

V3

Bio-Rad Explorer™
Rapid Blotting + V3 Western Workflow™
(Stain-Free Rapid Blotting)

BIO-RAD

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Introduction

This application note describes two methods for achieving rapid and robust results in western blotting:

- Rapid blotting using traditional SDS-PAGE technology coupled with innovative advances in transfer technology using the Trans-Blot® Turbo™ system
- V3 Western Workflow™ (stain-free rapid blotting), which incorporates stain-free technology into the rapid blotting protocol.

Both methods result in valuable time savings in the classroom, with the V3 Western Workflow providing the ability to immediately visualize, verify, and validate protein blotting results at several key checkpoints in the experimental procedure. For optimal speed and ease of use according to individual needs, the entire V3 Western Workflow, or portions thereof, can be applied to western blotting experiments to increase time savings.

The Bio-Rad Explorer™ kits used in this application — Comparative Proteomics Kit I: Protein Profiler Module (catalog #1662700EDU) and Comparative Proteomics Kit II: Western Blot Module (catalog #1662800EDU) — allow students to use SDS-PAGE to separate and analyze the protein profiles of muscle tissue from fish samples. By comparing the proteomes of divergent fish species and creating a phylogenetic tree based on the results, students can investigate evidence of biological evolution. Furthermore, by employing the immunodetection technique of western blotting to specifically identify myosin light chain from the hundreds of other proteins that comprise the fish muscle cell extracts, students can hypothesize how protein variation correlates with evolutionary relationships, as well as discuss what evolutionary conservation of a protein might suggest about the protein's function.

Learning Objectives

At the end of this exercise, students will be able to:

- Prepare an SDS-PAGE sample and perform vertical gel electrophoresis
- Understand the primary, secondary, and tertiary structure of proteins
- Identify major proteins found within muscle tissue
- Understand what antibodies are and how they function
- Perform immunodetection by western blotting
- Discern the conclusions that can be made from stained gels versus immunoblots
- Understand how protein variation supports evolutionary relatedness

Background Information for Instructors

Proteomics and the Study of Proteins

Proteomics was initially defined as the effort to catalog all the proteins expressed in all cells at all stages of development. That definition has now been expanded to include the study of protein functions, protein-protein interactions, cellular locations, expression levels, and posttranslational modifications of all proteins within all cells and tissues at all stages of development. Researchers in the proteomics field have discovered a number of modification systems that allow one gene to code for many proteins, and mechanisms that finely regulate the subcellular and extracellular locations and expression levels of proteins. These include alternative splicing of exons, use of different promoters, posttranscriptional modification, translational frameshifting, and posttranslational modification.

Muscle Proteins

Our most familiar daily movements, from walking to simply breathing, are driven by the interactions between specialized proteins in our muscle fibers. The basic contractile elements of muscle cells are the myofibrils that are bundled into muscle fibers, illustrated in Figure 1. Each myofibril consists of a linear

