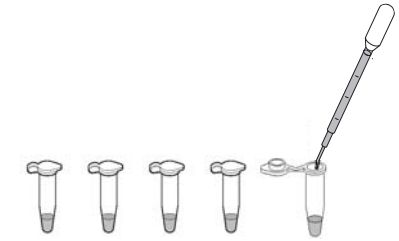
Comparative Proteomics Kit II: Western Blot Module – Quick Guide

Lesson 1 Quick Guide



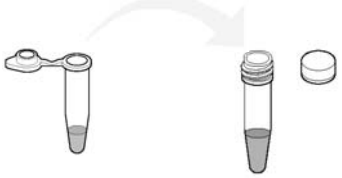
1 Label one 1.5 ml **fliptop** micro tube for each of five fish samples. Also label one **screw-cap** micro tube for each fish sample.

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 **screw-cap** microtube. Do not transfer the fish!

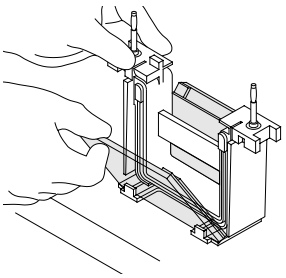
7. Heat the fish samples in screw-cap microtubes for 5 minutes at 95°C.

Lesson 2 Quick Guide

1. Set up Mini-PROTEAN 3 gel box.

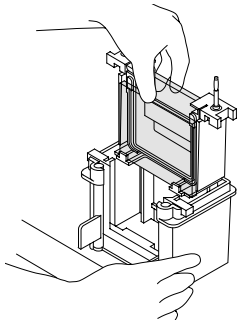
2. Prepare a Ready Gel cassette by cutting along the black line on the bottom of the cassette with a razor blade and pulling off the plastic strip, as indicated on gel cassette.

3. Remove the comb from the Ready Gel cassette.

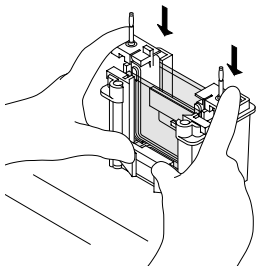


4. Place Ready Gel cassette into the electrode assembly with the short plate facing inward. Place a buffer dam or another Ready Gel cassette on the opposite side of the electrode assembly, with notch on buffer dam facing inward.

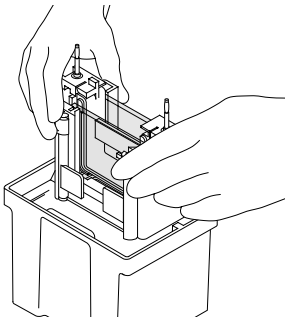
5. Slide gel cassette, buffer dam, and electrode assembly into the clamping frame.



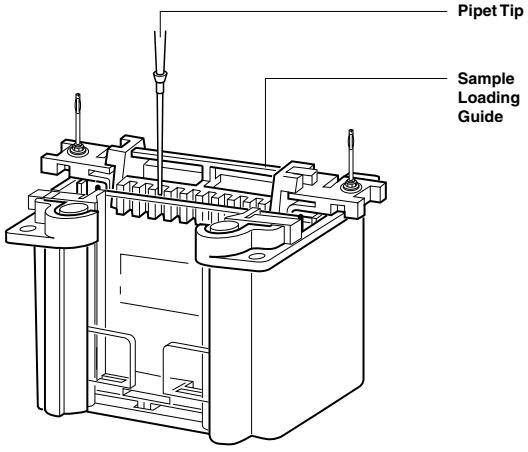
6. Press down the electrode assembly while closing the two cam levers of the clamping frame.



7. Lower the inner chamber into the mini tank.



8. Completely fill the inner chamber with 1x TGS gel running buffer, making sure the buffer covers the short plate (~150 ml).
9. Fill mini tank approximately 200 ml of 1x TGS gel running buffer.

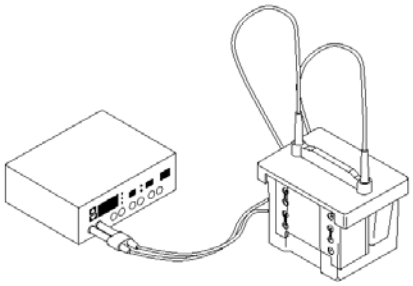


10. Plate sample loading guide on top of the electrode assembly.

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

12. Load your gel:

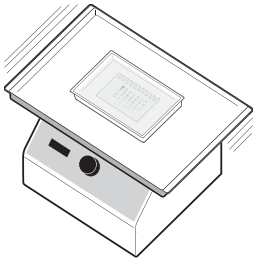
Lane	Volume	Sample
1 & 2	empty	Empty
3	5 µl	Precision Plus Protein Kaleidoscope prestained standards
4	5 µl	fish sample 1
5	5 µl	fish sample 2
6	5 µl	fish sample 3
7	5 µl	fish sample 4
8	5 µl	fish sample 5
9	5 µl	actin and mysin standard (AM)
10	empty	empty



13. Electrophorese for 30 minutes at 200 V in 1x TGS gel running buffer.

14. Proceed directly to Lesson 3, continue to step 15 of Lesson 2 to stain gels or store gels overnight at 4°C.

15. If the gels are to be stained, save 50 ml of 1x TGS gel running buffer.



16. Remove gel from cassette and transfer gel to a container with 25 ml Bio-Safe Coomassie stain/per gel and stain gel for 1 hour, with gentle shaking for best results.

17. Discard stain and destain gels in a large volume of water for at least 30 minutes to overnight, changing the water at least once. Blue-stained bands will be visible on a clear gel after destaining.

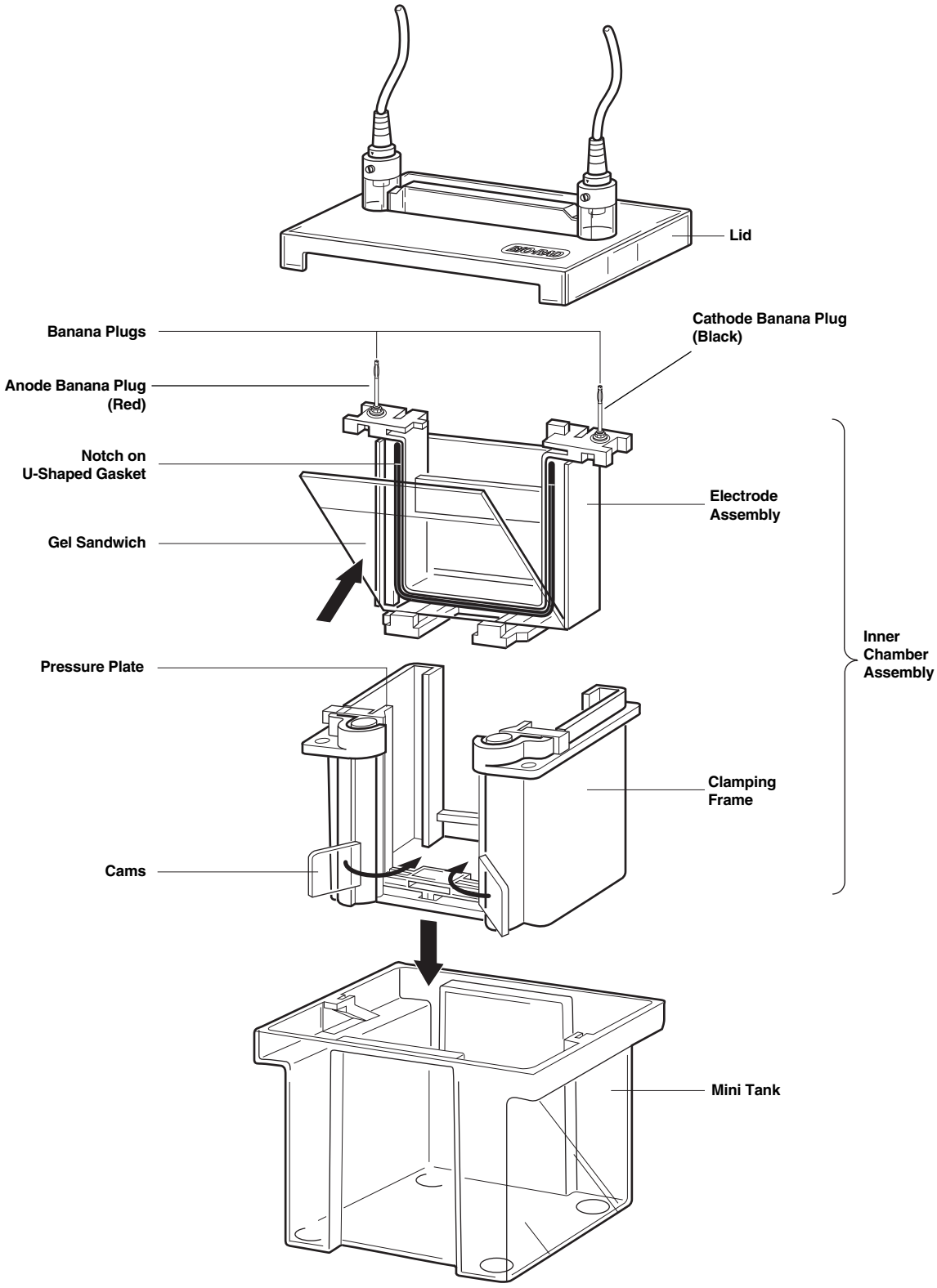
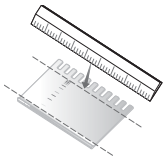
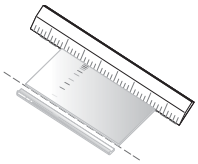
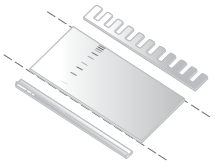


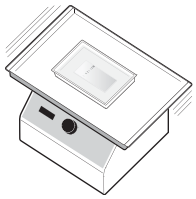
Fig. 4. Assembling the Mini-PROTEAN 3 cell.

Lesson 3 Quick Guide

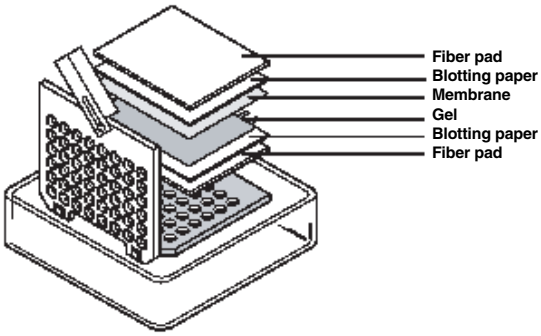
1. Using a ruler, chop the top and bottom off the gel.

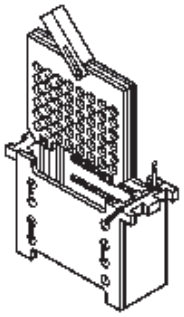
2. Equilibrate the gel in blotting buffer for 15 minutes on a rocking platform.



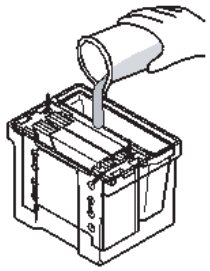
3. Soak fiber pads thoroughly in blotting buffer.
4. Mark the white nitrocellulose membrane with penciled (or black ball point pen) initials and prewet in blotting buffer along with the blotting paper.
5. Make the blotting sandwich:
   a. Add 1 cm depth of blotting buffer to container and insert plastic cassette with black side down.
   b. Lay a wet fiber pad on the black side of the cassette.
   c. Lay one wet blotting paper on the fiber pad and roll out air bubbles.
   d. Lay gel squarely on blotting paper and roll out air bubbles.
   e. Lay wet nitrocellulose membrane on the gel and roll out air bubbles.
   f. Lay one wet blotting paper on the membrane and roll out air bubbles.
   g. Lay a wet fiber pad on top of the blotting paper.



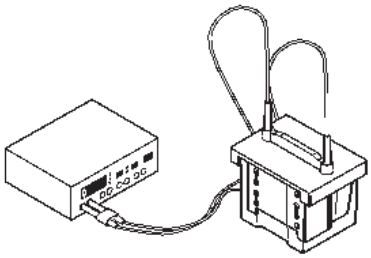
   h. Close the cassette and clamp together with the white clip.



6. Set up the Mini Trans-Blot module with the black side of the cassette next to the black side of the Mini Trans-Blot module. Add a frozen Bio-Ice module and fill with blotting buffer up to the white clip.



7. Place lid on tank, matching the power cords red-to-red and black-to-black, then blot at 20 V for 2.5 hours.



8. At this point the blots can be stored in the tanks submerged in blotting buffer at room temperature overnight or the sandwiches dismantled and the blots placed in blocker overnight at 4°C.

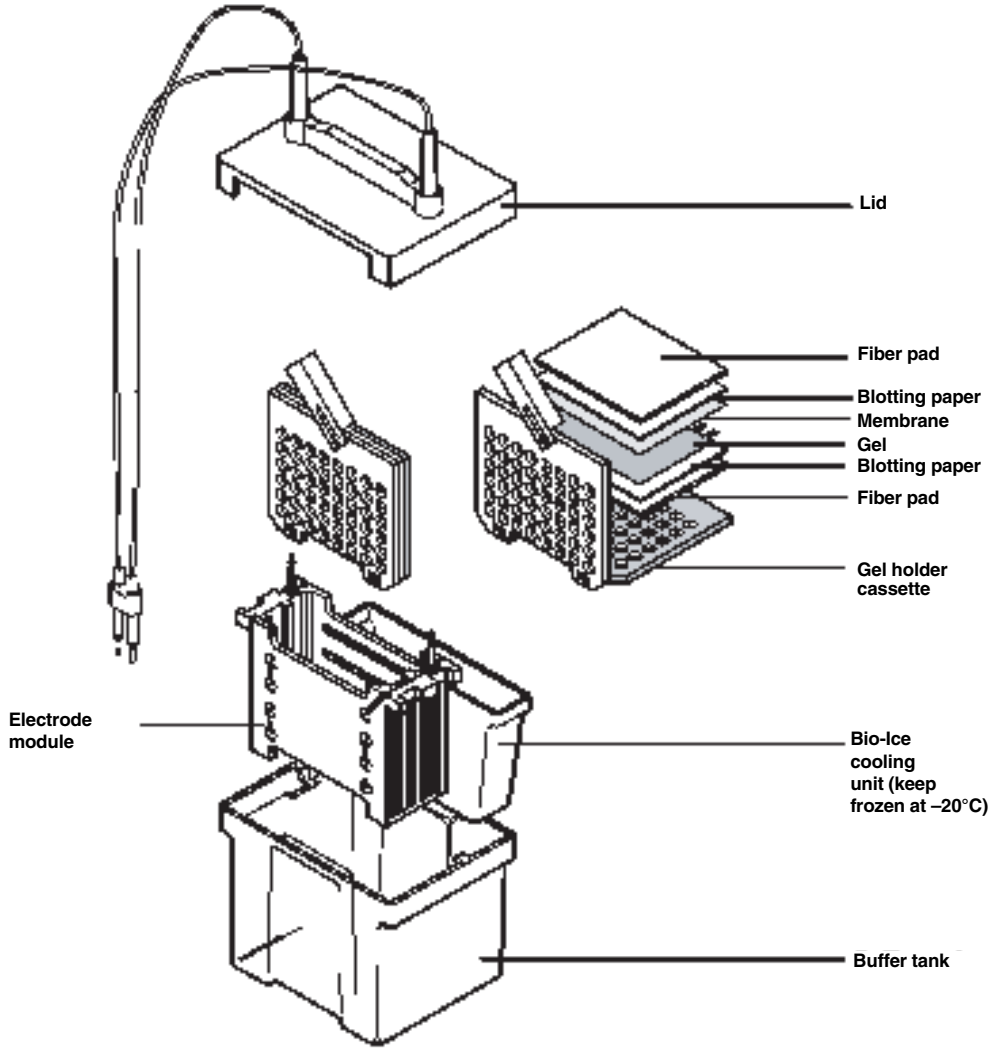
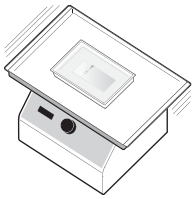


Fig. 5. Assembly of the Mini Trans-Blot cell.

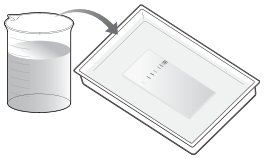
Lesson 4 Quick Guide

1. If not blocked overnight, immerse membrane in 25 ml blocking solution for 15 minutes to 2 hours at room temperature on a rocking platform.



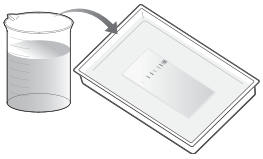
2. Discard blocking solution and incubate membrane with 10 ml of primary antibody for 10–20 minutes on rocking platform set to a faster setting to ensure constant coverage of the membrane.

3. Quickly rinse the membrane in 50 ml of wash buffer then discard the wash.
4. Add 50 ml of wash buffer to membrane for 3 minutes on rocking platform at a medium speed setting.



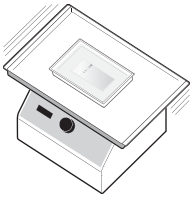
5. Discard the wash and incubate membrane with 10 ml of secondary antibody for 5–15 minutes on rocking platform set to a fast setting.

6. Quickly rinse the membrane in 50 ml of wash buffer and discard the wash.
7. Add 50 ml of wash buffer and wash membrane for 3 minutes on rocking platform on a medium speed setting.



8. Discard the wash and add 10 ml of HRP color detection reagent.

9. Incubate 10–30 minutes, either with manual shaking or on a rocking platform, and watch the color development.
10. Rinse the membrane twice with distilled water and blot dry with paper towel.
11. Air dry for 30 minutes to 1 hour and then cover in plastic wrap or tape in lab book.



Background

In 1990, the Human Genome Project was launched. The goal was to sequence all human nuclear DNA (e.g., the human genome). This endeavor took thirteen years to complete. Based on the complexity of the human organism, scientists first estimated that more than 100,000 human genes would be discovered. As it turns out, there are only about 20,000–25,000 genes needed to make us humans. That's only a few thousand more than are found in the genome of a worm. What's more, we share many common genes with worms – and with all species.

The Human Genome Project has completed the task of sequencing the entire human genome. Far from closing the book, this body of work opened up a whole new field called proteomics, which asks a far more important question: what do our genes do?

Genes encode proteins and proteins determine an organism's form, function, and phenotype and, as such, are the raw materials of natural selection and biological evolution. Changes in proteins reflect changes in the gene pool. Variations between organisms' protein profiles reflect physiological adaptations to different environments, 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 species – with new specialized functions:

Mutation—>Variation—>Specialization—>Speciation—>Evolution

Humans have evolved much larger genomes (3 billion base pairs) than worms (100 million base pairs) and are obviously more complex than a worm! So, how is it that humans and worms have similar numbers of genes? Recent discoveries have shown that in complex organisms a single gene can encode multiple proteins with very different functions. Organisms accomplish this by changing gene structure and function at the DNA, RNA, and protein levels by using a variety of DNA, RNA, and protein modification tools. For example, genes (DNA) may be silenced by methylation of their regulatory sequences, an epigenetic effect. Once genes are transcribed into RNA, the RNA transcript may be modified, edited, or shuffled resulting in changes to the nucleic acids that ultimately direct protein synthesis. Once the protein itself is translated from RNA, many modifications are possible. In fact, most proteins need some modifications to be able to perform their biological tasks. The result of this fine-tuning is over 1,000,000 different proteins that make us human.

Proteomics is the study of the function, structure, and interaction of proteins with each other and their environment. Proteomics aims to completely describe all proteins in an organism, a cell, or under specific environmental conditions. The Human Proteome Organization (HUPO) is an international attempt to catalog all human proteins and their functions – a daunting challenge for scientists.

A proteome is the collection of proteins that comprise a cell, a tissue, or an organism. Proteomes differ from cell to cell, tissue to tissue, and organism to organism. Unlike genomes, which are fixed blueprints that remain pretty much unchanged, proteomes are constantly changing through biochemical and environmental interactions. A single organism will have radically different protein expression patterns within the different cells and tissues of its body, and protein expression patterns will change at different stages in its life cycle and when exposed to different environmental conditions.

Antibodies are key proteins found in all animal immune systems. Animal immune systems generate antibodies that detect foreign invaders such as viruses, bacteria, and allergens and tag them for destruction. The ability of antibodies to act like magic bullets and home in and attach themselves to specific targets or epitopes makes them ideal for bioscience research, diagnostic tests, and medical therapies. Modern biological research has copied the way antibodies function in animal immune systems, adapting them for use in drug discovery and clinical diagnostic labs. Today, antibody-based “immunodetection” techniques are one of the most widely used tools used in proteomics research.

Western blotting is an immunodetection technique used by proteomic scientists to detect and quantify specific proteins in complex biological samples. First, proteins are extracted from a sample of cells or tissue. Extracted proteins are loaded into a sieving gel matrix and separated according to size using an electric current, that is, by electrophoresis. Proteins separated by electrophoresis are then transferred or “blotted” from the gel onto a paper-like membrane. A specific antibody, engineered to bind only to the protein of interest, is added to the membrane. This antibody is attached to a compound that causes a colored reaction, enabling scientists to detect and quantify a single protein of interest from hundreds of other proteins in a sample with high accuracy.

