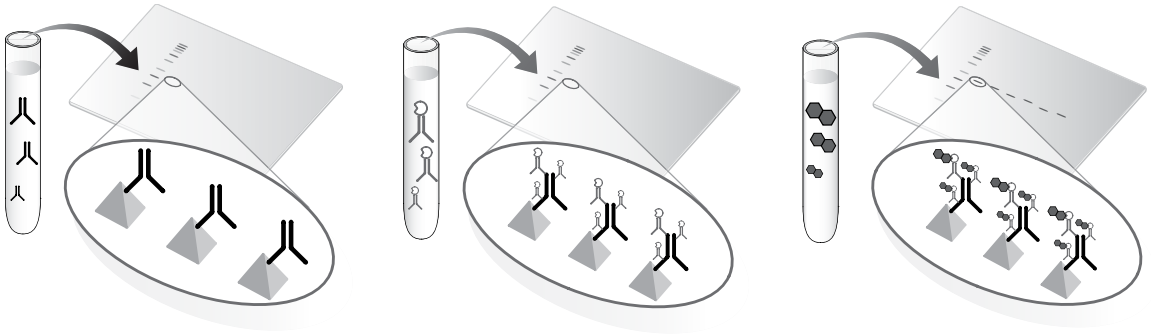

Bio-Rad Explorer™

Comparative Proteomics Kit II: Western Blot Module

Catalog Number
1662800EDU

explorer.bio-rad.com

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

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

BIO-RAD

Dear Educator:

Tapping nature's tool kit

Animal immune systems naturally generate antibodies when foreign invaders are detected and tag them for destruction. The ability of antibodies to act like magic bullets and target viral, bacterial, and allergenic antigens in the body also makes them ideal for hunting antigens in bioscience research and diagnostic tests.

Western blotting employs antibodies to pinpoint specific proteins of interest in complex protein mixtures such as cell extracts. Because of its accuracy, western blotting is used as the confirmatory diagnostic test for HIV and mad cow disease, or bovine spongiform encephalopathy.

Western blotting is used extensively in research to determine the presence of specific proteins, to quantify their expression levels, and to determine whether they have undergone genetic or posttranslational modifications. This surefire method categorically identifies proteins of interest based on two distinguishing features: molecular mass and antibody binding specificity.

This western blotting activity allows your students to take Bio-Rad's protein profiler kit to the next level. Students use western blotting to specifically identify myosin light chain from the hundreds of other proteins that comprise the muscle cell extracts of closely and distantly related species of fish.

In the first part of this laboratory (Protein Profiler Module), students generate protein profiles and visualize the unique arrays of proteins comprising the muscle tissues from each of their samples. From their protein gel results, students make educated guesses as to the identities of the proteins. Based on their relative molecular masses alone, however, these inferences remain guesses. Via western blotting, the protein bands in their polyacrylamide gels are transferred horizontally to a membrane and an anti-myosin antibody is employed to precisely identify which protein in each species' profile is myosin light chain.

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. However, differences in the molecular weights of the myosin light chains of different species are detectable via western blotting, leading students to hypothesize about how these variations relate to their evolutionary relationships. Using Internet-based bioinformatics databases, students can then compare their experimentally determined results to actual protein sequence data derived from DNA and RNA sequences and consider whether variations in myosins between species are due to “genetic” or “epigenetic” factors.

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

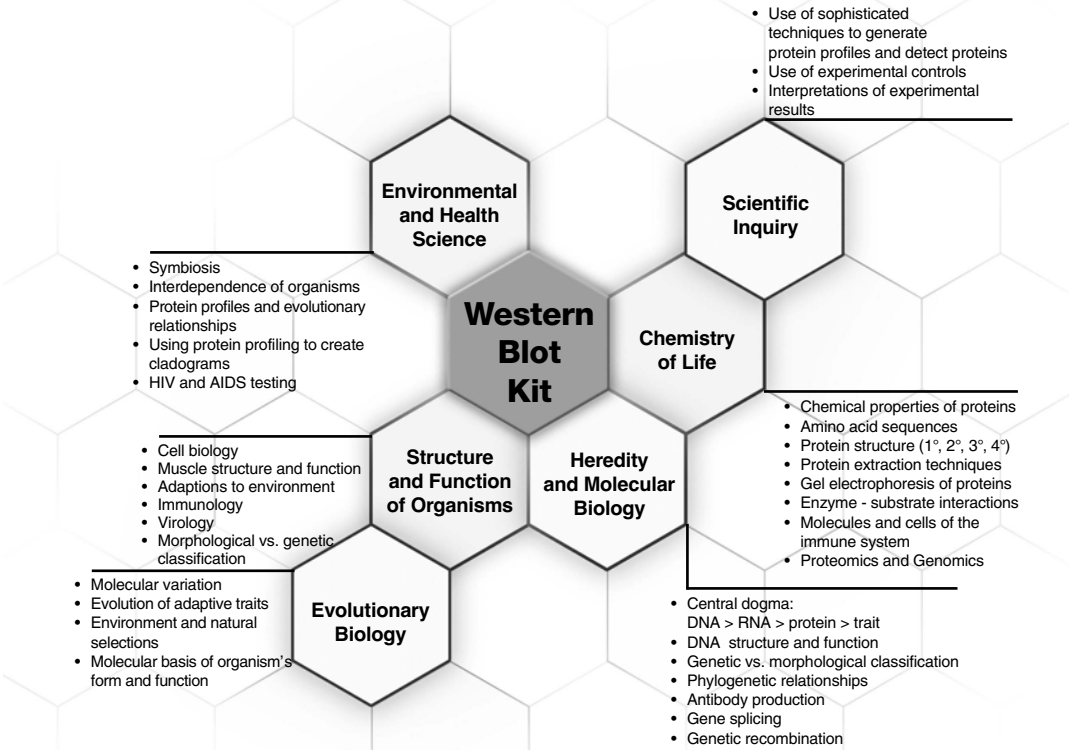


Table of Contents

Kit Inventory Checklist

This section lists required and recommended components for the western blot kit. Each kit contains materials for 8 workstations with a maximum of 4 students per workstation. Please use the checklist below to confirm inventory.

Kit Components	Number/Kit	(✔)
Primary antibody (anti-myosin light chain mouse monoclonal), lyophilized	1 vial	❒
Secondary antibody (goat anti-mouse polyclonal antibody conjugated to horseradish peroxidase (HRP)), lyophilized	1 vial	❒
HRP color detection reagent A	1 bottle	❒
HRP color detection reagent B	1 vial	❒
10x Tris-glycine	1 bottle	❒
Nonfat dry milk blocker	1 pack	❒
10x Phosphate buffered saline (PBS)	2 bottles	❒
10% Tween 20	1 bottle	❒
Nitrocellulose, 0.45 µm*	8 sheets	❒
Blotting paper*	16 sheets	❒
Reagent tubes	1 pack	❒
Curriculum/instruction manual	1	❒

*Nitrocellulose and blotting paper are shipped separately

Required Accessories	Number/Kit	(✔)
Comparative proteomics kit I: protein profiler module (#1662700EDU)	1	❒
4–20% Mini-PROTEAN® TGX™ precast gels, 10-well (#4561094EDU)	8*	❒
Vertical gel electrophoresis chambers (#1658005EDU)	4**	❒
Adjustable micropipet, 2–20 µl	8	❒
Power supply (#1645050EDU)	2–4***	❒
Water bath or heating block to reach 95°C (#166505EDU)	1	❒
Rocking platform or shaker (#1660709EDU)	1	❒
Reagent alcohol or ethanol	0.8–2 L	❒
Distilled water	8–12 L	❒
Fish samples	5	❒
Blade (for cutting fish)	8	❒
Containers (#1704089EDU)	8–16	❒
Foil to cover 8 reagent tubes	–	❒
Soft pencil	8	❒
Roller (#1651279EDU)	8	❒
Paper towel	16 sheets	❒
Marking pens	8	❒

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

** One Mini-PROTEAN® Tetra Cell can run one to two gels with one electrode assembly or up to four gels with the addition of a Mini-PROTEAN Tetra Companion Running Module (#1658038EDU).

*** See notes on power supplies for western blotting on page 19.

Recommended Accessories	Number/Kit	(✔)
Electroblotter (#1703924EDU)	4	❒

Refills Available Separately

Western blot temperature sensitive reagents bag (includes primary antibody, secondary antibody, HRP color detection reagents A and B) (1662801EDU)

Protein profiler temperature sensitive reagents bag (includes Precision Plus Protein™ Kaleidoscope™ prestained standards, 50 µl; actin & myosin standard, 500 µg; and DTT, 0.3 g) (#1662701EDU)

Laemmli sample buffer, 30 ml (#1610737EDU)

Precision Plus Protein Kaleidoscope standards, 500 µl (#1610375EDU)

Actin & myosin standard, 500 µg, lyophilized (#1660010EDU)

DTT, 1 g (#1610610EDU)

Primary antibody (anti-myosin light chain antibody), 200 µg, lyophilized, (#1662804EDU)

Secondary antibody (goat anti-mouse-HRP), 2 ml, frozen, quantity sufficient for 160 blots at 1:1,000 dilution (#1721011EDU)

Horseradish peroxidase (HRP) conjugate substrate kit, makes 2 L of HRP color detection solution (#1706431)

10x Tris-glycine-SDS, 1 L (#1610732EDU); 5 L (#1610772EDU)

10x Tris-glycine, 1 L (#1610734EDU); 5 L (#1610771EDU)

10x Phosphate buffered saline (PBS), 1 L (#1610780EDU); 100 ml (#1662403EDU)

10% Tween 20, 1 L (#1610781EDU); 5 ml (#1662404EDU)

Bio-Safe™ Coomassie stain, 1 L (#1610786EDU)

0.45 µm nitrocellulose, 7 x 8.4 cm, 10 sheets (#1620145EDU)

Thick blot paper, 7.5 x 10 cm, 50 sheets (#1703932)

Comparative Proteomics Kit I: Protein Profiler Module Contents, 1662700EDU:

Kit Components	Number/Kit	(✔)
Prot/Elect™ pipet tips for gel loading	1 rack	❒
1.5 ml fliptop micro test tube	2 packs	❒
1.5 ml screwcap micro test tube	1 pack	❒
Disposable 1 ml pipets (DPTPs)	3 packs	❒
Laemmli sample buffer, 30 ml (#1610737EDU)	1 bottle	❒
Precision Plus Protein Kaleidoscope prestained standards, 50 ul	1 vial	❒
10x Tris-glycine-SDS electrophoresis buffer, 1 L (#1610732EDU)	1 bottle	❒
Bio-Safe Coomassie stain for proteins, 200 ml	2 bottles	❒
Actin & myosin standard – 500 µg lyophilized (#1660010EDU)	1 vial	❒
DTT, 0.3 g	1 vial	❒
Gel staining trays	4	❒
Foam floats	8	❒
Curriculum/instruction manual	1	❒

Curriculum Fit

- Students develop abilities to conduct inquiry-based experiments
- Students learn laboratory skills technique commonly used in research
- Students formulate scientific explanations using data, logic, and evidence
- Students develop an understanding of biological evolution
- Students compare different forms of proteins that may give organisms an evolutionary advantage

Storage Instructions

Kit I – Protein profiler module (#1662700EDU): Open kit immediately upon receipt and place bag of temperature sensitive components (containing Precision Plus Protein Kaleidoscope prestained standards, actin & myosin standard, and DTT) in –20°C freezer. Store all other reagents at room temperature.

Kit II – Western blot module (#1662800EDU): Open kit immediately upon receipt and place bag of temperature sensitive components (containing HRP detection reagents A and B, anti-myosin light chain antibody and secondary antibody) in –20°C freezer. Store all other reagents at room temperature.

Time Line

Lesson 1 (45 minutes) – Students prepare muscle protein extracts (Instructor's advanced preparation 0.5–1 hour).

Lesson 2 (45 minutes) – Students load and run gels (Instructor's advanced preparation 0.25–1 hour). Staining the gels at this step is optional (see Appendix C for details).

Lesson 3* (45 minutes) – Students blot proteins to membrane (perform western blot)
* Note: It is preferable for lesson 3 to directly follow lesson 2 (instructor's advanced preparation 0.5–1 hour).

Prior to lesson 4, instructor puts membranes into blocker for 15 minutes to overnight.

Lesson 4 (45 minutes) – Students probe membrane for presence of myosin light chain (instructor's advanced preparation 1–2 h).

Note: If schedule permits, the entire laboratory can be conducted in a single 3 hour block.

Safety Issues

Eating, drinking, smoking, applying cosmetics, and wearing open toed shoes are prohibited in the work area. Wearing protective eyewear, gloves, and lab coats or other protective clothing is strongly recommended. Students should wash hands with soap before and after this exercise. If any solutions come into contact with eyes, then flush with water for 15 minutes. HRP color detection reagent A (4CN) is harmful if swallowed and will require immediate medical assistance. Nitrocellulose is highly flammable and should be kept away from direct flame or sparks. Although Bio-Safe Coomassie stain is not toxic, latex or vinyl gloves and protective clothing should be worn to keep hands and clothes from becoming stained. Please refer to the Material Safety Data Sheets (MSDS) available from Bio-Rad at (800) 4BIORAD in the US or at www.bio-rad.com for further information on reagents in this kit. Please consult your local environmental health and safety regulations for proper disposal.

Background for Instructors

Western Blotting Overview

Western blotting is a powerful tool used in scientific research and clinical diagnostic laboratories to identify specific proteins within a biological sample. This surefire method of identifying proteins is based on two distinguishing properties: molecular mass and antibody binding specificity. The western blot procedure in this kit is designed to identify myosin – a highly conserved muscle protein essential for survival in animals. The first step in the procedure employs protein electrophoresis to determine the molecular masses of the proteins within various fish muscle tissue samples. In the second step, proteins are transferred from the gel to a membrane and an antibody-based detection system is used to precisely identify which protein in each sample is myosin.

This western blotting activity illustrates how bioengineering has taken elements of the natural immune system and modified them for use in biological research and clinical diagnostic labs. Because of the sensitivity of the antibodies used in western blotting, this technique can be used to detect and identify trace amounts of proteins that would not be detectable using other methods.

In this activity, students begin by extracting proteins from the muscle tissues of different fish species. The lysis buffer used to break open or lyse the muscle cells contains the anionic detergent sodium dodecyl sulfate (SDS) and a strong reducing agent, dithiothreitol (DTT). SDS effectively coats all the proteins in the sample with negative charge and DTT breaks disulfide bridges, putting proteins into a linear, uniformly charged state which eases further analysis on western blots. The samples are then loaded into a vertical polyacrylamide gel electrophoresis system and proteins are separated according to their size, or molecular weight. (Please refer to the protein profiler module instruction manual for more detailed information about protein gel electrophoresis.)

Following electrophoresis, proteins are electrophoretically transferred horizontally from the gel onto a nitrocellulose membrane. Proteins, negatively charged by the SDS, migrate out of the gel and bind to the surface of the membrane creating a western, or protein blot. The proteins blotted onto the membrane form a mirror image of proteins separated in the original gel. Molecules on the surface of the membrane are now accessible for further analysis. Alternatively, although less efficient, capillary action can be used to transfer the proteins from the gel to the membrane. Please refer to Appendix B: Alternate Lesson 3 – Alternative Western Blotting Method Using Capillary Action.

The name **western blot** was given to the technique by W. Neal Burnette (Burnette 1981) and is a pun on the name Southern blot, a technique for DNA detection developed earlier by Edwin Southern. Detection of RNA is termed northern blotting.

Once proteins are transferred to the nitrocellulose membrane (the 'blot'), the next step is to probe the blot with an antibody that is specifically engineered to detect a particular protein of interest. First the blot is incubated in a protein-rich solution often derived from powdered milk protein. Powdered milk is used because it is inexpensive and easy to obtain. Incubating the blot with powdered milk effectively coats the remaining surface area of the membrane (where no proteins have been blotted) and blocks nonspecific protein binding sites.

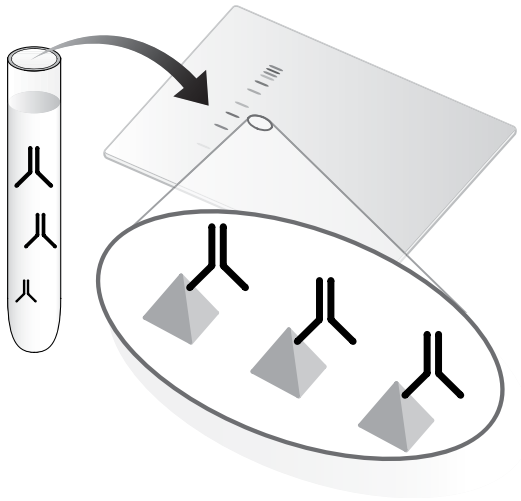
Next, the blot is incubated with a primary antibody. The primary antibody is designed to specifically recognize, or bind, the protein under study. In this kit, the primary antibody has been engineered to bind exclusively to myosin light chain proteins on the membrane. Following a quick rinse step, the membrane is then incubated with an enzyme-linked secondary antibody that has been engineered to bind specifically to the primary antibody.

A colorless colorimetric (color-producing) 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 purple precipitate that leaves visible deposits on the membrane at the precise location of the blotted myosin light chain proteins. The combined blotting and immunodetection procedure is used to determine the exact position of myosin. The precise molecular mass of myosin can then be determined for each sample by constructing a standard curve from the Precision Plus Protein Kaleidoscope prestained standards run alongside the protein samples in the gel. (Please refer to Appendix D for detailed instructions on generating standard curves for molecular weight determination of unknown proteins.)