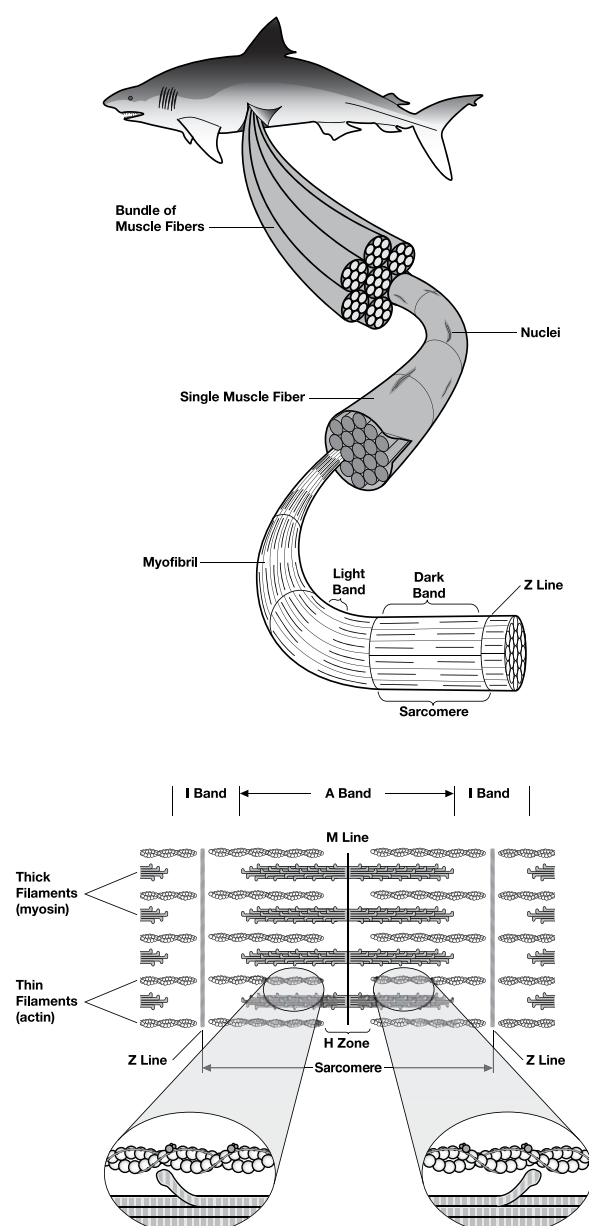


Fig. 1. Telescopic view of muscle structure. Thick myosin filaments and thin actin filaments form myofibrils, which are bundled together to make muscle fibers. Figure modified with permission (Campbell 1996).

series of contractile units called sarcomeres. Sarcomeres are precisely arranged assemblies of actin and myosin protein filaments. Thin overlapping filaments of actin are aligned with thick filaments of myosin in a parallel and partly overlapping manner. The sarcomere shortens when myosin hydrolyzes ATP (adenosine triphosphate) to slide along the actin filament, pulling the ends of the sarcomere toward each other. The combined contraction of many sarcomeres along a muscle fiber causes contraction of the entire muscle. It is important to note that, although actin and myosin are the major components, other proteins are also found in muscle tissue.

The actin and myosin standard included in the Protein Profiler Kit contains rabbit actin and myosin myofibrils that have been isolated from rabbit skeletal muscle. Actin (43 kD), myosin heavy chain (210 kD), three myosin light chains (15–25 kD), and tropomyosin (35 kD) are visible after gel electrophoresis and Bio-Safe™ Coomassie stain and destain procedures (see bulletin 10004530, Comparative Proteomics I: Protein Profiler I instruction manual, for complete protocols, available at www.explorer.bio-rad.com) or upon UV activation of stain-free gels. The myosin light chains vary in molecular weight between fish species and can be specifically identified during immunoblotting, which will be performed in this lab.

Numerous other proteins besides actin and myosin are also required for muscle contraction. While actin and myosin are highly conserved across all animal species, other muscle proteins show more variability. These variations in an organism's muscle proteins may reflect refinements of muscle function and performance that are adaptive to particular niches, environments, or physiological stresses. These differences in muscle proteins between species are one measure that can indicate evolutionary relatedness between species.

Evolutionary Trees and Fish Evolution

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

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

The data used to construct the evolutionary tree depicted in Figure 2 were obtained from the cladograms on the Tree of Life web page from the University of Arizona (www.tolweb.org). Please note that the field of phylogenetics is ever-changing and different methods used to construct a phylogenetic tree often result in differences between trees, hence the data on the Tree of Life web page may not concur exactly with “textbook” evolutionary trees.