This procedure will be performed in this laboratory!

Western blotting can categorically identify a specific protein among hundreds or thousands of other proteins within biological samples. This surefire method of identifying proteins is based on two distinguishing features of proteins: molecular mass and antibody binding specificity. Bioscience researchers use western blotting to identify proteins, quantify protein expression levels, and determine whether proteins have undergone posttranslational modification. Because it is so accurate, western blotting is the method of choice used to confirm positive test results for HIV, lupus, or bovine spongiform encephalopathy (BSE; mad cow disease).

This lab moves beyond DNA and into the new frontier of proteomics to explore evolution at the molecular level. You will generate protein fingerprint profiles from distantly and closely related species of fish and use western blotting to test the hypothesis that proteins can be indicators of genetic and evolutionary relatedness. Myosin is a major muscle protein essential for locomotion and survival in all animals. As such, the essential structure and function of myosin has remained relatively stable or “conserved" in all animals over evolutionary time.

Protein gel electrophoresis and western blotting will be used to specifically identify a subunit of a myosin light chain from the many thousands of proteins comprising the muscle tissues of different fish. Myosin light chain proteins will be compared from different species for variation, commonality, or evolutionary divergence.

Are there discernible differences between the myosin proteins extracted from the species you are investigating? What are they? How might these variations occur, and why? How might variations in myosin between species be used to determine their evolutionary relationships?

Muscle Proteins

All animal activity is dependent upon muscle proteins. From swimming and running to breathing and digestion, all movement is driven by interactions between specialized proteins comprising muscle fibers. Illustrated below are the basic contractile elements that comprise animal muscle cells. Functional units called “myofibrils” are bundled to form muscle fibers. Each myofibril consists of a linear series of contractile units called "sarcomeres".

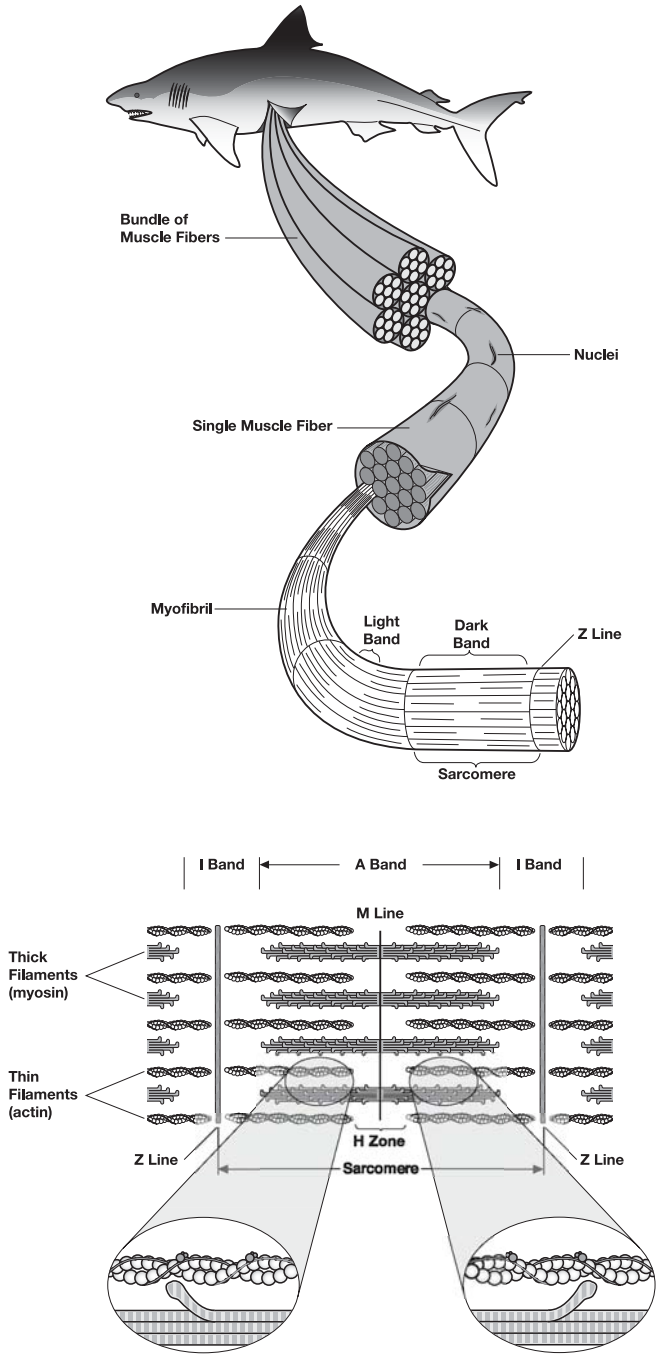


Fig. 6. Telescopic view of muscle structure: Thick myosin filaments and thin actin filaments form myofibrils, which are bundled together to make muscle fibers. (Figure modified from Campbell 1996 with permission.)

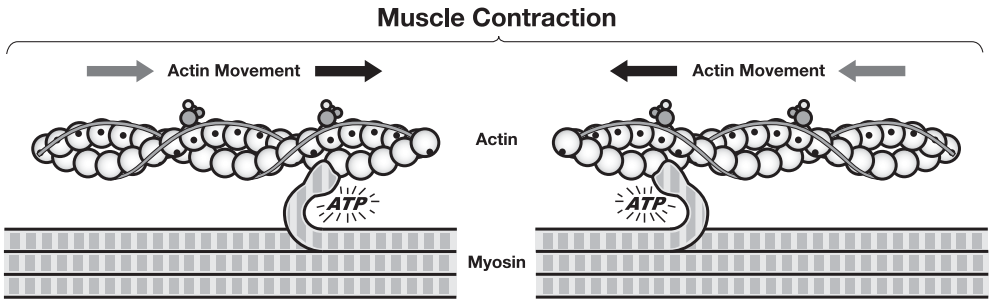


Fig. 7. Hydrolysis of ATP causes myosin and actin filaments to slide past one another, shortening the sarcomere and contracting the muscle. (Figure modified from Campbell 1996 with permission.)

Sarcomeres are precisely arranged assemblies of actin and myosin protein filaments. Up to fifty percent of skeletal muscle is comprised of myosin protein. Thin actin filaments are aligned with thick filaments of myosin in a parallel and partly overlapping manner. Myosin has a 3-D structure composed of six subunits: two myosin heavy chains with molecular masses of 200 kiloDaltons (kD) and four myosin light chains with molecular masses ranging from 15 to 25 kD. The heavy chains have a long tail, a neck, and a globular head region. The two heavy chain tails wind around each other and in turn encircle the tails of neighboring myosin molecules, weaving long cable-like structures that form tough myosin filaments. The head regions protruding from the cable filaments interact with thin actin filaments. Two myosin light chain proteins wrap around the neck of each myosin globular head region and help to regulate the contraction of the myosin protein.

The antibody in this experiment specifically binds to the myosin light chain proteins.

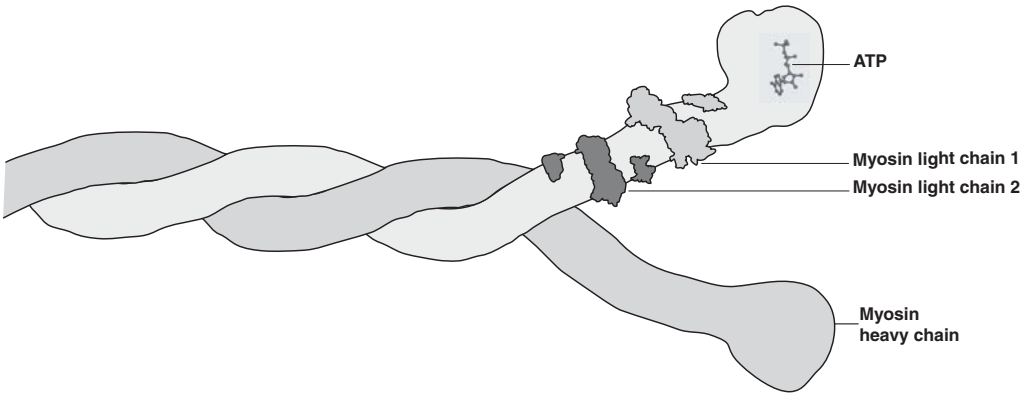


Fig. 8. Myosin protein structure.

The myosin head region contains a catalytic site and an actin-binding domain. The head region binds to actin and flexes at the neck region to pull the ends of the sarcomere together. Myosin obtains the energy for muscle contraction through enzymatic conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP). The combined mini-contractions of the countless sarcomeres composing a muscle fiber causes the macro-contraction of the entire muscle.

Other Muscle Proteins

Many other proteins are also required for muscle contraction in addition to actin and myosin. In a muscle tissue sample, the milieu of proteins that the antibody must sift through in order to bind myosin are found in the table below.

Table 1. 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

Conservation of Proteins

The basic actin-myosin protein interaction that first produced movement evolved in the most primitive organisms and has been passed on to every animal species descended from these ancient ancestors.

Variations between organisms' protein profiles reflect physiological adaptations to different environments, but they originate as chance DNA mutations. Changes in proteins reflect changes in the gene pool. Such random 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

Myosin: a Fight or Flight Protein?

Myosin (composed of 6 protein subunits) is a major muscle protein essential for locomotion and survival in all animals. As such, the primary structure or amino acid sequences of the protein subunits have remained relatively stable or "conserved" in all animals over evolutionary time. This is because any DNA mutation affecting the function of myosin, a protein essential for fight or flight, would likely decrease an organism's ability to survive and reproduce.

DNA—>RNA—>Protein—>Trait—>Evolution

The high degree of myosin conservation and stability across the animal kingdom means that an antibody that detects a myosin protein in chickens will also recognize myosin protein in a trout – even though these two species' common ancestor lived millions of years ago!

The antibody developed for this western blotting procedure can be used to detect myosin light chain protein subunits in most animal species: from fish to mammals and birds. The antibody recognizes a specific amino acid sequence (called an epitope) common to most myosin light chain proteins in most living animal species. If the antibody does not identify a myosin band in the extract from one of your samples, what can you deduce about that organism?

Even though most of the structures and all of the functions of myosin proteins have remained fairly stable through evolutionary time, slight structural variations have been introduced through random DNA mutations and posttranslational modifications and are detectable via western blotting – even among closely related species.

Do these slight variations in the proteins of each species reflect information about that species' genetic blueprint? Can they be mapped in reverse to construct an evolutionary tree?

Pre-lab Focus Questions

1. What are 5 proteins found in muscle? What do they do?

2. Draw, label, and describe the main quaternary structure of myosin, including all protein subunits.

3. Why has the structure of actin and myosin been conserved over millions of years?

4. How do variations in organisms occur in nature, and why? How does this contribute to biodiversity?

5. How might variations in proteins between species be used to determine their evolutionary relationships?

6. How can diverse species share so much common DNA sequence?

7. Can one gene encode more than one protein? How can two different proteins derived from the same gene have different sizes and have different functions?

Lesson 1: Protein Extraction From Muscle

Protein Structure

The primary structure of a protein is determined by its linear amino acid sequence. There are 20 common amino acids that are joined together by peptide bonds to form specific polypeptide sequences. Polypeptide chains form the primary structures of proteins. In addition to their linear amino acid content, all proteins exist in a three-dimensional (3-D) shape. How these 3-D shapes are formed is determined by environmental factors such as pH, temperature, hydrophilic and hydrophobic interactions, and protein-protein interactions. Hydrogen bonding can occur between the side chains of the individual amino acids, causing the polypeptide chain to bend and fold, leading to secondary structural changes. Tertiary structural changes are caused by covalent modifications to polypeptide chains that also encourage a 3-D shape. For example, the side chain of the amino acid cysteine is sulfur-rich and forms disulfide bonds or disulfide bridges (S-S) with the side chains of other cysteines. These disulfide bonds bend and loop polypeptide chains. Finally, quaternary structures can be observed when multiple polypeptide chains come together to make a single functional protein. For example, myosin, the protein in muscle examined in this laboratory, is a multi-subunit protein composed of six individual polypeptide chains.

All the interactions occurring at the primary, secondary, tertiary, and quaternary level produce the helices, pleated sheets, and other 3-D characteristics of biologically active proteins.

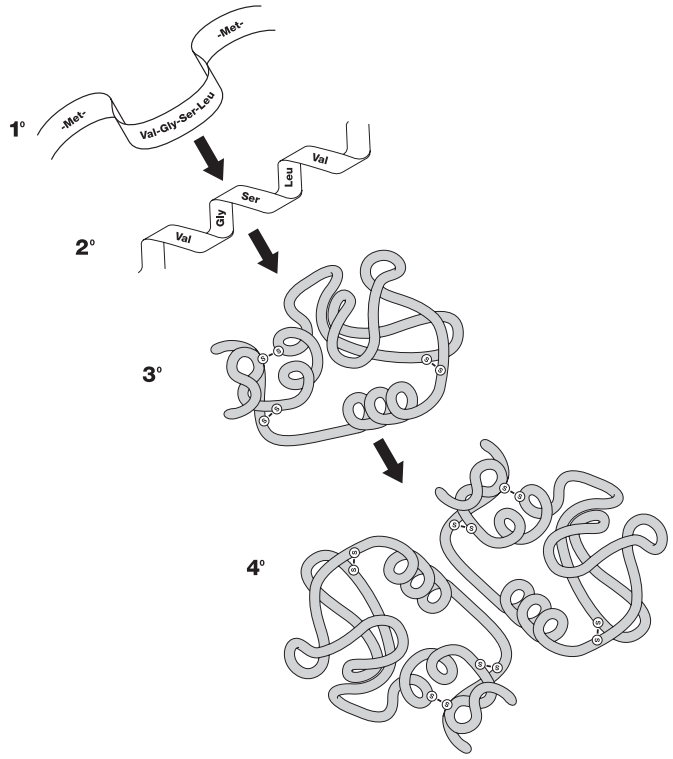


Fig. 9. Secondary (2°), tertiary (3°), and quaternary (4°) protein structure must be distrupted, or denatured, to accurately separate proteins by size.

Sample Preparation

In order to study a particular muscle protein, muscle tissue must first be broken down to release proteins from within the cells and the proteins must be denatured to their linear forms. This is because linear molecules move through the pores of a sieving gel matrix more efficiently than 3-D ones.

You will begin this laboratory by extracting proteins from the muscle tissues of different fish species. The cell membranes of all animals are composed mainly of lipid bilayer. The lysis buffer used to break open or lyse the muscle cells contains the ionic detergent sodium dodecyl sulfate (SDS) and a strong reducing agent called dithiothreitol (DTT). SDS effectively coats all the proteins in the sample with negative charge and DTT breaks the disulfide bridges that contribute to protein secondary, tertiary, and quaternary structure. SDS and DTT are contained in the lysis buffer (Laemmli sample buffer). Heating to 95°C further denatures proteins. Once extraction is complete, all the proteins in the sample are uniformly coated with SDS and carry equivalent negative charge density. SDS-PAGE electrophoresis can then be used to separate protein subunits, or polypeptides, based on their sizes. The Laemmli sample buffer also contains Tris – a buffer that maintains a constant pH, glycerol to add density to samples so they sink into the wells when loading the gel, and the dye Bromophenol Blue, to help visualize sample loading and to allow for tracking protein migration during electrophoresis.

Proteins migrate through the sieving gel matrix of the gel according to their size, which is determined by the number and kind of amino acids composing the primary structure of each polypeptide. The smaller the peptide, the more rapidly it migrates through the gel towards the positive electrode; larger peptides take longer to navigate through the gel. Similarly, denatured (linear) peptides can be more readily analyzed via gel electrophoresis than large 3-D complexes of proteins. The sieving properties of most gels are not capable of separating fully native (non-denatured) protein molecules.

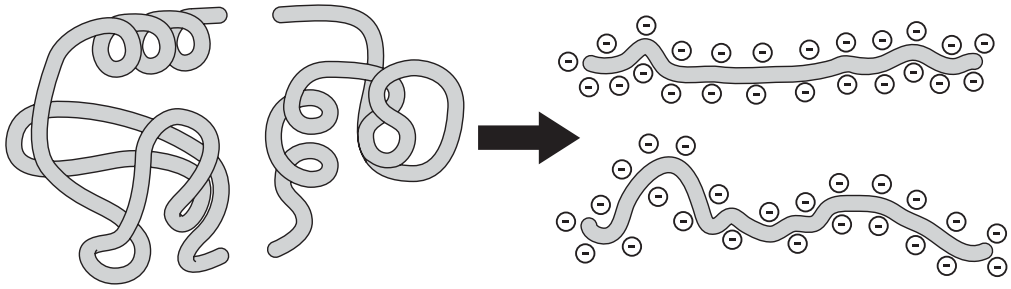


Fig. 10. The combination of heat and the detergent SDS denatures proteins for SDS-PAGE analysis.

Lesson 1: PROTOCOL

Prepare Muscle Protein Extracts

In this lab you will add tiny pieces of muscle to Laemmli sample buffer and manually disrupt the tissue by flicking the tubes. This flicking will release muscle specific proteins from the cells and into the sample buffer, unfold them, and add an overall negative charge to each protein. You will then heat the sample buffer containing the extracted proteins to 95°C in order to complete their denaturation into linear peptides.

Student Workstations

Material	Quantity
1.5 ml fliptop microtubes	5
1.5 ml screw-cap microtubes	5
1 ml disposable plastic transfer pipet	1
Muscle tissue samples	5 species
Marking pen	1
Laemmli sample buffer	1.5 ml
Knife or scissors to cut muscle extracts	1

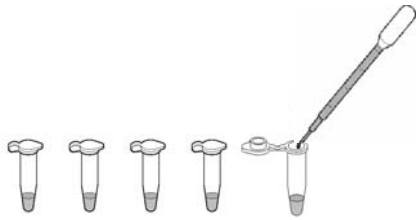
Common Workstation

Material	Quantity
Water bath set to 95°C	1

Procedure

1.1. Label one 1.5 ml fliptop microtube and one 1.5 ml screw-cap tube with the species of muscle tissue to be analyzed. There should be one fliptop and one screw-cap labeled tube for each sample being prepared for electrophoresis. It is best to label screw-cap tubes on the sides in case the caps are mixed up.

1.2. Add 250 µl of Laemmli sample buffer to each labeled **fliptop** tube.

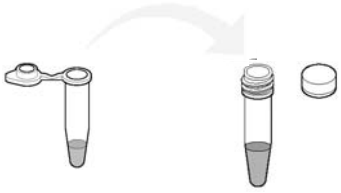


1.3. For each sample, obtain a piece of muscle tissue (avoid skin, fat, and bones) approximately 0.25 x 0.25 x 0.25 cm^3, and transfer it to the appropriately labeled microtube. Try to make the pieces of muscle tissue the same size for each sample. Close the lid.

1.4. Gently flick the microtube 15 times with your finger to agitate the tissue in the sample buffer. This flicking helps to mechanically disrupt the tissue.

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

1.6. Pour buffer containing the extracted proteins, but **not** the solid piece of muscle tissue from each fliptop tube, into a labeled 1.5 ml **screw-cap** tube. Note: It is not necessary to transfer all of the fluid to the screw-cap tube, since only a small volume (<20 µl) is actually needed for gel loading. It is essential not to transfer any chunks of muscle tissue to this tube.



1.7. Heat muscle extracts in screw-cap tubes for 5 minutes at 95°C to denature the proteins in preparation for electrophoresis.

1.8. Muscle extracts may be stored at room temperature for loading into gels to a maximum of 3–4 hours. Alternatively, these samples may be stored for future use at –20°C for up to several weeks.

Lesson 1: Focus Questions

1. Name four of the main ingredients of the Laemmli sample buffer. What does each do?

2. How many individual protein subunits make up the quaternary structure of one biologically active myosin protein? What are these proteins? What are their approximate molecular masses?

3. Why is it important to denature proteins before electrophoresis?

4. What effect does heating the sample have on the extracted material?

5. What is the difference between the primary and quaternary structures of proteins?

Lesson 2: Protein Gel Electrophoresis

Separating Proteins Using SDS-PAGE

In this investigation, polyacrylamide gel electrophoresis (PAGE) is used to separate proteins from the muscle tissue of different species. Using an electric current, proteins coated in SDS-containing sample buffer are separated in a sieving gel matrix that separates proteins by their size. A polyacrylamide gel is positioned in a buffer-filled chamber between two electrodes and muscle extracts are loaded into wells at the top of the gel. Then the electrodes are connected to a power supply that generates a voltage gradient from negative to positive down the gel. The SDS-coated, negatively charged proteins migrate through the gel toward the positively charged anode with the larger proteins migrating more slowly than the smaller proteins.

