below. Record your gel loading in the table below.

Lane	Volume	Sample Name
1	5 µl Stds	Precision Plus Protein Kaleidoscope prestained standard (Stds)
2	20 µl sample A	_____
3	20 µl sample B	_____
4	20 µl sample C	_____
5	20 µl sample D	_____
6	20 µl sample E	_____
7	20 µl AM	Actin & myosin standard (AM)
8	empty	none

7. Slide the cover of the chamber into place, and connect electrical leads to the power supply, anode to anode (red to red) and cathode to cathode (black to black). Make sure both electrical leads are attached to the same channel of the power supply.
8. Electrophorese at 100 V for 45 minutes. Shortly after the current is applied, the loading dye can be seen moving through the gel toward the positive side of the gel chamber.



9. When electrophoresis is complete, turn off the power supply, disconnect the leads from the inputs, and remove the lid from the gel box.
10. Carefully remove the gel tray and the gel from the gel box. Be careful, the gel is very slippery. Nudge the gel off the gel tray with your thumb and carefully slide it into the gel staining tray for fixing. Add 100 ml of gel fixative and place gel on rocking platform for 1 hour to overnight. Taking the extra time to fix the gels overnight results in much better resolution. Multiple gels can be fixed in the same container.



11. After fixing, pour off the gel fixative and add sufficient Bio-Safe Coomassie stain to cover the gel and leave to stain on a rocking platform for 2 hours to overnight. Multiple gels can be stained in the same container.
12. After staining, pour off the stain and rinse the gel with distilled water and then destain the gel on a rocking platform overnight with at least 3 changes of water. Note: you probably will not see any bands on the gel when you begin to destain. The bands will develop slowly as the gel destains. Destain until you see a nice contrast between the protein bands and the gel background.
13. If you wish to keep a permanent record of your gel, the gel can be dried between cellophane sheets in the manner described in lesson 3. Please note, however, that you must not use a gel drying oven for this process since the gels will melt. You can also peel away the cellophane once the agarose gel has dried; this is not possible with polyacrylamide gels.

Appendix B: Using databases to obtain real amino acid sequence data to create cladograms

In order to determine how closely related species are, scientists often will study amino acid sequences of essential proteins. Any difference in the amino acid sequence is noted and a phylogenetic tree is constructed based on the number of differences. More closely related species have fewer differences (i.e., they have more amino acid sequence in common) than more distantly related species.

There are many tools scientists can use to compare amino acid sequences of muscle protein. One such tool is the National Center for Biotechnology Information protein databases (http://www.ncbi.nlm.nih.gov/). By entering the amino acid sequence of a protein you are interested in, the BLAST search tool compares that sequence to all others in its database. The data generated provides enough information to construct cladograms.

The purpose of this activity is to use data obtained from NCBI to construct an evolutionary tree based on the amino acid sequences of the myosin heavy chain. In this example we have input a 60 amino acid sequence from myosin heavy chain of rainbow trout and then pulled out matching sequences using BLAST, which include chum salmon, zebra fish, common carp, and bluefin tuna, and then compared each of these sequences with each other.

You may either use the data provided below or go online and obtain data directly by performing BLAST searches. A quick guide to performing BLAST searches is given at the end of this activity.

The data below was obtained by entering a 60 amino acid sequence from the heavy myosin chain of rainbow trout (*Oncorhynchus mykiss*). The database search tool returned all sequences that were a close match. The results are formatted as such:

```
gi|755771|emb|CAA88724.1|  myosin heavy chain [Oncorhynchus mykiss]
        Length=698

Score =  119 bits (299),  Expect = 2e-26
Identities = 60/60 (100%), Positives = 60/60 (100%), Gaps = 0/60 (0%)

Query  1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL  60

VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL

Sbjct  1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL  60
```

The value for 'identities' is the number of amino acids exactly in common, the value for 'positives' is the number of amino acids that are similar to each other (such as serine and threonine), and the value for 'gaps' is the number of amino acid positions that are absent one of the sequences. 'Query' is the original trout sequence, 'Sbjct' is the aligned sequence, and the middle sequence shows the mismatches: a '+' indicates a positive and a space indicates a mismatch that is not a positive. There are resources on the NCBI web site to help you understand more about the information a BLAST search generates.