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

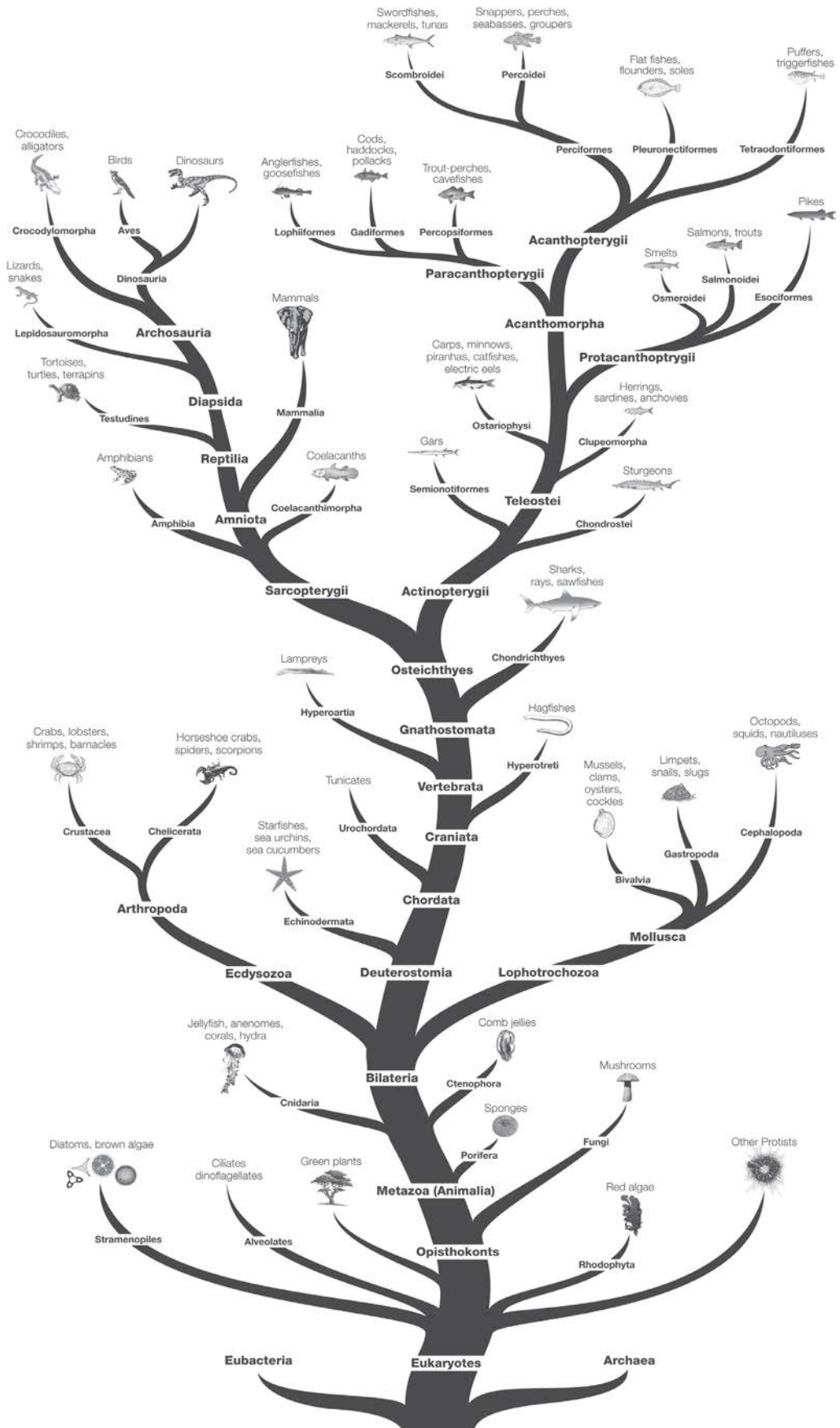


Fig. 2. Evolutionary tree showing the relationships of eukaryotes. Adapted from the Tree of Life web page from the University of Arizona (www.tolweb.org).

Hyperotreti (e.g. hagfish) are eel-like, jawless fishes that have a skull but no backbone.

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

Chondrichthyes (e.g. shark, ray, skate, and sawfish) have a cartilaginous rather than bony skeleton. Their skin is thick and without true scales, and they do not have swim bladders or lungs.

Osteichthyes (e.g. tuna and haddock) are the most diverse class of fish, having bony skeletons, true scales, paired fins, and movable rays in their fins and tail.

Sarcopterygians (e.g. lungfish) also include modern amphibians, reptiles, birds, and mammals. They form an important evolutionary link between fish and four-legged land animals.

Actinopterygians (e.g. sturgeon, mackerel, and anglerfish) is the subclass encompassing most modern ray-finned fish.

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

Protein Structures and Basic Properties

In this lab we will be examining the protein profiles of various fish species in order to compare and contrast them, and then use that information to make educated conclusions about their relatedness.

In contrast to DNA, which is quantified in terms of length (the number of base pairs), proteins are quantified in terms of their molecular weights relative to a hydrogen atom, in daltons. This is because DNA is composed of only four nucleotides, which are found in roughly equal proportions and are approximately the same molecular weight. Proteins, however, are composed of 20 amino acids with molecular weights from 89 to 204 daltons (the average is 110), and peptide chains that vary considerably in percentage of amino acid composition. One dalton equals the mass of one hydrogen atom, which is 1.66×10^{-24} grams. Most proteins have masses on the order of thousands of daltons, so the term kilodalton (kD) is used for protein molecular masses. Proteins range in size from several kilodaltons to thousands of kilodaltons, but most fall between the range of 10 kD and 220 kD.

