GelAir drying frame method:

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

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

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

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

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

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

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

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

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

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

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

Interpreting the bands collectively: Qualitative comparisons of protein profiles

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

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

3. What are possible explanations for this variation?

4. Which samples are most alike?

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

Post Laboratory - Quantitative Analysis of Data

The most similar species:

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

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

   _____ and _____

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

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

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

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

The least similar species:

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

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

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

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

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

Interpretation of Individual Bands

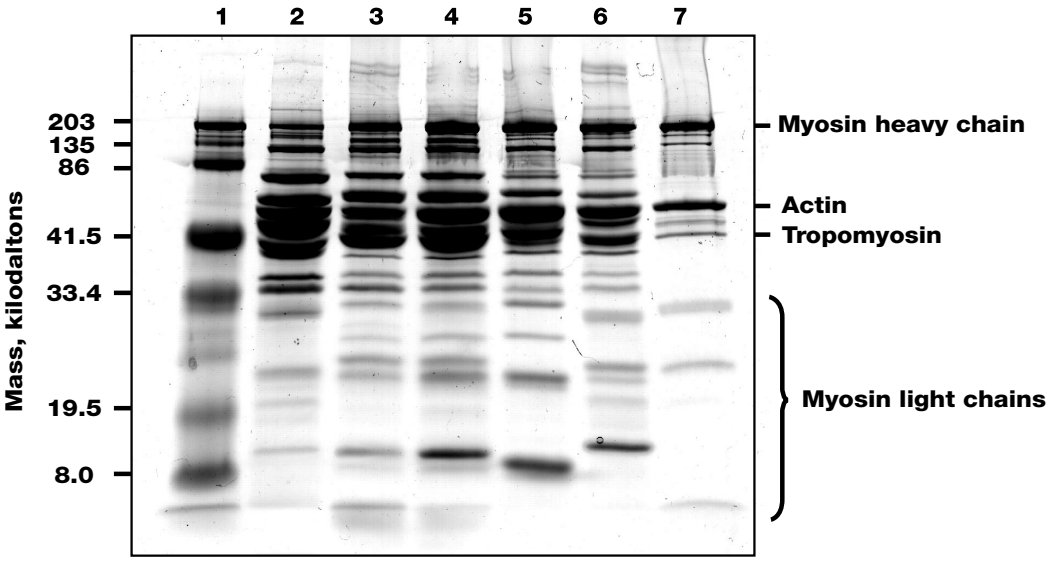
1. What do the relative positions of the bands on the gel indicate about the proteins in the bands?