The data below compares rainbow trout to salmon, zebra fish, carp, and tuna, and then compares salmon to zebra fish, carp, and tuna, then zebra fish to carp and tuna, and finally carp to tuna.

Use the data provided to determine how many amino acid differences exist between the organisms. Organize your data in charts.

Rainbow trout compared to Chum Salmon (*Oncorhynchus keta)*

```
gi|21623523|dbj|BAC00871.1|  myosin heavy chain [ Oncorhynchus keta]

        Length=1937

Score =  119 bits (299),  Expect = 2e-26
Identities = 60/60 (100%), Positives = 60/60 (100%), Gaps = 0/60 (0%)

Query  1
AKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL  60

VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL

Sbjct  1240
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL  1299
```

Rainbow Trout compared to Zebra Fish (*Danio rerio*)

```
gi|68360600|ref|XP_708916.1|  PREDICTED: myosin, heavy polypeptide 1, skeletal muscle
[ Danio rerio]

         Length=2505

Score =  108 bits (269),  Expect = 6e-23
Identities = 52/60 (86%), Positives = 57/60 (95%), Gaps = 0/60 (0%)

Query  1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL  60

VAKAK NLEKMCRTLEDQLSE+K+KNDEN+RQ+ND+S QRARL TENGEFGRQLEEKEAL

Sbjct  1240
VAKAKANLEKMCRTLEDQLSEIKSKNDENLRQINDLSAQRARLQTENGEFGRQLEEKEAL  1299
```

Rainbow Trout compared to Common Carp (*Cyprinus carpio*)

```
gi|806515|dbj|BAA09069.1|  myosin heavy chain [ Cyprinus carpio]

        Length=955

Score =  104 bits (259),  Expect = 8e-22
Identities = 51/60 (85%), Positives = 56/60 (93%), Gaps = 0/60 (0%)

Query  1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL  60

VAKAK NLEKMCRTLEDQLSE+KTK+DENVRQ+ND++ QRARL TENGEF RQLEEKEAL

Sbjct  259
VAKAKANLEKMCRTLEDQLSEIKTKSDENVRQLNDMNAQRARLQTENGEFSRQLEEKEAL  318
```

Rainbow Trout compared to Bluefin Tuna (*Thunnus thynnus*)

```
gi|1339977|dbj|BAA12730.1|  skeletal myosin heavy chain [ Thunnus thynnus]

         Length=786

Score =  104 bits (259),  Expect = 8e-22
Identities = 49/60 (81%), Positives = 57/60 (95%), Gaps = 0/60 (0%)

Query  1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL  60

VAK+KGNLEKMCRT+EDQLSELK KNDE+VRQ+ND++GQRARL TENGEF RQ+EEK+AL

Sbjct  88
VAKSKGNLEKMCRTIEDQLSELKAKNDEHVRQLNDLNGQRARLQTENGEFSRQIEEKDAL  147
```

Chum Salmon compared to Zebra Fish

```
gi|68360600|ref|XP_708916.1|  PREDICTED: myosin, heavy polypeptide 1, skeletal muscle
[ Danio rerio]

        Length=2505

Score =  108 bits (269),  Expect = 6e-23
Identities = 52/60 (86%), Positives = 57/60 (95%), Gaps = 0/60 (0%)

Query  1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL  60

VAKAK NLEKMCRTLEDQLSE+K+KNDEN+RQ+ND+S QRARL TENGEFGRQLEEKEAL

Sbjct  1240
VAKAKANLEKMCRTLEDQLSEIKSKNDENLRQINDLSAQRARLQTENGEFGRQLEEKEAL  1299
```

