Student Manual

Student Pre-laboratory Background Considerations

Consideration 1 – Is There Molecular Evidence for Evolution?

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

The Modern Theory of Evolution

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

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

Is There Proof?

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

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

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

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

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

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

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

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

Consideration 2 - What Are Proteins?

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

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

Example – Sickle Cell Anemia

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

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

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

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

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

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

The Transfer of Genetic Information: Transcription and Translation

β-globinA (β^A), wild type

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

β-globinA (β^S), sickle-cell mutant

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

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

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

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

Muscle Proteins – Universal Soldiers

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

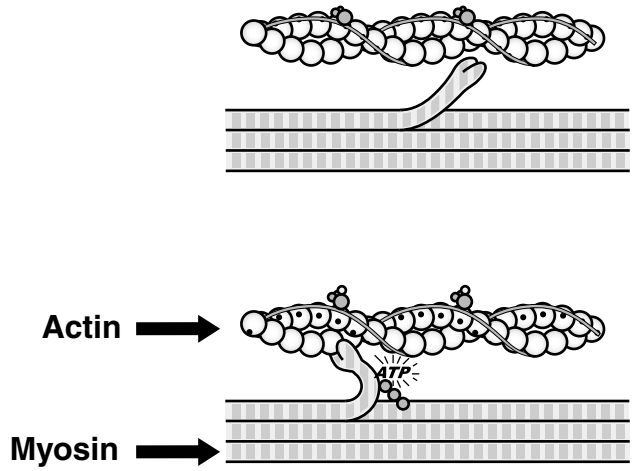
Characteristics of Actin and Myosin

Actin:

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

Myosin:

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



(Figure modified from Campbell 1996 with permission)

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

The Evolution of New Functions

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

Focus Questions

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

2. **Can you name 10?**

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

Consideration 3 – How Do You See a Protein Molecule?

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

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

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

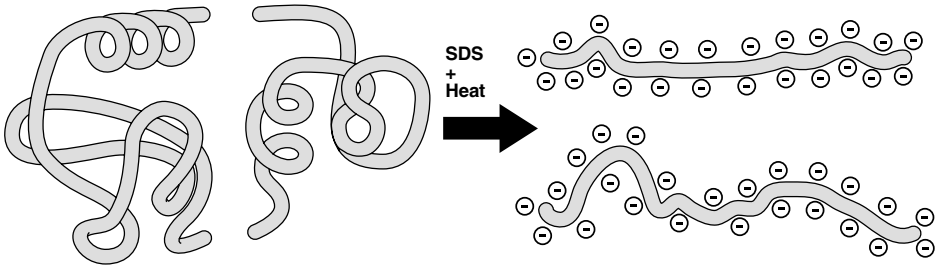


Figure 16. The combination of heat and the detergent SDS denatures proteins for SDS-PAGE analysis.

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

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

Consideration 4 - What Can Electrophoresis Tell Us about Proteins?

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

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

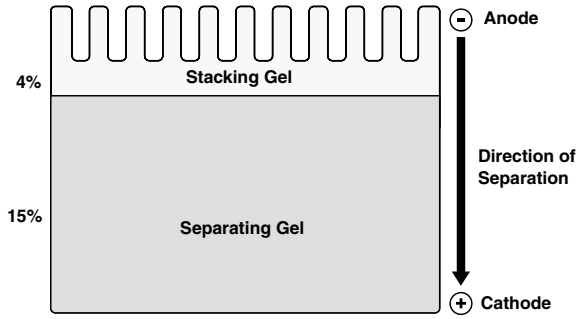


Figure 17. A simplified diagram of a polyacrylamide gel. The glass plates and supports that hold the gel upright are not shown. Mixtures of proteins are loaded into the "wells" at the top, and when the electric current is turned on, the proteins migrate through the gel, toward the bottom.