2. Are all of the bands of equal thickness?

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

Determining the Molecular Weights of Proteins

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



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

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

Construct Your Own Standard Curve

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

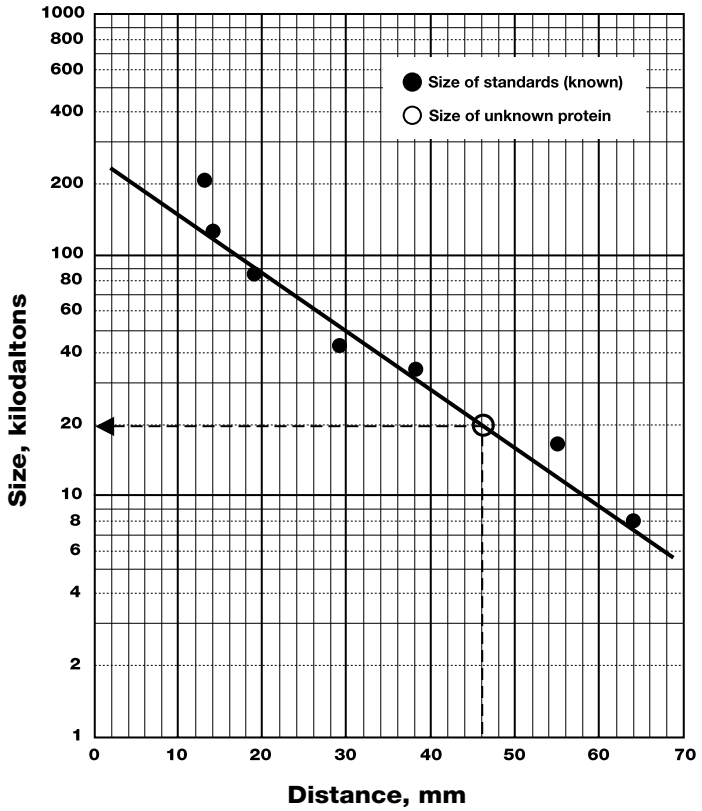
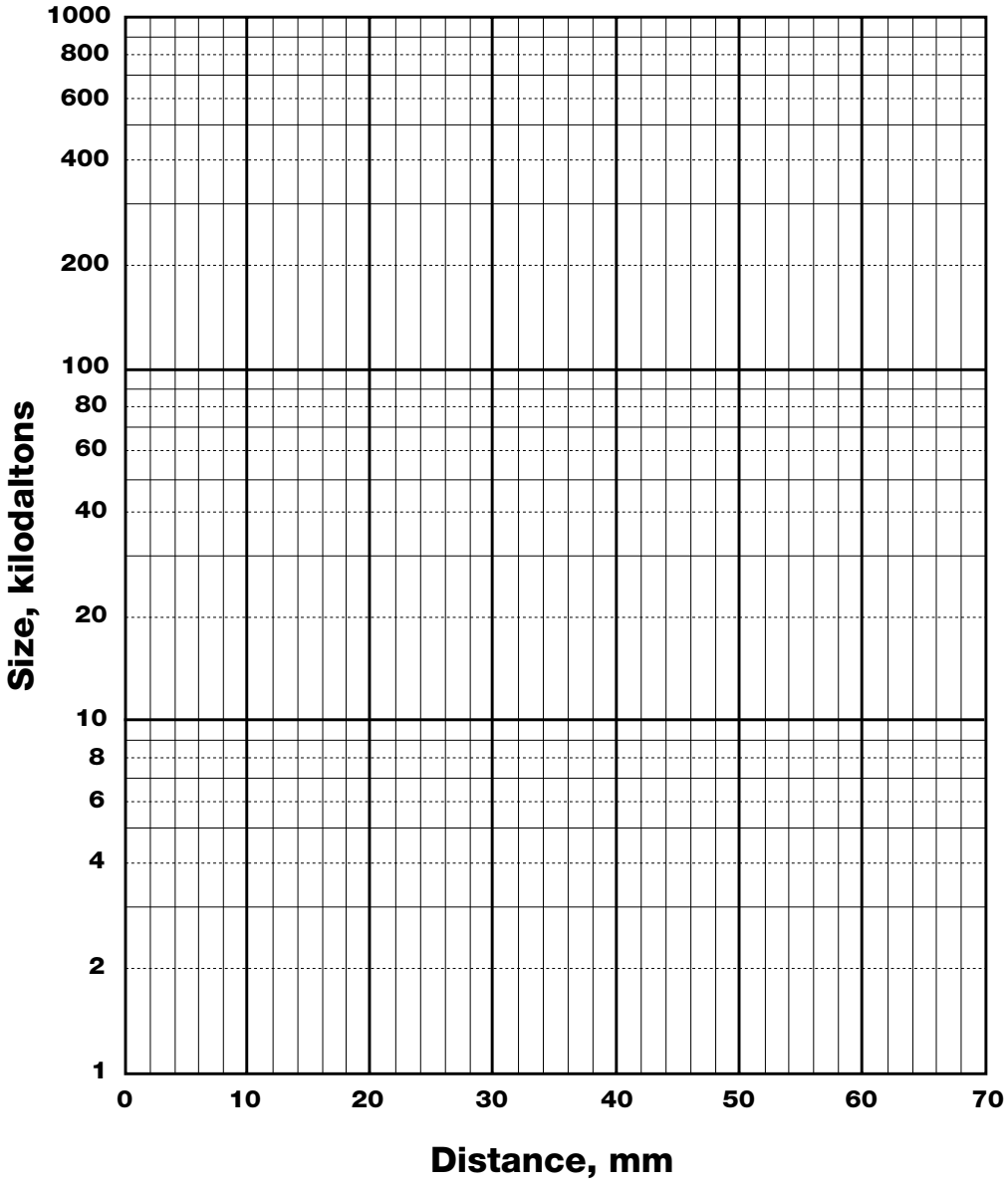


Figure 22. The distance migrated by each prestained protein was plotted against its size on semi-log graph paper to generate a standard curve for the gel.

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

Use the semi-log paper provided here:

Semi-Log Graph Paper



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

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

   Actin: _____ Myosin: _____

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Conclusions

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

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

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

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

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

Glossary

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

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

Appendix: SDS-PAGE Chemistry

Why This Technique Works So Well...

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

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

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

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

Casting Polyacrylamide Gels

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

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

References:

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

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

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

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

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

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

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

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

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

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

Further Reading:

Books and journal articles

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

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

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

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

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

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

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

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