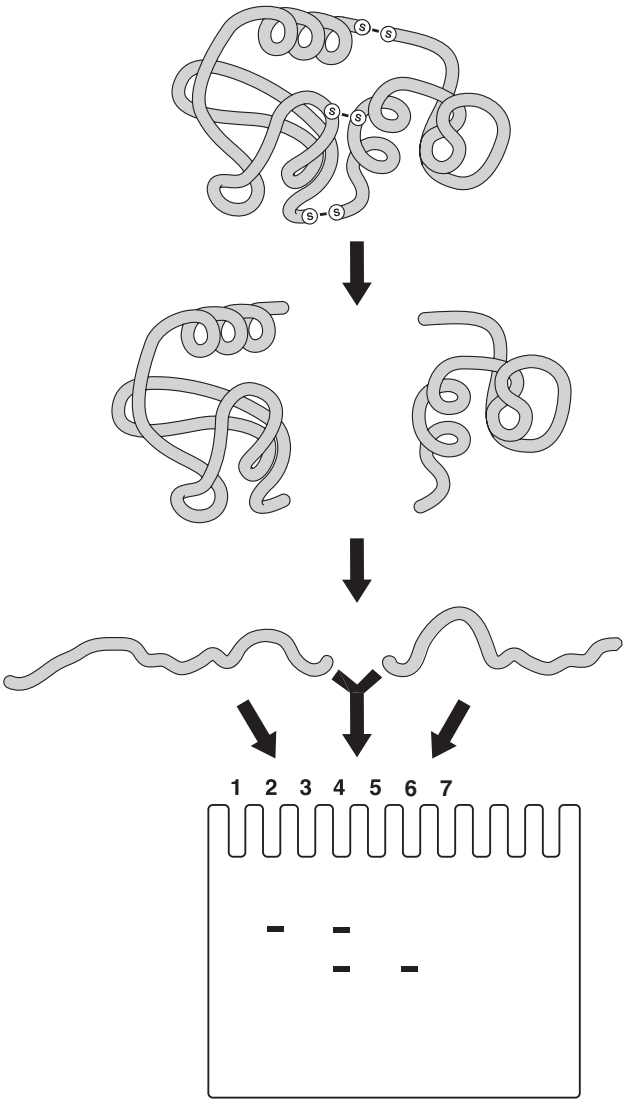


Fig. 11. A quaternary protein complex denatured with reducing agents, heat, and SDS, can be separated into individual proteins and resolved by size using SDS-PAGE.

Once the electric current is applied, the SDS-coated proteins begin their race toward the positive electrode. Smaller proteins move through the gel more quickly than the larger ones and over time proteins will be separated according to size.

Protein size is quantified in **Daltons**, a measure of molecular mass. One Dalton is defined as the mass of a hydrogen atom, which is 1.66×10^{-24} grams (g). Most proteins have masses of thousands of Daltons, therefore the term **kiloDalton** (kD) is often used to describe protein molecular mass. Given that the average mass of an amino acid is 110 Daltons, the predicted mass of a protein can be approximated from the number of amino acids it contains.

- Average amino acid = 110 Daltons
- Approximate molecular mass of protein = number of amino acids x 110 Daltons

Monitoring Invisible Proteins During Electrophoresis

While it is not possible to visualize the proteins in the muscle extracts while the gel is running, Precision Plus Protein Kaleidoscope prestained protein standards are designed to be watched. These genetically engineered proteins have dyes covalently bound to them and resolve into multi-colored bands that move down the gel during electrophoresis. The blue tracking dye in the sample buffer can also be used to monitor the progress of the run. The blue dye is negatively charged and smaller than most known proteins, so it is drawn toward the positive electrode slightly ahead of the proteins. If the electric current is left on for too long, the standards, the dye, and the proteins will eventually run off the bottom of the gel. Keep an eye on the progress of the tracking dye and the protein standards to monitor the extent of electrophoresis.

Experimental Controls

There are two types of controls used in this lab. The visible prestained standards are used to monitor the progress of proteins during the electrophoresis and blotting procedures. These standards are run through the gel and are transferred along with the unknown samples during the blotting procedure. The prestained standards are finally used to determine the molecular weights of the myosin light chain proteins on the western blots.

The molecular weights (sizes) of prestained standard protein sizes are as follows:

Color	Size, kD
Blue	250
Purple	150
Blue	100
Pink	75
Blue	50
Green	37
Pink	25
Blue	20
Blue	15
Yellow	10

The actin & myosin standard is a mixture of rabbit myofibrils and contains actin, myosin, tropomyosin, and trace amounts of other muscle filament proteins. The primary antibody in this kit is designed to detect myosin light chain. This control sample serves as a positive experimental control for the immunodetection procedure.

Lesson 2: PROTOCOL

Separate Proteins by Polyacrylamide Gel Electrophoresis

In the first lesson, proteins were extracted, denatured, and imparted with a negative charge. Now the proteins in your samples will be separated according to their molecular weights via protein gel electrophoresis.

Student Workstations

Materials	Quantity
Muscle extracts from lesson 1	5 species
Actin & myosin standard, 10 µl	1 vial
Precision Plus Protein™ Kaleidoscope™ standards, 6 µl	1 vial
15%, 10 well, Ready Gel® precast gel	1
1–20 µl adjustable-volume micropipet	1
Prot/Elec™ pipet tips for gel loading	7 tips
Mini-PROTEAN® 3 electrophoresis module (gel box)	1 per 2 gels
1x Tris-glycine-SDS (TGS) gel running buffer	500 ml per gel box
Power supply (200 V constant) to be shared between workstations	1
Sample loading guide for 10 well comb	1 per gel box
Buffer dam (only required if running 1 gel/box)	1
Staining trays	1 per 2 gels
Bio-Safe™ Coomassie stain (optional)	50 ml per 2 gels

Common Workstation

Material	Quantity
Water bath set at 95°C	1
Rocking platform (if staining gels)	1
Distilled water for gel destaining (optional)	2 L

Procedure

2.1. Reheat frozen muscle extracts and actin & myosin standards at 95°C for 2–5 minutes to redissolve any precipitated detergent.

Note: If muscle extracts were prepared today, there is no need to reheat them.

2.2. Assemble gel boxes. Use the pictorial guide in the quick guide to properly insert the Ready Gel precast gels into the vertical electrophoresis module if the instructor has not already preassembled them.

Before loading gels, ensure that the buffer in the inner chamber is well above the top of the smaller plate. If the buffer is at a low level then there may be a leak; consult with the instructor. If there is a leak, the outer chamber of the gel box can be filled to above the small inner plates in order to equalize the buffer levels in both reservoirs.

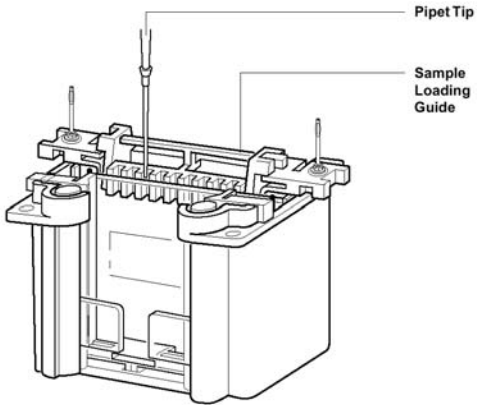
2.3. If available, place a yellow sample loading guide on top of the electrode assembly. This guide will direct the pipet tip to the correct position for loading each sample in a well.

2.4. Record order of samples loaded into gel in the table below:

Well	Volume	Sample Name
1	Empty	none
2	Empty	none
3	5 µl prestained standards	Precision Plus Protein Kaleidoscope prestained protein standard
4	5 µl extract 1	
5	5 µl extract 2	
6	5 µl extract 3	
7	5 µl extract 4	
8	5 µl extract 5	
9	5 µl standard	Actin & myosin standard (AM)
10	Empty	None

a. Load 5 µl of prestained standards gently into well # 3 using a thin gel-loading tip.

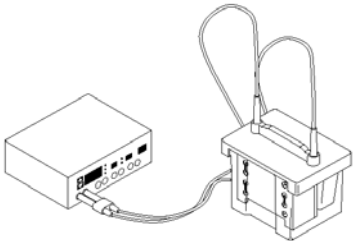
Note: The fine barrel of the gel loading tips releases liquid more slowly than normal tips. You must therefore release the plunger of the micropipet very slowly when taking up samples and when loading them; otherwise the correct volume will not be loaded.



b. Using a fresh tip each time, load 5 µl of each of the muscle extracts gently into separate wells as designated in the table above.

c. Using a fresh tip, load 5 µl of the actin & myosin standard gently into well # 9.

2.5. After loading all samples, remove the yellow sample loading guide (if used), place lid on the tank, and insert leads into the power supply by matching red-to-red and black-to-black. Set the voltage to 200 V and run the gels for 30 minutes. Watch the colored prestained standard proteins separate and monitor the blue tracking dye to assess how electrophoresis is progressing.



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

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

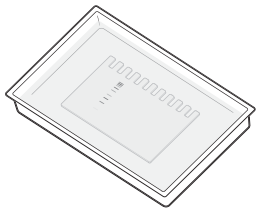
2.8. Ideally the blotting step should be performed directly following gel electrophoresis. If blotting cannot be performed immediately following electrophoresis, gels may be stained with Bio-Safe Coomassie stain to fix and stabilize the proteins in the gels for up to 24 hours. Alternatively gels may be stored in their cassettes at 4°C overnight. However, the storage without staining will result in thicker, less defined myosin bands due to the diffusion of proteins within the gel over time.

If the gels are to be stained before blotting then follow these instructions:

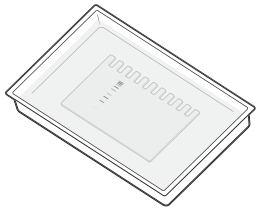
2.9. Save 50 ml of gel running buffer per gel for the next lesson.

2.10. Remove gel from the cassette. Lay gel cassette flat on bench with the short plate facing up.

Note: Do not touch the gel with ungloved hands. Carefully pry apart the gel plates using gloved fingertips. The gel will usually adhere to one of the plates. To detach gel from plate, transfer to a tray containing tap water. The gel may also be lifted directly (and gently!) from the plate and placed into the water. If there is sufficient time, then rinse the gel 3 times with tap water for 5 minutes; this will increase band clarity. Otherwise, place gels directly into stain after a quick rinse.

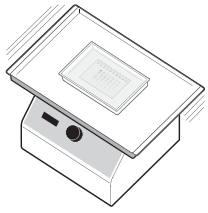


2.11. Carefully replace rinse with 50 ml of Bio-Safe Coomassie stain per 2 gels.



2.12. Stain gels for 1 hour, with shaking if available.

2.13. Discard the stain, rinse the gels with distilled water and add a large volume of distilled water to destain the gel overnight with shaking. Change the water at least once during destaining.



Lesson 2: Focus Questions

1. Why are proteins treated with ionic detergent (SDS), reducing agents (DTT), and heat before SDS-PAGE?

2. Why do SDS-coated proteins migrate in an electric field?

3. What is the purpose of using experimental controls? What purpose do the actin & myosin standards serve? The prestained standards?

4. The molecular mass of myosin light chain 1 is approximately 22 kD, myosin heavy chain is 200 kD and actin is 42 kD. Which proteins will migrate fastest through the gel? Why?

5. Draw a gel below and mark the relative positions of myosin light chain 1, myosin heavy chain, and actin from the actin and myosin standard after electrophoresis.

Lesson 3: Perform Western Blotting

Overview of Blotting

In the previous two steps, proteins were extracted from muscle tissue, then separated according to their sizes via electrophoresis. The rest of the laboratory focuses on using antibodies to identify myosin light chain proteins in the muscle extracts. The separated muscle proteins are currently embedded within a flimsy and fragile gel. To probe the samples with the myosin-specific antibody, proteins must first be transferred or "blotted" from within the gel onto the surface of a membrane. A membrane is more stable and longer lasting than a gel and proteins bound to the surface of a membrane are more accessible to antibodies. This procedure is called western blotting.

Proteins are electrophoretically transferred from the gel onto a nitrocellulose membrane. Proteins, still negatively charged from the SDS, migrate out of the gel and bind to the surface of the membrane, creating a mirror image of proteins separated in the original gel.

Once proteins are transferred to the nitrocellulose membrane (the 'blot'), the next step is to probe the blot with an antibody that has been specifically engineered to detect the protein of interest. But first, the blot must be incubated in a protein-rich solution such as one derived from powdered milk protein. Incubating the blot with milk protein effectively coats the entire surface area of the membrane where no proteins have been blotted and blocks nonspecific protein binding sites.

Next the blot is incubated with an antibody engineered to bind only to myosin light chain proteins (the primary antibody). Following a quick rinse, the membrane is incubated with an enzyme-linked secondary antibody that has been engineered to bind specifically to the primary antibody. Finally, a colorless colorimetric enzyme substrate is added to the membrane in solution. The enzyme that is linked to the secondary antibody oxidizes the colorimetric substrate into an insoluble colored precipitate, leaving a visible deposit on the membrane at the precise location of the blotted myosin light chain proteins.

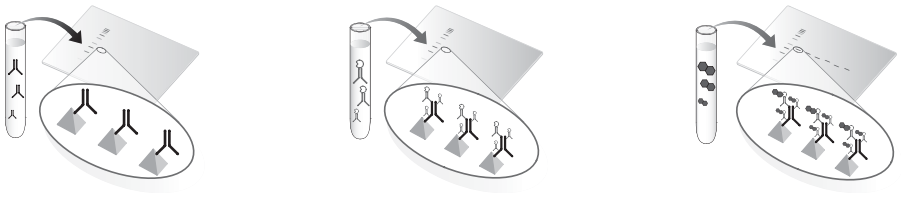
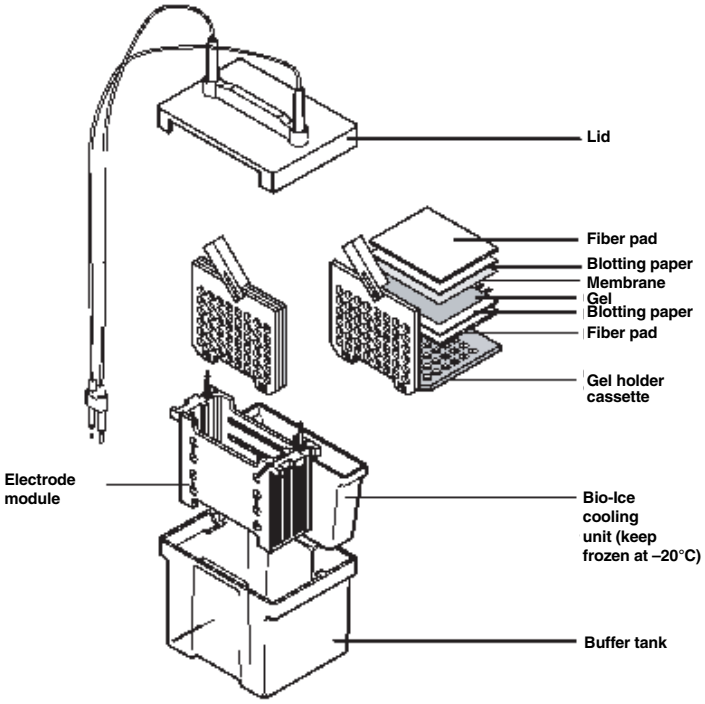


Fig. 12. Overview of Immunodetection on the blot. The membrane is incubated with the primary antibody, followed by incubation with the secondary antibody, and lastly the substrate is added.

Western Blot Reagents and Equipment

Mini Trans-Blot apparatus: the Mini Trans-Blot is specifically designed to pass electric current horizontally through the gel, forcing the negatively charged proteins to migrate out of the gel onto the nitrocellulose membrane.



The Mini Trans-Blot module is designed to fit into the Mini-PROTEAN 3 gel electrophoresis tank and lid. If a Mini Trans-Blot is not available, follow the alternative protocol for transferring the proteins using capillary action described in Appendix B.

Nitrocellulose membranes: Nitrocellulose acts as a solid support for proteins bound to its positively charged surface. These durable membranes can undergo multiple wash and incubation steps, and provide a white background on which to visualize the color development at the site of the protein of interest only. Please avoid touching the membrane with ungloved hands as this may produce protein-rich fingerprints! Restrict contact with the membrane to outer edges or use forceps to handle. Each white nitrocellulose membrane is packaged between two protective sheets of blue paper.

Blotting paper: Blotting paper is used to support the gel and nitrocellulose and to protect them from the fiber pads during assembly and electrophoresis. The blotting paper also facilitates a uniform flow of buffer and current through the gel. Blotting paper is made of 100% cotton fiber and does not contain any additives that may interfere with the blotting process.

Fiber pads: Fiber pads press the gel and nitrocellulose together tightly and uniformly, eliminate air bubbles, and allow efficient transfer of proteins out of the gel and onto the membrane. The pads must be thoroughly cleaned and rinsed in distilled water before use to remove contaminants.

Blotting buffer: The 1x blotting buffer is composed of 2.5 mM Tris, 19.2 mM glycine, and 20% ethanol and is pH 8.3. It contains tris to maintain pH, glycine ions to transmit current, and ethanol to facilitate protein binding to the nitrocellulose.

Blocker: This solution is 5% nonfat dried milk powder in phosphate buffered saline (PBS) and 0.025% Tween 20. All surface area unoccupied by proteins transferred from the gel needs to be “blocked” prior incubation with the primary antibody by incubating with a blocking

agent such as this milk solution. Without this blocking step, the primary antibody can randomly adhere to the membrane and obscure or weaken the specific antibody (anti-myosin) signal. PBS (1 mM sodium phosphate, 15 mM NaCl, pH 7.4) provides the ideal pH and salt conditions for maintaining milk protein binding integrity. Tween 20 is a detergent that helps keep nonspecifically bound antibody from adhering to the membrane.

Setting Up for Protein Blotting

After running the polyacrylamide gel, the gel must be equilibrated in blotting buffer to remove excess SDS. Proteins can then be transferred from the gel to a protein-binding nitrocellulose membrane. The blot is set up as a sandwich in a plastic cassette partially submerged in blotting buffer. The figure below illustrates the sandwich construction consisting of a fiber pad at the bottom followed sequentially by a layer of blotting paper, the gel, the membrane, another layer of blotting paper – and the final fiber pad. It is imperative that no air bubbles exist between the blotting paper, the gel, or the membrane since bubbles will prevent proteins from being transferred. After adding each layer to the sandwich, a roller is used to push out any air bubbles – starting at one end of the membrane/gel/paper and rolling to the other. The sandwich is then clamped together in the plastic cassette.

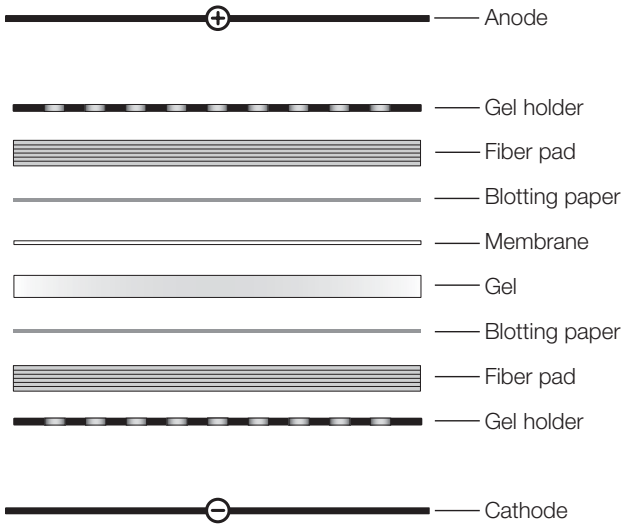


Fig. 13. Schematic of western blot. The current is conducted through the blotting buffer and negatively charged proteins migrate from the gel onto the protein binding membrane.

It is important that the sandwich be oriented with the black edge of the cassette facing down. The cassette is then submerged in blotting buffer in the transfer tank, aligning the clear plastic side to the red electrode and black to black with color-coded electrodes of the blotting module. This orientation will ensure that the negative current runs from the gel toward the membrane. Similarly to running proteins vertically through the gel during the electrophoresis, here the current will force the negatively charged proteins horizontally out of the gel and onto the surface of the membrane.

Lesson 3: PROTOCOL

Western Blotting

Now that the muscle extract proteins are separated, they must be transferred out of the gel and blotted onto a membrane support in order to facilitate antibody detection of myosin. In this lesson, a sandwich will be carefully prepared from the gel and membrane, an electric current will be passed through it, and proteins will ultimately be blotted onto a nitrocellulose membrane in a mirror image configuration to that found in the gel.

Student Workstations

Material	Quantity
Blotting buffer	500 ml
Blotting paper	2
Nitrocellulose membrane	1
Mini Trans-Blot/Mini-PROTEAN 3 tank and lid	1 per 2 workstations
Red & black Mini Trans-Blot inner module	1 per 2 workstations
Frozen Bio-Ice™ unit	1 per 2 workstations
Hinged black and clear plastic sandwich cassette	1
Fiber pads	2
Power supply to be shared between workstations	1
Roller (pencil, test tube, or pipet)	1
Soft pencil	1
Containers	2
Blocker (optional)	25 ml

Common Workstation

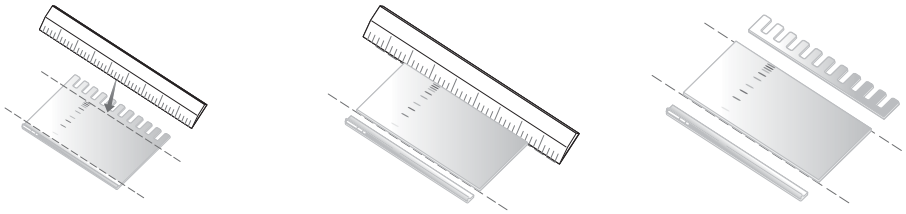
Material	Quantity
Rocking platform	1

3.1. Prepare gel for blotting:

Option A: If the gel was not stained in the previous lesson, then remove it from the cassette. Lay the gel cassette flat on the bench with the short plate facing down. Using fingertips, carefully pry apart the gel plates. The gel will usually adhere to one of the plates.