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

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

Determining the Sizes of Proteins via Electrophoresis

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

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

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

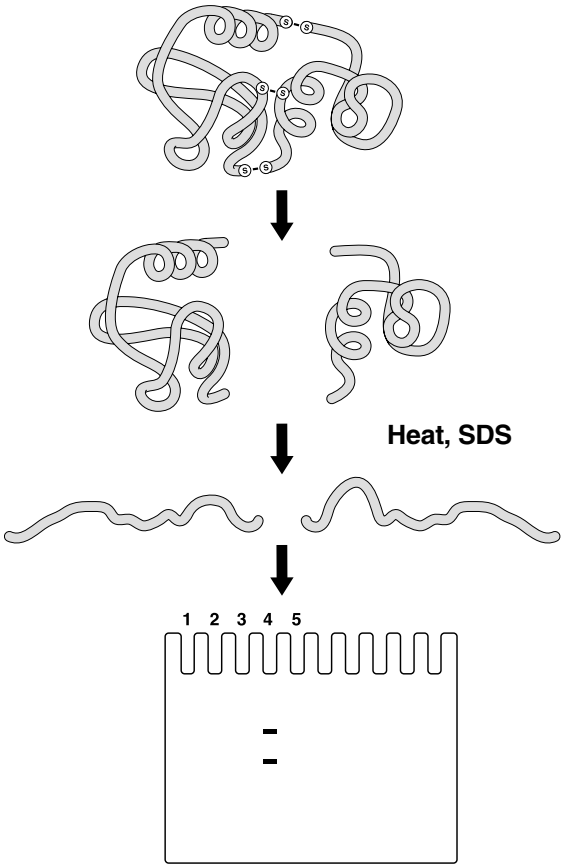


Figure 18. As soon as the electric current is applied, the SDS-coated proteins begin their race toward the positive electrode. The smaller proteins can move through the gel more quickly than the larger ones, so over time, the proteins will be separated according to their sizes.

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

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

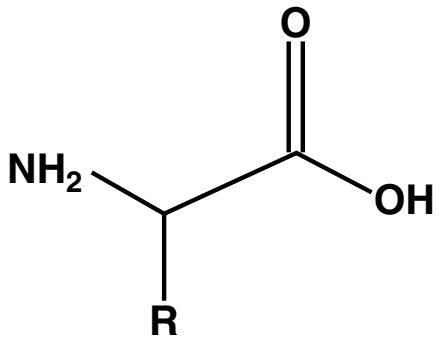


Figure 19. Chemical structure of an amino acid.

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

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

Consideration 5 – What Can Proteins Tell Me about Evolution?

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

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

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

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

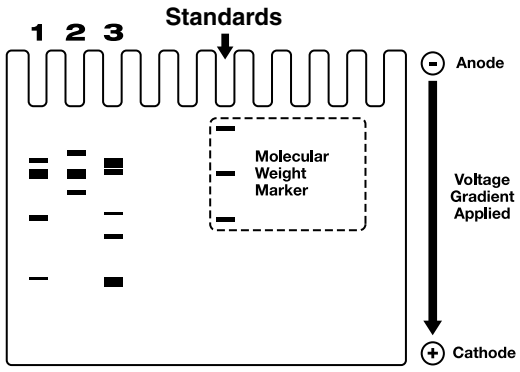
   Lane 2 is loaded with a mixture of 3 proteins composed of 200 amino acids, 250 amino acids, and 350 amino acids.

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

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

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

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



Consideration 6 - Are Proteins a Window to DNA?

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

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

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

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

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

Focus Questions:

1. How many mRNAs are produced by each gene?

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

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

Let's review...