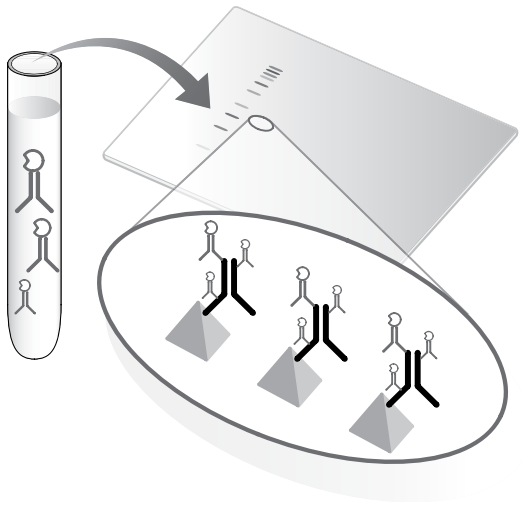
Why is it necessary to transfer the proteins from the gel to the nitrocellulose membrane? Why can't myosin be detected by applying antibodies directly on the gel? First, since the proteins are contained within the gel and embedded within the polyacrylamide matrix, antibodies would have difficulty reaching the proteins. Second, the gel is fragile and can easily break during analysis (as some students may unfortunately discover while performing this lab!) while a membrane is more stable and durable. Lastly, the membrane can be stripped of antibodies and reprobed several times.

Immunodetection: Step-by-Step

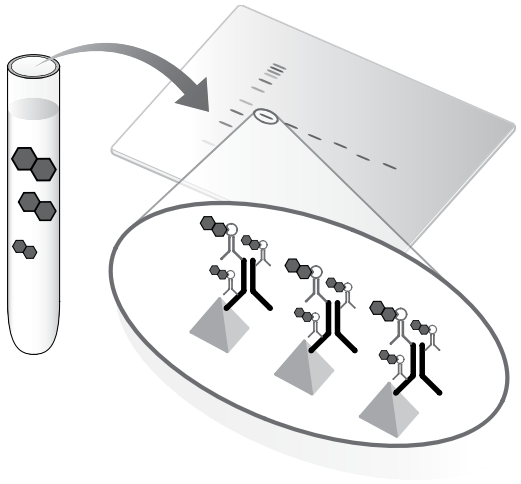
Primary antibody is added to the membrane and incubated to allow the antibody to bind to the myosin protein on the membrane. The unbound antibody is then washed away.

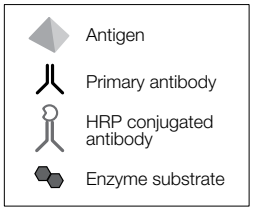


Secondary antibody is added to the membrane and incubated to allow the secondary antibody to bind to the primary antibody. The unbound secondary antibody is then washed away.



Colorimetric 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.





Western Blotting Applications in the Real World

Western blotting provides information on the identity, size, and quantity of proteins. This information is useful for many applications including disease diagnosis, agriculture, and biomedical research.

Disease Diagnosis

The enzyme-linked immunosorbent assay (ELISA) is widely used as the initial screening test for diseases such as HIV, lupus, and Lyme disease. Because it is inexpensive and easy to automate, an ELISA can be used to examine hundreds of samples in one run. But, when positive results for HIV or Lyme disease are returned following an ELISA, western blotting is used by medical clinicians to confirm the result.

ELISA assays identify proteins by virtue of their antibody binding specificity, while western blots identify proteins by both their molecular mass and their antibody binding specificities. Without additional information such as size, an ELISA is not as reliable as western blotting. Occasionally, antibodies used in an ELISA will bind to multiple proteins from different sources. For example, medical conditions such as Lyme disease, syphilis, and lupus sometimes test positive in ELISA assays designed to detect HIV, and visa versa. The reason for this is that the different protein markers associated with these diseases can share common sequences of amino acids that may be recognized by the same primary antibody – even though the proteins may be very different in terms of mass and function. The short sequence of 5–10 amino acids recognized by an antibody is called an **epitope**.

Protein markers from Lyme disease, lupus, or syphilis that may cause a false positive result in an ELISA HIV test are unlikely to have the same molecular mass as an HIV antigen. Thus, a patient blood sample that tests positive for HIV in an ELISA assay must always be retested via western blotting to confirm the molecular mass of the viral protein. So, if a patient has tested positive for HIV in an ELISA, but in reality has lupus, a western blot will display an antibody binding signal, but it will not be the correct molecular mass for HIV antigen. Therefore, the test would be negative for HIV.

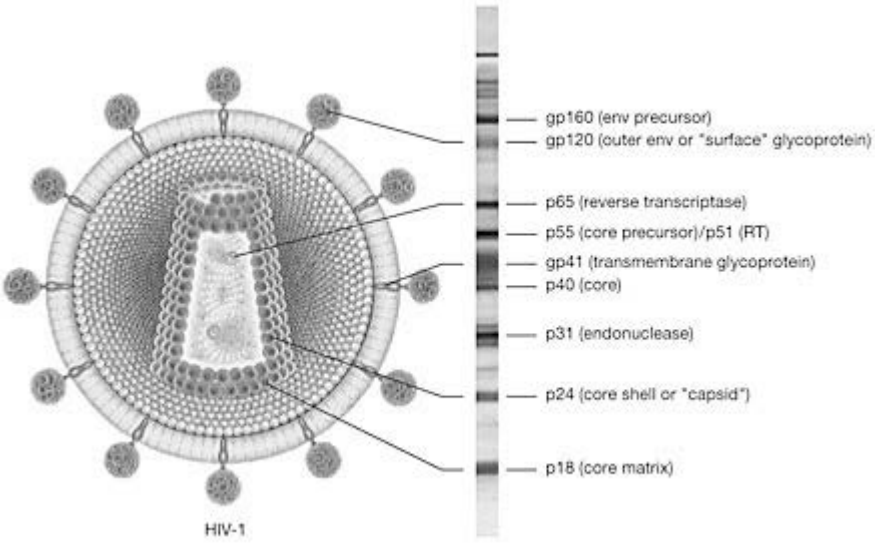


Fig. 1. Bio-Rad's Clinical Diagnostics Group produces a commercial western blot kit to test blood samples for antibodies specific for HIV proteins. HIV proteins are separated on a gel and western blotted. A sample of patient's blood is incubated with the membrane and if the patient has been exposed to HIV, antibodies from their blood will bind to multiple HIV proteins. The figure shows a cartoon of the HIV virus and a western blot designed to detect antibodies to nine HIV viral proteins.

So, why not test all HIV samples by western blotting instead of ELISA? Western blotting is costly, time consuming, and not easily automated. Since ELISA has a low false negative rate it is a convenient and inexpensive test to rule out HIV and other diseases. A western blot is only needed to confirm a positive ELISA.

Agricultural Applications

Western blotting is used extensively in agriculture to verify results obtained from ELISA tests – especially when a positive result will impact the agricultural community. For example, western blotting may be used to confirm a positive ELISA test for bovine spongiform encephalopathy (BSE or mad cow disease). The consequences of a false positive test for mad cow disease case may severely impact an entire country's cattle trade. Western blotting is also an important tool in crop science used to detect and quantify protein associated with genetically modified crops. Protein markers in crop science are used to evaluate such things as crop yield, pest resistance, and nutritional value.

Biochemical and Biomedical Research Applications

Because western blotting can determine the presence of a specific protein, its quantity and molecular mass, it is used extensively to understand protein structure and function in biochemical and medical research.

Once a protein is synthesized from its DNA blueprint, it may undergo **posttranslational modifications** that can alter its three-dimensional structure and function. Based on environmental and metabolic conditions, such posttranslational modifications can take many forms. For example, proteins may have sugar or carbohydrate-based compounds added to them in a process called **glycosylation**. Western blotting can determine whether glycosylation has occurred by revealing changes in the molecular weight of a protein; these changes can be characteristic of a particular physiological condition. For example, a surplus of glucose present in diabetes results in excessive glycosylation of proteins in the eye leading to cataract formation (Altan 2003). Western blot analysis of eye proteins can be used to diagnose a diabetic condition because heavily glycosylated proteins from diabetic patients have increased molecular mass compared to the same proteins from a “normal” nondiabetic patient.

Antibodies can be generated to selectively recognize a huge variety of specific posttranslational modifications. For example, proteins are sometimes cleaved or broken into smaller products posttranslation. Detecting whether a protein has been cleaved can be performed by engineering antibodies that recognize the original amino- or carboxy-terminal ends of the original full-length protein. Alzheimer's disease results from aberrant and excessive posttranslational cleavage of a β-amyloid precursor protein in brain cells which causes protein precipitates that kill these cells, resulting in severe memory loss (Sisodia 1992).

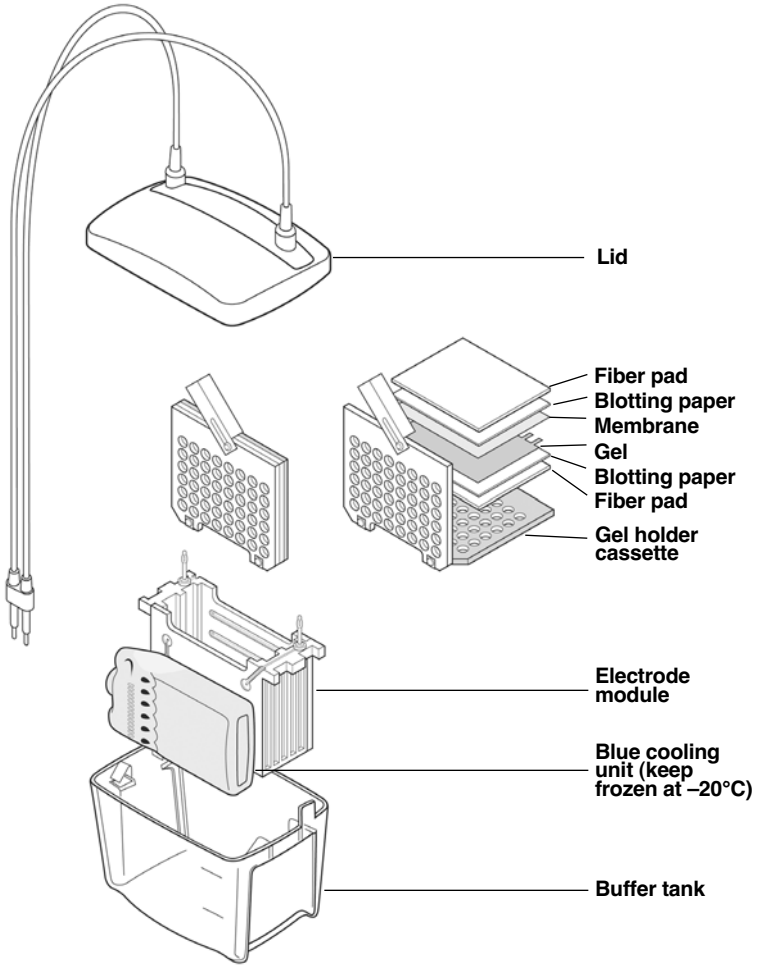
Phosphorylation of proteins is a common posttranslational regulatory event that can alter protein function and localization. In fact, phosphorylation is the process thought to regulate the cleavage of β-amyloid precursor protein in Alzheimer's disease (Gandy and Greenhard, 1994). Antibodies can be engineered to bind to a protein only when a single specific amino acid of that particular protein has been phosphorylated. Therefore, western blotting is also used to determine the nature and extent of protein phosphorylation under different physiological conditions.

More detailed information about posttranscriptional and posttranslational modifications can be found in the protein profiler instruction manual, which is freely available for download at explorer.bio-rad.com.

Western blotting is routinely used to help understand how new drugs modify protein structure and function. In biotechnology and pharmacological research, candidate drugs are screened using western blotting to determine their effects on protein mediated signaling pathways within cells. For example, samples treated with drugs designed to block phosphorylation in Alzheimer's disease are then blotted and probed with an antibody specific for phosphorylated proteins to determine whether the treatment reduced their protein phosphorylation levels.

Western Blot Reagents and Equipment

Tetra blotting module apparatus: the Tetra blotting module 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 Tetra blotting module (#1703924) is designed to fit into the Mini-PROTEAN® Tetra cell gel electrophoresis tank and lid. If a Tetra blotting module is not available, follow the alternative protocol for transferring the proteins using capillary action as 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 washing 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: Blotting buffer is supplied as a 10x solution and must be diluted with distilled water. Ethanol is added according to directions in the "Instructor's Advance Preparation" section. The 1x blotting buffer is composed of 25 mM Tris, 192 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. Methanol can be used to substitute for ethanol. However, ethanol is less hazardous and requires less current to obtain the same transfer efficiency. Exclusion of alcohol or inclusion of SDS (e.g., using Tris-glycine-SDS buffer instead of tris-glycine) will reduce the transfer efficiency! Chilling blotting buffer to 4°C before use is recommended and will reduce current generation during blotting.

Blocker: This solution must be prepared according to directions in the "Instructor's Advance Preparation" section. The final concentration of the blocking solution will be 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 "blocked" by incubating with this milk solution prior to incubation with the primary antibody. 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.

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 same primary antibody provided in this kit. 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 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.

Primary antibody: The primary antibody is designed to specifically recognize, or bind, the protein under study. The primary antibody in this kit is a monoclonal mouse anti-myosin light chain antibody, derived from a mouse hybridoma cell line (see Appendix A for details on monoclonal antibodies and hybridomas). This antibody was made by injecting chicken myosin protein into mice.

Secondary antibody: The secondary antibody is designed to bind specifically to the primary antibody. The secondary antibody in this kit is a polyclonal goat anti-mouse antibody conjugated to an enzyme called horseradish peroxidase (HRP). Secondary antibody is produced by injecting goats with primary mouse antibodies. The secondary goat-anti-mouse antibodies are purified from goat serum, and chemically linked or conjugated to HRP. HRP is the enzyme that catalyzes oxidation of a colorimetric substrate in order to permit visualization of the protein of interest.

Colorimetric substrate: The colorimetric substrate in this kit is based on 4-chloro-1-naphthol (4CN). When 4CN is oxidized by HRP in the presence of hydrogen peroxide, this colorless solution forms a purple 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. Store in a foil-wrapped container prior to use.

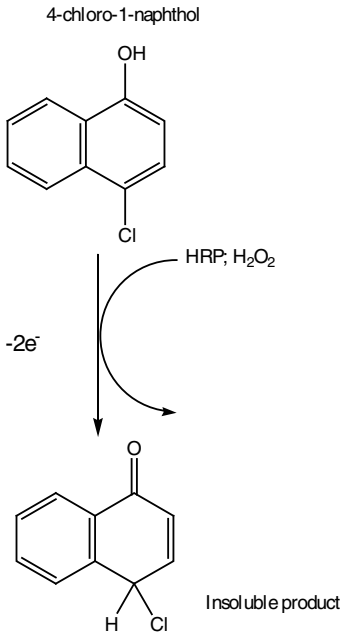


Fig. 2. Colorimetric detection: Oxidation of 4CN by HRP.

Incubation times: This kit protocol has been designed to allow students to perform a western blot within a 45 minute period.

The rate of antibody binding to the myosin light chain proteins on the membrane depends on antibody concentration and on the length of incubation. This kit has been optimized to function best according to the protocol provided. Incubating the membrane for longer times will result in stronger band intensities. Incubating the membrane with reduced or increased antibody concentrations will decrease or increase band intensities, respectively. Increasing the antibody concentration or incubation times may also increase undesired background binding. In research labs, western blots are often performed using more dilute reagents and longer incubation times.

Additional background information on immunology, antibodies, and myosin proteins is located in Appendix A of this instruction manual. Background information on SDS-PAGE, fish evolution, and muscle structure can be found in the protein profiler instruction manual. This manual and the protein profiler instruction manual are available to download from the Bio-Rad web site at explorer.bio-rad.com.

Instructor's Advance Preparation

These instructions are designed for setting up 8 student workstations consisting of up to 4 students per workstation.

Lesson 1: Protein Extraction

Prior to SDS-PAGE and western blotting, students prepare protein extracts from fish muscle tissue samples. This protein extraction protocol differs slightly from Lesson 1 of the protein profiler module in that the reducing agent, dithiothreitol (DTT), is required during protein extraction when western blotting will be conducted. DTT is optional when the protein profiler module is run as a stand-alone lab. If DTT is not used, background high molecular weight bands will likely develop above the main myosin band due to aggregation of myosin with other proteins. All reagents for conducting protein extraction and SDS-PAGE are contained in the protein profiler module – kit I.

Note: Protein electrophoresis (lesson 2) may begin immediately following this lesson. However, for the best results, we recommend that western blotting (lesson 3) be conducted immediately following protein electrophoresis.

Required Materials for Reagent Preparation (8 Student Workstations)

Materials	Where Provided	Quantity
Laemmli sample buffer	Kit I	30 ml
DTT	Kit I	0.3 g
1.5 ml flip-top micro tubes	Kit I	8
1 ml disposable plastic transfer pipet (DPTP)	Kit I	1
Muscle tissue samples	Instructor's own	5 species
Blade for cutting muscle tissue	Instructor's own	1

Prepare and Aliquot Reagents:

Muscle tissue

Cut each muscle tissue sample into eight square chunks roughly 0.5–1 cm, place on card or plastic and label with each species' name.