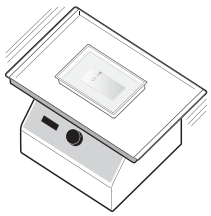
Option B: If the gel was stained in the previous lesson, incubate it for 15 minutes in gel running buffer (TGS) reserved from the previous lesson with gentle rocking. Longer incubation in TGS will not harm the experiment.

Using a ruler or a plastic ID-type card (shown below), carefully chop away wells and the 4% stacking gel located just above the top band of the prestained standards. At the bottom of the gel, chop away the ridge located below the blue line derived from the Laemmli sample buffer. Take great care to chop straight down into the gel rather than slicing across the gel which will cause tearing. If the gel does tear, it can be pieced together in step 3.5d.



3.2. Equilibrate the gel:

Transfer the gel to a tray containing blotting buffer. If the gel is adhered to the plate allow the liquid to detach the gel from the plate. Incubate the gel in blotting buffer for at least 15 minutes. Longer equilibration times will not harm the experiment. While the gel is equilibrating, prepare the materials for the blotting sandwich.

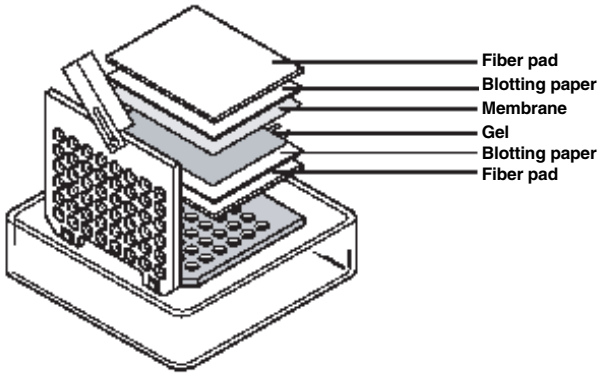


3.3. Soak the fiber pads thoroughly in blotting buffer and squeeze buffer into them to ensure they are thoroughly soaked.

3.4. Write your initials on a corner of the white nitrocellulose membrane with a pencil. Prewet the blotting paper and nitrocellulose membrane in blotting buffer.

3.5. Make the blotting sandwich:

a. Add approximately 1 cm depth of blotting buffer in a container large enough to fit the plastic gel holder cassette. Place the gel holder cassette into the container with the black side immersed in the buffer and the clear side outside of the buffer as shown in the figure below.



b. Lay one wet fiber pad flat on the black plastic cassette.

c. Place a piece of wet blotting paper onto the fiber pad and roll out any air bubbles. Ensure that there is sufficient buffer to just cover the blotting paper. The liquid assists in squeezing air bubbles out of the sandwich.

d. After equilibration, place gel squarely onto blotting paper. Wet the roller and carefully roll over the gel to push out air bubbles. It is very important to eliminate air bubbles between the gel and blotting paper.

e. Carefully place the wetted nitrocellulose membrane squarely onto the gel with the side with your initials facing down. Try to move the membrane as little as possible once it has been placed on the gel, since proteins begin to blot immediately and ghost bands may form if the membrane is moved. Roll out air bubbles between the gel and membrane. It is very important to eliminate air bubbles between the gel and membrane.

f. Place a second sheet of wet blotting paper on top of the nitrocellulose membrane and roll out any air bubbles. It is very important to eliminate any air bubbles between the membrane and the blotting paper.

g. Place the second wet fiber pad onto the blotting paper.

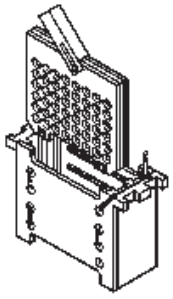
h. Fold the clear plastic half of the cassette over the sandwich and clamp it to the black plastic half by sliding over the white clip. This tight fit will squeeze the sandwich together. Keep the sandwich cassette partly submerged in blotting buffer.

3.6. Set up the Mini Trans-Blot apparatus:

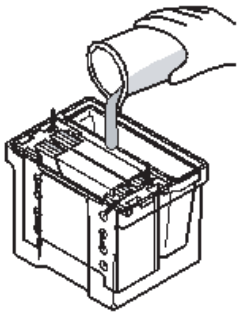
a. Place the red and black Mini Trans-Blot module into the gel tank with the black side in the center of the tank and the banana plugs protruding up in the middle.

b. Place the frozen Bio-Ice module into the gel tank.

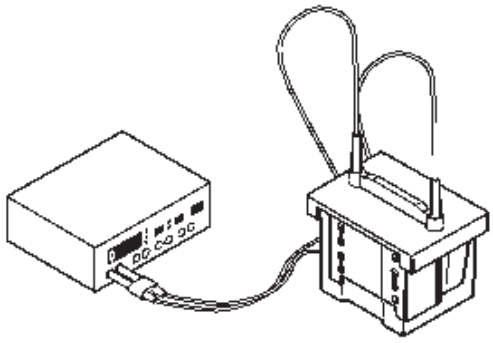
c. Slide the cassette containing the blotting sandwich into the red and black Mini Trans-Blot module; the black side of the sandwich must be facing the black side of the Mini Trans-Blot module and the clear side of the sandwich facing the red side of the module. Two sandwich cassettes fit into each module. It is crucial for this apparatus to be correctly oriented or the blot will not be successful.



d. Fill tank with blotting buffer up to the level of the white clip. Blotting buffer used in the previous preparation steps should be used in the tank and topped up with fresh buffer. Ensure that the black side of the sandwich cassette is facing the black side of the Mini Trans-Blot module and the frozen Bio-Ice unit is in place. Place the lid on the tank; match the red plug on the lid with the red plug on the Mini Trans-Blot module.



3.7. Attach the Mini Trans-Blot to a power supply and ensure a red-to-red and black-to-black match. For unstained gels, run the blot at 20 V for 2.5 hours. For stained gels, run the blot at 20 V for 15 hours. It is possible to run unstained gel blots for 30 minutes at 100 V; however electroblotting generates very high current (160–220 mA/tank). Consequently, the power supply must have a high current capacity. Refer to the instructor's advance preparation section of this manual for more information and for the specifications of Bio-Rad's power supplies. It is preferable to use low-voltage blotting as this will avoid overloading the power supply.



3.8. Once blotting is complete, immunodetection may be performed immediately. Alternatively, blotting modules can be left overnight in their tanks at room temperature or sandwiches can be dismantled and the membranes can be placed in blocker at 4°C overnight. If blots need to be stored for longer periods, they can be stored submerged in blotting buffer or wash buffer at 4°C for up to 1 week.

Lesson 3: Focus Questions

1. Why are proteins blotted from the polyacrylamide gel to a membrane?

2. Why is it important for the gel to be in complete contact with the membrane without any air bubbles?

3. Why do proteins migrate from the gel to the membrane?

4. Can you think of a way to determine if the transfer of a stained gel has been successful? An unstained gel?

Lesson 4: Immunodetection for Myosin Light Chains

Using Antibodies to Identify Proteins

Immunology is the study of the immune system and how the body protects itself against disease. Over 100 years ago, biologists discovered that animals' internal immune systems respond to invasion from foreign entities by provoking an immune response that begins with the production of proteins called antibodies. Any foreign invader that elicits antibody production is called an antigen. Like magic bullets, antibodies seek out and attach themselves to invading entities, flagging these foreigners for destruction by other cells of the immune system. Antigenic invaders may consist of any molecule foreign to the body, including components of infectious agents like bacteria, viruses, and fungi. Astonishingly, there are between 10^6 and 10^{11} unique antibodies circulating in blood with each one recognizing a different antigen. Antibodies comprise up to 15% of total blood serum protein!

Tapping Nature's Tool Kit

Because of its accuracy, western blotting is used to confirm positive test results for HIV, lupus, or bovine spongiform encephalopathy (BSE or mad cow disease) following initial screening using high-throughput enzyme-linked immunosorbent assay (ELISA).

The human immune system generates antibodies that detect foreign invaders such as viruses, bacteria, and allergens and tag them for destruction. The ability of antibodies to act like magic bullets and home in on specific targets makes them ideal for bioscience research, diagnostic tests, and medical therapies.

Western blotting can pinpoint a specific protein among hundreds or thousands of other proteins within biological samples. This surefire method of identifying proteins is based on two distinguishing features of proteins: molecular mass and antibody binding specificity. Bioscience researchers use western blotting as a tool to investigate proteomes: to identify proteins, quantify protein expression levels, and determine whether proteins have undergone genetic or posttranslational modifications.

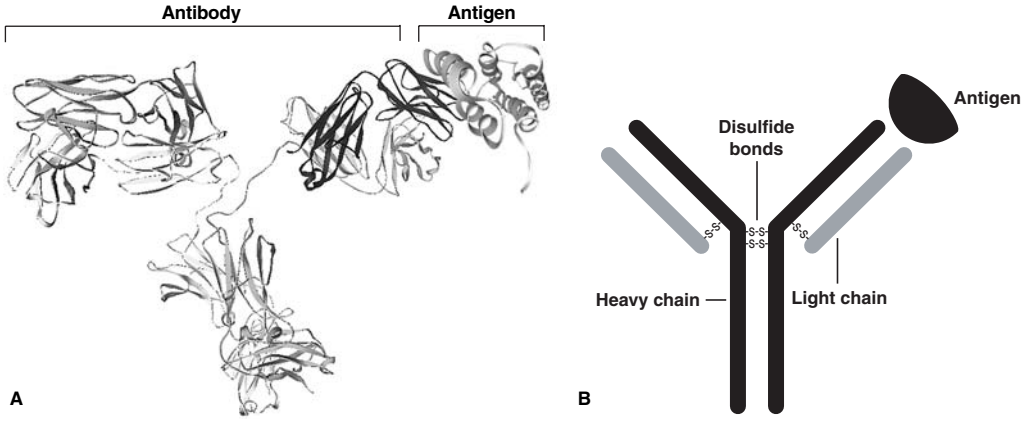


Fig. 14. A) Structure of IgG bound to the HIV capsid protein p24 as determined by X-ray crystallography (Harris et al. 1998, Momany et al. 1996). These structures can be downloaded from the Protein Data Bank (www.pdb.ufmg.br, Berman et al. 2000) using the PDB identification codes 1IGY and 1AFV and manipulated using free online software such as Rasmol and Protein Explorer. **B)** A commonly used representation of an antibody bound to an antigen.

The immune system's natural ability to generate unique antibodies is an invaluable mechanism that has been taken advantage of to advance modern biological research and drug discovery. Since a crucial component in biological research involves the ability to track and identify proteins, antibodies have been used as flagging devices to identify and localize whatever protein is being studied. For example, scientists design a specific antibody that will recognize a disease-associated protein. These custom-made antibodies can then be used in experiments to characterize the protein's function. For example, antibodies may be used to identify the presence and quantity of a protein involved in a disease state or they can be used to determine whether drug treatments affect the disease-associated protein.

What Is Immunodetection?

Since antibodies seek out and bind to specific proteins, they are ideal tools for proteomic research when proteins need to be identified and analyzed. Immunodetection is the term used for laboratory methods that use antibodies to detect proteins. In this lesson, an antibody that is specific for myosin light chain will be used to detect myosin from among the thousands of proteins immobilized on the membrane, much like finding a needle in a haystack.

Antigen: An antigen is by definition any substance that is recognized by an antibody. In this experiment the antigen consists of two proteins: myosin light chain 1 (MLC1) and myosin light chain 2 (MLC2). Both are recognized by the primary antibody. MLC1 is one of the essential myosin light chains. MLC2 is known as the myosin regulatory light chain. Although myosin light chain protein from fish muscle tissue is designated as the central focus in this laboratory activity, the primary antibody in this kit will also detect myosin light chain proteins in many other species including human, mouse, rabbit, chicken, and frog, allowing students to run independent research projects investigating muscle proteins from other species.

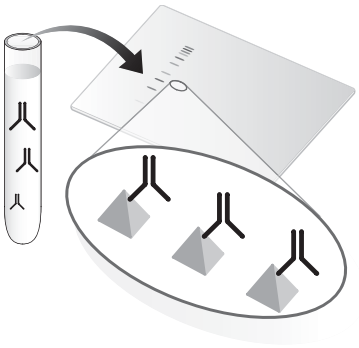
How Are Antibodies Made?

When exposed to a foreign entity (e.g., molecules, cells, or tissues) most animals generate an immune response and produce antibodies. Each antibody recognizes only a single antigen. The antigen in this experiment is myosin light chain, which is in the proteins extracted from the fish muscle. Animals such as goats, rabbits, and mice can be injected with an antigen to stimulate antibody production. Over time, antibodies will accumulate in the blood serum and can be purified for use in the laboratory. In an immunoassay, the antibodies produced in this way to identify antigens are called primary antibodies.

Secondary antibodies recognize and bind to primary antibodies in an immunoassay. Secondary antibodies are prepared by injecting primary antibodies produced from one species of animal into another species so that the foreign species will provoke an immune response. For example, if the desired product is a secondary antibody that will recognize a mouse-derived primary antibody, then mouse antibodies are injected into a different animal such as a goat. Following the goat's immune response, its serum will contain antibodies that recognize and bind to any mouse-derived antibodies. Secondary antibodies are frequently tagged (or conjugated) so that they can be made visible. In this experiment, the secondary antibody is conjugated to horseradish peroxidase (HRP), an enzyme that when in the presence of its substrate, 4CN, produces a purple/gray precipitate that deposits color on the membrane at the precise location where the antigen-primary antibody-secondary antibody complex is bound.

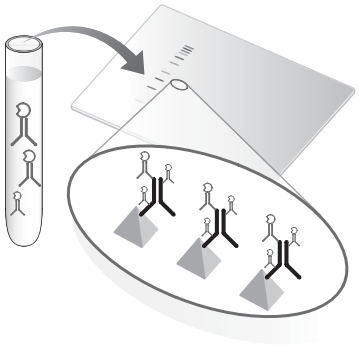
Immunodetection Step by Step

Primary antibody is added to the blot and incubated to allow the antibody to bind to the myosin protein on the membrane. The unbound antibody is then washed away.



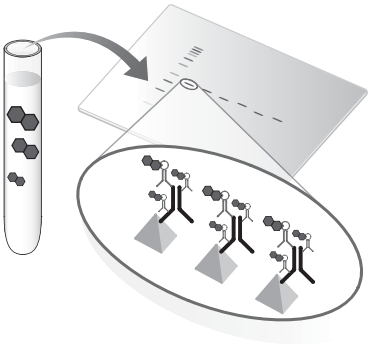
The primary antibody provided is a monoclonal mouse anti-myosin light chain antibody. This antibody was made by injecting purified chicken myosin protein into mice and generating an immortalized antibody producing cell line (a hybridoma) from one mouse that constantly produces the same antibody.

Secondary antibody is added to the blot and incubated to allow the secondary antibody to bind to the primary antibody. The unbound secondary antibody is then washed away.

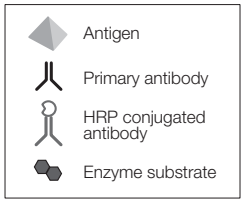


The secondary antibody is a polyclonal goat anti-mouse antibody conjugated to HRP. Secondary antibody was produced by injecting goats with primary mouse antibodies. The secondary goat anti-mouse antibodies were purified from goat serum, and chemically linked or conjugated to HRP. HRP is the enzyme that catalyzes oxidation of the colorimetric substrate so the protein of interest can be identified.

Colorimetric (color-producing) enzyme substrate is added to the membrane and incubated to allow color to develop. Purple/gray bands will develop on the membrane exactly where the myosin protein bands are located.



The colorimetric substrate in this kit is 4-chloro-1-naphthol (4CN). When oxidized by HRP in the presence of hydrogen peroxide, this colorless solution forms a purple/gray precipitate that binds to the membrane at the antigen location. Note: The HRP color detection reagent is light sensitive and must be kept in the dark at all times.



Lesson 4: PROTOCOL

Immunodetection

In this final series of steps, antibodies will be used to detect one specific protein from the thousands on the membrane.

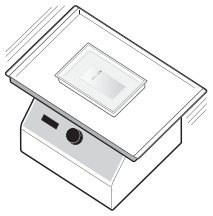
Student Workstations

Material	Quantity
Blocker (if membranes have not been blocked already)	25 ml
Ready-to-use primary antibody, anti-myosin antibody	10 ml
Ready-to-use secondary antibody, goat anti-mouse HRP antibody	10 ml
HRP color detection substrate	10 ml
Wash buffer	200 ml
Distilled water	100 ml
Paper towel	2 sheets
Incubation tray	1
Container for waste liquid	1

4.1. When the blot is finished, dismantle the sandwich. Only handle outer edges of the membrane.

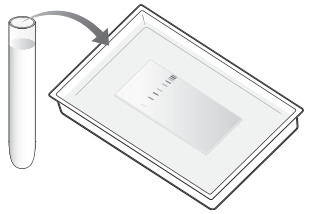
4.2. Wearing gloves and only handling corners, peel the membrane off the gel and check for the presence of the prestained standards on the membrane. Inform the instructor if the colored bands are not visible.

4.3. Place the membrane in a staining tray containing 25 ml of blocking solution with the prestained standards facing up. Place the tray on a rocker for 15 minutes to 2 hours at room temperature (or overnight at 4°C). Note: the membrane cannot be “over-blocked”. However, the milk used in the blocker can spoil and the resulting microbial growth can degrade proteins on the membrane.



4.4. Pour off blocking solution.

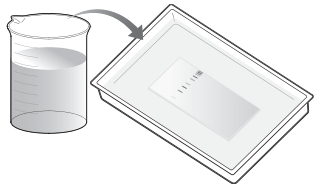
4.5. Add 10 ml anti-myosin primary antibody to the tray and place on the rocker for 10–20 minutes at room temperature or overnight at 4°C. Longer incubation times will result in more intense bands. Adjust rocker to a faster setting if necessary to ensure the antibody solution is constantly washing over the membrane.



4.6. Pour off the anti-myosin primary antibody.

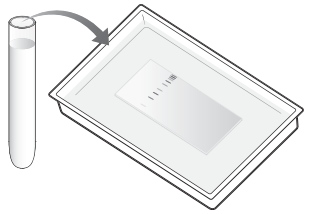
4.7. Rinse the membrane in approximately 50 ml of wash buffer and pour off.

4.8. Add another 50 ml of wash buffer to the membrane and place on rocker for 3 minutes. Longer wash times will not harm the experiment. Reduce rocker speed if splashing occurs. If necessary, the membrane can be stored overnight in wash buffer at 4°C.



4.9. Pour off wash buffer.

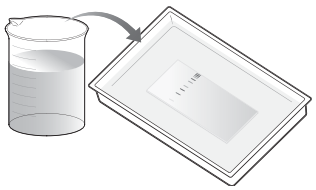
4.10. Add 10 ml secondary antibody to membrane and place on rocker for 5–15 minutes. Longer incubation times will result in more intense bands. Adjust rocker to a faster setting if necessary to ensure the antibody solution is constantly washing over the membrane.



4.11. Pour off the secondary antibody solution.

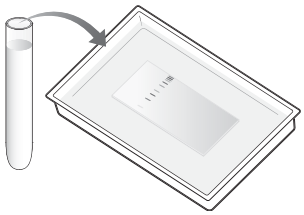
4.12. Rinse the membrane in approximately 50 ml of wash buffer and pour off.

4.13. Add another 50 ml of wash buffer to membrane and place on rocker for 3 minutes. If necessary, the membrane can be stored overnight in wash buffer at 4°C.



4.14. Pour off wash buffer.

4.15. Add 10 ml of HRP color detection reagent, place on rocker, and allow at least 10 minutes for bands to develop.



4.16. Once the bands have developed, discard the detection reagent and rinse the membrane twice in distilled water, pat it gently between sheets of paper towel, and air-dry for up to one hour. Air-drying and exposing to light for a longer period may cause the bands to fade and the membrane to turn yellow. Tape membrane into notebook or wrap in plastic and store in the dark. Handle the membrane very carefully – it is fragile when dry.

4.17. Draw or tape results in the empty space below and label the sizes of the Precision Plus Protein Kaleidoscope prestained protein standards, the actin & myosin standard, and the samples. Estimate the size of the myosin light chains from your samples.