Chum Salmon compared to Common Carp

```
gi|806515|dbj|BAA09069.1|  myosin heavy chain [ Cyprinus carpio]

         Length=955

Score =  104 bits (259),  Expect = 8e-22
Identities = 51/60 (85%), Positives = 56/60 (93%), Gaps = 0/60 (0%)

Query  1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL  60

VAKAK NLEKMCRTLEDQLSE+KTK+DENVRQ+ND++ QRARL TENGEF RQLEEKEAL

Sbjct  259
VAKAKANLEKMCRTLEDQLSEIKTKSDENVRQLNDMNAQRARLQTENGEFSRQLEEKEAL  318
```

Chum Salmon compared to Bluefin Tuna

```
gi|1339977|dbj|BAA12730.1|  skeletal myosin heavy chain [ Thunnus thynnus]

        Length=786

Score =  104 bits (259),  Expect = 8e-22
Identities = 49/60 (81%), Positives = 57/60 (95%), Gaps = 0/60 (0%)

Query  1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL  60

VAK+KGNLEKMCRT+EDQLSELK KNDE+VRQ+ND++GQRARL TENGEF RQ+EEK+AL

Sbjct  88
VAKSKGNLEKMCRTIEDQLSELKAKNDEHVRQLNDLNGQRARLQTENGEFSRQIEEKDAL  147
```

Zebra Fish compared to Common Carp

```
gi|806515|dbj|BAA09069.1|  myosin heavy chain [ Cyprinus carpio]

        Length=955

Score =  108 bits (271),  Expect = 4e-23
Identities = 53/60 (88%), Positives = 59/60 (98%), Gaps = 0/60 (0%)

Query  1
VAKAKANLEKMCRTLEDQLSEIKSKNDENLRQINDLSAQRARLQTENGEFGRQLEEKEAL  60

VAKAKANLEKMCRTLEDQLSEIK+K+DEN+RQ+ND++AQRARLQTENGEF RQLEEKEAL

Sbjct  259
VAKAKANLEKMCRTLEDQLSEIKTKSDENVRQLNDMNAQRARLQTENGEFSRQLEEKEAL  318
```

Zebra Fish compared to Bluefin Tuna

```
gi|1339977|dbj|BAA12730.1|  skeletal myosin heavy chain [ Thunnus thynnus]

        Length=786

Score =  102 bits (253),  Expect = 4e-21
Identities = 47/60 (78%), Positives = 57/60 (95%), Gaps = 0/60 (0%)

Query  1
VAKAKANLEKMCRTLEDQLSEIKSKNDENLRQINDLSAQRARLQTENGEFGRQLEEKEAL  60

VAK+K NLEKMCRT+EDQLSE+K+KNDE++RQ+NDL+ QRARLQTENGEF RQ+EEK+AL

Sbjct  88
VAKSKGNLEKMCRTIEDQLSELKAKNDEHVRQLNDLNGQRARLQTENGEFSRQIEEKDAL  147
```

Common Carp compared to Bluefin Tuna

```
gi|1339977|dbj|BAA12730.1|  skeletal myosin heavy chain [ Thunnus thynnus]

Length=786

Score =  104 bits (259),  Expect = 9e-22
Identities = 49/60 (81%), Positives = 57/60 (95%), Gaps = 0/60 (0%)

Query  1
VAKAKANLEKMCRTLEDQLSEIKTKSDENVRQLNDMNAQRARLQTENGEFSRQLEEKEAL  60

VAK+K NLEKMCRT+EDQLSE+K K+DE+VRQLND+N QRARLQTENGEFSRQ+EEK+AL

Sbjct  88
VAKSKGNLEKMCRTIEDQLSELKAKNDEHVRQLNDLNGQRARLQTENGEFSRQIEEKDAL  147
```

1. Construct a table of your data containing the number of amino acid differences between each of the different fish.

	Rainbow Trout	Chum Salmon	Zebra Fish	Common Carp	Bluefin Tuna
Rainbow Trout	**0**				
Chum Salmon	**X**	**0**			
Zebra Fish	**X**	**X**	**0**		
Common Carp	**X**	**X**	**X**	**0**	
Bluefin Tuna	**X**	**X**	**X**	**X**	**0**

2. Which two fish share the most amino acids in their myosin heavy chains based on your data?

3. Which two fish share the fewest amino acids?

4. Are there any fish that share more amino acids with each other than each does with the two fish in question one? If yes, which fish?