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

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

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

3. **Changes in DNA produce:**

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

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

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

5. Look at the evolutionary tree below.

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

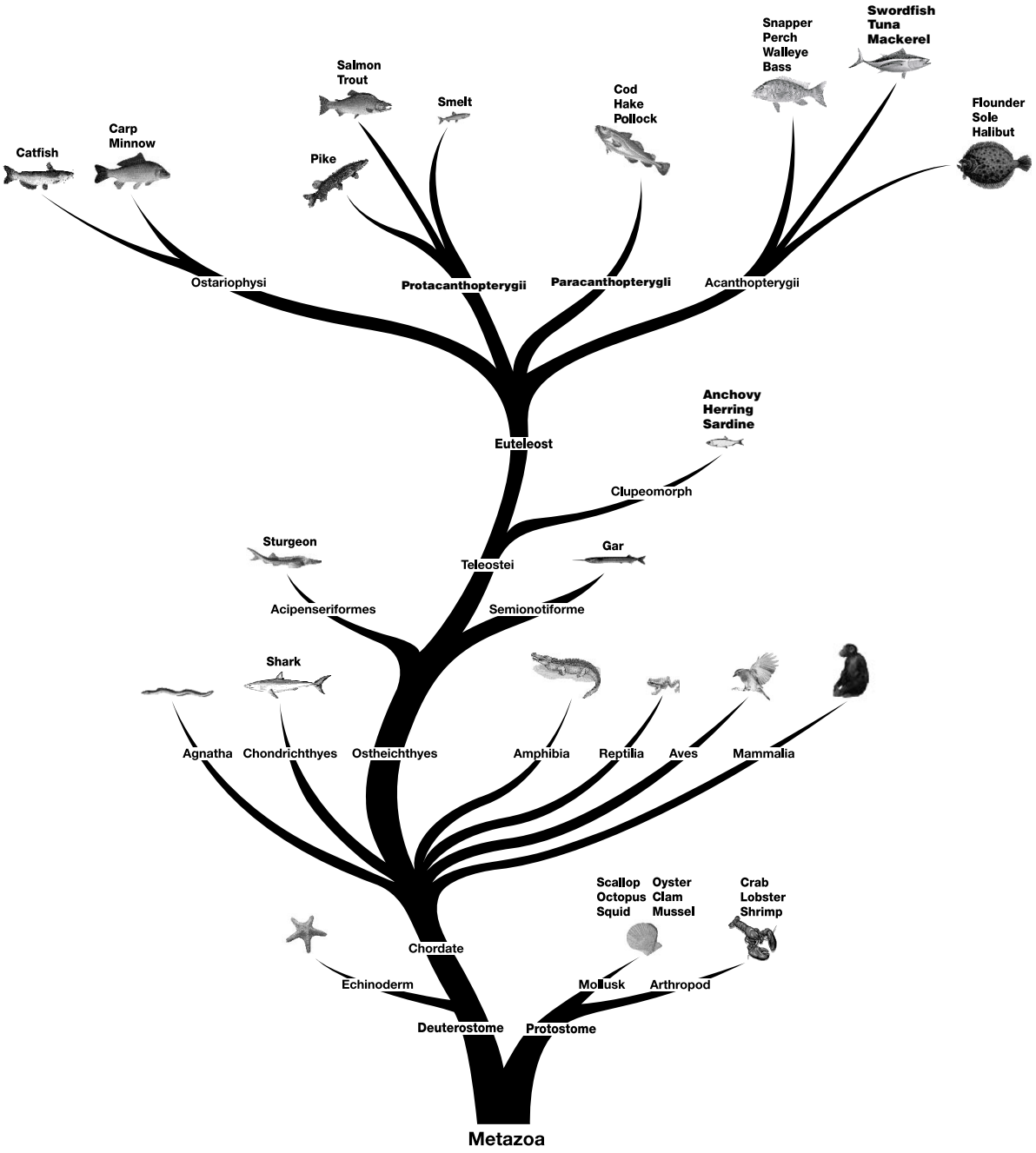


Figure 20. Fish evolutionary tree based on Ichthylogy Web Resources and the Tree of Life.

Evolutionary Tree of Fishes

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

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

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

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

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

Laboratory Day 1

What Is the Question?

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

Do Biomolecules Reflect Evolutionary Relationships?

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

Laboratory Day 1 - Sample Preparation: Muscle Protein Extraction

Purpose of this laboratory:

Prepare protein extracts of muscle tissue from fish samples

Procedure Overview:

1. Extract proteins from tissue
2. Denature proteins

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

Laboratory Checklist (✔)

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

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

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

Laboratory Day 1

Step 1: Sample Preparation – Muscle Protein Extraction

Procedure

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

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

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

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

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

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

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

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

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

Focus Questions:

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

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

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

Laboratory Day 2

Electrophoresis

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

Electrophoresis: gel loading, running, and staining

Purpose of this laboratory:

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

Procedure Overview:

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

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

Laboratory Checklist (✔)

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

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

**You may already have aliquots at student stations

Laboratory Day 2

Step 1: Prepare Samples, Electrophoresis Gels and Gel Boxes

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

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

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

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

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

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

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

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

Laboratory Day 2

Step 2: Load and run gels

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

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

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

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

Record your samples here:

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

Laboratory Day 2

Step 3: Stain and Visualize the Proteins

Gel staining:

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

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

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

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

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

Focus Questions

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

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

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

Laboratory Day 3

Gel Preservation

Purpose of this laboratory:

Examine data and make a permanent record

Procedure Overview:

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

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

Laboratory Checklist (✔)

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

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

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

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

GelAir drying frame method:

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

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

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

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

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

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

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

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

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

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

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

Interpreting the bands collectively: Qualitative comparisons of protein profiles

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

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

3. What are possible explanations for this variation?

4. Which samples are most alike?

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

Post Laboratory - Quantitative Analysis of Data

The most similar species:

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

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

   _____ and _____

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

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

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

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

The least similar species:

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

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

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

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

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

Interpretation of Individual Bands

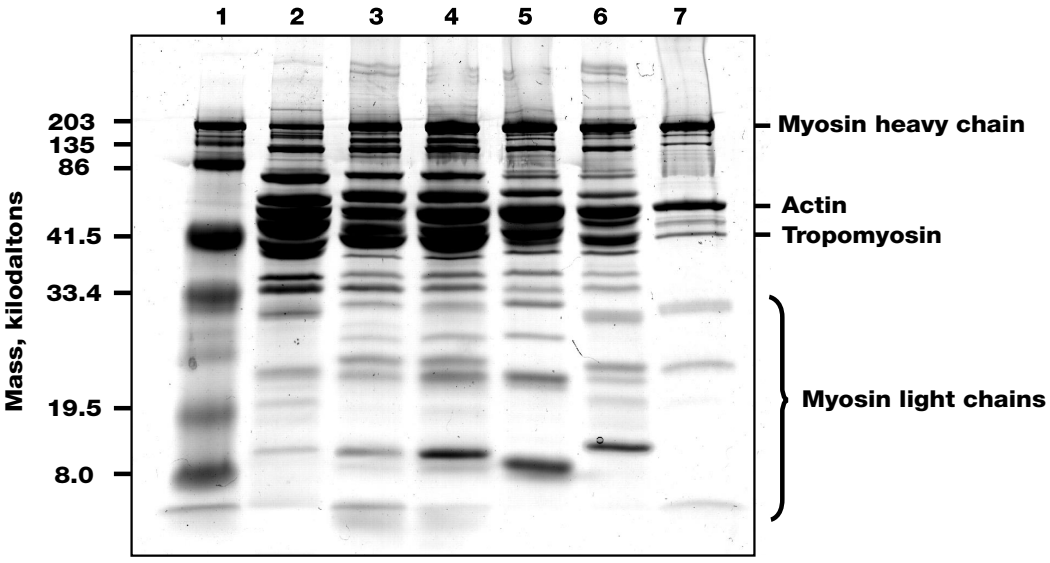
1. What do the relative positions of the bands on the gel indicate about the proteins in the bands?