Prestained Color	Standard Mass (kD)
Blue	250
Purple	150
Blue	100
Pink	75
Blue	50
Green	37
Pink	25
Blue	20
Blue	15
Yellow	10

Focus Questions: Lesson 4

4.1. Describe how a specific protein can be identified from a mixture of proteins.

4.2. Name three other methods used to analyze proteins besides western blotting and immunodetection.

4.3. What information does the western blot provide for each sample?

4.4. Are myosin proteins the same or different sizes across species? How are protein sizes calculated?

4.5. How can this information be used to explain structural (and perhaps evolutionary) differences in animal species?

4.6. Explain why the secondary antibody is used.

4.7. Describe how to make an antibody to detect another muscle protein such as dystrophin.

Appendix A: Further Background

Immunological Terminology and Concepts

Immunology is the study of the immune system. The body protects itself from infection using physical and chemical barriers, antibodies that circulate in the blood and immune cells that attack foreign substances and invading microorganisms. Some types of immune cells adapt to remember or recognize specific invaders in case of future attacks. A person is born with certain immunological defenses collectively termed **innate immunity** that includes circulating cells called macrophages and natural killer cells. These defenses do not change with exposure to pathogens and do not have specificity for particular pathogens (e.g., organisms that cause disease such as bacteria, viruses, fungi, infectious proteins called prions, and parasites).

Passive immunity is the acquisition of antibodies from an external source such as mother to infant, or from post-exposure vaccines such as for rabies. The passive immunity response lasts only a few weeks and is not altered by multiple exposures.

Acquired or adaptive immunity refers to a unique immune response toward specific foreign substances that is initiated upon first contact. In other words, initial contact with an invader triggers an individualized immune response that is repeated and magnified in subsequent exposures to the same pathogen. Acquired immunity is split into two categories: humoral immunity and cell-mediated immunity. **Humoral immunity** involves the production of antibodies that circulate in the bloodstream and lymphatic system and which bind specifically to foreign antigens. **Cell-mediated immunity** involves the production of T lymphocytes (T cells) that bind and destroy infected cells.

Components of the Acquired Immune Response

An immune response to an invader displaying a foreign body or "antigen" generates **antibody** production by B lymphocytes (also known as B cells). Each B lymphocyte produces a unique antibody that recognizes a single shape on an antigen called an **epitope** and thus helps the **immune cells** (e.g., B cells, T cells, and macrophages) to recognize and attack foreign invaders. All non-immune-compromised individuals have circulating antibodies and lymphocytes that collectively recognize a huge number of antigenic substances.

Antigens can be microorganisms (e.g., viruses and bacteria), microbial products (e.g., toxins produced by some bacteria, or protein components of the microbes), foreign proteins, DNA and RNA molecules, drugs, and other chemicals.

Antibodies, also called immunoglobulins (Ig), are produced by B cells and can remain attached to B cells or become free-floating. There are five classes of immunoglobulins: IgG, IgM, IgA, IgE, and IgD. IgG is the most abundant immunoglobulin in internal body fluids, comprising about 15% of total serum protein in adults, and each IgG molecule can bind two antigen molecules. IgM is also in serum and is responsible for the primary immune response. IgA is found in external secretions such as tears, saliva, milk, and mucosal secretions of the respiratory, genital, and intestinal tracts and is a first line of defense against invading microorganisms. IgA is also the only antibody passed from mother to infant. IgD may be involved in regulating the immune response, and IgE plays a major role in allergic reactions.

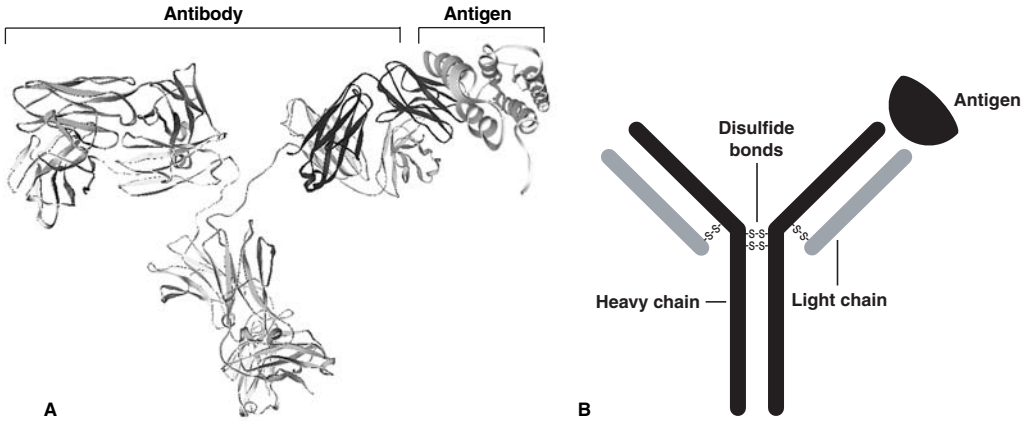


Fig. 15. A) Structure of IgG bound to the HIV capsid protein p24 as determined by X-ray crystallography (Harris et al. 1998, Momany et al. 1996). These structures can be downloaded from the Protein Data Bank (www.pdb.ufmg.br, Berman et al. 2000) using the PDB identification codes 1IGY and 1AFV and manipulated using free online software such as Rasmol and Protein Explorer. **B)** A commonly used representation of an antibody bound to an antigen.

An **epitope** is the specific section of an antigen that is recognized by an antibody. Antigens have multiple epitopes and so can be recognized by multiple antibodies. For example, an HIV virus particle (virion) has many potential epitopes on its surface that can be recognized by many different antibodies; one particular antibody may recognize an amono acid sequence at the amino terminus of p24, an HIV capsid protein, while another may recognize the carboxy terminus of p24. Most epitopes can be thought of as 3-D surface features of an antigen molecule. Exceptions may be linear epitopes, which are determined by the amino acid sequence (the primary structure) rather than by the tertiary or quaternary structure of a protein. Antibodies may have different specificities for a linear epitope on a denatured protein than a 3-D epitope on the native protein.

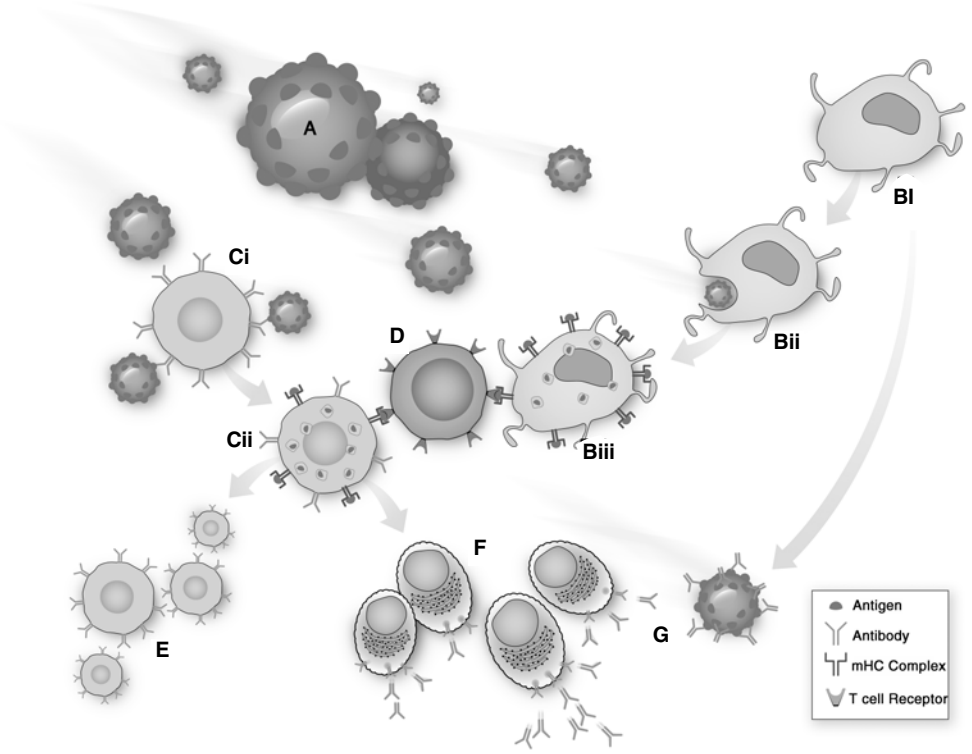


Fig. 16. Summary of immune cell and pathogen interactions.

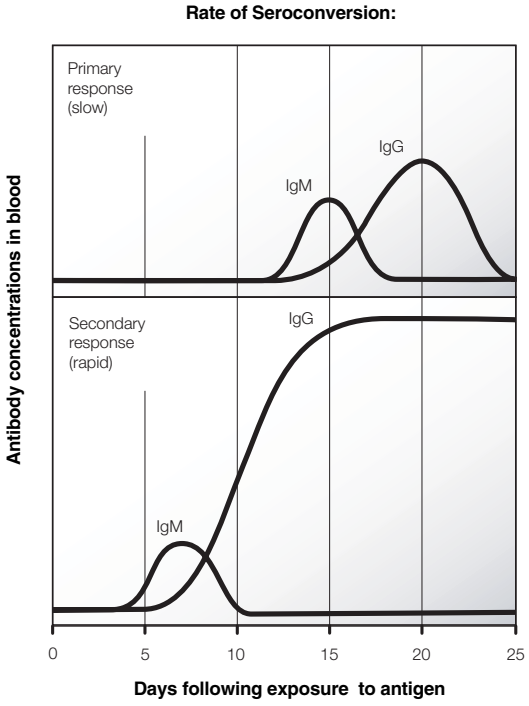
Immune cells are the soldiers of the acquired immune response. Macrophages (Fig 16. Bi) engulf foreign cells, pathogens and molecules (A) from the blood (Bii), and present antigens on their cell surfaces via their major histocompatibility (MHC) complexes to be recognized by T cells (Biii). T cells (D) attract more immune cells to the site of infection causing inflammation. Like macrophages, B cells (C) present antigens on their surface to attract T cells (Cii). T cells recognize antigens through their T cell receptors. T cells kill whole cells that are infected by a virus to prevent the spreading of further infection. T cells also stimulate the proliferation of B cells that have bound to an antigen and form both memory B cells (E) that are part of the secondary immune response and plasma cells (F) that secrete antibodies. Secreted antibodies label pathogens (G) making it easier for other immune cells to find and destroy them. Both B and T cells are white blood cells or lymphocytes; T cells mature in the thymus and B cells mature in bone marrow. B and T lymphocytes have the ability to rearrange their DNA to produce a huge number of diverse antibodies and T cell receptors respectively.

Why We Need an Immune System

Even bacteria have a rudimentary innate immune system; they make restriction enzymes that destroy foreign bacterial virus DNA (bacteriophages), protecting their own DNA through methylation. Our immune system is at work every day, protecting us from thousands of potential threats, but it is so efficient that we usually don't notice it.

Immune Response

When immunized with a foreign substance (either by vaccination or through natural exposure), an individual mounts the primary response. Within 1–2 weeks, there is an increase in antibody production against the antigen, dominated by the IgM class of antibodies. IgM production is followed by production of IgG, followed by a decrease in antibody levels. Another exposure to the same antigen will result in a larger and more rapid immune response. In the secondary response, IgM is made within days, followed within two weeks by a much larger production of IgG than in the primary response. Other classes of immunoglobulin may also be produced. IgG persists in the blood for a much longer time than in the primary response. Antibody production may continue for months or even years.



Tapping Nature's Toolkit: Putting Antibodies to Use

Immunoassays

In recent years the expansion of technology to produce antibodies has yielded a myriad of new applications that take advantage of antibody binding specificity. The basis of all immunoassays is the specific binding of an antibody to its antigen, and there are many ways that this targeting can be used.

Enzyme-linked immunosorbent assay (ELISA) is a powerful and widely used diagnostic tool in human and veterinary medicine, food testing, and agriculture. ELISAs are performed in polystyrene plates with 96 or 384 wells per plate and an automated plate reader is used to detect the colored signal from positive samples. ELISAs are inexpensive, rapid, and allow quantitation of antigen or antibody levels. An ELISA can either directly test for the

presence of a disease antigen, or indirectly test for exposure to a disease by assaying a patient's blood for antibodies to the disease, which will only be present if the patient has been in contact with the disease – an antibody ELISA. In an antibody ELISA, a preparation or extract of the disease agent is adsorbed (bound) to polystyrene wells in a plate and then incubated with a patient's blood serum and any unbound serum antibodies are washed away. Enzyme-linked anti-human antibodies are then added to the wells, and they will bind any of the patient's antibodies that bound to the disease agent. These secondary antibodies are detected by adding a colorimetric substrate for the enzyme that changes color if the enzyme is present. If the patient did not have antibodies to the disease agent, no enzyme-linked antibodies will have bound and the substrate will remain colorless.

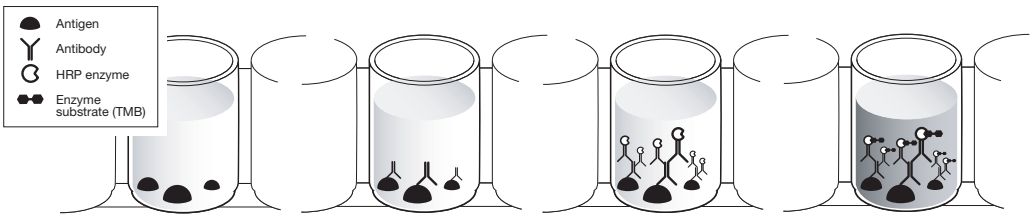


Fig. 17. Antibody capture ELISA.

An antibody ELISA tests whether a patient has antibodies to the disease agent, but does not identify what antigen the patient's antibodies detect. Thus, it is possible for a patient's serum to react with the preparation of the disease agent even if they haven't been exposed because the disease agent may share common proteins or antigens with a disease the patient has been exposed to. This is called a false positive. Some medical conditions such as Lyme disease, syphilis, and lupus can cause a false positive result in an ELISA for HIV.

Western blotting is often used in the clinic to confirm positive ELISA results for critical tests like HIV and Lyme disease. Similar to an antibody ELISA, a diagnostic western blot is usually indirect and tests whether a patient has antibodies to the disease agent, but it also identifies which proteins from the disease the patient has generated antibodies against. In a diagnostic western blot, a preparation of the disease agent is first separated on a polyacrylamide gel to separate the disease proteins by molecular mass and blotted onto a membrane. The patient's serum is then incubated with the membrane and any antibodies that bind to disease agent proteins are identified using an enzyme-linked secondary antibody and substrate that develops bands on the membrane. The molecular weights of these bands are then calculated and compared to the known molecular weights of the disease proteins and thus identify which specific disease proteins the patient has been exposed to. Most diagnostic western blots have a minimum number of disease agent proteins that the patient's serum must react with before the patient is given a positive diagnosis. Western blotting is more technically demanding to perform and interpret than other assays, thus in a clinical setting it is mainly used to verify positive results obtained by inexpensive and automated ELISAs rather than as a front-line test.

Western blotting is one of the most popular techniques in biological research. It allows scientists to identify, quantify, and determine the size, activity levels, or cleavage status of their proteins of interest. In contrast to the indirect diagnostic HIV western blot that identifies unknown antibodies in a patient's serum using known antigens, in research, western blots are usually direct assays that identify unknown antigens in a protein extract using a known antibody.

The western blotting procedure has several steps: First, samples are run through a gel matrix (i.e., SDS-polyacrylamide gel electrophoresis (PAGE)) that separates proteins by molecular mass. Proteins separated in SDS-PAGE gels are then blotted to the surface of a

solid support such as a nitrocellulose membrane using an electrical current. Next, the membrane is incubated with a primary antibody specific for the protein of interest. Later, an enzyme-linked secondary antibody binds the primary antibody and localizes the complex by oxidizing a colorimetric substrate that produces a colored band on the membrane. Alternatively, the oxidized substrate may emit light (chemiluminescent substrate) that is detected as a band on photographic film. The size and abundance of the protein is determined by comparing the position and intensity of the band to known protein standards that are run in parallel.

Another type of immunoblotting is called **dot blotting**, in which a sample is spotted directly onto a membrane. Dot blotting is used for rapid screening of a large number of samples. This technique provides a quick answer to whether a particular protein or antigen is present since many samples may be spotted on a membrane and processed simultaneously. However, like ELISA, this method only identifies proteins by antibody specificity, not molecular mass.

Immunostaining uses specific binding to localize antigens within intact organelles, cells, tissues, or whole organisms, and can also be used to distinguish one cell type from another. Immunostaining uses both antibody specificity and cellular localization to identify antigens, which like the western blot, makes it a useful confirmatory diagnostic test. Pathologists can identify cancer cells using immunostaining. Cancer cells frequently look identical to normal cells under the microscope, but when they are immunostained, variations in the amount and kinds of cell surface proteins are revealed. Studying this information can help diagnose cancer and help physicians decide on treatment regimens. Immunostaining is also used in research to deepen our understanding of where proteins function in a cell in order to find out how cancer cells cause harm.

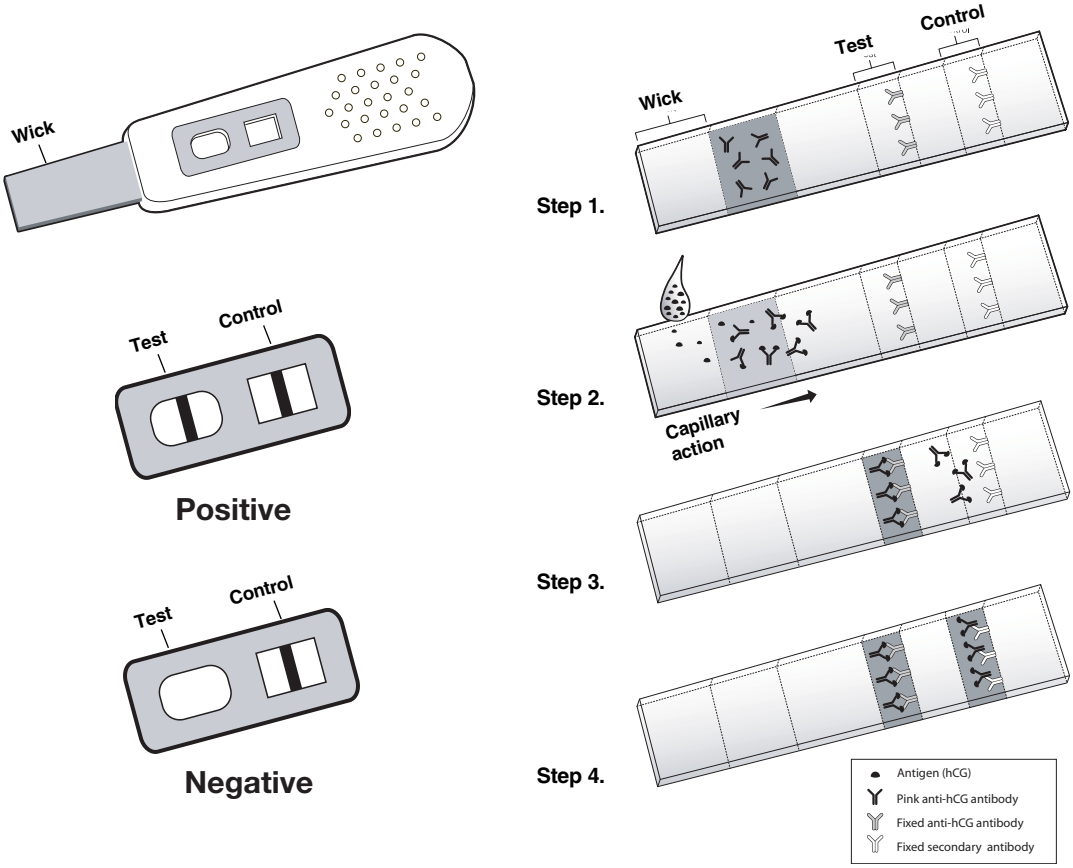
Another area of research would be immunostaining of plant seedlings at different stages of maturation to track the change in a protein's abundance and localization as the plant grows. Antibodies for immunostaining are labeled with either fluorescent molecules or enzymes that produce colored signals upon addition of a substrate.

A special application of immunostaining is fluorescence-activated cell sorting (FACS), in which a population of cells is stained with a fluorescently labeled antibody and then physically separated into labeled and unlabeled cells. The cell sorter uses lasers and an electrostatic charge to sort the cells. Cell sorters can separate as many as 30,000 cells per second!

Dipstick tests are immunochromatography assays that yield rapid positive or negative results, but again only use antibody specificity for antigen identification. One of the antibodies in the test is labeled with a colored compound such as colloidal gold, which produces a pink band in the test strip. Dipstick tests are used for determining pregnancy, illegal drug use (e.g., marijuana, cocaine, and methamphetamines), and the presence of infectious agents (e.g., HIV, plague, *E. coli* O157, and *Legionella*).