Laemmli sample buffer

Add entire vial of DTT to 30 ml of Laemmli sample buffer (final concentration of DTT will be 70 mM). Label eight 1.5 ml flip-top micro tubes as sample buffer (SB). Dispense 1.5 ml Laemmli buffer into each tube, store at –20°C until use.* Laemmli buffer with DTT may be stored at –20°C for up to 3 months.

*Note: This solution is stored at –20°C due to DTT instability at room temperature. However, SDS is also contained in the buffer, and may precipitate at –20°C. Therefore, warm the solution to room temperature and mix thoroughly prior to use.

Student Workstations

Material Needed for each Workstation	Where Provided	Quantity
1.5 ml fliptop microtubes	Kit I	5
1.5 ml screw-cap microtubes	Kit I	5
1 ml DPTP	Kit I	1
Laemmli sample buffer	Prepared by Instructor	1.5 ml
Foam float	Kit I	1
Muscle tissue samples, labeled	Prepared by Instructor	5 species
Marking pen	Instructor's own	1
Knife or scissors to cut muscle tissue samples	Instructor's own	1

Common Workstation

Material Required	Quantity
Water bath set to 95°C	1

Lesson 2: Protein Electrophoresis

Prior to western blotting, students separate muscle protein extracts via SDS-PAGE. For the best results when blotting proteins from SDS-PAGE gels, we recommend loading **5 µl** of each muscle sample extract per well in the electrophoresis step. Because antibody detection is much more sensitive than total protein staining, loading 5 µl of protein per lane rather than the 10 µl recommended for SDS-PAGE when using the protein profiler kit alone produces better resolution and tighter bands on blots.

Optimally, the blotting step is conducted immediately following gel electrophoresis. Delaying blotting may result in the proteins diffusing somewhat within the gel. Waiting overnight to blot gels will result in the myosin bands appearing thicker and less well defined on the blot.

If blotting cannot be performed immediately following electrophoresis, gels may be stained with Bio-Safe Coomassie stain to partially fix and stabilize the proteins in the gels for up to 24 hours. This also 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. More information on this staining method is located in Appendix C.

If there is insufficient time to either stain the gels or to set up the blotting sandwiches directly after running the gel, then gels may be stored in their cassettes at 4°C overnight.

Required Materials for Reagent Preparation (for eight workstations)

Material	Where Provided	Quantity
Actin and myosin standard, 500 mg lyophilized	Kit I	1 vial
Precision Plus Protein Kaleidoscope prestained protein standards, 50 µl	Kit I	1 vial
10x Tris-glycine-SDS (TGS) running buffer	Kit I	200–400 ml
Bio-Safe Coomassie stain (optional)	Kit I	200 ml
Distilled or deionized water	Instructor's own	2–4 L
1.5 ml screwcap microtubes	Kit I	9
1.5 ml fliptop microtubes	Kit I	8
1 ml DPTP (or 200–1,000 µl adjustable-volume micropipet and tips)	Kit I	1
1–20 µl adjustable-volume micropipet and tips	Instructor's own	1
Water bath set to 95°C	Instructor's own	1

Prepare and Aliquot Reagents

Actin and myosin standard

Add 500 µl of Laemmli sample buffer to the vial of freeze-dried actin and myosin standard to rehydrate the proteins. Incubate at room temperature for 20–30 minutes. Transfer the rehydrated actin and myosin sample to a labeled screwcap tube and heat for 5 minutes at 95°C.

Label eight 1.5 ml screwcap tubes as actin and myosin (AM) on their sides. Dispense 10 µl of rehydrated and preheated actin and myosin standard into each tube. Rehydrated actin and myosin standard may be stored at –20°C for up to 12 months after heating.

TGS gel running buffer	**Tris-Glycine-SDS (TGS) running buffer**. One Mini-PROTEAN Tetra cell with two gels requires 700 ml of 1x TGS running buffer. One Mini-PROTEAN Tetra cell using the companion running module to run four gels requires 1.1L of 1x TGS running buffer. To make 3 L of 1x TGS running buffer mix 300 ml of 10x TGS with 2,700 ml of distilled water. Store at room temperature. Tip: you may want to prepare 1–2 L of extra 1x TGS buffer in case your gel boxes leak after assembly. If you do have a leak, the outer chamber of the gel box can be filled to above the inner small plates, to equalize the buffer levels in both reservoirs. This requires approximately 1,200 ml of 1x TGS buffer per gel box and is a more convenient fix than reassembling the apparatus mid-lesson.
Precision Plus Protein Kaleidoscope prestained protein standard	Label eight 1.5 ml flip-top tubes Precision Plus Protein Kaleidoscope standards. Dispense 6 µl of prestained standards into each tube.

Student Workstations

Materials Needed for Each Workstation	Where Provided	Quantity
Fish samples from lesson 1	Prepared by Instructor or students	5 tubes
Actin & myosin standard, 10 µl	Prepared by Instructor	1 tube
Prestained standards, 6 µl	Prepared by Instructor	1 tube
4–20% Mini-PROTEAN TGX precast gel*	Purchased separately	1
1–20 µl adjustable-volume micropipet or 5 µl fixed-volume pipet	Instructor's own	1
Prot/Elec pipet tips for gel loading	Kit I	7 tips
Mini-PROTEAN Tetra cell electrophoresis module (gel box)	Instructor's own	1 per 2 gels**
1x TGS running buffer	Prepared by Instructor	500 ml per gel box
Power supply to be shared between workstations	Instructor's own	1
Sample loading guide for 10-well comb (optional)	Part of Mini-PROTEAN Tetra cell	1 per gel box
Buffer dam (only required if running 1 gel per box)	Part of Mini-PROTEAN Tetra cell	1
Bio-Safe Coomassie stain (optional)	Kit I	50 ml per 2 gels

* TGX precast gels have a 3 month shelf life and so should be ordered as required.

** One Mini-PROTEAN Tetra cell can run one to two gels with one electrode assembly or up to four gels with the addition of a Mini-PROTEAN Tetra Companion Running Module.

Common Workstation

Materials Required	Quantity
Water bath set to 95°C	1
Rocking platform (if staining gels)	1
Distilled water for destaining (optional)	2 L

Lesson 3: Western Blotting

Note: If a Tetra blotting module or another brand of electroblotting apparatus is not available, an alternative protocol can be used to blot proteins by capillary action and is described in Appendix B. Separate advance preparation directions for this alternate procedure are also described in Appendix B.

In this lesson, students transfer or "blot" proteins from their gels onto a nitrocellulose membrane support using electric current. The blotted membranes are then placed into blocking solution and the detection procedure continues (lesson 4) or membranes can be stored in blocking solution at 4°C overnight. 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. Fifteen minutes at room temperature is the required minimum blocking protocol. Alternatively, the blotted membranes can be left in their gel cassettes in the tanks overnight if necessary and then put into blocker the following morning. If blotted membranes need to be stored for longer periods they can be stored in blotting buffer or wash buffer at 4°C for up to 1 week.

To perform a western blot, students first construct a sandwich placing the gel and the membrane between blotting paper. An electric current is passed through the sandwich forcing the negatively charged proteins to move out of the gel and onto the protein-binding nitrocellulose membrane.

Staining the gel after blotting with Bio-Safe Coomassie contained in kit 1 can be used to demonstrate the efficiency of the blotting procedure. For unstained gels, we recommend blotting for 2.5 hours at 20 V or 30 min at 100 V. For stained gels, we recommend blotting for 15 h (or overnight) at 20 V. Shortening the blotting run time may result in some high molecular weight proteins remaining in the gel since the high percentage of acrylamide tends to trap larger proteins. Similarly, if blotting is continued beyond the suggested run duration, then smaller proteins, including myosin, may pass through the membrane and be lost.

Note: The blue cooling unit needs to be filled with water and then frozen prior to beginning this lesson.

Tip: It is vital that students form tight, air bubble-free sandwiches, especially at the gel/membrane interface.

Tip: It is imperative that the membrane and gel be ordered and aligned correctly. The negative electrode (black) should be closest to the gel and the positive electrode (red) should be closest to the membrane. Similarly, it is prudent to check that the blotting cassette is inserted into the blotting tank appropriately, and that the blotting chamber lid and leads are attached correctly. Follow the directions closely with respect to the formation of the sandwich and its placement in the Tetra blotting module. After blotting, ensure that each blot has visible colored size standards. If the standards are not visible then the blot may have been performed in the wrong direction meaning that the proteins have migrated away from the membrane and into the blotting buffer.

Required Materials for Reagent Preparation (for 8 workstations)

Material	Where Provided	Quantity
10x Tris-glycine buffer	Kit II	400 ml
10x phosphate buffered saline (PBS)	Kit II	40 ml
10% Tween 20	Kit II	1 ml
Dry blocker	Kit II	Pack (20 g)
Distilled water	Instructor's own	3,500 ml
Ethanol/reagent alcohol (specially denatured alcohol (SDA) formula 3A)	Instructor's own	800 ml
Blue cooling unit	Part of Tetra blotting module	1

Prepare and Aliquot Reagents:

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**	2,800 ml 400 ml 800 ml **4,000 ml**	Transfer buffer used in the blotting tank. Ethanol, methanol, or isopropyl alcohol may be substituted for reagent alcohol. Store at room temperature or 4°C for up to 6 months.
Blocker: 5% dry blocker in wash buffer	Distilled water 10x PBS 10% Tween 20 Dry blocker **TOTAL**	359 ml 40 ml 1 ml 20 g (pack) **400 ml**	Used to block membranes and dilute antibodies. Ensure dry blocker is fully dissolved in solution before use. Store at 4°C for up to 48 hours.
Blue cooling units			Freeze prior to the lab.

Student Workstations

Materials needed for each Workstation	Where Provided	Quantity
Blotting buffer	Prepared by instructor	500 ml
Blotting paper	Kit II	2
Nitrocellulose membrane	Kit II	1
Mini-PROTEAN® Tetra cell tank and lid	Instructor's own	1 per 2 workstations
Red & black Tetra blotting inner module	Part of Tetra blotting module	1 per 2 workstations
Frozen blue cooling unit	Part of Tetra blotting module	1 per 2 workstations
Hinged black and clear plastic sandwich cassette	Part of Tetra blotting module	1
Fiber pads	Part of Tetra blotting module	2
Power supply* to be shared between workstations	Instructor's own	1 per 2–8 workstations*
Roller (pencil, test tube, or pipet)	Instructor's own	1
Soft pencil (black ballpoint pen also works)	Instructor's own	1
Containers for blot prep and assembly	Kit I and instructor's own	2
Blocker	Prepared by instructor	25 ml

*See explanation of power supply requirements below

Common Workstation

Materials Required	Quantity
Rocking platform	1

Power Supplies

Electroblotting generates much higher current than electrophoresis; thus, attention should be paid to the properties of the power supply available since many power supplies have limited current capacity. Using too high a voltage setting may overload your power supply. For this laboratory blotting for 50 volt hours, i.e., 2.5 hours at 20 volts, is recommended. Faster blotting is possible, e.g. 30 minutes at 100 V; however, the load on your power supply may need to be reduced by connecting just one or two Tetra blotting module tanks to each power supply. As a rough estimate, each Tetra blotting module tank set to 100 V generates 160–230 mA of current. Chilling blotting buffer prior to blotting reduces current. In contrast, contaminants in the buffer, including salts or minerals from the water, the use of methanol instead of ethanol, the absence of the blue cooling unit, and increased buffer temperature increase current. It is preferable to use low-voltage blotting instead of overloading the power supply. Power supplies specifically made for high-current applications like blotting do exist such as Bio-Rad's PowerPac™ HC and PowerPac Universal. Use the following table as a guide to gauge optimal run conditions:

Recommended blotting conditions for Bio-Rad power supplies:

Model	Max. Voltage (V)	Max. Current (mA)	Max. Power (W)	Max. No. Blotting Modules	Run Voltage (V)	Run Time (min)
PowerPac Mini*	Fixed 100 or 200	200	40	Not recommended		
PowerPac Basic power supply**	300	400	75	1–2	100	30
				4	20	150
PowerPac HC power supply	250	3,000	300	4	100	30
PowerPac Universal power supply	500	2,500	500	4	100	30
PowerPac HV power supply**	5,000	500	400	2	100	30
				4	20	150

*The PowerPac Mini is not recommended for this lesson because it does not have variable voltage. Consequently, if the current rises above the 200 mA limit for this power supply, the safety features built into the unit may cause it to blow a fuse since it cannot lower its voltage to compensate for high current.

**If the current goes beyond the maximum for these units, the voltage will be lowered to compensate. If this rise in current occurs, then the time of blotting should be increased such that blotting has occurred for approximately 50 V–hr.

Lesson 4: Immunodetection

In this lesson students probe their blocked membranes with antibodies specifically engineered to bind only to myosin light chain proteins, then probe again with secondary antibodies linked to the HRP enzyme for the anti-myosin antibody to visualize the myosin light chains. Lastly, students will use a colorimetric substrate to visualize and locate the antibody complex bound to myosin light chains.

Required Materials for Reagent Preparation (for 8 Workstations)

Material	Where Provided	Quantity
Freeze-dried anti-myosin light chain (primary) antibody	Kit II	1 vial
Freeze-dried goat anti-mouse-HRP secondary antibody	Kit II	1 vial
HRP color detection reagent A	Kit II	10 ml
HRP color detection reagent B	Kit II	0.6 ml
10x PBS	Kit II	160 ml
10% Tween 20	Kit II	3.75 ml
Distilled water (plus 1 ml of sterile distilled water—optional)	Instructor's own	2.5 L
Blocker	Prepared by Instructor	160 ml
14 ml tubes	Kit II	24 tubes
Foil	Instructor's own	To cover 8 x 14 ml tubes and HRP color detection preparation container

Prepare Reagents:

Reagent to Prepare	Stock Reagents	Volume of Stock Reagent	Notes
Wash buffer: 1x phosphate buffered saline with 0.025% Tween 20 (PBST)	Distilled water 10x PBS 10% Tween 20 **TOTAL**	1,350 ml 150 ml 3.75 ml **1504 ml**	Wash buffer is used to wash membranes and rehydrate antibodies. Store at room temperature for up to 2 weeks.
Prepare HRP color detection reagent	Distilled water 10x PBS HRP color detection reagent A HRP color detection reagent B **TOTAL**	90 ml 10 ml 10 ml 0.6 ml **110.6 ml**	This reagent should be prepared within 1 hour prior to use. HRP color reagent is light sensitive and must be prepared and stored in a dark or foil wrapped container. Ensure thorough mixing of reagents.
Aliquot HRP color detection reagent	Wrap eight 14 ml tubes in foil to protect from light and label "Enzyme Substrate". Aliquot 10 ml per workstation into 14 ml **foil-wrapped** tubes.		
Ready-to-use primary antibody* (anti-myosin antibody)	1. Make a 200x stock solution** of primary antibody by adding 0.5 ml of wash buffer to the freeze-dried anti-myosin light chain antibody, close the stopper, and shake to mix. 2. Label eight 14 ml tubes "Primary antibody" or "Anti-myosin antibody". 3. Mix 0.5 ml of 200x stock of anti-myosin antibody with 100 ml of blocker. Mix well. 4. Aliquot 10 ml of ready to use anti-myosin antibody into the 14 ml tubes.		
Ready-to-use secondary antibody*	1. Make a 200x stock solution** of secondary antibody by adding 0.5 ml of wash buffer to the freeze-dried goat anti-mouse-HRP secondary antibody, close the stopper, and shake to mix. 2. Label eight 14 ml tubes "Secondary antibody". 3. Mix 0.5 ml of 200x stock of secondary antibody with 100 ml of blocker. Mix well. 4. Aliquot 10 ml of ready to use secondary antibody into the 14 ml tubes.		

*Store ready-to-use antibodies at 4°C for up to 48 h. Allow tube to reach room temperature before use. Antibodies may be reused while the milk in the blocker is still good.

**Store 200x stock antibodies at 4°C. If 200x stock antibodies are to be stored for longer than 24 hours (max. 2 weeks), use sterile 1x PBS to rehydrate antibodies. 1 ml of sterile PBS is comprised of 0.1 ml 10x PBS plus 0.9 ml sterile distilled water.

Student Workstations

Materials Needed for Each Workstation	Where Provided	Quantity
Blocker (if membranes have not already been blocked)	Prepared by Instructor in Lesson 3	25 ml
Ready-to-use primary antibody (anti-myosin antibody)	Prepared by Instructor	10 ml
Ready-to-use secondary antibody	Prepared by Instructor	10 ml
HRP color detection substrate	Prepared by Instructor	10 ml
Wash buffer	Prepared by Instructor	200 ml
Distilled water	Instructor's own	100 ml
Paper towel	Instructor's own	2 sheets
Incubation tray*	Kit I or instructor's own	1
Container for waste liquid (should hold at least 2 L)	Instructor's own	1

*Incubation trays are provided in kit I. Trays can be shared between two workstations with two membranes incubated in each tray. Always use 10 ml of antibody solution per membrane. When two membranes are incubated together in one tray use 20 ml of antibody solution per tray. As an alternative, incubation trays commonly used in research labs are the lids and bases of pipet tip boxes since the membrane is almost the same size and can be completely covered with small volumes of antibody solution. If using an alternative incubation tray, make sure that the blot can be covered by the antibody solution in whatever tray you choose. Ensure all incubation trays are thoroughly washed before use to prevent contamination of membranes and immunodetection reagents.