5. Construct a cladogram based on this data:

6. The myosin heavy chain of white croaker (*Pennahia argentata*) (BAB12571) has the following amino acid differences with the five fish above.

	Rainbow Trout	**Chum Salmon**	**Zebra Fish**	**Common Carp**	**Bluefin Tuna**
White Croaker	4	4	11	9	11

Add this fish to your cladogram and explain why you placed it where you did.

Taxonomic data can be derived from many sources: DNA sequences, protein sequences, morphology, and paleontology. Classification of organisms derives from these sources. Inconsistencies in the phylogenetic trees generated between molecular and taxonomic data emphasize why data from different sources is required to generate phylogenetic trees and why there is still much dispute in the field of phylogenetics on the correct placement of organisms within phylogenetic trees. Bear in mind that myosin heavy chain is around 1900 amino acids in length and our molecular data is based on a 60 amino acid region – just 3% of the entire protein. The amount of work required to process the small amount of data provided here also emphasizes the need for skilled bioinformaticists to process and analyze the vast amount of data generated by genomic and proteomic research.

Examine the taxonomic classification of the fishes below. The large phylogenetic tree figure will be useful for this exercise.

Rainbow Trout (*Oncorhynchus mykiss*)
Vertebrata; Euteleostomi; Actinopterygii; Neopterygii; Teleostei; Euteleostei; Protacanthopterygii; Salmoniformes; Salmonidae; Oncorhynchus.

Chum Salmon (*Oncorhynchus ket*a)
Vertebrata; Euteleostomi; Actinopterygii; Neopterygii; Teleostei; Euteleostei; Protacanthopterygii; Salmoniformes; Salmonidae; Oncorhynchus.

Zebra Fish (*Danio rerio*)
Vertebrata; Euteleostomi; Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Cypriniformes; Cyprinidae; Danio.

Common Carp (*Cyprinus carpio*)
Vertebrata; Euteleostomi; Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Cypriniformes; Cyprinidae; Cyprinus.

Bluefin Tuna (*Thunnus thynnus*)
Vertebrata; Euteleostomi; ctinopterygii; Neopterygii; Teleostei; Euteleostei; Neoteleostei; Acanthomorpha; Acanthopterygii; Percomorpha; Perciformes; Scombroidei; Scombridae; Thunnus.

White Croaker (*Pennahia argentata*)
Vertebrata; Euteleostomi; ctinopterygii; Neopterygii; Teleostei; Euteleostei; Neoteleostei; canthomorpha; Acanthopterygii; Percomorpha; Perciformes; Percoidei; Sciaenidae; Pennahia.

7. Construct a phylogenetic tree based on the taxonomic classification of the fishes above.

8. Does the taxonomic classification support the molecular data? Please explain your answer.

9. What reasons might there be for a discrepancy between the molecular data and the taxonomic classification.

10. Why do scientists need to examine multiple data sets before determining evolutionary relatedness?

Quick Guide to BLAST searching

Please note, this is a quick guide to obtain a list of fish myosin sequences, there are many refinements you can make to your search and many different ways to use BLAST searches. Further information can be found on the NCBI web site.

1) Go to http://www.ncbi.nlm.nih.gov/ and choose BLAST
2) Choose Protein BLAST.
3) Enter your myosin sequence into the search box.

Rainbow Trout Myosin Heavy Chain Protein Sequence (CAA88724):

VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL

4) Leave the other fields as found and hit the BLAST button.
5) A new window should pop up. Hit the Format button.
6) After a short wait the BLAST results window will come up and may well be hundreds of pages long — don't worry. There should be a long list of sequences that produced significant alignments. Although the search may pick up hundreds of sequences, they are in order of homology, so the ones you are interested in should be in the first 25 or so.
7) Further down the BLAST results page, after the list of sequences, each sequence will be aligned with the original trout sequence (as shown in the example) so that you can see how the two compare.
8) To compare your second fish, say bluefin tuna, with the other fish, you must perform a second BLAST search with the tuna sequence to obtain the protein alignments of tuna with the other fish. Alternatively, you can align 5 protein sequences yourself from your original search in a word processing document (use Courier font, this aligns sequences because all the letters are the same width) and have your students manually compare them.