A molecule's electrical charge and its mass affect its mobility through a gel during electrophoresis. The ratio of charge to mass is called charge density. Since every protein is made of a unique combination of amino acids, each of which may have a positive, negative, or neutral charge, the net charge of each protein is naturally different. The inherent charges of proteins must be removed as a factor affecting migration in order for polyacrylamide gel electrophoresis to be effective as a method of protein molecular weight determination. The intrinsic charges of proteins are obscured by placing a strongly anionic (negatively charged) detergent, sodium dodecyl sulfate (SDS), in both the sample buffer and the gel running buffer. SDS binds to and coats the proteins and also keeps them unfolded in relatively linear chains. In this form, proteins migrate in a polyacrylamide gel as if they have equivalent negative charge densities, and mass becomes the main variable affecting the migration rate of each protein (note: posttranslational modifications such as glycosylation can also affect protein migration). This technique is called SDS-polyacrylamide gel electrophoresis, or SDS-PAGE.

To effectively determine the molecular weights of proteins, the secondary (2°), tertiary (3°), and quaternary (4°) structures of the protein complexes within a protein extract are disrupted prior to electrophoresis. These structures are illustrated in Figure 3. The process of structural disruption is called denaturation.

- **Primary structure** — denatured linear chain of amino acids
- **Secondary structure** — domains of repeating structures, such as β -pleated sheets and α -helices
- **Tertiary structure** — 3-dimensional shape of a folded polypeptide, maintained by disulfide bonds, electrostatic interactions, hydrophobic effects
- **Quaternary structure** — several polypeptide chains associated together to form a functional protein