Common Workstation

Material	Quantity
Rocking platform	1 or more

Analysis of Blots

Below are examples of how the membranes should appear when blotting unstained gels or gels stained with Bio-Safe Coomassie before blotting, respectively. The lane containing the Precision Plus Protein Kaleidoscope prestained protein standards should appear on one side and a single purple/gray band should appear in each lane containing the fish muscle protein extracts in the size range between 25 kD and 20 kD. This single band is the myosin light chain 1. There may also be a smaller and fainter second band between 20 kD and 15 kD. This band is myosin light chain 2 and is often not visible. Protein bands in some samples may also appear as doublets (two bands close together), which may indicate the presence of different isoforms of these protein subunits. Divergent species (such as shellfish) may not react with the antibody. This provides an opportunity for discussion on how protein size and structure can be altered as a consequence of evolution and how subtle changes in a protein's structure may affect the epitope (see definition of epitope in Appendix F – Glossary) recognized by the antibody, thus making antibody binding specificity an indicator of protein homology. Please refer to Appendix D for bioinformatics lesson extensions for suggestions about how to investigate protein sequence homology.

Fig. 3. Result from unstained gel. **Result from stained gel.**

Troubleshooting Blot Analysis

First-time users: For students performing western blotting for the first time, it may be instructive to follow the Bio-Safe Coomassie gel staining protocol in Appendix C. The staining protocol 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 antibody specificity. Troubleshooting is also easier using this protocol since the success of each step is witnessed as it is performed.

Extra bands: Nonspecific background bands may sometimes appear on the membrane even when DTT is added to the Laemmli sample buffer. Ensure that muscle extracts are heated to 95°C prior to loading the gel. In addition, the membrane must be incubated with blocking solution prior to the immunodetection step.

Overloading SDS-PAGE gels may contaminate neighboring samples and is often caused by taking excessively large chunks of tissue into the extraction step. Overloading gels with protein can also cause annoying background bands, halo bands, or faint shadow bands. For this reason we recommend loading only 5 µl of each sample per well when blotting is to follow electrophoresis.

Vertical lines on development with substrate: Minute pieces of muscle may be transferred to the screw-cap tube. If these are loaded on the gel, they may cause streaks to appear upon development with the enzyme substrate. To prevent this, centrifuge the samples after heating (to ensure the SDS is in solution) and prior to loading on the gel.

Halo bands: If after immunodetection, bands appear thick with dark edges and are pale at the center, then samples may have been overloaded. Using excessively large chunks of muscle tissue in the extraction step or loading 10 µl instead of the recommended 5 µl onto the gel can cause overloading. When blotting, more is not always better!

No Precision Plus Protein Kaleidoscope prestained protein standards and no bands appear on the blot: The gel may have been transferred in the wrong direction. This problem has several potential causes: 1) setting up the sandwich with inappropriate configuration within the cassette; 2) inserting the cassette with the black side of the cassette next to the red wall of the Tetra blotting module instead of the black wall, 3) placing the lid on the Tetra blotting module the incorrect way, and 4) connecting the electrode leads incorrectly to the power supply.

Prestained standards are present but there no other bands: Primary and secondary antibodies may have been mixed up or accidentally omitted. The HRP color detection reagent may have been overexposed to light (reagent turns a brown color when it degrades).

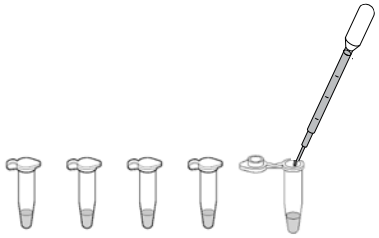
Bands have small white circles or holes in them: Small blank circles where bands should be indicate that air bubbles were trapped between the membrane and gel and prevented transfer of proteins in those regions.

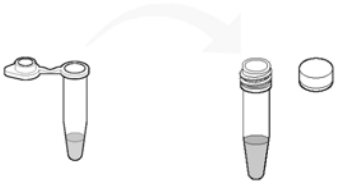
Bands are smiling or frowning: Smiles or frowns usually indicate a problem with the gel or the gel running buffer. The buffer level in the inner chamber of the gel apparatus may be too low due to a leak. Alternatively, the buffer may be the wrong concentration or the wrong buffer may have been used. The proper gel running buffer is provided in kit I; it consists of TGS supplied as a 10x concentrated solution to be diluted 1 to 10 in distilled water. Buffer problems can usually be detected by monitoring the migration of the loading dye during electrophoresis. The dye front in all lanes should migrate downward through the gel at a constant rate, forming a straight line across the gel as it goes. Expired gels may also affect the shape of the dye front and the banding pattern. Check the expiration dates on polyacrylamide gels as they have a 3-month shelf life.

Bands are pale: Pale bands indicate possible blotting problems. The blotting sandwiches must be very carefully constructed. The gel and membrane must be in direct, full contact without air bubbles and the components of the sandwich must be rolled out at each step of construction from the bottom layer up. The reagents in this kit have been optimized to produce clear bands within a 45-minute period. Extending the recommended minimum incubation times indicated in the manual will result in more intense bands.

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.

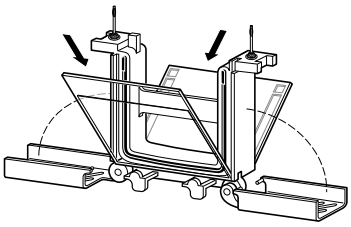
Lesson 2 Quick Guide

1. Set up Mini-PROTEAN® Tetra cell gel box.

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

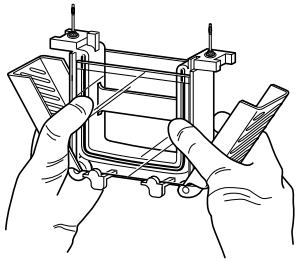
3. Remove the comb from the TGX gel cassette.

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



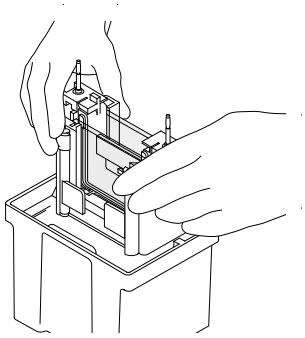
QUICK GUIDE

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



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

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



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

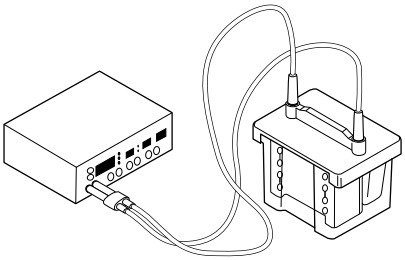
8. Fill mini tank with approximately 550 ml of 1x TGS electrophoresis buffer until the buffer reaches the line indicated by "2 Gels" on the front of the tank.

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

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

11. Load your gel:

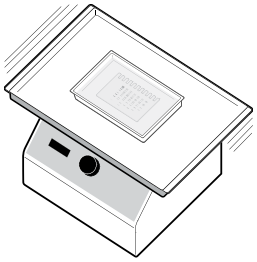
Lane	Volume	Sample
1 & 2	empty	Empty
3	5 µl	Precision Plus Protein Kaleidoscope prestained standards
4	5 µl	fish sample 1
5	5 µl	fish sample 2
6	5 µl	fish sample 3
7	5 µl	fish sample 4
8	5 µl	fish sample 5
9	5 µl	actin and mysin standard (AM)
10	empty	empty



12. Electrophorese for 30 min at 200 V in 1x TGS gel running buffer.

13. Either (a) proceed directly to Lesson 3, (b) continue to step 14 of Lesson 2 to stain gels or (c) store gels overnight at 4°C.

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



15. 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.

16. Discard stain and destain gels in a large volume of water overnight, changing the water at least once. Blue-stained bands will gradually become visible after around 1 h of destaining.

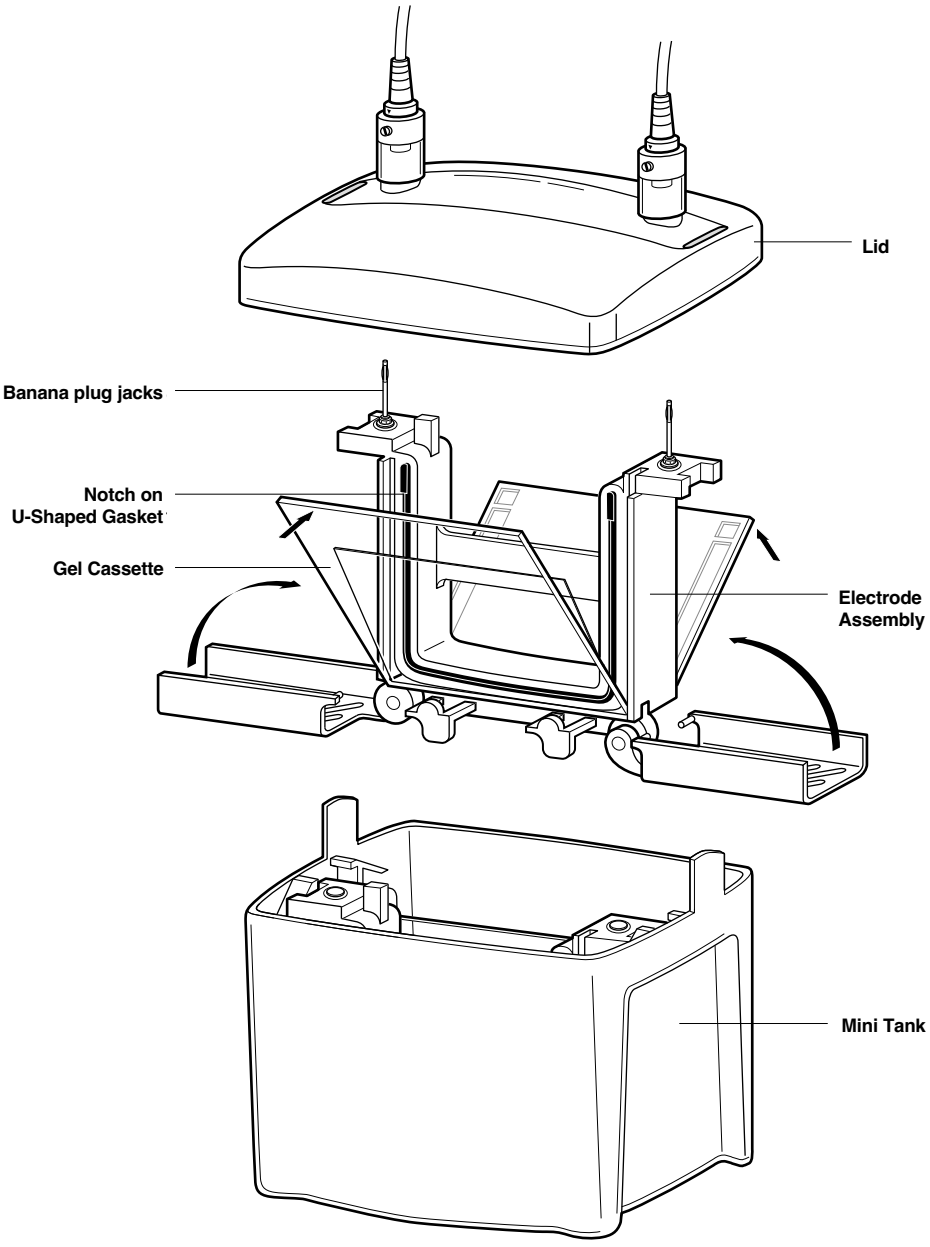
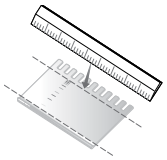
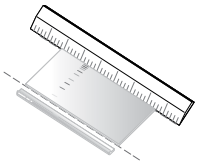
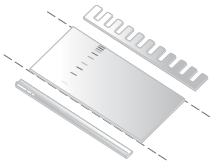


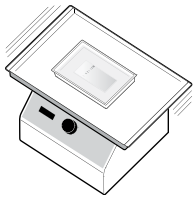
Fig. 4. Assembling the Mini-PROTEAN Tetra cell.

Lesson 3 Quick Guide

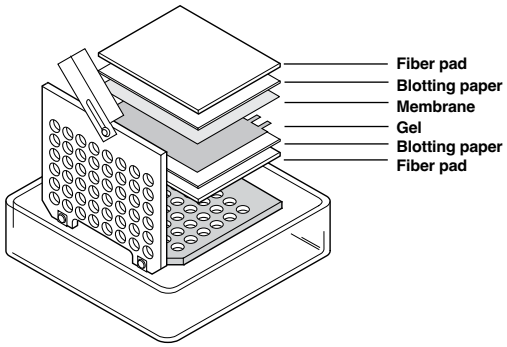
1. Using a ruler, chop the top and bottom off the gel.

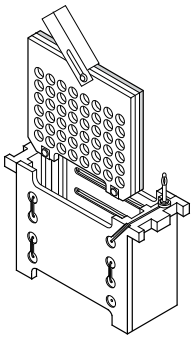
2. Equilibrate the gel in blotting buffer for 15 minutes on a rocking platform.



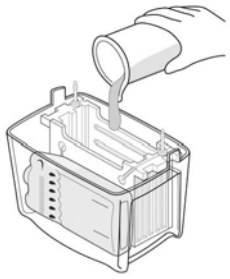
3. Soak fiber pads thoroughly in blotting buffer.
4. Mark the white nitrocellulose membrane with penciled (or black ball point pen) initials and prewet in blotting buffer along with the blotting paper.
5. Make the blotting sandwich:
   a. Add 1 cm depth of blotting buffer to container and insert plastic 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.



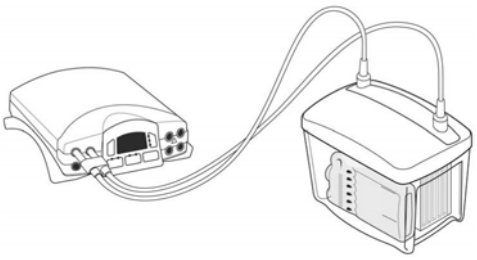
   h. Close the cassette and clamp together with the white clip.



6. Set up the Tetra blotting module with the black side of the cassette next to the black side of the Tetra blotting module. Add a frozen blue cooling unit 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.

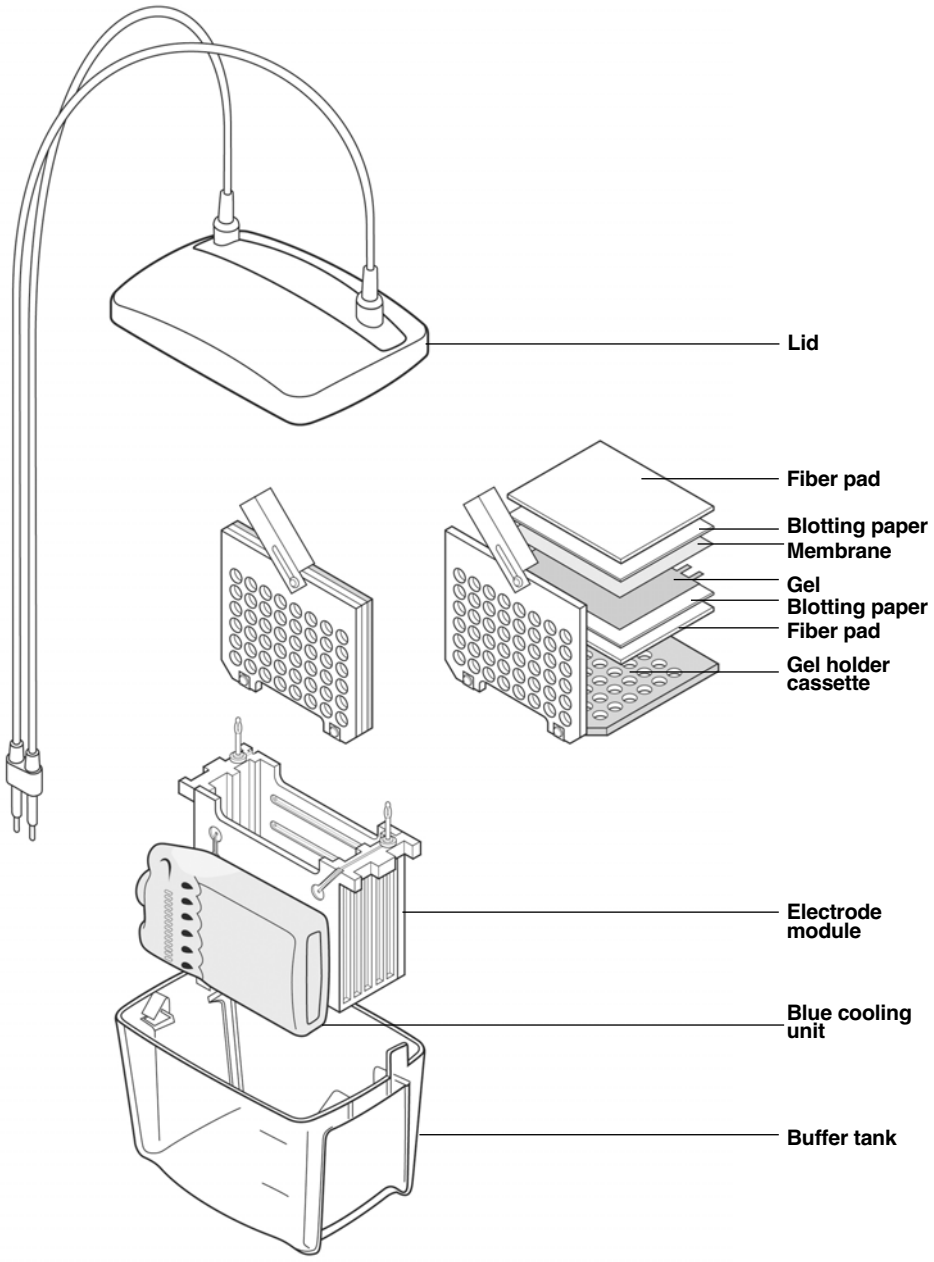
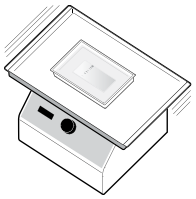


Fig. 5. Assembly of the Mini Trans-Blot® cell.