Appendix C: Glossary

Actin – major muscle protein organized into thin filaments

Amino acids – basic building blocks of proteins

Anode – positive electrode

Bioinformatics – use of data storage and analysis technologies to extract meaningful information from large quantities of biological data

BME (β-mercaptoethanol) – a reducing agent that breaks disulfide bonds

Cathode – negative electrode

Charge density – ratio of charge to mass of a protein

Cladogram – tree-like relationship-diagrams that demonstrate the evolutionary relatedness between organisms

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 x 10–24 g

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

DTT (dithiothreitol) – a reducing agent that breaks disulfide bonds

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

Gel electrophoresis – technique used to separate molecules that carry electric charges. The molecules separate from each other according to the different rates at which they migrate through an electric field set up in a gel soaked in a chemical solution.

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

Genomics – the study of all the nucleotide sequences in the chromosomes of 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) – 1,000 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

Polypeptide – a chain of amino acids

Posttranscriptional modification – alterations to mRNA that allow one gene to code for many proteins, such as alternate splicing

Posttranslational modification – alterations of proteins after they are synthesized by the cell, such as phosphorylation or cleavage

Protein – a functional assembly of one or more polypeptides, made of sequences of amino acids

Protein folding – the process by which a protein bends and twists to achieve its normal three-dimensional shape

Proteome (protein complement expressed by a genome) – the complete protein profile found under given conditions in a biological sample

Proteomics – the study of the proteome in specific cells, tissues, organs, organ systems, or organisms during a specific time period (e.g., during development)

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 which acts as adaptor molecule between mRNA and an amino acid

APPENDIX C

Appendix D: Teacher Answer Guide

Student Questions – Pre-Lab Activity

Answers to this activity will vary depending on the types of fish chosen.

Focus Questions – Alternative Pre-Lab Activity

1. Name three proteins found in muscle

Potential answers include: titin, dystrophin, filamin, myosin heavy chain, spectrin, M1/M2, M3, C protein, nebulin, α-actin, gelsolin, fimbrin, actin, tropomyosin, troponin (T), myosin light chains, troponin (I), troponin (C), thymosin.

Answers to questions 2–4 will vary depending on the types of fish chosen.

Focus Questions – Lesson 1

1. Why did you add Laemmli sample buffer to your fish samples?

The Laemmli sample buffer has multiple functions. A buffering agent helps keep the sample at the correct pH. SDS is a detergent that coats each protein in a uniform series of negative charges so that the proteins can migrate according to size rather than their charge. Glycerol makes the sample denser than the running buffer so the sample will not easily float out of the well. Bromophenol Blue is a dye that provides a visual for tracking movement through the gel. DTT is a reducing agent that breaks disulfide bonds allowing the protein to be completely unfolded and prevents peptide chains from binding to each other.

2. What was the purpose of heating the samples?

The heat helps to denature the protein tertiary and quaternary structures, so that the proteins become less three dimensional and more linear.

3. How are the proteins extracted from the fish samples?

The Laemmli sample buffer contains detergent that breaks open the cell membranes of the muscle cells. By flicking the tubes, the muscle tissue was also mechanically disrupted. These two things released proteins from the muscle tissue.

4. Have all the proteins been extracted from the fish sample or some still left after the extraction? How could you test your hypothesis?

Not all proteins will be extracted during this process. You can test this by adding more sample buffer to the fish sample, repeating the extraction steps, and then separating them by SDS-PAGE.

Focus Questions – Lesson 2

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

SDS is a negatively charged molecule that sticks to the polypeptide chain and adds a negative charge to the protein that is proportional to its length. The SDS-coated proteins move away from the negative charge towards the positive charge.