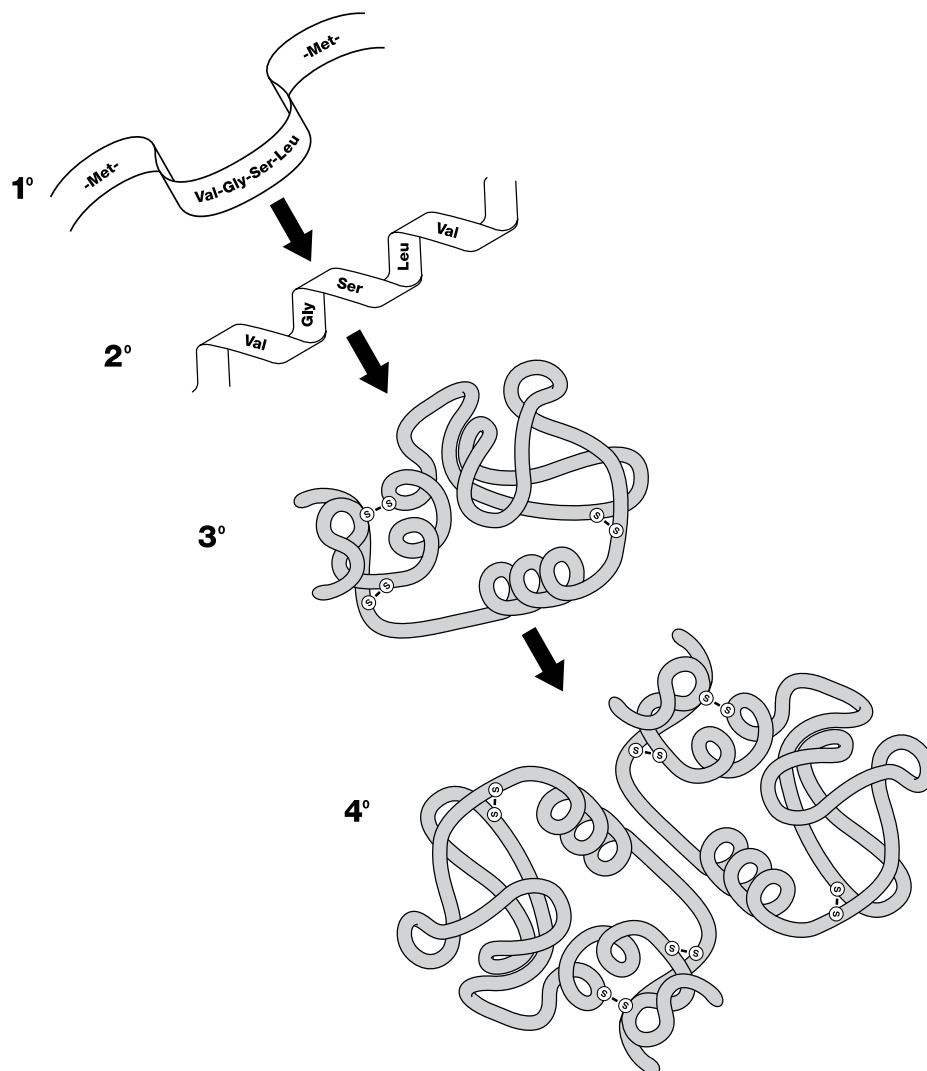


Fig. 3. Secondary (2°), tertiary (3°), and quaternary (4°) protein structures must be disrupted, or denatured, to accurately separate proteins by size.

A reducing agent, such as β -mercaptoethanol (BME) or dithiothreitol (DTT, used in this lab), is added to ensure complete breakage of disulfide bonds within single polypeptide chains, and between different polypeptide chains. BME and DTT both have an unpleasant smell, and can reduce any proteins they come into contact with. Caution is advised and proper protective equipment (gloves and safety glasses) is recommended when performing this lab.

Three factors – heat, ionic detergent, and reducing agent – completely disrupt the 2°, 3°, and 4° structures of proteins and protein complexes, resulting in linear chains of amino acids, shown in Figure 4. These molecules snake through the gel at rates proportional to their molecular masses.

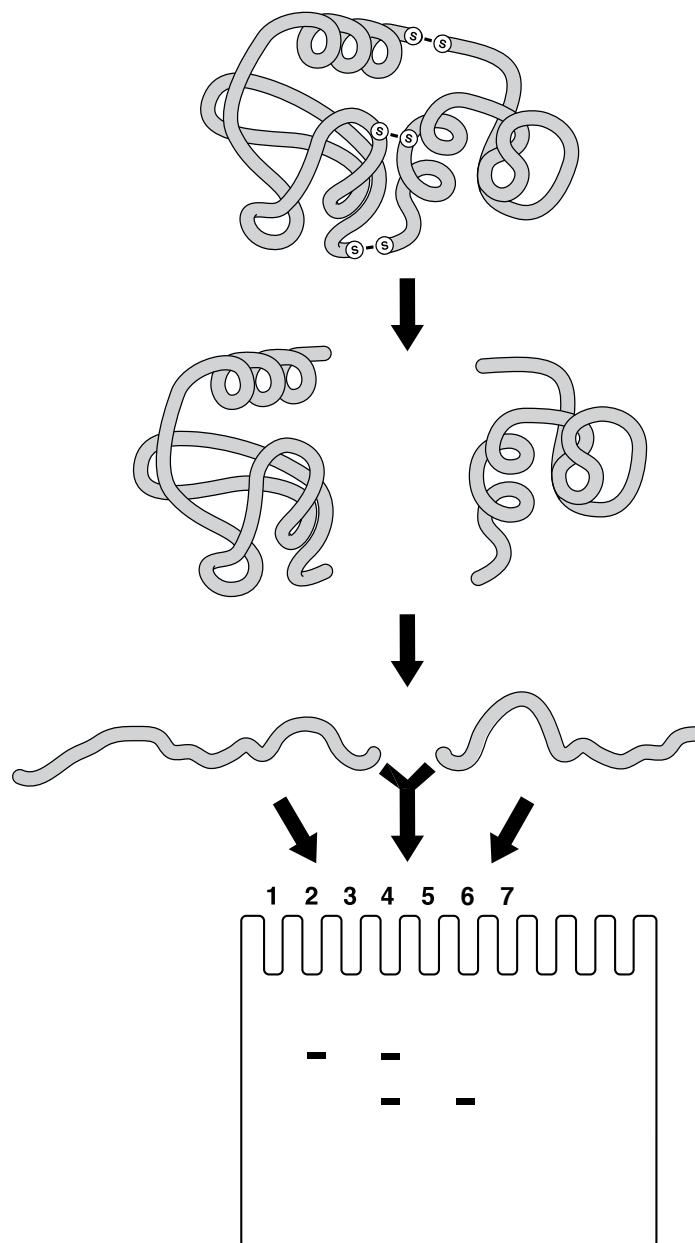


Fig. 4. A quaternary protein complex denatured with reducing agents, heat, and SDS, can be separated into individual proteins and resolved by size using SDS-PAGE.