Home pregnancy tests detect levels of human chorionic gonadotropin (hCG), a hormone that appears in the blood and urine of pregnant women within days of fertilization. A home dipstick test usually consists of a strip of absorbent material contained within the plastic portion of the test and a wick that is wetted with the material to be tested. In a home pregnancy test, the bottom of the absorbent strip closest to the wick is coated with mouse monoclonal anti-hCG antibody labeled with colloidal gold to make it pink (step 1). When the wick is dipped in urine, the urine will migrate up the absorbent strip via capillary action, carrying the pink anti-hCG antibody with it. If hCG is present, the pink antibody will bind the hCG (step 2). The test zone (the window) of the test consists of two narrow bands of antibodies fixed in place. The first band consists of a different type of anti-hCG antibody (a polyclonal antibody made in goats) which will bind to hCG that is already complexed with

the pink mouse antibody and make a pink stripe (step 3). If there is no hCG present in the urine, the pink antibodies will not bind to the first band of antibodies since they only bind hCG. The second band in the test zone is a built-in control. There is an excess of pink antibodies in the strip and they continue to migrate up the strip past the first stripe. The second band of antibodies consists of anti-mouse antibodies that bind specifically to the pink mouse antibodies, whether or not they are bound to hCG, to make another pink stripe (step 4). If no pink stripe appears in the control zone, the test did not function properly.



Immunotherapy

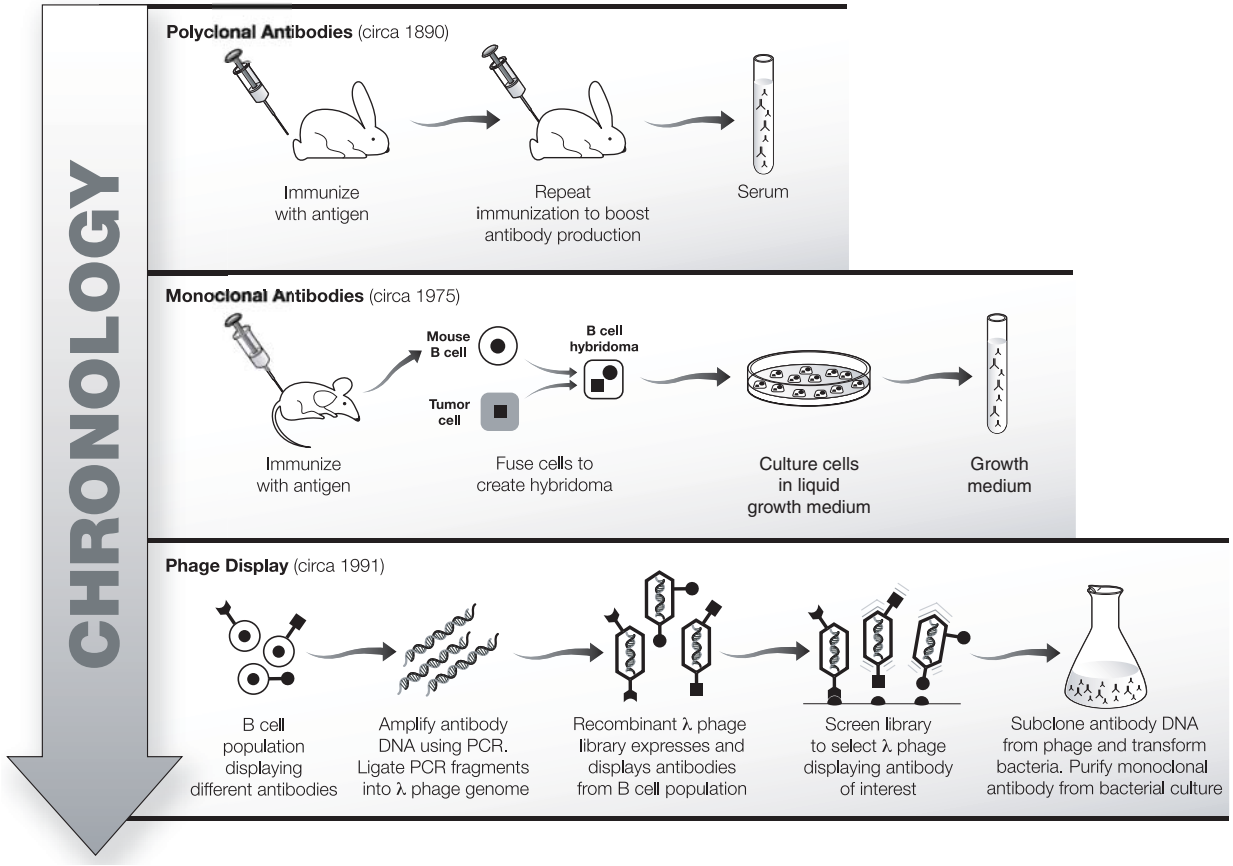
Although antibodies have traditionally been used to diagnose diseases, they are now being used as therapies against cancer. "Humanized" mouse monoclonal antibodies can now be genetically engineered in bacteria, so that they may be used in humans without eliciting an immune response. In addition, human antibody genes have been genetically engineered into the genomes of mice and bacteria in order to produce actual human antibodies.

Monoclonal antibodies can be injected into patients to seek out cancer cells, potentially leading to disruption of cancer cell activities, or to enhance the immune response against the cancer. Humanized monoclonal antibodies have been used effectively to help treat certain cancers. An antibody called Rituxan is used in the treatment of non-Hodgkin's lymphoma, while Herceptin is used against certain breast cancers.

Scientists are studying ways of linking cytotoxic drugs, toxins, or radioisotopes to antibodies to enhance their effectiveness against cancer cells. In this case, the antibodies would function as a targeted drug delivery mechanism, like a guided missile capable of seeking out cancer cells.

Manufacturing Antibodies

Antibodies used in diagnostics and research can be manufactured in the laboratory. While most antibodies are still produced from animals or cells, revolutionary methods using recombinant DNA technology are currently being developed. There are two types of traditionally produced antibodies: **polyclonal** and **monoclonal**.



Polyclonal antibodies have been used for over 100 years and are generated by immunizing an animal, usually a rabbit, goat, or sheep, and obtaining its serum. For example, purified HIV gp120 protein can be injected into a goat to generate antibodies against the many epitopes of gp120 – remember that mammals will produce many different antibodies to the multiple epitopes of an antigen. Blood containing antibodies is drawn from the goat and the cells of the blood are removed, leaving the serum. The product is called an **antiserum** towards gp120, and can be used either in this form or the antibodies may be purified from it to increase potency. These antibodies are "polyclonal" or derived from many (*poly*) B cell clones (*clonal*) in the goat's blood, so they recognize multiple epitopes on an antigen, meaning that multiple antibodies can bind to a single antigen. Polyclonal antiserum has the advantage of being simple and inexpensive to produce, but has the disadvantage that no two batches, even produced by the same animal, will be exactly the same.

Monoclonal antibodies were developed for clinical use around 30 years ago. For antibody applications such as diagnostic tests, polyclonal antibodies have too much variability to reliably identify proteins of interest. In these cases, one unique antibody generated from a single B cell clone is preferable. B cell clones producing single antibodies can be isolated from the spleens of immunized mice and used in diagnostic tests. Unfortunately, these cells

die after a few weeks, limiting production of the large amounts of antibody generally needed for research and commercial applications. However, B cells can continue to live and produce antibodies indefinitely if they are fused with immortalized tumor-like cells. This fusion generates hybrid cells or a hybridoma that generates monoclonal antibodies with almost no batch-to-batch variability. Monoclonal antibodies recognize a single epitope on an antigen and so each antigen can only be bound by a single antibody, which can lead to a weak signal that requires amplification by indirect detection (see below).

Genetically Engineering Antibodies

Antibodies act like magic bullets and home in on their targets, making them ideal candidates for medical therapies. For example, an antibody that recognizes a tumor antigen can be attached to chemotherapy drugs or radioactive molecules and bind specifically to targeted tumor cells, sparing the patient many of the side effects of conventional chemotherapy or radiation treatment. Antibodies made in animals cannot be used for therapeutic applications since the human body identifies them as foreign bodies and elicits an immune response to destroy them. Recombinant DNA technology can be used to produce antibodies that are not foreign to the human immune system and can be used as therapeutic agents in people. Using genetic engineering to manufacture antibodies also obviates the need to sacrifice laboratory animals. Two of the methods used to engineer antibodies are described below.

Hybridoma Immortalization

Recombinant DNA technology can camouflage the antigen recognition site of a mouse monoclonal antibody within a human antibody by combining parts of the mouse and human antibody genes. Bacteria transformed with this DNA are capable of producing humanized monoclonal antibodies indefinitely, with the added bonus that culturing bacteria requires much less time and expense then the culture of a mouse hybridoma cell line.

Phage Display

Libraries of billions of potentially useful antibodies are being created by inserting shuffled antibody genes from billions of human B cells into the genomes of bacteriophage lambda (λ) (bacteriophage, or phage, are viruses that infect bacteria; lambda phage is a specific species of phage), so that the lambda phage display the binding sites from human antibodies on their surfaces. This **phage library** is screened to find a phage that binds to a specific antigen and can then be used as an antibody. Alternatively, DNA from the selected phage can be cloned into a human antibody gene and transformed into bacteria. Large amounts of the antibody can then be produced for therapeutic use. Phage display is on the cutting edge of immunotherapy.

Labeling and Detecting Antibodies

Antibodies are used as labeling tools in diagnosis and research. Antibodies are covalently linked or conjugated to chemical labels that emit detectable signals that are visible to the researcher. Fluorescently labeled antibodies allow you to localize an antigen in a cell using high-tech fluorescent microscopy. Antibodies can also be linked to enzymes that oxidize a colorimetric (color-producing) substrate, producing visible color only where the enzyme-linked antibody has bound. Enzyme-linked antibodies are commonly used in microscopy, ELISA, and western blotting.

Detection of antibodies

Antibody targets or antigens can be detected directly by labeling the antibody specific for the antigen (the primary antibody) and looking for signal.

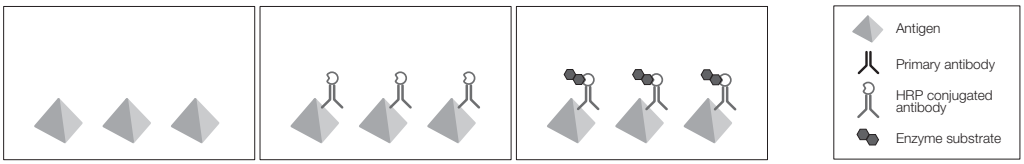


Fig. 18. Direct detection.

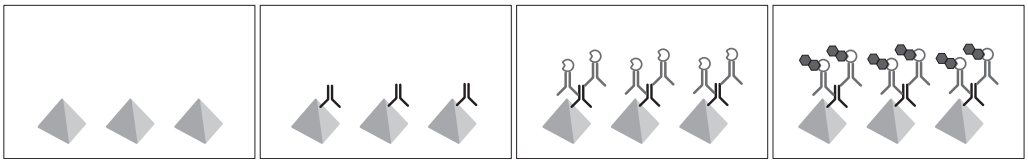


Fig. 19. Indirect detection.

However, labeling every primary antibody scientists might wish to use is time-consuming and costly. Indirect detection is the more common approach used to visualize antigens and relies on polyclonal secondary antibodies that recognize primary antibodies. Injecting antibodies from one animal species into a different species produces secondary antibodies that recognize any antibody from that animal. For example, if the primary antibody is derived from mouse, secondary antibodies are generated in a goat by immunizing it with mouse antibodies. Goat polyclonal anti-mouse IgG is purified from the goat serum and linked to an enzyme for detection. Hence, the primary antibody is effectively an antigen to the secondary antibody. Indirect detection also reduces the amount of costly primary antibody required for an immunoassay since inexpensive polyclonal secondary antibodies recognize multiple epitopes on the primary antibody. Thus, more label accumulates around the antigen and amplify the signal 10 to 50 times. Secondary antibodies are commercially available, either unlabeled or with a wide variety of fluorescent or enzymatic labels for many applications.

Myosin

Skeletal muscle on average is 40–50% myosin. Each myosin protein has a long tail that winds around other myosin molecules to form thick filaments and two globular heads that stick out of the filament and pull the myosin along actin filaments by binding to and releasing actin, which results in muscle contraction. Myosin obtains the power for muscle contraction through enzymatic conversion of ATP to ADP and inorganic phosphate, which releases energy. Myosin is composed of six subunits. Two myosin heavy chains, so named because they have molecular masses of 200 kiloDaltons (kD) and four myosin light chains, that have molecular masses that range from 15 to 25 kD. The heavy chains have two regions, a tail, where the two heavy chains wind around each other, and a globular head region, which has both an ATPase and an actin binding domain. Two myosin light chains wrap around the "neck" of each head region. Each myosin head contains one essential and one regulatory myosin light chain. There are two types of myosin essential light chains, myosin light chain 1 (MLC1) (22–25 kD) and myosin light chain 3 (MLC3) (15–18 kD). These are also called alkali light chains. There is only one type of myosin regulatory light chain, myosin light chain 2 (MLC2) (18-22 kD). The antibody in this western blot is specific for MLC1 and MLC2.

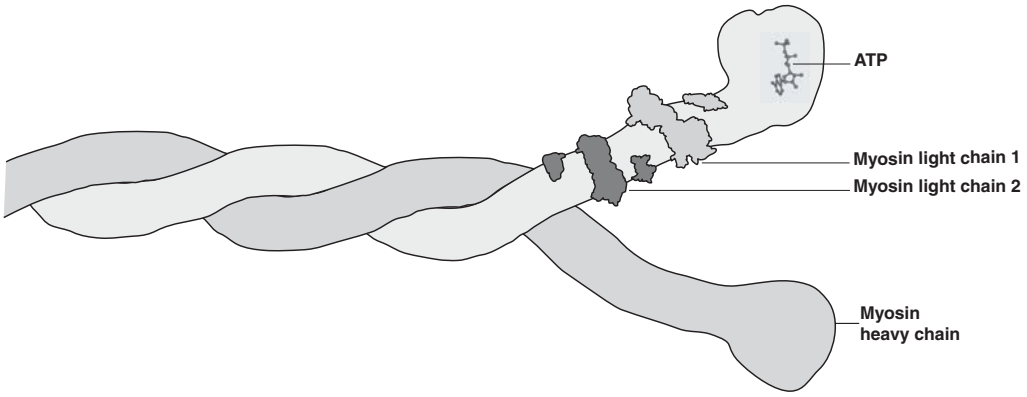


Fig. 20. Depiction of myosin protein structure.

The Role of Myosin in Muscle Contraction

The sarcomere is shortened, and thus the muscle contracted, in a cyclic process of myosin binding, sliding and releasing actin thin filaments. The motion is caused by changes in protein shape in the myosin head and neck regions when they are in different energy states. Myosin heads, when not bound to actin and with ATP in their active site, are in a low-energy state and their neck region is flexed. When the ATPase domain in the myosin head hydrolyzes ATP, this provides energy and causes the myosin neck region to straighten and the head to bind to actin. When ADP and inorganic phosphate are released, the energy is also released, causing the myosin to change back to its low-energy conformation. Thus, the neck region flexes again and in doing so, slides the actin towards the center of the sarcomere. This movement is called a power stroke. The myosin head then dissociates from the actin and binds another ATP molecule ready for the next cycle (Vale and Milligan 2000, Reedy 2000).

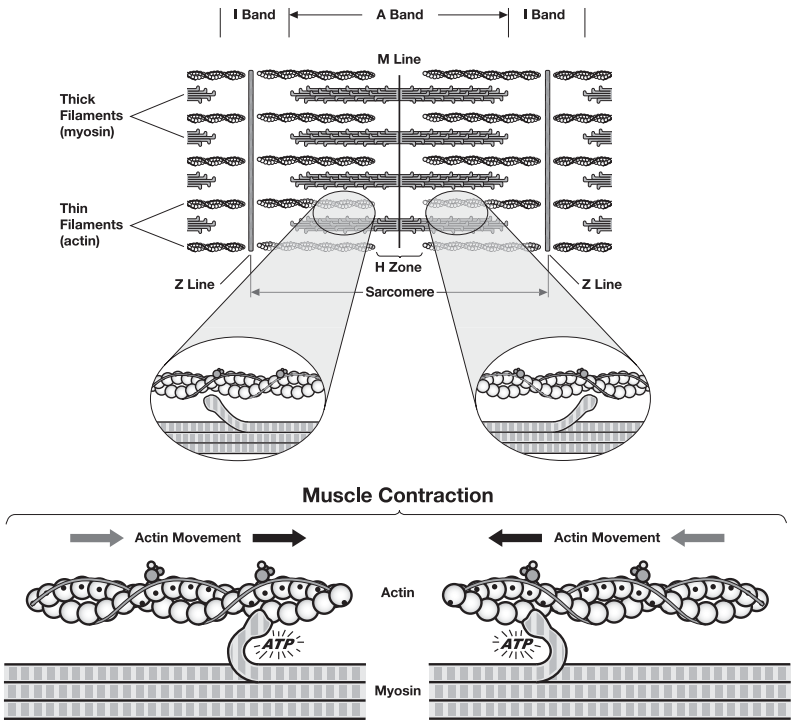


Fig. 21. Depiction of a sarcomere, relaxed actin and myosin, and contracted actin and myosin.

Myosin light chains form part of the lever arm of the protein, which is vital for myosin flexing (akin to biceps and triceps being necessary for arm flexing) and plays a role in regulating myosin function.

The myosin "thick filament" is comprised of approximately 300 myosin proteins whose long tail regions intertwine to form a cable – the filament. The myosin head regions protrude out from the filament and are constantly binding, sliding, and releasing the actin thin filament, contracting both the sarcomere and the muscle as a whole.

Myosin and Proteomics

Interestingly, myosin itself provides an example of the complexity that proteomic scientists are attempting to unravel. Myosin light chains (MLC) 1 and 3 in vertebrates such as chickens and rats (but not in fish) are alternative splice variants of the same gene. They are expressed by two different promoters and share exons 5 through 9, but MLC 1 contains exons 1 and 4 whereas MLC 3 has exons 2 and 3. In fish, MLC 1 and MLC 3 are derived from 2 different genes (Hirayama et al. 1997). Moreover, within a single fish species, different isoforms of MLC 2 can also exist depending on the environmental condition of the fish. It is still unclear whether these isoforms are derived from the same gene (Hirayama et al. 1998). MLC 2 is phosphorylated by myosin light chain kinase, which regulates myosin protein contraction in smooth muscle. However, in skeletal muscle, MLC 2 is always phosphorylated, and the function of this posttranslational modification is unclear.

SDS-PAGE separates myosin into its subunits. By staining the gel, one can predict which bands are the myosin subunits based on their molecular masses and the actin-myosin control. However, to definitively distinguish MLC 1 and MLC 2, a western blot must be performed.

Conservation of Proteins

The basic actin-myosin protein interaction that first produced movement evolved in the most primitive organisms and has been passed on to every animal species descended from these ancient ancestors.

Variations between organisms' protein profiles reflect physiological adaptations to different environments, but they originate as chance DNA mutations. Changes in proteins reflect changes in the gene pool. Such random 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

Myosin: a Fight or Flight Protein?

Myosin (composed of 6 protein subunits) is a major muscle protein essential for locomotion and survival in all animals. As such, the primary structure or amino acid sequences of the protein subunits have remained relatively stable or "conserved" in all animals over evolutionary time. This is because any DNA mutation affecting the function of myosin, a protein essential for fight or flight, would likely decrease an organism's ability to survive and reproduce.

DNA—>RNA—>Protein—>Trait—>Evolution

The high degree of myosin conservation and stability across the animal kingdom means that an antibody that detects a myosin protein in chickens will also recognize myosin protein in a trout – even though these two species' common ancestor lived millions of years ago!

The antibody developed for this western blotting procedure can be used to detect myosin light chain protein subunits in most animal species: from fish to mammals and birds. The antibody recognizes a specific amino acid sequence (called an epitope) common to all myosin light chain proteins in most living animal species. If the antibody does not identify myosin in an organism, such as shellfish, it implies that the epitope on the myosin molecule (presupposing the organism possesses a myosin gene at all) is so different from the original myosin antigen that the antibody does not recognize it. Although trout and chickens diverged millions of years ago, shrimp and chickens diverged even longer ago!

Even though most of the structures and all of the functions of myosin proteins have remained fairly stable through evolutionary time, slight structural variations have been introduced, through random DNA mutations and posttranslational modifications and are detectable via western blotting – even among closely related species.

Do these slight variations in the proteins of each species reflect information about that species' genetic blueprint? Can they be mapped in reverse to construct an evolutionary tree? This can be investigated further in Appendix D, lesson extension 2.

Appendix B: Alternate Lesson 3 – Alternative Western Blotting Method Using Capillary Action

Instructor's Advance Preparation

In this lesson students transfer, or blot, the separated proteins from the gels to a nitrocellulose membrane support using capillary action – a process that takes at least 2 days. Blotted membranes will then be put into blocking solution for 15 minutes to 2 hours at room temperature or at 4°C overnight prior to lesson 4. Please note that this method is less efficient than electroblotting and will result in thicker, less defined bands on the blot, due to diffusion of the proteins within the polyacrylamide matrix. This method may be used with Bio-Safe Coomassie-stained or unstained gels.

Capillary Action Blot

This method is similar to the Southern blot procedure where DNA is transferred by capillary action to a membrane, except western blotting involves transferring proteins instead of DNA. In both procedures, the blotting buffer travels from the reservoir upwards through the wick, gel, and membrane then into the stack (or tower) of paper towels. The blotting buffer moves by capillary action, carrying the proteins with it. The nitrocellulose binds proteins naturally as they are carried from the gel.