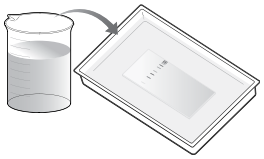
Lesson 4 Quick Guide

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.



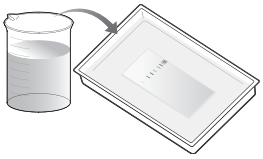
2. Discard blocking solution and incubate membrane with 10 ml of primary antibody for 10–20 minutes on rocking platform set to a faster setting to ensure constant coverage of the membrane.

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.



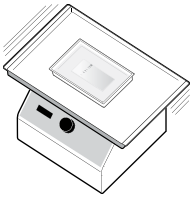
5. Discard the wash and incubate membrane with 10 ml of secondary antibody for 5–15 minutes on rocking platform set to a fast setting.

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".

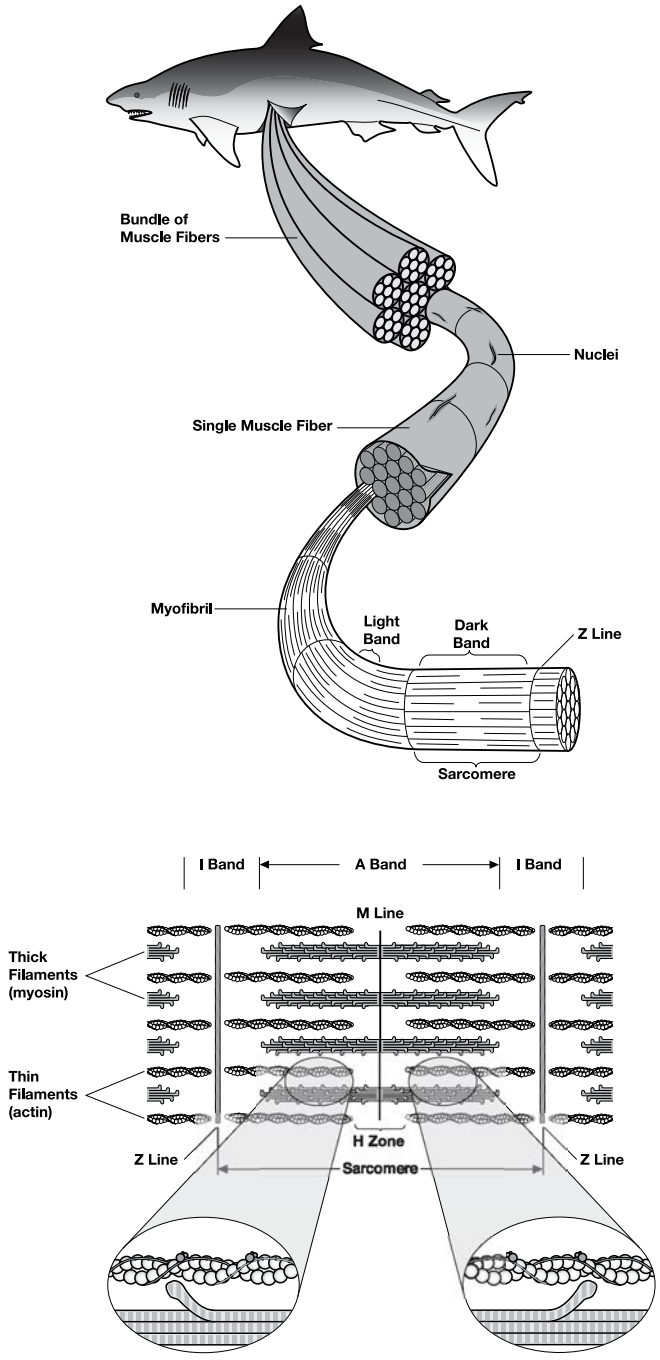


Fig. 6. Telescopic view of muscle structure: Thick myosin filaments and thin actin filaments form myofibrils, which are bundled together to make muscle fibers. (Figure modified from Campbell 1996 with permission.)

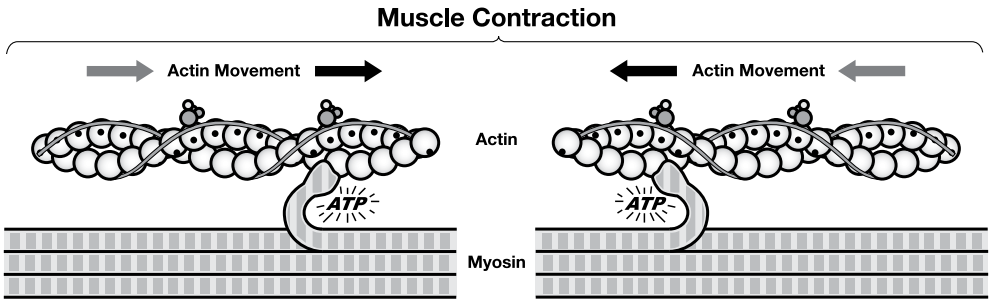


Fig. 7. Hydrolysis of ATP causes myosin and actin filaments to slide past one another, shortening the sarcomere and contracting the muscle. (Figure modified from Campbell 1996 with permission.)

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.

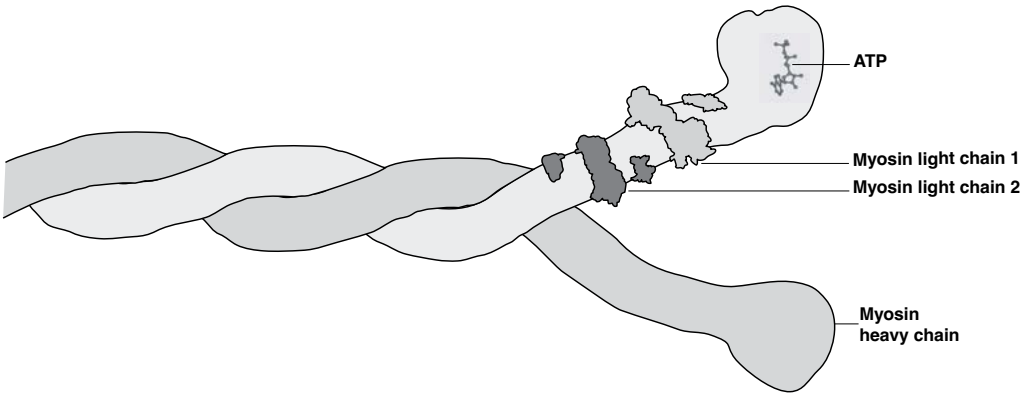


Fig. 8. Myosin protein structure.

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.

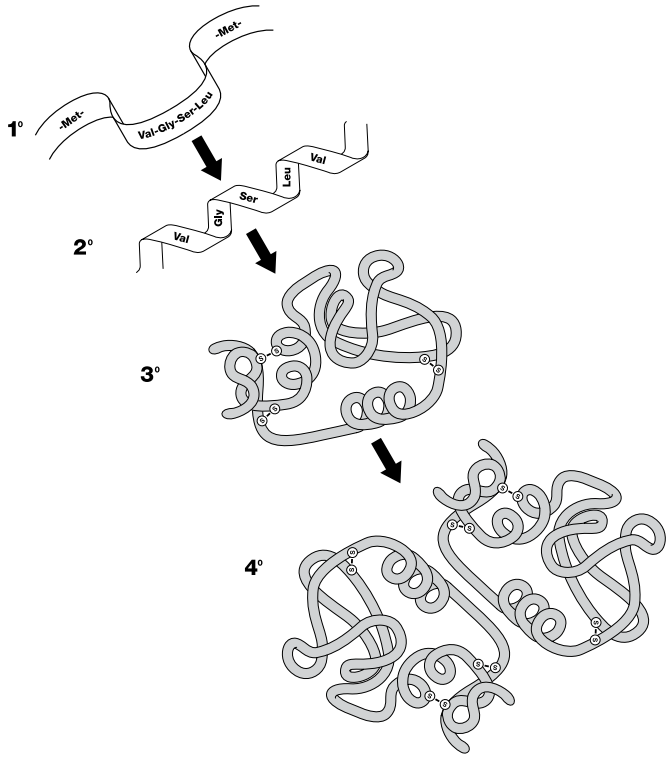


Fig. 9. Secondary (2°), tertiary (3°), and quaternary (4°) protein structure must be distrupted, or denatured, to accurately separate proteins by size.

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.

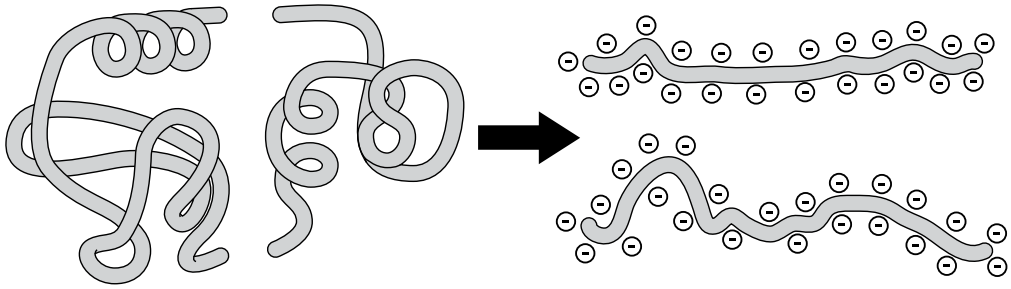


Fig. 10. The combination of heat and the detergent SDS denatures proteins for SDS-PAGE analysis.

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

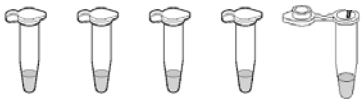
Common Workstation

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.

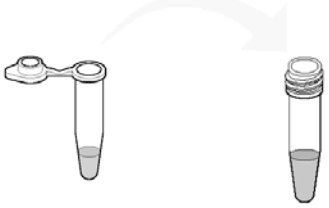


1.3. For each sample, obtain a piece of muscle tissue (avoid skin, fat, and bones) approximately 0.25 x 0.25 x 0.25 cm^3, and transfer it to the appropriately labeled microtube. Try to make the pieces of muscle tissue the same size for each sample. Close the lid.

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.

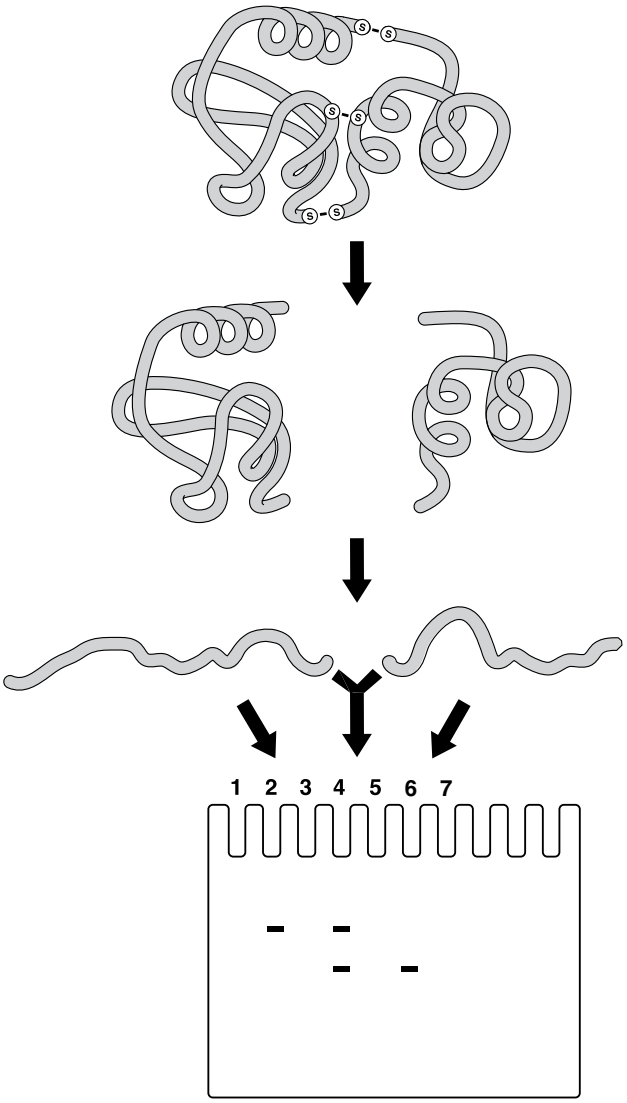


Fig. 11. A quaternary protein complex denatured with reducing agents, heat, and SDS, can be separated into individual proteins and resolved by size using SDS-PAGE.

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
4–20% Mini-PROTEAN TGX,10-well, precast gel	1
1–20 µl adjustable-volume micropipet	1
Prot/Elec™ pipet tips for gel loading	7 tips
Mini-PROTEAN Tetra cell electrophoresis module (gel box)	1 per 2 gels
1x Tris-glycine-SDS (TGS) gel running buffer	700 ml per gel box
Power supply (200 V constant) to be shared between workstations	1
Sample loading guide for 10 well comb	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 TGX 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.

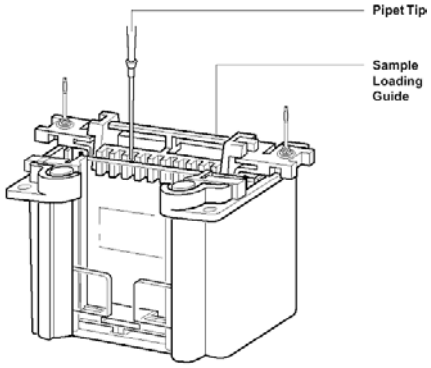
2.3. If available, place a yellow sample loading guide on top of the electrode assembly. This guide will direct the pipet tip to the correct position for loading each sample in a well.

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 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 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.

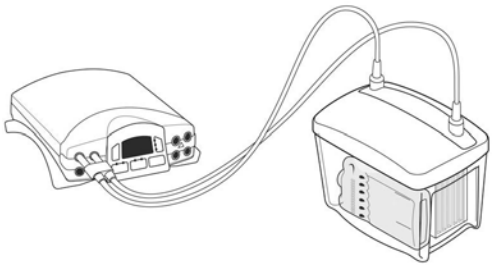
Note: The fine barrel of the gel loading tips releases liquid more slowly than normal tips. You must therefore release the plunger of the micropipet very slowly when taking up samples and when loading them; otherwise the correct volume will not be loaded.



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.

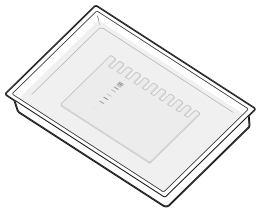
2.8. Ideally the blotting step should be performed directly following gel electrophoresis. If blotting cannot be performed immediately following electrophoresis, gels may be stained with Bio-Safe Coomassie stain to fix and stabilize the proteins in the gels for up to 24 hours. Alternatively gels may 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:

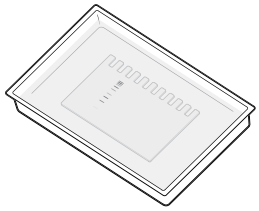
2.9. Save 50 ml of gel running buffer per gel for the next lesson.

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.

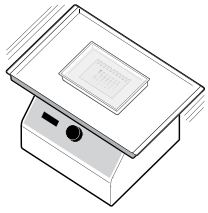


2.11. Carefully replace rinse with 50 ml of Bio-Safe Coomassie stain per 2 gels.



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.

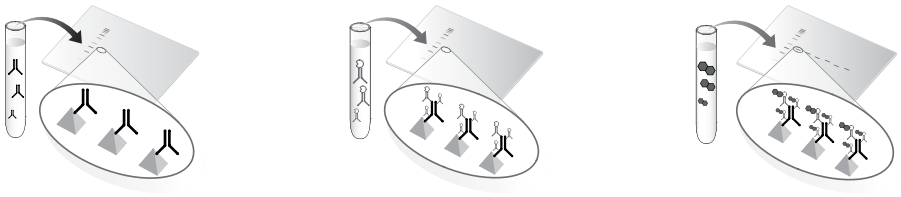
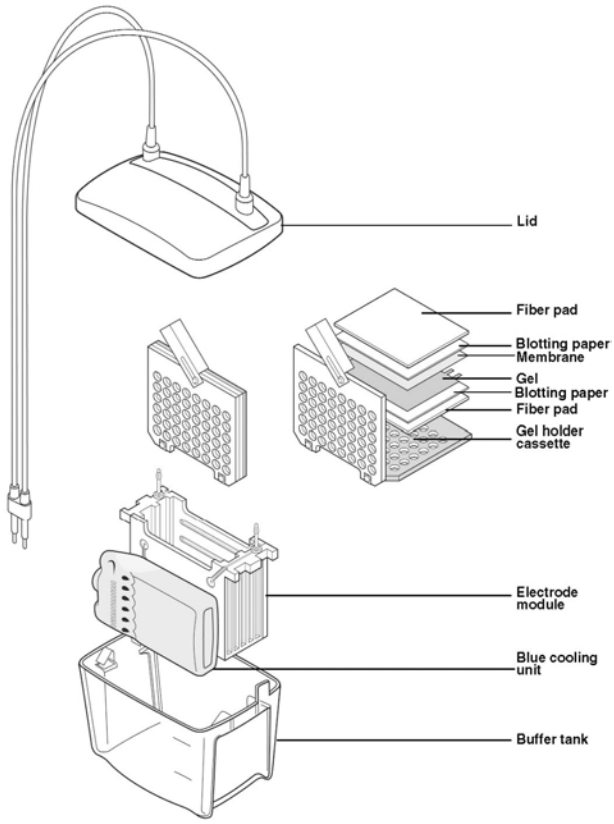


Fig. 12. Overview of Immunodetection on the blot. The membrane is incubated with the primary antibody, followed by incubation with the secondary antibody, and lastly the substrate is added.