Using Polyacrylamide Gel Electrophoresis to Separate Proteins

Proteins are usually separated using polyacrylamide gels rather than agarose gels, which are more commonly used to separate DNA. This is because most proteins are much smaller than DNA fragments and polyacrylamide gels have pore sizes similar to the sizes of proteins. The gel matrix formed by polyacrylamide is much tighter than agarose and able to resolve much smaller molecules.

Polyacrylamide gel electrophoresis (PAGE) uses two phases of polyacrylamide. One phase consists of an upper stacking gel, typically of 4% acrylamide, and the other consists of a lower resolving gel of a higher percentage of acrylamide (this lab uses a resolving gradient gel from 4–20%). This is called a discontinuous system and results in all of the proteins in a sample separating, or resolving, at the same time (Laemmli 1970). Since sample volumes can vary from lane to lane, forming vertically narrow or broad bands in the wells, not all proteins in a sample enter the gel simultaneously. However, the low

percentage of the stacking gel allows proteins to migrate rapidly and be compressed at the edge of the denser resolving gel, regardless of their sizes. The samples of mixed proteins are thus concentrated into uniformly thin bands in each lane before they move into the denser resolving gel and begin to separate from one another according to their molecular weights.

Proteins within the gel are not visible unless they have been prestained with covalently attached dyes, as is the case with the Precision Plus Protein™ Kaleidoscope™ standards provided in the Comparative Proteomics Kit I: Protein Profiler Module. In order to visualize the separated protein samples, gels are usually subject to a staining and destaining procedure with Bio-Safe Coomassie Blue stain. The stain binds specifically to proteins and not to other macromolecules such as DNA or lipids. After destaining, distinct blue bands appear on the gel, each band representing on the order of 10^{12} molecules of a particular protein that have migrated to that position; the higher the amount of protein, the more intense the blue staining.

Stain-Free Gels

Stain-free technology offers an alternative to staining and destaining procedures, saving significant time, effort, and reagents. This technology uses a unique in-gel chemistry that is available only in Bio-Rad's TGX Stain-Free™ precast gels. The gel formulation incorporates a trihalo compound that, when exposed to UV irradiation, activates a covalent reaction between the compound and tryptophan amino acids within the proteins in the gel, resulting in UV-induced fluorescence. Activation of the trihalo compounds in the gel adds a 58 dalton moiety to available tryptophan residues and is required for protein visualization. Because proteins undergo this modification after electrophoretic separation, the mobility and apparent size of the proteins in the gel are not affected. The sensitivity of the stain-free system is comparable to the sensitivity achieved by Coomassie Blue staining for all proteins whose amino acid content is at least 1.5% tryptophan; superior sensitivity to Coomassie Blue staining is possible for proteins with 3% tryptophan or greater. (See also bulletin 5974, Mini-PROTEAN® and Criterion™ TGX Stain-Free™ Precast Gels.) Following a short 2–5 minute activation time on a stain-free enabled imager (for example the Gel Doc™ EZ imaging system), rapid fluorescent detection of proteins is achieved. Furthermore, these gels can then be used for western blotting, whereas protein transfer of Coomassie Blue stained gels is generally not recommended. This is because Coomassie Blue stain typically contains components that “fix” proteins in the gel, which makes them less prone to diffusion. Unfortunately, this also makes protein transfer to a solid support significantly less efficient.

Western Transfer

Following gel electrophoresis, the separated proteins can be transferred to a solid support where they are immobilized and can be investigated further with greater ease of manipulation. Synthetic membranes, also referred to as blots, are a commonly used type of solid support. The most frequently used synthetic membranes are made from nitrocellulose or polyvinylidene difluoride (PVDF).

While there are different methods that can be employed to transfer protein samples from a gel to a membrane, the most common method uses electrophoretic transfer. In addition to being less time-consuming than diffusion methods, electrophoretic transfer preserves the high-resolution separation of proteins achieved during PAGE. One type of transfer system in this category is the tank blotting system. In tank blotting, the gel and membrane are submerged in transfer buffer inside a tank. A nonconducting cassette holds the membrane in close contact with the gel and the cassette assembly is submerged into a tank of conducting transfer buffer. A second type of transfer system in the same category is the semi-dry transfer system, so-named because of the limited amount of buffer required. In semi-dry transfer protocols, the gel and membrane are sandwiched between buffer-wetted filter papers that are in direct contact with flat-plate electrodes. While both methods perform well for protein transfer to a membrane, the semi-dry setup achieves higher intensity blotting conditions, which means transfer can occur more rapidly. Additionally, semi-dry blotting eliminates the need for large volumes of transfer buffer. The Trans-Blot® Turbo™ transfer system used in this lab is a semi-dry transfer system. A comparison of tank blotting versus Trans-Blot Turbo blotting workflows is illustrated in Figure 9.