Tips

- Ensure that the buffer moves entirely through the gel without being absorbed by the blotting paper overlapping the edges of the gel. Therefore, a waterproof barrier must be formed around the gel edges (using plastic wrap) to prevent overlaying blotting paper sheets from having contact with each other.
- Maintain a continuous flow of buffer through the gel; this means the blotting buffer reservoir may need to be replenished and/or wet paper towels from the stack may need to be replaced with dry ones during the course of the blot.
- It is vital that no air bubbles exist between the gel and membrane. Air bubbles will prevent blotting of the protein and result in holes in the protein complement on the membrane.
- It is recommended that two gels be blotted side by side, to conserve buffer and create a more stable tower with a wider base.
- Stabilize towers by taping around the weight and container. As the paper towel stack soaks up the buffer, the tower becomes less stable.
- Covering the buffer in the reservoir with plastic wrap will prevent the buffer from evaporating, especially if the room is warm.
- Once the procedure is completed, check each membrane for visible prestained standards in order to confirm a successful blot.

Required Materials for Reagent Preparation

(for Eight Workstations)	Where Provided	Quantity
10x Tris-glycine buffer	Kit II	1 L
10x Phosphate buffered saline (PBS)	Kit II	40 ml
10% Tween 20	Kit II	1 ml
Dry blocker	Kit II	pack
Distilled water	Instructor's own	7 L
Ethanol/reagent alcohol (specially denatured alcohol (SDA) formula 3A)	Instructor's own	2 L

Prepare and aliquot:

Reagent to Prepare	Stock Reagents	Volume of Stock Reagent	Notes
Blotting buffer: 1x Tris-glycine with 20% ethanol	Distilled water 10x Tris-glycine Reagent alcohol **TOTAL**	7 L 1 L 2 L **10 L**	Blotting buffer used for blotting. Methanol or isopropyl alcohol can be substituted for reagent alcohol. Store at room temperature for up to 6 months.
Blocker: 5% dry blocker in wash buffer – Make this the day the blot is due to finish.	Sterile distilled water 10x PBS 10% Tween 20 Dry blocker **TOTAL**	359 ml 40 ml 1 ml 20 g **400 ml**	Used to block membranes and to dilute antibodies. Ensure dry blocker has fully dissolved in solution before using. Store at 4°C for up to 48 hours.

Student Workstations for Performing Capillary Action Blot

Material	Where Provided	Quantity Per Workstation
Blotting buffer	Prepared by Instructor	1 L
Blotting paper	Kit II	2
Nitrocellulose membrane	Kit II	1
Paper towel	Instructor's own	6–10 cm stack
Wick (paper towel or blotting paper strip long enough to cover the platform and reach the bottom of the container on both sides)	Instructor's own	1
Plastic barrier (such as plastic wrap or rubber strips)	Instructor's own	1
Large container	Instructor's own	1
Platform that fits in large container (upside-down agarose gel casting trays or gel staining trays are good for this)	Instructor's own	1
Weight (500 g to 1 kg) (a bottle of water works if actual weights are not available)	Instructor's own	1
Roller (pencil, test tube, pipet)	Instructor's own	1
Soft pencil	Instructor's own	1
Tape	Instructor's own	
Blocker – to be made once blotting is complete	Prepared by instructor	25 ml

Student Protocol

Alternate Lesson 3: Western Blotting Using Capillary Action Protocol

In this lesson, separated proteins will be transferred or "blotted" from the gel to a membrane that will eventually be probed for myosin using specific antibodies. First, a sandwich consisting of a gel and nitrocellulose membrane will be prepared in order to facilitate capillary action of blotting buffer and proteins penetrating through the layers.

Note: Towers are more stable with two gels blotted side by side on the same platform sharing the paper towel.

Student Workstations for Performing Capillary Action Blot

Materials Required	Quantity
Blotting buffer	1 L
Blotting paper	2
Nitrocellulose membrane	1
Paper towel	6–10 cm stack
Wick (paper towel or blotting paper strip long enough to cover the platform and reach the bottom of the container)	1
Plastic barrier (such as plastic wrap or rubber strips)	1
Large container to hold platform	1
Medium container to wet membranes	1
Platform that fits in large container	1
Weight (500 g to 1 kg)	1
Roller (pencil, 5 ml test tube, pipet)	1
Soft pencil	1
Tape	1

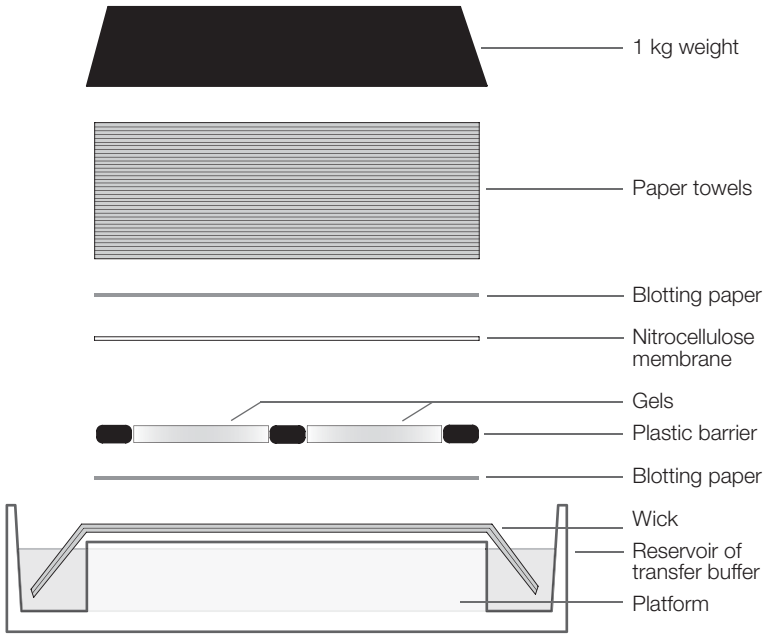
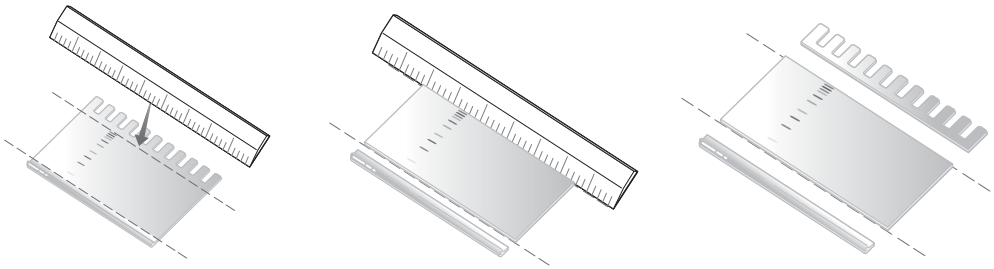


Fig. 22. Capillary action blot.

3.1. Prepare gel for blotting:

If the gel was not stained in the previous lesson, then remove it from the cassette. Lay the gel cassette flat on the bench with the short plate facing down. Using fingertips, carefully pry apart the gel plates. The gel will usually adhere to one of the plates.

On a stained or unstained gel, use a ruler or a plastic ID-type card (shown below), to carefully chop away wells and the 4% stacking gel located just above the top band of the prestained standards. At the bottom of the gel, chop away the ridge located below the blue line derived from the Laemmli sample buffer. Take great care to chop straight down into the gel rather than slicing across the gel, which will cause tearing. If the gel does tear, it can be pieced together in step 3.8.



3.2. Equilibrate the gel:

Transfer the gel to a tray containing blotting buffer. If the gel is adhered to the plate, allow the liquid to detach the gel from the plate. Incubate the gel in blotting buffer for at least 15 minutes. Longer equilibration times will not harm the experiment. While the gel is equilibrating, prepare the materials for the blotting sandwich.

3.3. Use a pencil to initial a corner of the white nitrocellulose membrane.

3.4. Wet two pieces of blotting paper and the nitrocellulose membrane in blotting buffer.

Refer to Figure 22 to help build the blotting tower.

3.5. Place the platform into the container and add 2–3 cm of blotting buffer so that it is 0.5 cm lower than the height of the platform.

3.6. Wet the wick (paper towel or blotting paper) in blotting buffer and lay it over the platform so that the ends are in the blotting buffer. The wick acts to pull buffer from the reservoir and conduct the buffer to the blotting paper. As the buffer moves through the gel and membrane, it will carry the proteins along with it.

3.7. Wet a piece of blotting paper, lay it over the wick, and remove any bubbles using the roller.

3.8. Lay the gel on the blotting paper and roll out any air bubbles. Keep the gel wetted with buffer; this will ease manipulation.

3.9. Place a plastic barrier (such as strips of plastic wrap) around the edges of the gel to prevent the buffer from soaking up through the edges of the blotting paper that surrounds the gel. Cover the entire surface of the wick, platform, and blotting paper that is not covered by the gel with plastic. Do not cover the gel with plastic or let the plastic overlap onto the gel.

3.10. Place the wet nitrocellulose membrane carefully on top of the gel. The nitrocellulose will probably overlap with the plastic wrap. Use the roller to ensure that there are no air bubbles between the gel and membrane. Try to move the membrane as little as possible once it has been placed on the gel, since proteins begin to blot immediately and ghost bands may form if it is moved.

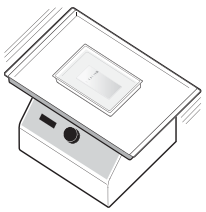
3.11. Place a wet piece of blotting paper on top of the nitrocellulose membrane. Use the roller to ensure that there are no air bubbles between the membrane and the blotting paper.

3.12. Place a 10–15 cm stack of dry paper towels on top of blotting paper. Ensure that paper towels do not come into contact with the reservoir of blotting buffer. If necessary cut the paper towels to size.

3.13. Place a 0.5–1 kg weight on top of paper towels. Tape the weight in place to prevent the tower from toppling over.

3.14. Leave the tower in place for two days, topping off the reservoir of blotting buffer as needed and replacing soaked paper towels (without disturbing the blotting paper) to ensure continual capillary action through the gel. The tower may be left over the weekend, but ensure the reservoir doesn't run dry for at least 48 hours. Blotting buffer used in the preparation steps should be used to top off the reservoir.

3.15. When the blot is completed, lesson 4 may be performed directly. Alternatively, the membrane may be put in 25 ml of blocker at 4°C overnight, or if a longer storage period is required, the membranes may be stored submerged in blotting buffer or wash buffer at 4°C for up to 1 week.



Appendix C: Blotting a Bio-Safe Coomassie Stained Gel

After destaining and analyzing the gel from kit I: protein profiler module, but before drying the gel, a western blot may be performed. This allows visualization of all the proteins within the gel and helps illustrate the principles of electrophoresis, blotting, and immunodetection since students can monitor the progress of the proteins throughout the stages of the experiment. After blotting, proteins on the membrane will be a blue mirror image of their position in the gel. Following antibody incubation and developing the membrane with a colorimetric substrate, myosin will appear as purple bands against the blue band background. Students will see how the antibody picks out one specific protein from the milieu found in muscle tissue. Note: Blue bands will fade slightly during the immunodetection steps.

Why is a western blot not normally performed on a Bio-Safe Coomassie-stained gel? Researchers are most interested in the quickest and most efficient blotting procedure and as such the extra steps required to stain the gel, along with the reduction in blotting efficiency due to the partial fixation of proteins in the gel are undesirable. In addition, reversible stains, such as Ponceau S, that will reversibly stain proteins on a membrane are available to check blotting efficiency.

If a western blot is being performed for the first time, it may be useful to use this protocol since it allows the status of the gel to be assessed prior to and during the blotting procedure. It is easier for students to grasp the concept of immunodetection if they can see the full complement of proteins on the blot prior to visualizing the antibody specificity. Troubleshooting is also easier using this protocol since the success of each step is witnessed as it is performed.

The modifications to the regular blotting protocol are stated below. These modifications are inserted as options in the western blotting protocol described in lessons 2 and 3.

Modification 1: Following staining and destaining with Bio-Safe Coomassie, but before drying the gel, an extra step is required. This is described as option B in step 3.1 of lesson 3. Bio-Safe Coomassie stain strips the negative charge from proteins in the gel that was acquired from SDS-containing Laemmli sample buffer. To replace the negative charge, incubate the gel in Tris-glycine-SDS (TGS) gel running buffer for at least 15 minutes before equilibrating in blotting buffer. Gels can remain in the TGS for a few hours if necessary.

Modification 2: Bio-Safe Coomassie stain partially fixes the proteins in the gel; therefore the blot must be run longer than the usual protocol to allow enough time for the electric current to pull the proteins out. Run the blot for 15 hours (e.g., overnight), at 20 V. This information is provided in step 3.7 of lesson 3. Most power supplies have timer functions.

Note: If protein stains other than Bio-Safe Coomassie stain are used to stain the gel, such as regular Coomassie stain, this protocol may not work because other stains may have stronger fixation properties.

Appendix D: Lesson Extensions

Lesson Extension 1: Determining the Molecular Mass of Myosin for Each Sample

In this lesson, the molecular mass of myosin is determined using Precision Plus Protein Kaleidoscope prestained protein standards. The term "standard" is used for known markers of molecular size used in experiments. These results may be recorded in the table below. A standard curve will be used to determine the molecular masses of different myosins by comparing the mobility of unknown proteins with protein standards run in the same gel. Although the prestained standards proteins range from 10–250 kD, a 15% polyacrylamide gel is designed to optimally separate low molecular mass proteins (under 50 kD). The standard curve will be derived from the 37, 25, 20, 15 and 10 kD protein standards.

Rough estimates of myosin molecular weight can be made by eye, by comparing the position of the myosin band on the gel with the prestained standards. Do this and record the estimations in the table at the end of this activity. After calculating the molecular weights using a standard curve at the end of this activity, compare the calculated values to the original estimates.

Construct a Standard Curve

Draw a horizontal line ~2 mm above the largest (250 kD) marker of the prestained standards. This line approximates the top of the resolving gel. Use this line as the starting point to measure and record distance in the table below from the line to the 37, 25, 20, 15 and 10 kD bands in the prestained standards. Accuracy to 0.5 mm is required.

Prestained Standard	Molecular Weight (Mr)	Log Mr	Distance Migrated (mm)
Green	37	1.57	
Pink	25	1.40	
Blue	20	1.30	
Blue	15	1.18	
Yellow	10	1.00	

A linear relationship exists between the mobilities of the proteins and $\log_{10}$ of the molecular weight*. Plot a graph using semi-log graph paper with the molecular weight (Mr) on the y-axis (log) and the distance migrated on the x-axis (linear). Alternatively, using linear graph paper, plot the log molecular weight (log Mr) on the y-axis and the distance migrated on the x-axis. The slope and intersept of this graph can be used to determine the linear equation for the graph: $y = mx + b$, where m is the slope and b is the intersept. This equation can then be used to calculate the Mr of myosin for each species. Another option is to use a graphing computer program to generate the chart, make a line of best fit (or a trend-line) through these points, and formulate an equation to calculate the Mr.

*Note: For maximum accuracy, standard curves should be plotted using the log Mr against relative migration (R_f) values. R_f is the distance the protein has migrated divided by the distance from the top of the resolving gel to the ion front (usually estimated as the Bromophenol Blue dye front or if this has disappeared off the blot; estimate it at 5 mm below the 10 kD standard). However, the method described above is sufficient to determine band sizes for the purpose of this lesson without adding complexity.

Finally, measure and record the distance migrated by each of the myosin bands. Determine the Mr either by reading values directly from the graph or by using the linear equation.

Note: One standard curve will not suffice for the entire class as each gel will run slightly differently. A new standard curve must be plotted for each gel run.

Sample Name	Estimated Mr From Blot	Distance Migrated (mm)	Calculated Mr

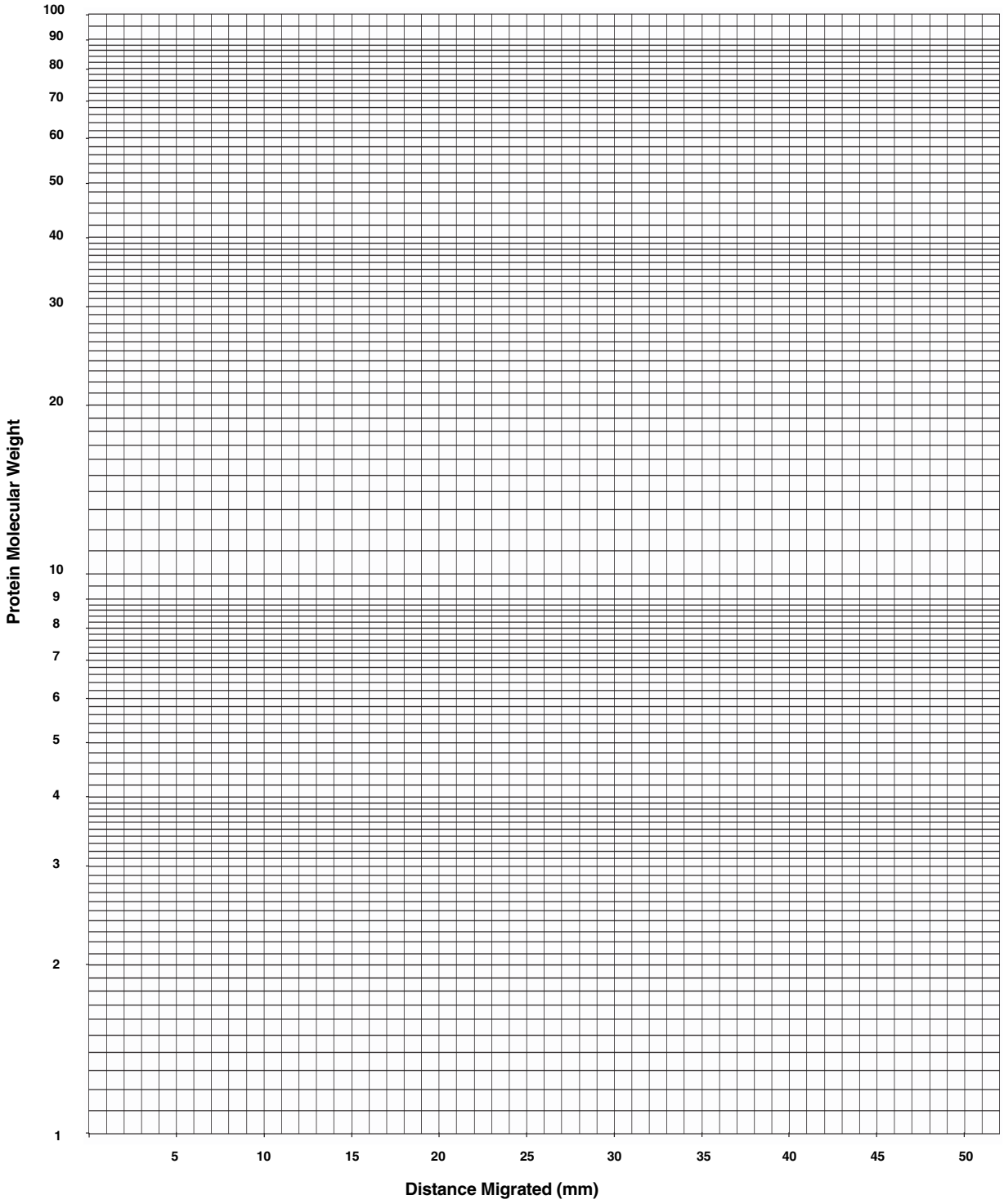


Fig. 23. Two-cycle semi-logarithmic graph paper to construct curve of the protein molecular weight against the distance migrated.