Western Blot Reagents and Equipment

Tetra blotting apparatus: the Tetra blotting module 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 Tetra blotting module is designed to fit into the Mini-PROTEAN® Tetra gel electrophoresis tank and lid. If a Mini Tetra blotting module 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 25 mM Tris, 192 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.

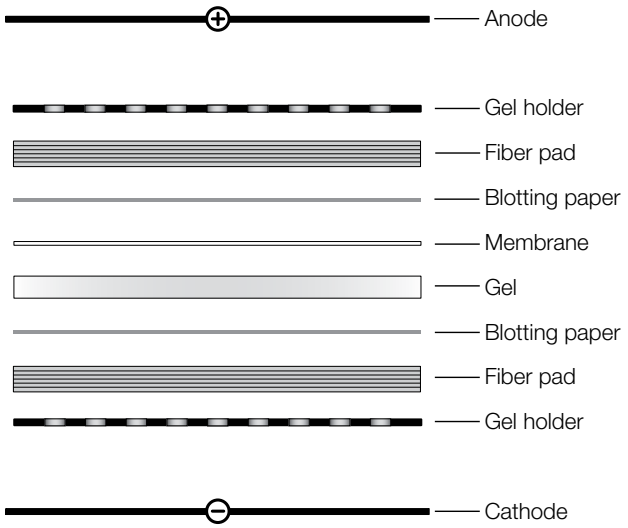


Fig. 13. Schematic of western blot. The current is conducted through the blotting buffer and negatively charged proteins migrate from the gel onto the protein binding membrane.

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 Tetra tank and lid	1 per 2 workstations
Red & black Mini Trans-Blot inner module	1 per 2 workstations
Bio-Ice™ unit, frozen	1 per 2 workstations
Hinged black and clear plastic sandwich cassette	1
Fiber pads	2
Power supply to be shared between workstations	1
Roller (or pencil, test tube, or pipet)	1
Soft pencil	1
Containers	2
Blocker (optional)	25 ml

Common Workstation

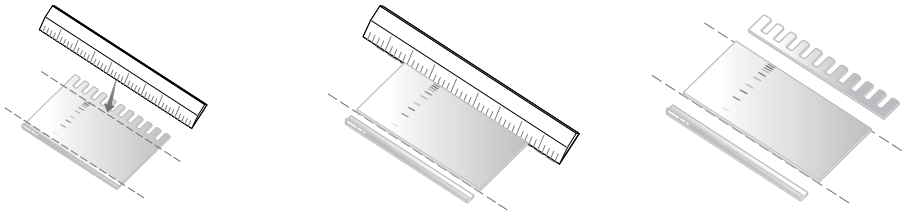
Material	Quantity
Rocking platform	1

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.

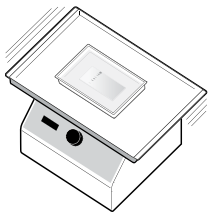
Option B: If the gel was stained in the previous lesson, incubate it for 15 minutes in gel running buffer (TGS) reserved from the previous lesson with gentle rocking. Longer incubation in TGS will not harm the experiment.

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.

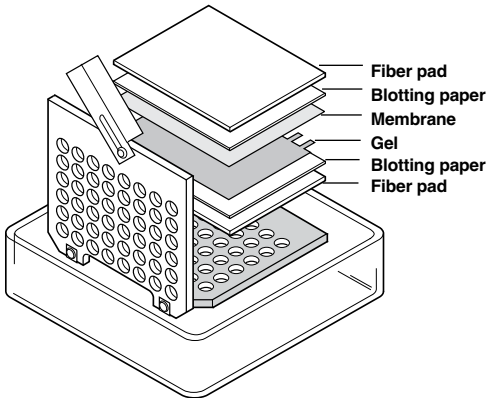


3.3. Soak the fiber pads thoroughly in blotting buffer and squeeze buffer into them to ensure they are thoroughly soaked.

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.

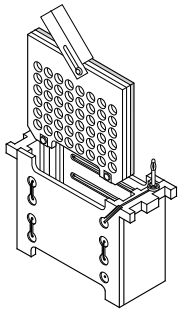
h. Fold the clear plastic half of the cassette over the sandwich and clamp it to the black plastic half by sliding over the white clip. This tight fit will squeeze the sandwich together. Keep the sandwich cassette partly submerged in blotting buffer.

3.6. Set up the Tetra blotting apparatus:

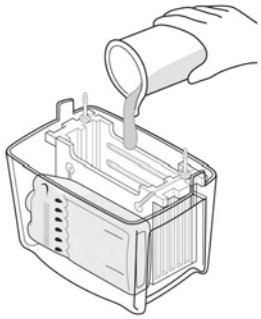
a. Place the red and black Tetra blotting module into the gel tank with the black side in the center of the tank and the banana plugs protruding up in the middle.

b. Place the frozen blue cooling unit into the gel tank.

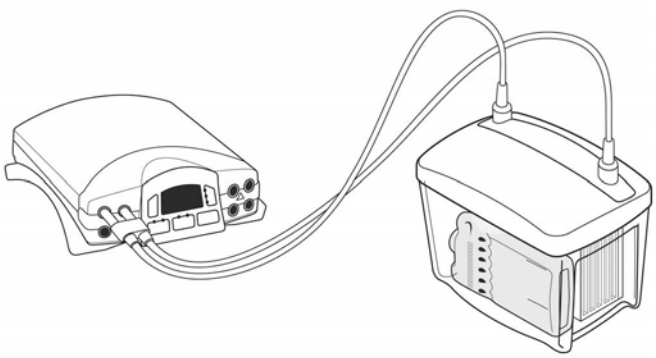
c. Slide the cassette containing the blotting sandwich into the red and black Tetra blotting module; the black side of the sandwich must be facing the black side of the Tetra blotting module and the clear side of the sandwich facing the red side of the module. Two sandwich cassettes fit into each module. 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 Tetra blotting module and the frozen blue cooling unit is in place. Place the lid on the tank; match the red plug on the lid with the red plug on the Tetra blotting module.



3.7. Attach the Tetra Blotting module 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.

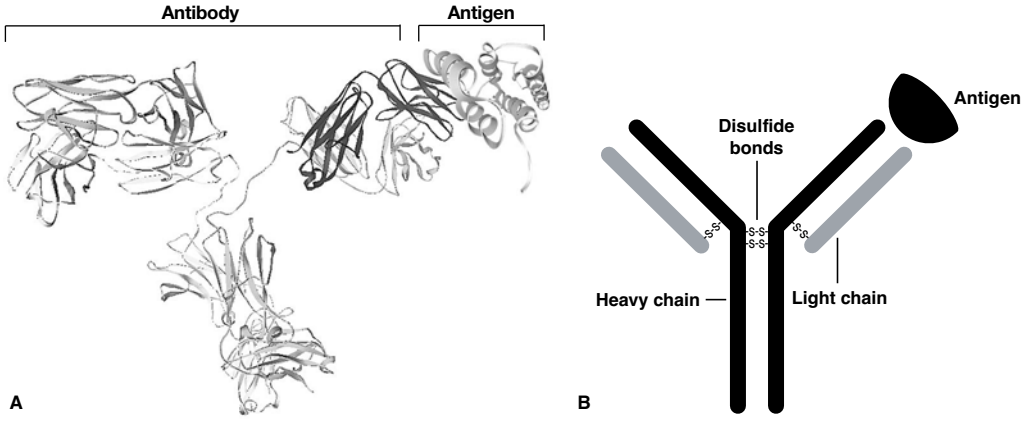


Fig. 14. A) Structure of IgG bound to the HIV capsid protein p24 as determined by X-ray crystallography (Harris et al. 1998, Momany et al. 1996). These structures can be downloaded from the Protein Data Bank (www.pdb.ufmg.br, Berman et al. 2000) using the PDB identification codes 1IGY and 1AFV and manipulated 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.

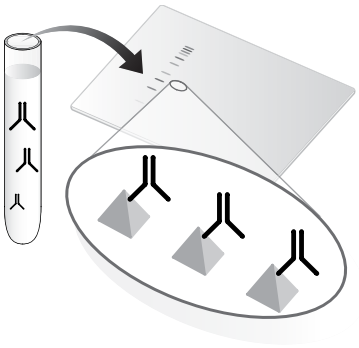
How Are Antibodies Made?

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.

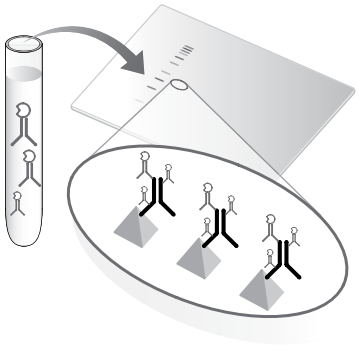
Immunodetection Step by Step

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.



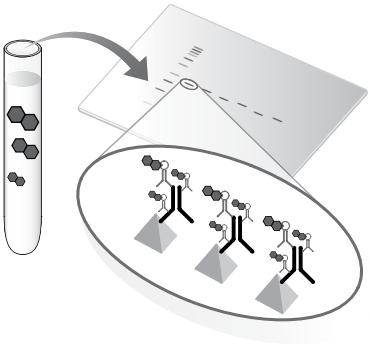
The primary antibody provided is a monoclonal mouse anti-myosin light chain antibody. This antibody was made by injecting purified chicken myosin protein into mice and generating an immortalized antibody producing cell line (a hybridoma) from one mouse that constantly produces the same antibody.

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.

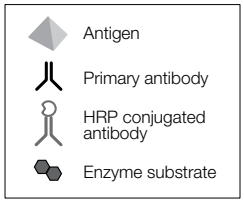


The secondary antibody is a polyclonal goat anti-mouse antibody conjugated to HRP. Secondary antibody was produced by injecting goats with primary mouse antibodies. The secondary goat anti-mouse antibodies were purified from goat serum, and chemically linked or conjugated to HRP. HRP is the enzyme that 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.

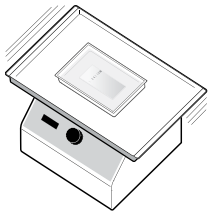
Student Workstations

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.

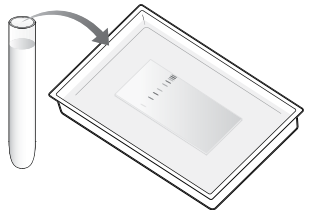
4.2. Wearing gloves and only handling corners, peel the membrane off the gel and check for the presence of the prestained standards on the membrane. Inform the instructor if the colored bands are not visible.

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.

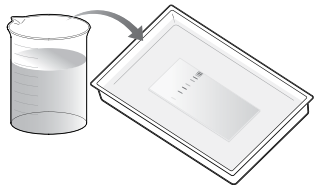
4.5. Add 10 ml anti-myosin primary antibody to the tray and place on the rocker for 10–20 minutes at room temperature or overnight at 4°C. Longer incubation times will result in more intense bands. Adjust rocker to a faster setting if necessary to ensure the antibody solution is constantly washing over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



4.6. Pour off the anti-myosin primary antibody.

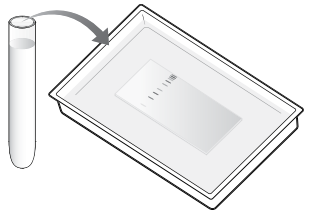
4.7. Rinse the membrane in approximately 50 ml of wash buffer and pour off.

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.

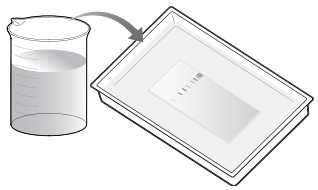
4.10. Add 10 ml secondary antibody to membrane and place on rocker for 5–15 minutes. Longer incubation times will result in more intense bands. Adjust rocker to a faster setting if necessary to ensure the antibody solution is constantly washing over the membrane.



4.11. Pour off the secondary antibody solution.

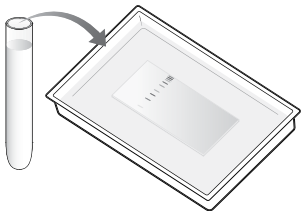
4.12. Rinse the membrane in approximately 50 ml of wash buffer and pour off.

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.

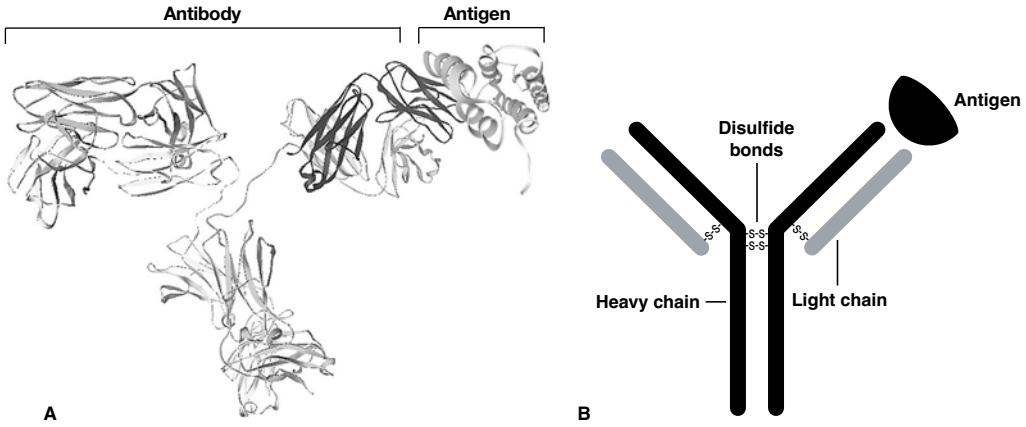


Fig. 15. A) Structure of IgG bound to the HIV capsid protein p24 as determined by X-ray crystallography (Harris et al. 1998, Momany et al. 1996). These structures can be downloaded from the Protein Data Bank (www.pdb.ufmg.br, Berman et al. 2000) using the PDB identification codes 1IGY and 1AFV and manipulated 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 amino 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.

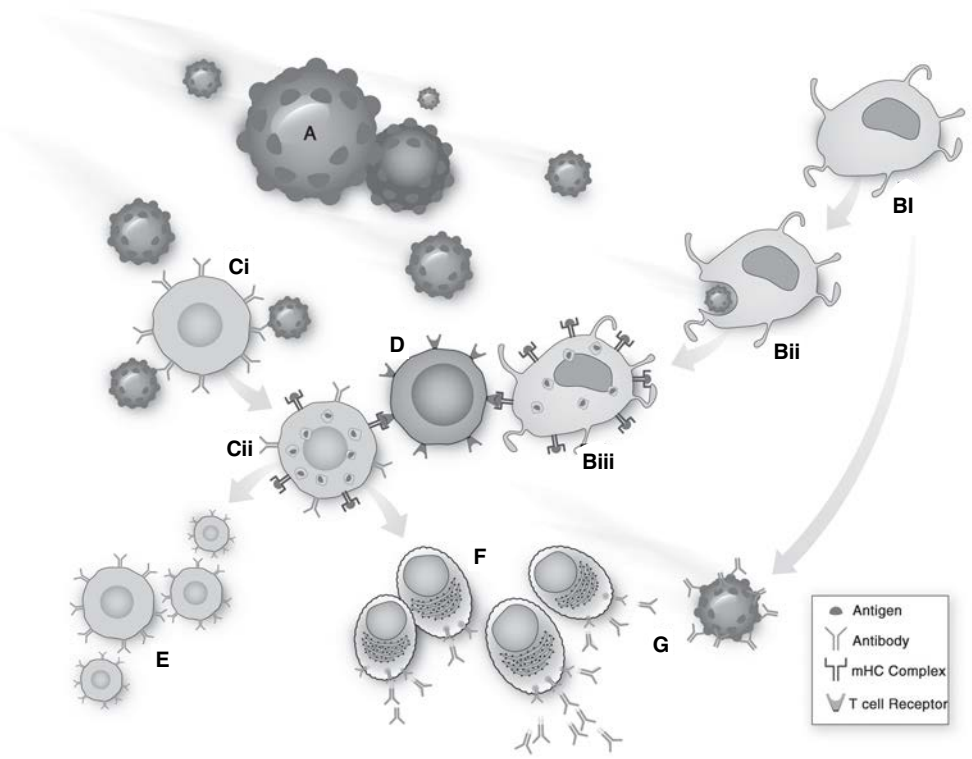


Fig. 16. Summary of immune cell and pathogen interactions.