2. Are all of the bands of equal thickness?

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

Determining the Molecular Weights of Proteins

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



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

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

Construct Your Own Standard Curve

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

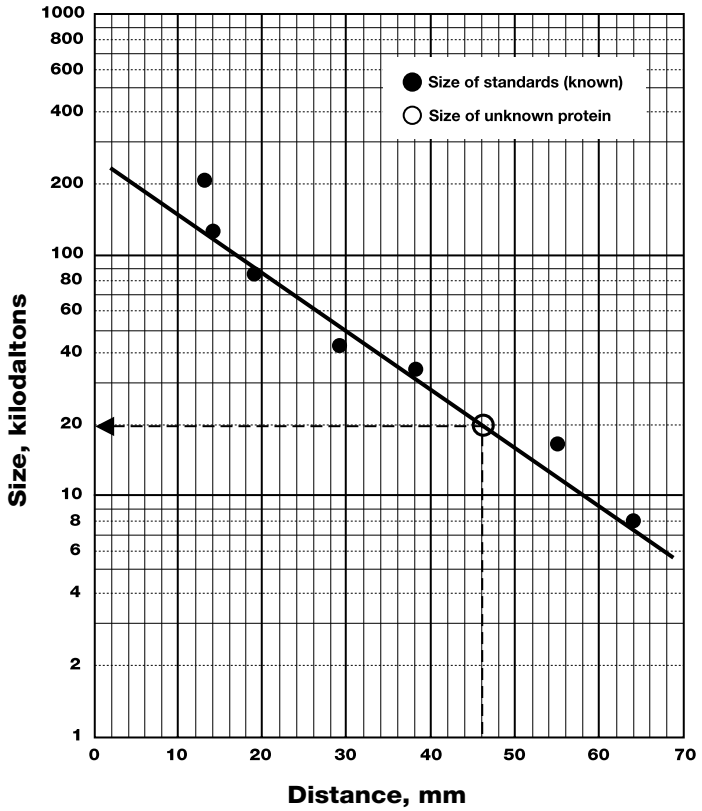
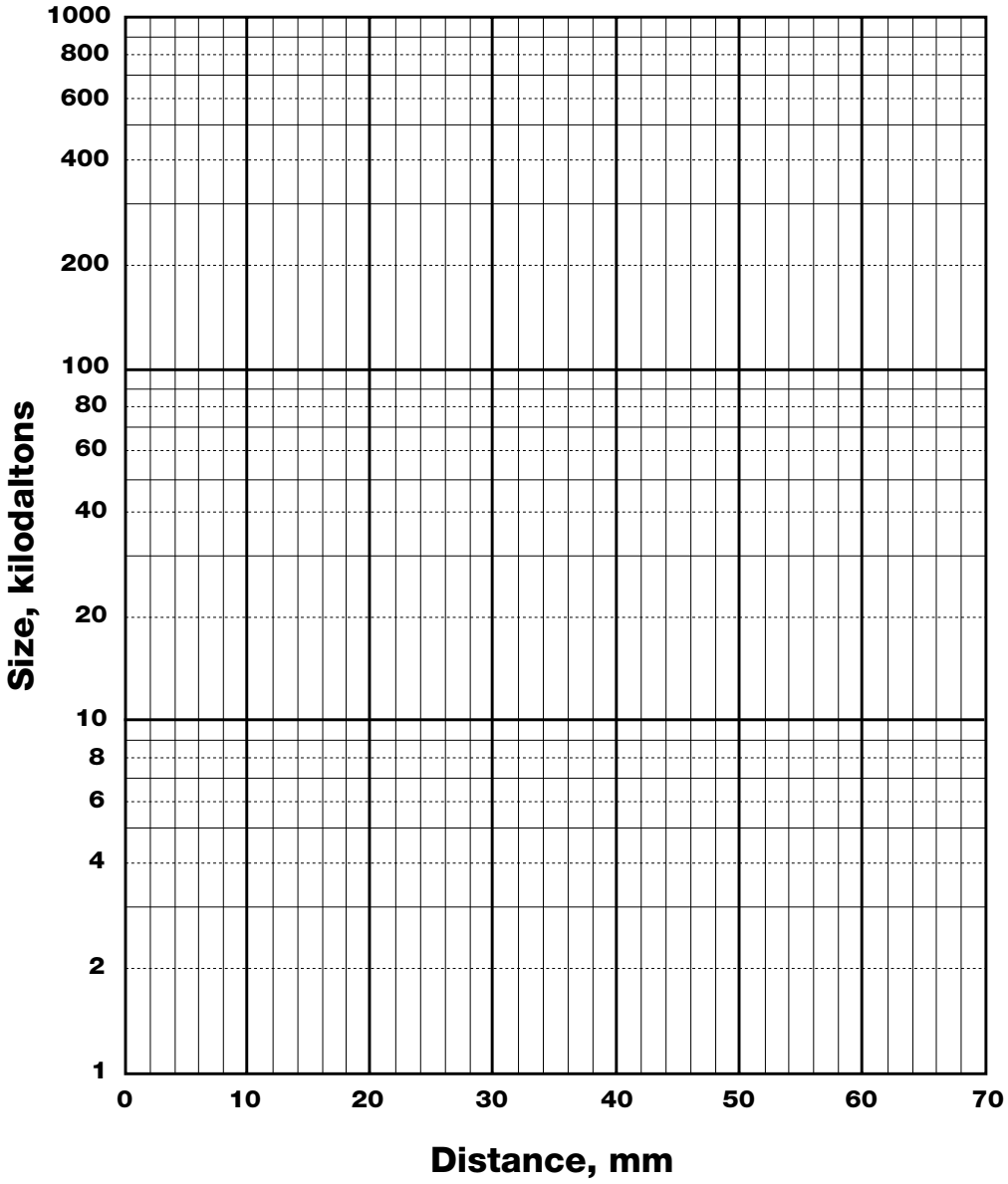


Figure 22. The distance migrated by each prestained protein was plotted against its size on semi-log graph paper to generate a standard curve for the gel.

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

Use the semi-log paper provided here:

Semi-Log Graph Paper



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

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

   Actin: _____ Myosin: _____

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Conclusions

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

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

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

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

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

Glossary

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

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

Appendix: SDS-PAGE Chemistry

Why This Technique Works So Well...

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

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

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

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

Casting Polyacrylamide Gels

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

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

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Notices