2. What is the purpose of the actin & myosin standards and the Precision Plus Protein Kaleidoscope prestained standard?

The actin & myosin standard contains two common muscle proteins that have already been isolated. They serve as a good reference to locate actin and myosin proteins in the samples

and act as a positive control for gel analysis. The proteins in the Precision Plus Protein Kaleidoscope prestained standard have known molecular weights. The prestained standard provides a size reference so that a standard curve can be drawn. Sample proteins are then compared to this standard curve to calculate the molecular weights of the unknown proteins in the muscle samples.

3. Which proteins will migrate farthest? Why?

The smallest proteins will migrate the furthest because they are able to move through the gel matrix more easily.

4. What is the purpose of the stain?

Proteins are invisible in the gel. The stain contains dye molecules that stick to the proteins and make them visible.

Focus Questions – Lesson 3

Answers to questions 1, 2, and 4 will vary depending on the types of fish chosen.

3. Give an explanation for why you think the protein profiles of some fish species share more bands than other fish species.

Variations in an organism's muscle protein profile may reflect refinements of muscle function and performance that are adaptive to particular niches, environments, or physiological stresses.

Student Questions - Appendix B

1. Construct a table of your data containing the number of amino acid differences between each of the different fish.

	Rainbow Trout	Chum Salmon	Zebra Fish	Common Carp	Bluefin Tuna
Rainbow Trout	0	0	8	9	11
Chum Salmon	X	0	8	9	11
Zebra Fish	X	X	0	7	13
Common Carp	X	X	X	0	11
Bluefin Tuna	X	X	X	X	0

2. Which two fish share the most amino acids in their myosin heavy chains based on your data?

Trout and salmon

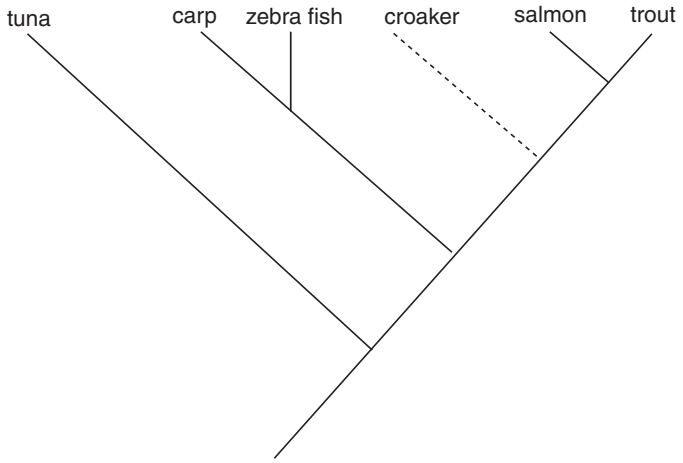
3. Which two fish share the fewest amino acids?

Tuna and zebra fish

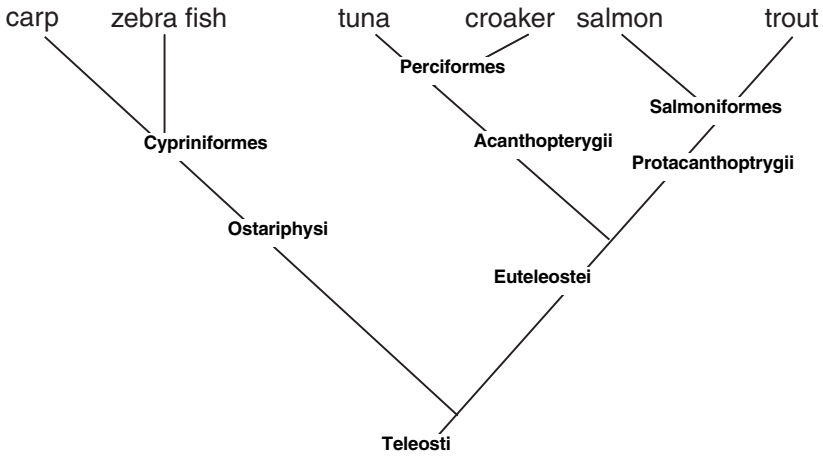
4. Are there any fish that share more amino acids with each other than to than to the two fish in question one? If yes, which fish?