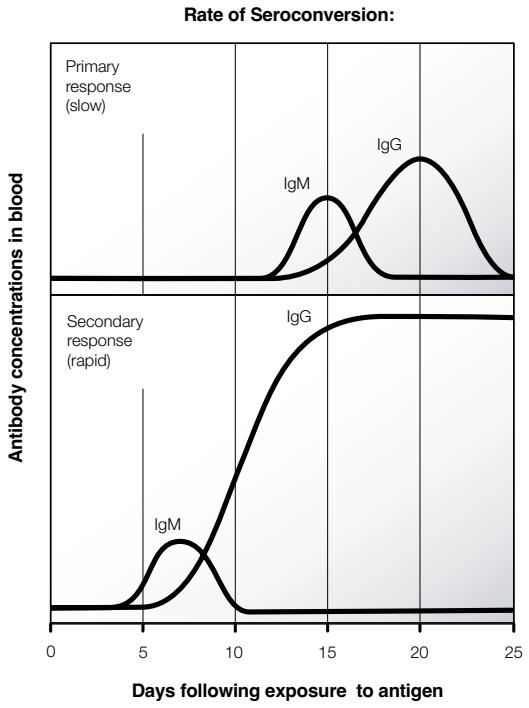
Immune cells are the soldiers of the acquired immune response. Macrophages (Fig 16. Bi) engulf foreign cells, pathogens and molecules (A) from the blood (Bii), and present antigens on their cell surfaces via their major histocompatibility (MHC) complexes to be recognized by T cells (Biii). T cells (D) 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.

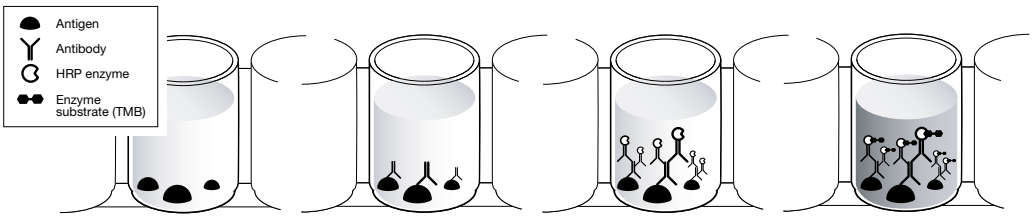


Fig. 17. Antibody capture ELISA.

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.

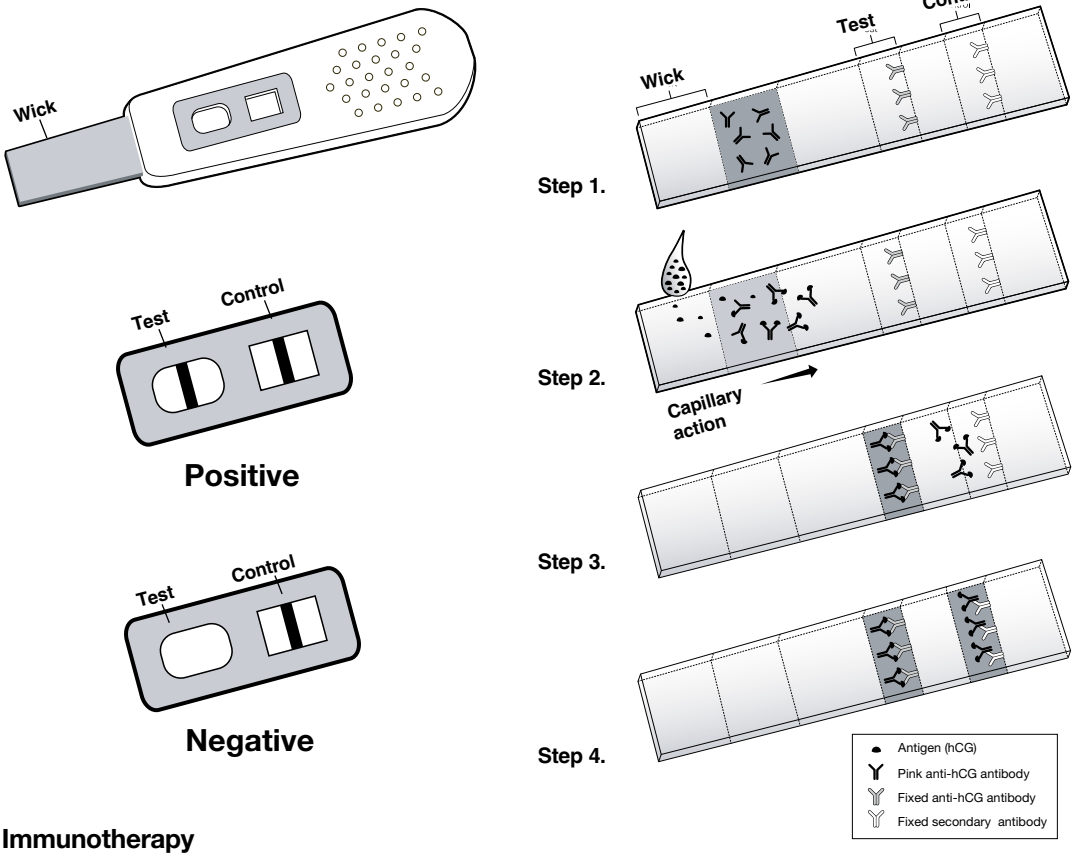
Another area of research would be immunostaining of plant seedlings at different stages of maturation to track the change in a protein's abundance and localization as the plant grows. Antibodies for immunostaining are labeled with either fluorescent molecules or enzymes that produce colored signals upon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

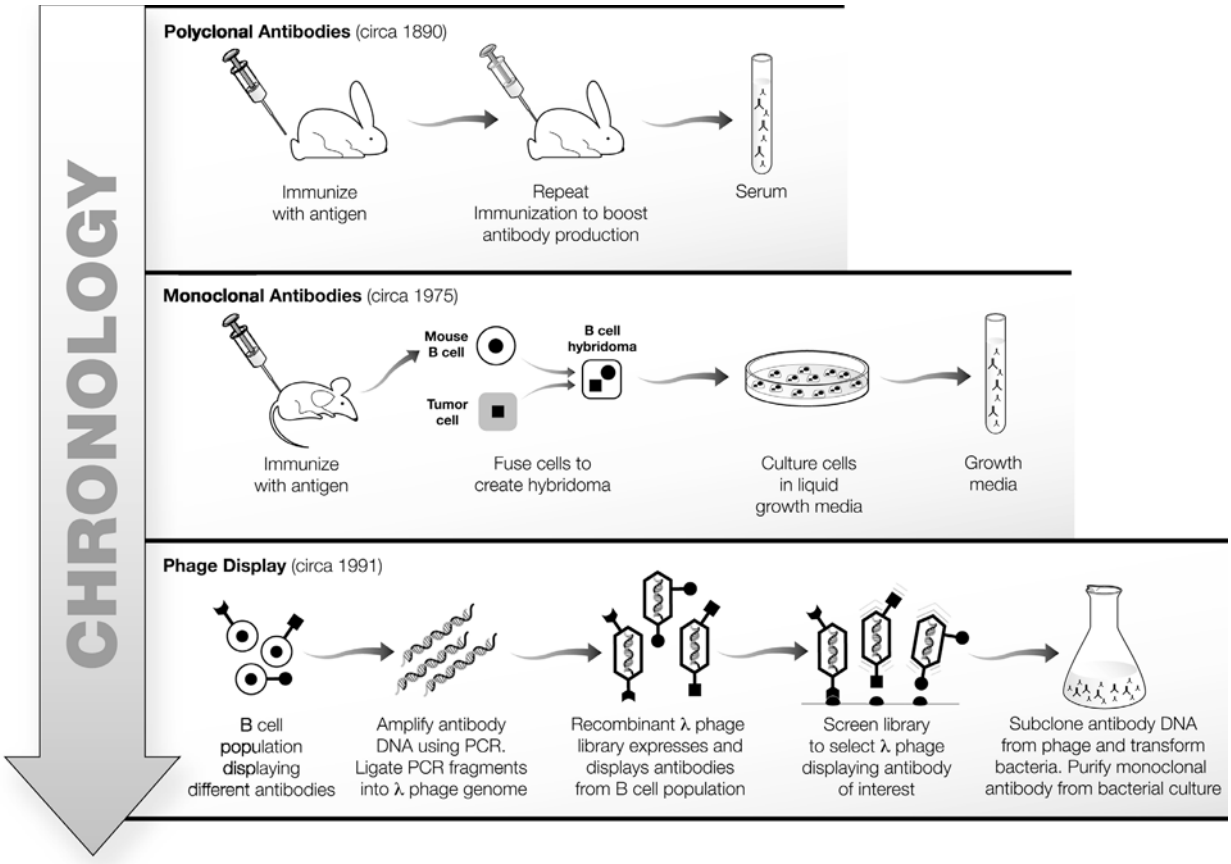
Although antibodies have traditionally been used to diagnose diseases, they are now being used as therapies against cancer. "Humanized" mouse monoclonal antibodies can now be genetically engineered in bacteria, so that they may be used in humans without eliciting an immune response. In addition, human 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.

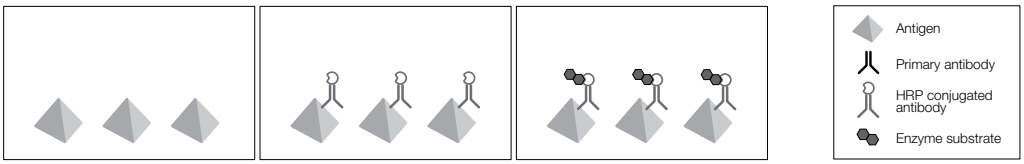


Fig. 18. Direct detection.

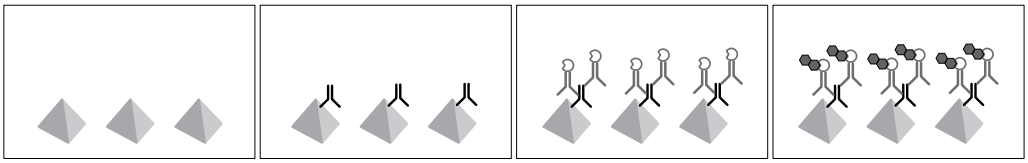


Fig. 19. Indirect detection.

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.

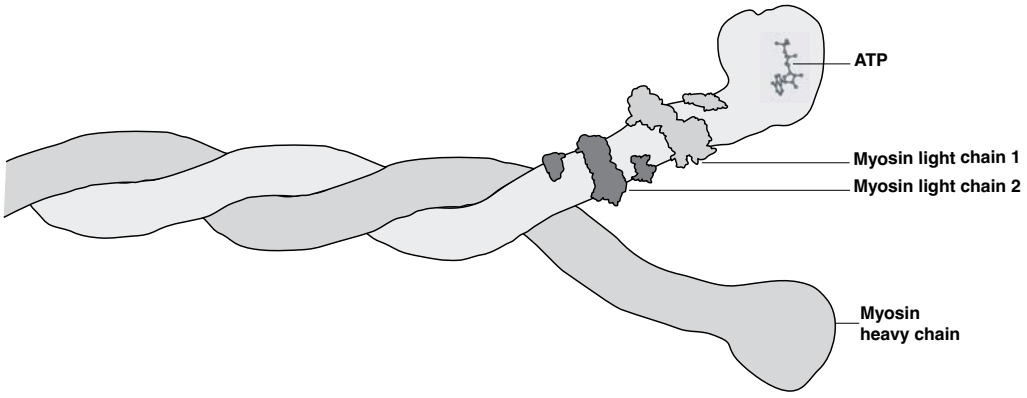


Fig. 20. Depiction of myosin protein structure.

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).

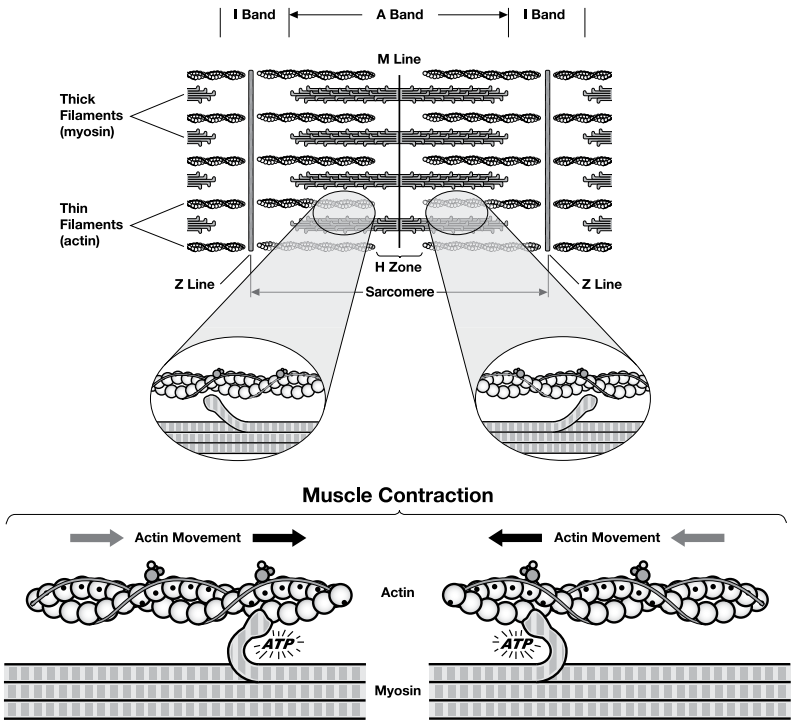


Fig. 21. Depiction of a sarcomere, relaxed actin and myosin, and contracted actin and myosin.

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:	Distilled water	7 L	Blotting buffer used for blotting.
1x Tris-glycine with Methanol	10x Tris-glycine	1 L	
20% ethanol	Reagent alcohol	2 L	or isopropyl alcohol can be substituted for reagent alcohol. Store at room temperature for up to 6 months.
	TOTAL	**10 L**	
Blocker:	Sterile distilled water	359 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.
5% dry blocker in wash buffer – Make this the day the blot is due to finish.	10x PBS	40 ml	
	10% Tween 20	1 ml	
	Dry blocker	20 g	
	TOTAL	**400 ml**	

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 (or 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 (or pencil, 5 ml test tube, pipet)	1
Soft pencil	1
Tape	1

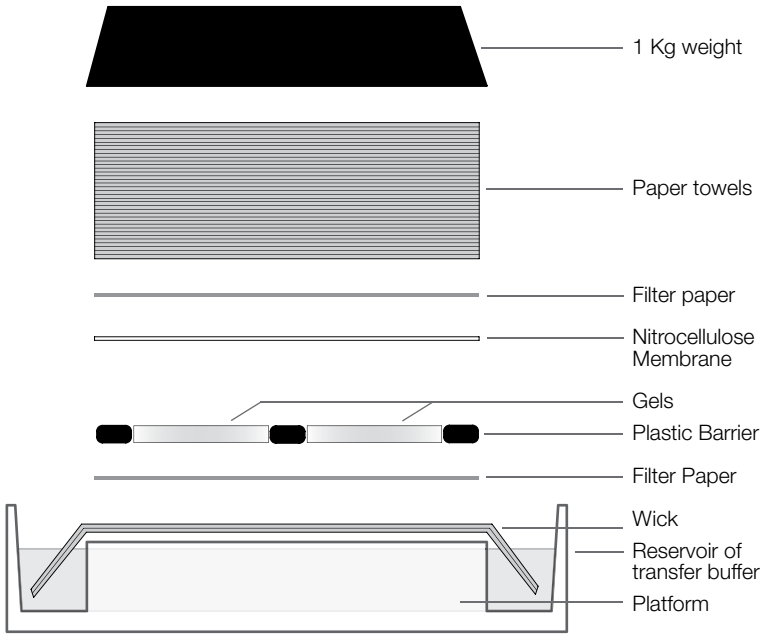
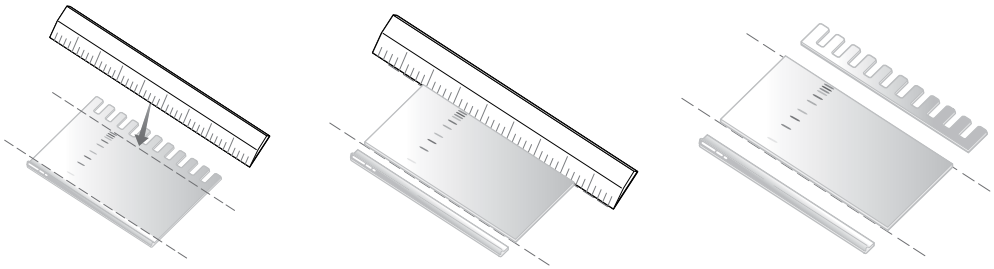


Fig. 22. Capillary action blot.

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.

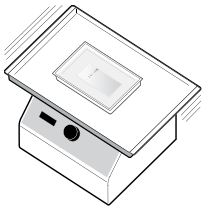
3.11. Place a wet piece of blotting paper on top of the nitrocellulose membrane. Use the roller to ensure that there are no air bubbles between the membrane and the blotting paper.