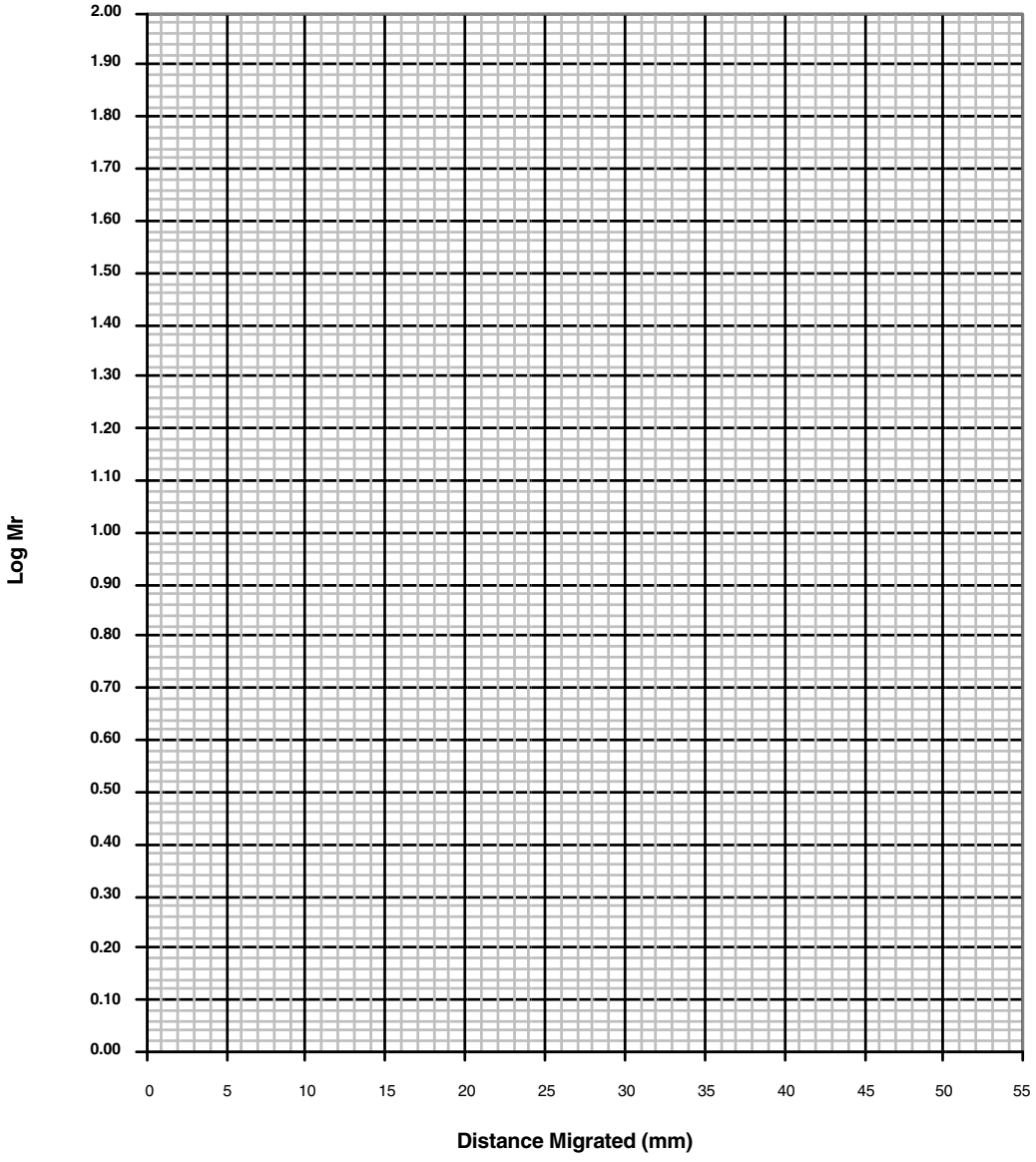


Fig. 24. Linear graph paper to construct a standard curve of the log molecular weight (log Mr) against the distance migrated.

Lesson Extension 2: Comparing Results With Published Data

Myosins from different fish species examined will vary in size. Is this size variation due to differences in the gene sequences as a result of "genetic drift" (e.g., the amount of random change in gene sequences)? Perhaps it is due to changes in posttranscriptional RNA or posttranslationally modified proteins? Does evidence for divergence exist at the molecular level? How far can proteins diverge before they hit an evolutionary dead end? Can the essential regulatory regions of myosin be determined?

One way to further investigate these intriguing questions is by performing some bioinformatics research. The National Center for Biology Information (NCBI), in the National Library of Medicine (NLM) at the National Institutes of Health (NIH) maintains databases of DNA, RNA, and protein sequence information generated by scientists all around the world. The NCBI Entrez sequence database (http://www.ncbi.nlm.nih.gov/entrez) can be used to determine whether the fish myosins under investigation have been sequenced. Protein sequences (in the protein database) are usually deduced from mRNA sequences. These databases are constantly being updated as scientists around the world continuously submit new data. Unfortunately, it is unlikely that the myosins from all the species chosen in this study have already been sequenced since a database search in January, 2006 revealed only 17 myosin protein sequences derived from fish. This paucity of data indicates that there is much to discover and students from this class may be the first to determine the size of myosin in a particular species.

Use the published fish myosin sequences to determine which factor is a better determinant of relatedness: protein size or protein sequence. Appendix B from kit I: protein profiler module contains a lesson for how to construct a cladogram using protein sequence homology. Below there are five protein sequences referenced and aligned for myosin light chains (MLC) 1 and 2 from five fish species that may be used to construct a cladogram based on sequence homology. Can deductions about the essential regulatory regions of myosin be made from the sequence alignment?

The molecular masses given below are deduced from the mRNA transcripts that translate into the amino acid sequences of these proteins; they were not determined by SDS-PAGE analysis. Do the results from the preceding lesson match these predicted protein sizes? If not, consider why the molecular mass of a protein determined by gel analysis may differ from that predicted by the mRNA sequence. What factors other than sequence affect the mass of a protein? Also consider how these theories could be tested.

	MLC1		MLC2	
Fish	**Predicted Molecular Mass (kD)**	**Protein Accession #**	**Predicted Molecular Mass (kD)**	**Protein Accession #**
Carp (*Cyprinus carpio*)	21.13	BAA12731	18.89	BAA89704
Bluefin tuna (*Thunnus thynnus*)	21.28	BAA95123	18.99	BAA95125
Scad (*Trachurus trachurus*)	20.61	BAA95135	19.03	BAA95137
Sardine (*Sardinops melanostitctus*)	21.83	BAA95138	19.42	BAA95140
Walleye/pollock (*Theragra chalcogramma*)	21.25	BAA95143	19.05	BAB18578

Alignment of MLC1 from five fish species:

```
BAA12731 (1) MAPKKDAK-KPEP-AKKAEP---APAPAPAPAPEAPPK-PAAVDLSGVKVDFNQDQLEDYREAFGLFDRVG
BAA95123 (1) MAPKKDAK-APAKKAEPAKKAEPAPAPAPAPEPAPAPAAPAAVDLSAVKVEFSADQIEDYKEAFGLFDRVG
BAA95135 (1) MAPKKDAK-APAKKAEP------APAPAPAPEPAPVPAAPAAVDLSAVKIEFSPDQVEDYKEAFGLFDRVG
BAA95138 (1) MAPKKDAKPAPAKKAEPAKKAEPAKKEEPLPEPPPKPA-PAAVDLSAVKVEFTPDQIEDYREAFGLFDRLG
BAA95143 (1) MAPKKDVK-APAAAAKKAEP---AKKVEPAPEPVAVPA-PKTVDLSAVKVDFTPDQMEDYREAFGLFDRVG

Section 2
BAA12731 (66) DNKVAYNQIADIMRALGQNPTNKEVTKILGNPTADEMANKRVDFEGFLPMLQFVVNSPNKATYEDYVEGLR
BAA95123 (71) DNKVAYNQIADIMRALGQNPTNKDVAKLLGMPSAEDMTNKRVEFEGFLPMLQTIINSPNKAGYEDYVEGLR
BAA95135 (65) DNKVAYNQIADIMRALGQNPTNKEVAKMLGTPSAEDMANKRVEFEGFLPMLQTIINSPNKAGYEDYVEGLR
BAA95138 (71) DNKVAYNQIADIMRALGQNPTNKEVKHILGNPSPEDMAGKRIEFEQFLPMLQTVVNNPNKAQFEDYVEGLR
BAA95143 (67) DNKVCYNQIADIMRALGQNPTNKEVKAILGNPSDEDMNSKRVDFEGFLPMMQTIVNSPNKGTLDDYVEGLR

Section 3
BAA12731 (137) VFDKEGNGTVMGAELRIVLSTLGEKMTEVEIDALMQGQEDENGCVNYEAFVKHIMSV
BAA95123 (142) VFDKEGNGTVMGAELRIVLSTLGEKMTEAEIDALMQGQEDESGCVNYEAFVKHIMSV
BAA95135 (136) VFDKEGNGTVMGAELRIVLSTLGEKMTEAEIDALMTGQEDESGGVNYEAFVKHIMSV
BAA95138 (142) VFDKEGNGTVMGAELRIVLSTLGEKMNEAEVDALMTGQEDENGCVNYEAFVKHIMSV
BAA95143 (138) VFDKEGNGTVMGAELRIVLSTLGEKMTEAEIDALMQGQEDENGCINYESFVKHIMSI
```

How to Use NCBI Databases

The NCBI Entrez protein sequence database can be used to search for fish myosins using search terms related to the proteins of interest. However, navigating through the NCBI databases can be difficult; it is important to use search terms that are wide enough to include what is needed and yet narrow enough to avoid trawling through hundreds of hits. For example, “myosin light chain” may result in 800 hits. If this search is refined by adding a class of ray-finned fish "Actinopterygii, myosin light chain", then results may be narrowed down to around 150 hits. Using the terms “carp, myosin light chain” may retrieve 8 hits, but does not retrieve carp myosin light chain 2 (BAA89704) because “myosin”, “light” and “chain” need to be separated with commas to allow the search to include “myosin regulatory light chain”, another term used for myosin light chain 2.

A simpler alternative to using the NCBI protein database to search for proteins by their names is “BLAST” – a search tool that will retrieve protein sequences that are homologous to an input sequence, in this case, a known fish myosin sequence. A drawback to this method is that an assumption is made that fish myosins will be homologous to each other. Any fish myosin sequences that differ significantly from the input sequence may not appear in the search results; but then such divergence would undermine evolutionary theory!

Quick Guide to BLAST Searching

This quick guide is designed to obtain a list of fish myosin sequences. BLAST searches may be conducted in multiple ways; further information may be found on the NCBI web site.

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

Carp Myosin Light Chain 1 Protein Sequence (BAA12731):

```
mapkkdakkp epakkaepap apapapapea ppkpaavdls gvkvdfnqdq ledyreafgl
fdrvgdnkva ynqiadimra lgqnptnkev tkilgnptad emankrvdfe gflpmlqfvv
nspnkatyed yveglrvfdk egngtvmgae lrivlstlge kmteveidal mqgqedengc
vnyeafvkhi msv
```

Other fish myosin proteins can be found and used by searching the NCBI Entrez Protein database.

4) Hit the BLAST button without modifying any fields.
5) Hit the Format button on the new window.
6) After a short wait, the BLAST results window will appear and may be several hundred pages. There should be a long list of sequences that produced significant alignments. Although this search may detect hundreds of sequences, they are listed in order of homology, which means only the first 25 or so hits will be relevant.
7) Click on the accession number link to open the page that describes the organism, protein, and protein sequence with links to the mRNA sequence.
8) Investigate BLAST search data further:
    a. To find out how myosins vary between taxonomic classes, there will probably be sequences from other species such as mice (*Mus musculus*), frogs (*Xenopus laevis*), and humans located further down the list. However, remember that the NCBI database is designed for and used by experienced scientists, which is why the data is complex. There are multiple myosin genes and multiple entries for myosin genes for each species, so it can be confusing.
    b. Further down the BLAST results page, sequences will be aligned with the original for comparison. The value given for "identities" is equal to the number of identical amino acids, the value for “positives” is derived from the number of amino acids that are similar to each other (e.g., serine and threonine), and the value for “gaps” is the number of amino acid positions that are absent from one of the sequences.
9) There are resources on the NCBI web site to help understand BLAST search results.

Appendix F: Glossary

Actin – a major muscle protein organized into thin filaments

Adsorb – adhesion of molecules to the surface of a solid with which they are in contact

Amino acid – molecules that form the building blocks of proteins. Most organisms construct proteins from a particular set of 20 amino acids, although several dozen other amino acids are found in nature.

Anode – positive electrode

Antibody – immunoglobulin protein formed in response to a challenge of the immune system by a foreign agent. Antibodies bind to specific antigens.

Antigen – any agent that provokes an acquired immune response and is bound specifically by either antibodies or T cells

Antiserum – blood serum containing antibodies raised against a specific antigen

beta-Mercaptoethanol (BME) – a chemical that can cleave disulfide bonds and protect sulfhydryl groups

Blot – in molecular biology, a blot is a method of transferring proteins, DNA or RNA, onto a solid support such as a nitrocellulose membrane. In many instances, blotting is performed after gel electrophoresis, where molecules from the gel are transferred or "blotted" onto the blotting membrane. In the case of proteins, this kind of blot is called a 'western blot'

Cathode – negative electrode

Charge density – the ratio of charge to mass of a protein

Clone – in the context of cell biology, "a clone" is a cell or group of cells that are all derived through cell division from the same parent cell and thus have identical genetic data. In the context of molecular biological techniques, "to clone" means to obtain a fragment of DNA from a genome and ligate it into another piece of DNA, such that the ligated DNA will contain an identical copy of that gene fragment.

Cladogram – a sketched tree that represents historical branching relationships among species. The depicted branch lengths in a cladogram are arbitrary; only the branching order signifies ancestral history.

Codon – a set of three DNA nucleotides that code for an amino acid

Colorimetric substrate – colorless reagents that create a colored product when the reaction producing them is catalyzed by an enzyme are termed colorimetric substrates. For example, 4-chloro-1-napthol (4CN) produces a purple product when oxidized by horseradish peroxidase. The term **chromogenic** is also used in this context.

Conjugate – a substance formed by the covalent bonding of two types of molecules, such as horseradish peroxidase linked or "conjugated" to an antibody

Capillary action – the movement of liquid molecules upwards against the force of gravity. Capillary action is caused by the adhesive intermolecular forces between the liquid (blotting buffer) and a solid (the blotting paper and paper towels) that are stronger than the cohesive intermolecular forces within the liquid.

Dalton (D) – a unit of molecular mass equal to the mass of a hydrogen atom, 1.66×10^{-24} gm

Denature – to disrupt a protein's 3-D structure

Deoxyribonucleic acid (DNA) – the genetic material of nearly all life forms. DNA is used to store the genetic information of all living creatures (except RNA viruses) that cells need in order to propagate, replicate DNA, and produce proteins.

Disulfide bond – the S-S bond between amino acids in a polypeptide chain; contributes to tertiary and quaternary structure of proteins

Dithiothreitol (DTT) – a chemical that can cleave disulfide bonds by reducing disulfides to dithiols and prevents the oxidation of thiol groups

Electroblotting – the use of an electric current to blot molecules from a gel onto a solid support

ELISA – see Enzyme-linked Immunosorbent Assay

Enzyme – a protein that facilitates or "catalyzes" a chemical reaction without itself being altered in the process. The molecule that an enzyme catalyzes is called its substrate. Enzymes are classified and frequently named on the basis of the reactions that they catalyze. For example, a peroxidase catalyzes the oxidation of its substrate.

Epigenetic – something that affects a cell, a tissue or an organism's RNA or protein expression without directly affecting its DNA (genome)

Epitope – the part of a foreign organism or its proteins that is being recognized by the immune system and targeted by antibodies, cytotoxic T cells, or both – also called antigenic determinant. Most epitopes can be thought of as 3-D surface features of an antigen molecule. Exceptions are linear epitopes, which are determined by the amino acid sequence (the primary structure) rather than by the tertiary structure of a protein. Epitopes can be mapped using ELISA techniques.

Enzyme-linked immunosorbent assay (ELISA) – an immunological assay that involves adsorbing proteins to multi-well polystyrene microplates then using antibodies to probe for specific proteins. Enzyme-linked antibodies are used to oxidize a substrate causing a quantifiable color change in the microplate wells. Frequently used in disease diagnosis.

Exon – in a gene region, the active coding region for translating amino acids (compare to intron)

Fingerprint – a distinct pattern of bands on a protein gel, used to identify a sample or species

Gene – a segment of DNA that contains information on hereditary characteristics such as hair color, eye color, and height, as well as susceptibility to certain diseases. A working subunit of DNA. Each gene contains the code for specific products; typically, a protein such as an enzyme. Many genes encode multiple proteins

Genome – the entire complement of genes in an organism

Genomics – a global study of genes, their functions, and their origins

Genotype – the entire genetic identity of an individual that may or may not translate into specific outward characteristics

Homologous – in the context of proteins, a sequence that resembles another previously characterized sequence closely enough to suggest common genetic ancestry. In molecular biology, homologous is commonly used to mean similar, regardless of genetic relationship.

Homology -— similarity between different genes due to common ancestry

Horseradish peroxidase (HRP) – an enzyme frequently used to label secondary antibodies. HRP oxidizes substrates such as 4CN for colorimetric detection.

Immune cell – any cell of the immune system, including lymphocytes (B and T cells) and macrophages

Immunogen - any agent that provokes an immune response. Immunogens that trigger a response from the acquired immune system are called antigens

Immunoglobulin (Ig) – general term for all types of antibodies, it refers to a specific amino acid structure or domain found in all antibodies

Immunology – the study of the immune system, or the system that protects the body from foreign substances, cells, and tissues by producing an immune response

Intron – a noncoding region of a gene that consequently does not translate into amino acids (compare to exon)

Kilodalton (kD) – 1,000 Daltons

Linear epitope – a linear epitope is an epitope that is recognized by antibodies by its linear sequence of amino acids, or primary structure. In contrast, some antibodies recognize an epitope that has a specific 3-D shape and its protein structure.

Lymphocyte – a type of white blood cell in the immune system that includes thymus-derived T cells and bone marrow-derived B cells

Messenger RNA (mRNA) – the template or message derived from a gene that is translated into peptide sequences. An intermediary between DNA and protein synthesis.

Macrophage – a type of white blood cell that engulfs foreign materials and antigens in a process called phagocytosis. Macrophages serve two primary functions: 1) removing foreign cells and molecules from the blood; and 2) processing antigens and presenting them on cell surfaces as a flag for destruction.

Membrane – a solid paper-thin support that proteins, DNA, or RNA are bound to during respective western, Southern, or northern blotting procedures

Myosin – a major muscle protein organized into thick filaments

Native – the natural structure of a protein or protein complex, as found within the organism, rather than the denatured form after treatment with detergent

Nitrocellulose – a synthetically nitrated derivative of cellulose; it is made into porous membrane filters to immobilize DNA, RNA, or protein, which can then be probed with a labeled sequence or antibody. Used in Southern, northern, and western blotting procedures involving DNA, RNA, and proteins.

PAGE – polyacrylamide gel electrophoresis

Phylogeny – the evolutionary relationship of species based on lineage and history of descent

Peptide – a molecule comprised of two or more amino acids

Posttranslational modification – after synthesis, additional modifications made to a protein that influence or determine its function, such as phosphorylation, glycosylation, or protein cleavage

Profile – a distinct pattern of bands on a protein gel, used to identify a sample or species

Protein – a functional assembly of one or more polypeptides

Proteomics – the study of proteins and their functions

Phenotype – the observable traits or characteristics of an organism, for example hair color, weight, or the presence or absence of a disease. Phenotypic traits are not necessarily derived from genetic traits and can be influenced by the environment.

Primary antibody – in an immunoassay, the primary antibody binds a specific antigen, conferring specificity to the assay

Ribonucleic acid (RNA) – a chemical found in the nucleus and cytoplasm of cells; it is the intermediary between DNA and protein synthesis and is also involved in other chemical activities of the cell. The structure of RNA is similar to that of DNA. There are several classes of RNA molecules, including messenger RNA, transfer RNA, ribosomal RNA, and other small RNAs, each serving a different purpose.

SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis; a form of electrophoresis where samples are treated with SDS to denature proteins and provide a uniform charge to mass ratio

Secondary antibody – in an immunoassay, an antibody that recognizes the primary antibody, and is conjugated to an enzyme that can catalyze a reaction to produce a colored product

Serum (plural, sera) – the clear fluid obtained when solid components such as red and white blood cells are removed from whole blood

Substrate – the target molecule for an enzyme

Transcription – the synthesis of mRNA from DNA genetic information

Translation – the production of a peptide from messenger RNA (mRNA)

Transfer RNA (tRNA) – RNA that acts as an adaptor molecule between mRNA and amino acids

Western – see blot

Appendix G: References

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Further Reading: Books and Journal Articles

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SDS-PAGE & Western Blotting Internet Resources:

http://www.mcb.uct.ac.za/manual/MolBiolManual.htm: Molecular Biology Techniques Manual, Dept Microbiology University of Cape Town, Cape Town, South Africa

http://www.ruf.rice.edu/~bioslabs/studies/sds-page/gellab2.html: Experimental Biosciences Introductory Laboratory Course, Rice University, Houston, TX

Evolution Internet Resources

http://tolweb.org/tree/ 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.fishbase.org: Global information system on fish

Bioinformatics Internet Resources

http://www.ncbi.nlm.nih.gov/: National Institutes of Health, National Center for Biotechnology Information bioinformatics databases

http://workbench.sdsc.edu: Bioinformatics tools from University of California, San Diego

www.wwpdb.org: Protein data bank, database of protein structural information

Bio-Rad Technical Bulletins (request from your local Bio-Rad office or download from www.bio-rad.com)

Ready Gel Resource Guide-bulletin 2144

Protein Blotting Guide-bulletin 2895

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Germany *089 318 84 0* ***Greece*** *30 210 777 4396* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 455 8800* ***India*** *91 124 4029300/5013478* ***Israel*** *03 963 6050*
Italy *39 02 216091* ***Japan*** *03 5811 6270* ***Korea*** *82 2 3473 4460* ***Mexico*** *55 5200 05 20* ***The Netherlands*** *0318 540666* ***New Zealand*** *64 9415 2280*
Norway *23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 095 721 14 04* ***Singapore*** *65 6415 3188* ***South Africa*** *27 0861 246 723*
Spain *34 91 590 5200* ***Sweden*** *08 555 12700* ***Switzerland*** *061 717 95 55* ***Taiwan*** *886 2 2578 7189/2578 7241* ***United Kingdom*** *020 8328 2000*

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