Yes, carp and zebra fish

5. and 6. Construct a cladogram based on this data:



7. Construct a phylogenetic tree based on the taxonomic classification of the fishes above.



8. Does the taxonomic data support the molecular data? Please explain your answer.

The trees do not entirely match. Both trees show a close relationship between salmon and trout, and zebra fish and carp. However, tuna is in the same sub-phylum (Euteleostei) as salmon and trout, yet this does not concur with the molecular data and croaker is in the same order as tuna (Perciformes) and yet the amino acid sequence of croaker's myosin is much closer to salmon than tuna.

9. What reasons might there be for a discrepancy between the molecular data and the taxonomic classification?

The taxonomic classification is based on many types of data: morphological, developmental and molecular, while our molecular data is based on a 60 amino acid sequence from a 1900 amino acid long protein and so is a very limited data set.

10. Why do scientists need to examine multiple data sets before determining evolutionary relatedness?

The statistical relevance of data grows as the size of the data set increases. The 60 amino acid segment of myosin heavy chain constitutes just 3% of the myosin heavy chain molecule, which is around 1,900 amino acids long. Performing a BLAST search with a larger portion of the molecule generates a cladogram with different relationships, demonstrating that the 60 amino acid piece is not large enough to provide a full picture of relatedness. However, even if the full-length myosin were compared, that is just a single protein out of the thousands generated by the organism. The data would be much stronger if the sequences of multiple proteins were compared and stronger still if molecular data were used with other types of classification data such as morphological data.

Appendix E: References

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

Clamp M, Fry B, Kamal M, Xie X, Cuff J, Lin MF, Kellis M, Lindblad-Toh K and Lander ES. Distinguishing protein-coding and non-coding genes in the human genome, PNAS 104 (49), 19428–19433 (2007)

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)

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

Maas S and Rich A, Changing Genetic Information Through RNA Editing, BioEssays 22, 790–802 (2000)

Further Reading: Books and Journal Articles

Alberts et al., Molecular Biology of the Cell, 4th edition, Garland Publishing, Inc., New York (2002)

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

http://www.tolweb.org
Tree of Life Web Project from University of Arizona

http://evolution.berkeley.edu/evosite/evohome.html
Understanding Evolution for Teachers from UC Berkeley

http://www.ncbi.nlm.nih.gov/
National Institutes of Health National Center for Biotechnology Information – Bioinformatics Databases

http://workbench.sdsc.edu
Bioinformatics tools from UC San Diego

http://www.hupo.org
Human Proteome Organisation (HUPO)

www.FishBase.net
Database of fish species

http://animaldiversity.ummz.umich.edu
Animal diversity website from the University of Michigan, Museum of Zoology

Notices

BIO-RAD

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *800 424 6723* ***Australia*** *61 2 9914 2800* ***Austria*** *01 877 89 01* ***Belgium*** *09 385 55 11* ***Brazil*** *55 31 3689 6600*
Canada *905 364 3435* ***China*** *86 21 6169 8500* ***Czech Republic*** *420 241 430 532* ***Denmark*** *44 52 10 00* ***Finland*** *09 804 22 00*
France *01 47 95 69 65* ***Germany*** *089 31 884 0* ***Greece*** *30 210 777 4396* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 459 6100* ***India*** *91 124 4029300*
Israel *03 963 6050* ***Italy*** *39 02 216091* ***Japan*** *03 6361 7000* ***Korea*** *82 2 3473 4460* ***Malaysia*** *60 3 2117 5260* ***Mexico*** *52 555 488 7670*
The Netherlands *0318 540666* ***New Zealand*** *64 9 415 2280* ***Norway*** *23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700*
Russia *7 495 721 14 04* ***Singapore*** *65 6415 3170* ***South Africa*** *27 861 246 723* ***Spain*** *34 91 590 5200* ***Sweden*** *08 555 12700*
Switzerland *061 717 95 55* ***Taiwan*** *886 2 2578 7189* ***Thailand*** *66 2 6518311* ***United Kingdom*** *020 8328 2000*

Sig 0211

10004530 Rev F