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

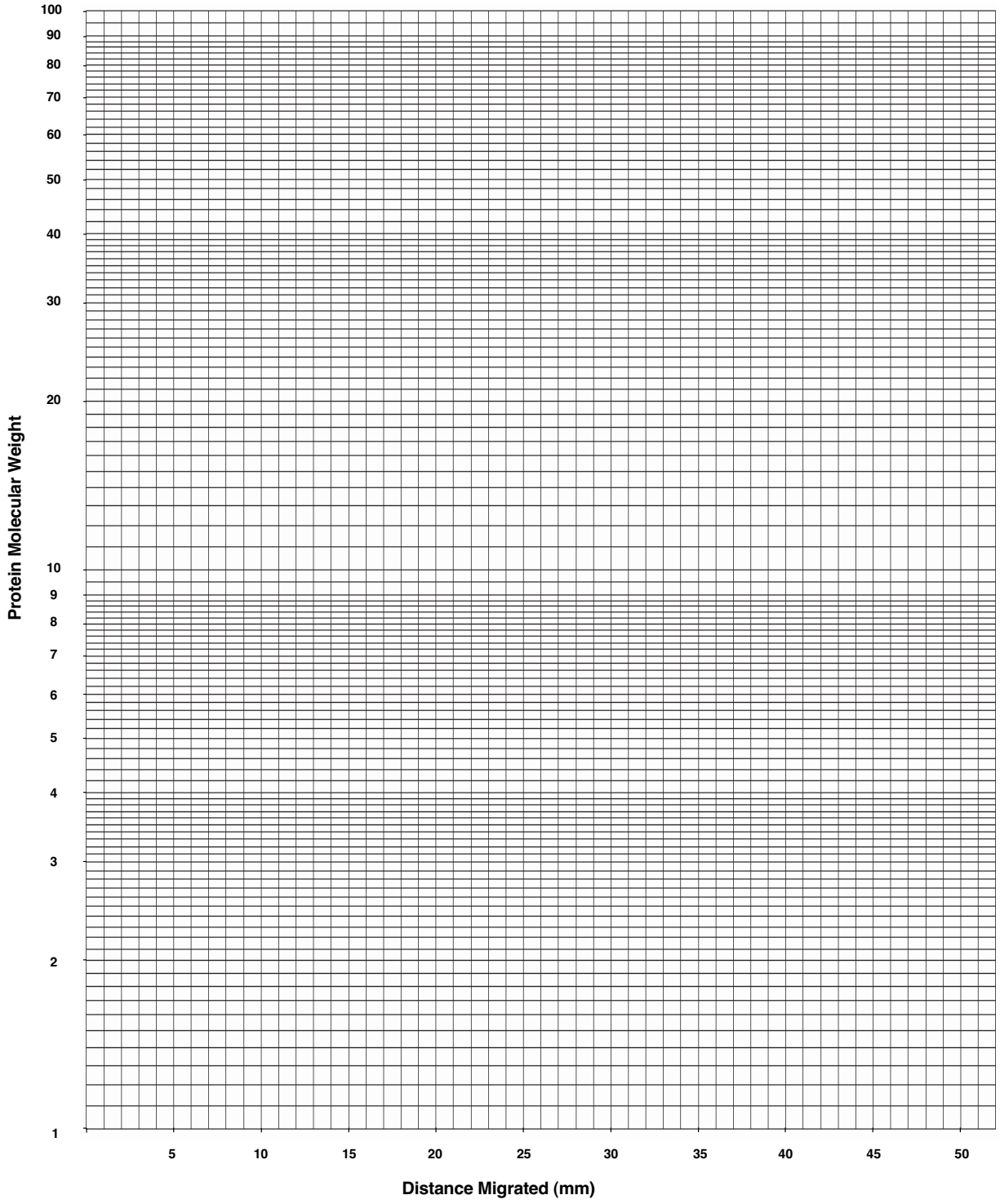


Fig. 23. Two-cycle semi-logarithmic graph paper to construct curve of the protein molecular weight against the distance migrated.

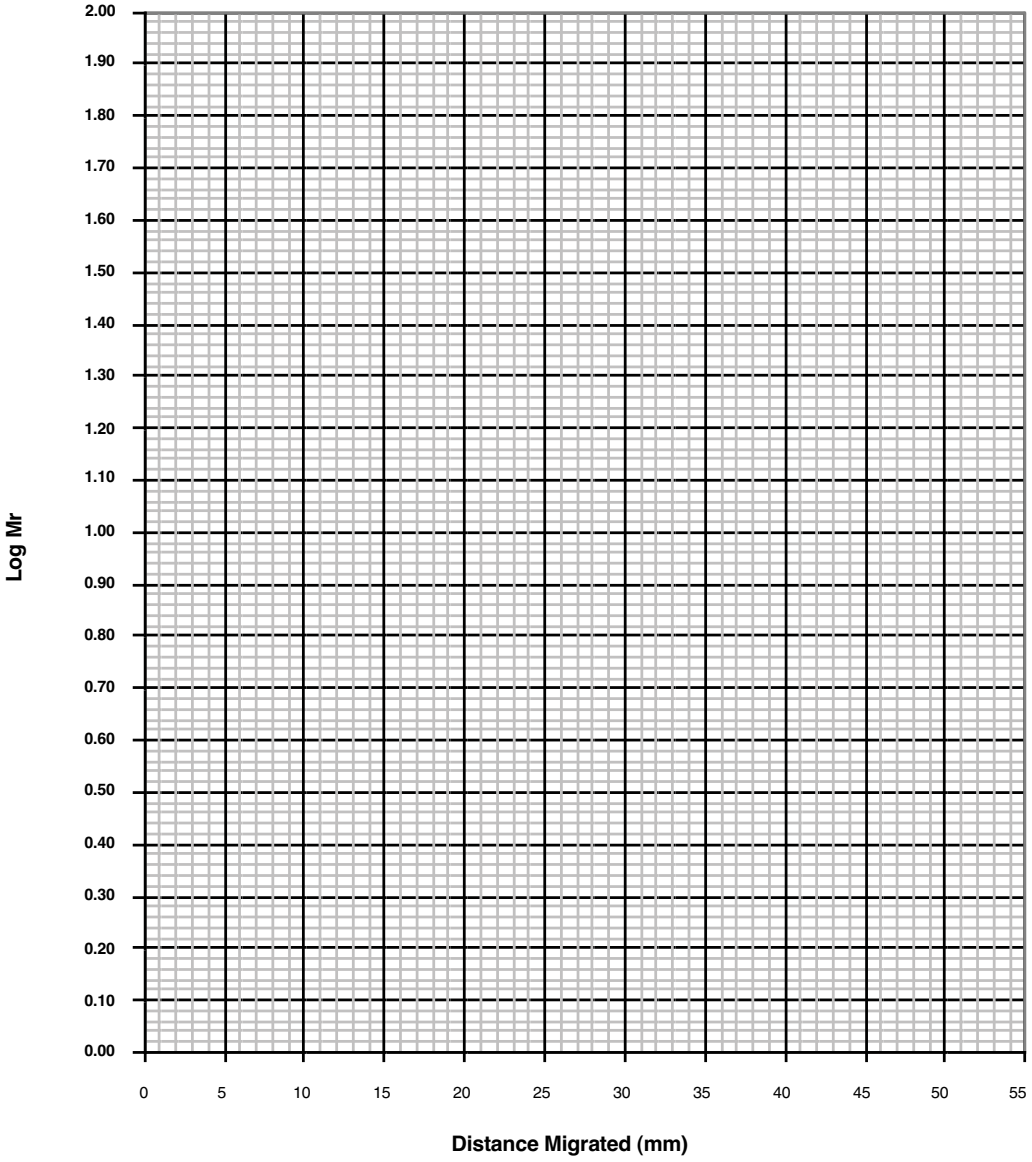


Fig. 24. Linear graph paper to construct a standard curve of the log molecular weight (log Mr) against the distance migrated.

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 November 2008 revealded myosin light chain protein sequences derived from only 29 fish species. 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 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 E: Teacher Answer Guide

Focus Questions – Pre-Lab Activity

1. **What are 5 proteins found in muscle?**

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

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

   Myosin is composed of 2 heavy chains and 4 light chains. The heavy chains are comprised of a long tail region and a globular head. Two different myosin light chains (either myosin light chain 1 + myosin light chain 2 or myosin light chain 2 + myosin light chain 3) are wrapped around the neck of each heavy chain. Refer to Figure 20.

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

   Muscle contraction is vital to the survival of an organism. Other less vital proteins such as skin pigments can change without compromising survival, and allow an animal to explore new environments. If actin and myosin don't function properly, the animal will die because it can't breathe, move, or beat its heart. Also muscle contraction relies on the interaction of multiple proteins; therefore, modifying one of those proteins must be beneficial for interacting with all the others. Thus, only a very small number of mutations in the actin and myosin genes will be advantageous and therefore retained, making their evolution very slow.

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

   An organism's DNA is constantly being mutated. Environmental and selective pressures cause advantageous gene mutations to be retained. This selective advantage means that over time the sequence of genes that started out the same in the common ancestor change. As environments change and promote changes in organisms, the creation of new species, and the loss of other species, gradually occurs.

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

   More closely related species would share more proteins than more distantly related species and those proteins would have more similar sequences and structures than those of more distant relatives.

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

   The same gene, or gene function, may be essential for life in many different species.

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?**

   Yes; many possibilities exist – posttranscriptional modifications, such as alternatively spliced RNA, mRNA editing, posttranslational modifications, such as cleaved proteins, modified proteins, phosphorylation, glycosylation, acetylation, all of which can change protein mass.

Focus Questions – Lesson 1

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

   Tris – buffer, maintains pH.

   Sodium dodecyl sulfate (SDS) – ionic detergent that solubilizes proteins and coats them with a negative charge thereby denaturing them into linear peptides.

   Dithiothreitol (DTT) – breaks disulfide bonds by reduction to destroy tertiary protein structure and linearize proteins.

   Bromophenol blue – a tracking dye that runs ahead of the proteins in the sample.

   Glycerol – adds density to samples for ease of loading to make samples sink into the wells.

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?**

   Six: 2 myosin heavy chains (200 kD) and 4 myosin light chains (15–25 kD).

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

   Denaturing linearizes proteins. Linear proteins can more readily migrate through the gel matrix and be separated according to protein mass.

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

   Heat helps to denature proteins.

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

   The primary structure is the linear sequence of amino acids that comprise the protein. The quaternary structure describes multi-subunit proteins and describes how the different peptides that comprise the protein interact with one another.

Focus Questions – Lesson 2

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

   The SDS, DTT, and heat denature the proteins, together destroying their secondary, tertiary, and quaternary structures so that they will migrate through the gel as a linear molecule. The SDS also confers a negative charge to the proteins so that they will migrate through the gel according to their mass rather than their native charge.

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

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

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

   Controls help to interpret results and to determine if there have been any experimental errors. The actin and myosin standard is a positive control for the antibody used to help locate actin, myosin heavy chain, tropomyosin, and the myosin light chains on a stained gel or myosin on a blot. The Precision Plus Protein Kaleidoscope prestained protein standards allow assessment and calculation of the sizes of bands on the blot.

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

   The smaller protein (myosin light chain) will migrate fastest because smaller proteins can move through the gel matrix more quickly.

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

Focus Questions – Lesson 3

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

   The gel is fragile and would be difficult to work with. The membrane is sturdier and longer lasting. The proteins are embedded in the gel and are more accessible to antibodies when blotted to the surface of the membrane.

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

   The proteins blot directly out of the gel onto the membrane. Proteins cannot jump over any bubbles between the gel and the membrane, so bubbles result in an uneven blot with no antibody binding in that region.

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

   Proteins are negatively charged from the SDS in the Laemmli sample buffer. Within an electric field, proteins migrate out of the gel because they are pushed away from the negative charge on the gel side and drawn toward the positive charge on the membrane side. Similarly to electrophoresis, proteins are carried out of the gel to the membrane during electroblotting. If capillary action was used instead, then the proteins were pulled out of the gel along with the blotting buffer moving from the reservoir, through the gel, the membrane and up the tower of paper towels. The proteins do not pass through the membrane because nitrocellulose binds proteins and has very small pores trapping them to its surface.

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

   For a stained gel, if the transfer has been successful, the blue stained proteins will show up on the membrane and no blue stained proteins should be visible on the gel. For an unstained gel, if the transfer has been successful the Precision Plus Protein Kaleidoscope prestained protein standards will be visible on the membrane. Alternatively, the membrane could be stained for proteins after transfer.

Focus Questions – Lesson 4

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

   Antibodies are used to identify specific proteins from a mixture. Many different methods such as western blotting and ELISA use antibodies to identify proteins. There are other methods for identifying proteins, such as electrophoresis and chromatography combined with mass spectrometry or protein sequencing.

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

   There are several other methods used to analyze proteins: high-pressure liquid chromatography (HPLC), size exclusion chromatography, hydrophobic interaction chromatography, centrifugation, SDS-PAGE, native PAGE, mass spectrometry, peptide sequencing, immunostaining, ELISA, dot blotting, and dipstick tests.

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

   The blot identifies myosin proteins and their location enables us to determine their molecular weights.

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

   A standard curve can be drawn using the known sizes and distances migrated of the Precision Plus Protein Kaleidoscope prestained protein standards. This curve can then be used to determine the sizes of the myosin proteins by comparing the distance migrated on the gel to the standard curve.

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

   Differences in amino acids can cause variations in molecular mass and structure. Changes in structure can provide evolutionary advantages.

6. **Explain why the secondary antibody is used.**

   The secondary antibody detects the primary antibody and has a color-producing enzyme linked to it that forms a visible precipitate when the colorimetric substrate is added, so the antigen (myosin) can be identified.

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

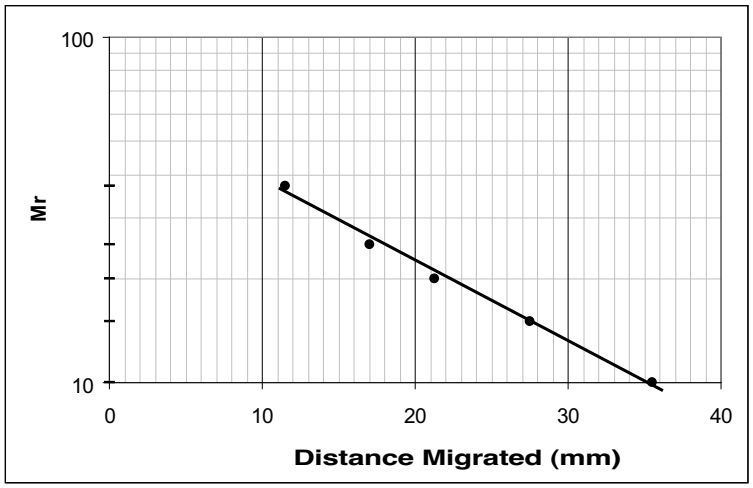
   A polyclonal antibody can be made by injecting purified dystrophin into a mammalian model (mouse, goat, or rabbit). The mammal will produce antibodies against the protein that will be present in the blood of the animal. The blood can be drawn from the animal and purified into serum by removal of the blood cells and platelets. The serum can be used directly, or the antibody can be further purified. Other answers could include the more complex process of making a monoclonal antibody, or screening a phage display library. Details on these processes are included in Appendix A.

Student Questions – Appendix D: Lesson Extension 1

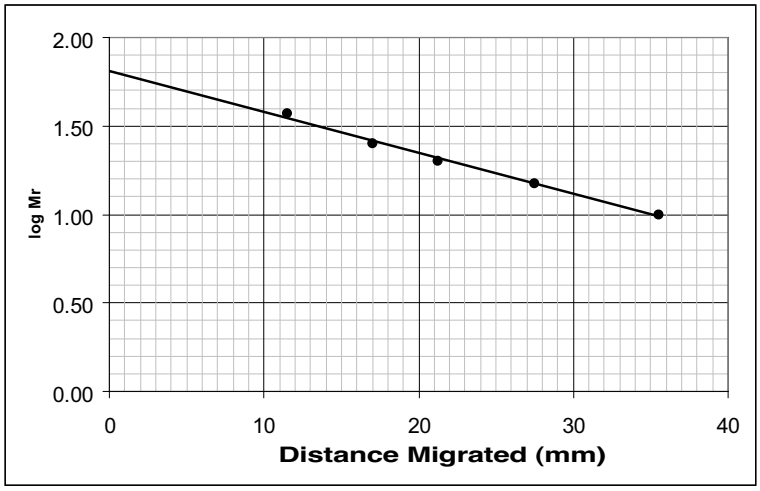
Construct a Standard Curve Site

Below are two examples of graphs generated from western blot data.

This semi-log graph shows data that was obtained from a western blot using a 15% gel. For a myosin band that has migrated 18.5 mm between the 20 and 25 kD standards, a line is drawn from the x-axis at 18.5 mm then across to the y-axis. The Mr is estimated to be 24 kD.



In this graph, data was obtained from the same western blot as above. The slope is 0.8/36 which equals 0.022 and the intercept is 1.8, thus the linear equation is y = -0.022x + 1.8. For a myosin band that has migrated 18.5 mm to between the 20 & 25 kD standards, the estimated logMr (y) is 1.37, and thus the Mr of the myosin is 23.4. The r2 value for this graph is 0.99, where r2 is the correlation coefficient measuring the strength of the relationship between the distance migrated and Mr. A perfect r2 relationship of 1.0 indicates that distance migrated can exactly determine the Mr of these proteins without error; therefore, an r2 = 0.99 being near 1.0 is excellent.



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

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SDS-PAGE & Western Blotting Internet Resources:

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.pdb.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)

Mini-PROTEAN® TGX™ Precast Gels Instruction Manual, bulletin M1658100

Protein Blotting Guide, bulletin 2895

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