Immunodetection

Why would one want to transfer proteins to a membrane? What information can be determined from experiments performed on proteins that have been transferred to a membrane? It is not possible to definitively identify unknown proteins in an SDS-PAGE gel without additional confirming information. In an experiment like the one in this lab, each protein extract contains a complex mixture of proteins. The different proteins appear as distinct blue-stained bands on a gel stained with Bio-Safe Coomassie Blue stain, or black bands when imaging stain-free gels on the Gel Doc EZ imager. From the positions and intensities of these bands, we can determine the size and relative abundance of the proteins, but we can only make educated guesses about the identity of each protein based on available references. For example, since the samples are all from muscle tissue, you may correctly assume that there would be large quantities of the predominant muscle proteins such as actin and myosin. The actin and myosin standard provided in the Protein Profiler Kit helps to identify these proteins. The Precision Plus Protein Kaleidoscope standards are used to determine the molecular masses of the unknown proteins and to help monitor the progress of the run.

Even when the molecular weight of a protein is known, and is used as a criterion for identification, there are two possible sources of error. First, bands that migrate almost identically on a gel may actually be different proteins of very similar sizes. Second, proteins of very similar composition, function, and evolutionary origin may be different in molecular weight, because of variations acquired as they evolved, or due to posttranslational modifications. Definitive identification of a protein requires mass spectrometry, sequencing, or immunodetection. Immunodetection methods, such as western blotting, use antibodies that specifically recognize the proteins of interest. Such antibodies can provide positive identification.

In this lab, myosin light chain proteins can specifically be identified using immunodetection. It is also possible to discover differences in the molecular weights of these proteins in previously uninvestigated fish species. After polyacrylamide gels are run, all the proteins are transferred to a membrane by passing an electric current through the gel, causing the negatively charged proteins to migrate from the gel and bind to the membrane. Then an antibody that specifically binds to myosin light chains is added and incubated with the membrane. A second antibody that specifically binds the first or primary antibody is linked to a color-producing enzyme and incubated with the membrane. A substrate is then added that reacts with the enzyme-linked secondary antibody and the enzymatic reaction causes color development on the membrane only at the specific position of the bound myosin light chains. This detection method is depicted in Figure 5. Because immunodetection uses antibodies which, in this lab, specifically bind to myosin light chain protein, this technique provides certainty about the position and identity of the myosin light chains on the blot.

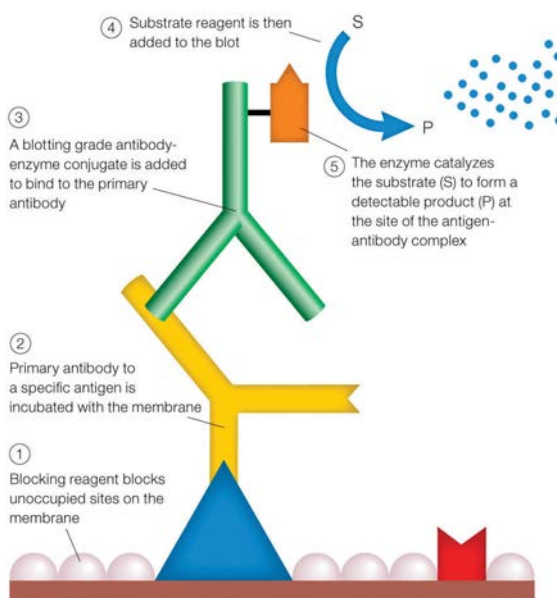


Fig. 5. Specific enzymatic detection of membrane-bound proteins.

Experimental Protocol

Purpose

In this lab, myosin light chain proteins will be identified from muscle tissue of various fish species using the rapid, improved, and streamlined V3 Western Workflow™ (stain-free rapid blotting) outlined in Figure 6. The Protein Profiler and Western Blot Modules contain the necessary reagents for completing the gel electrophoresis and immunoblotting laboratories.

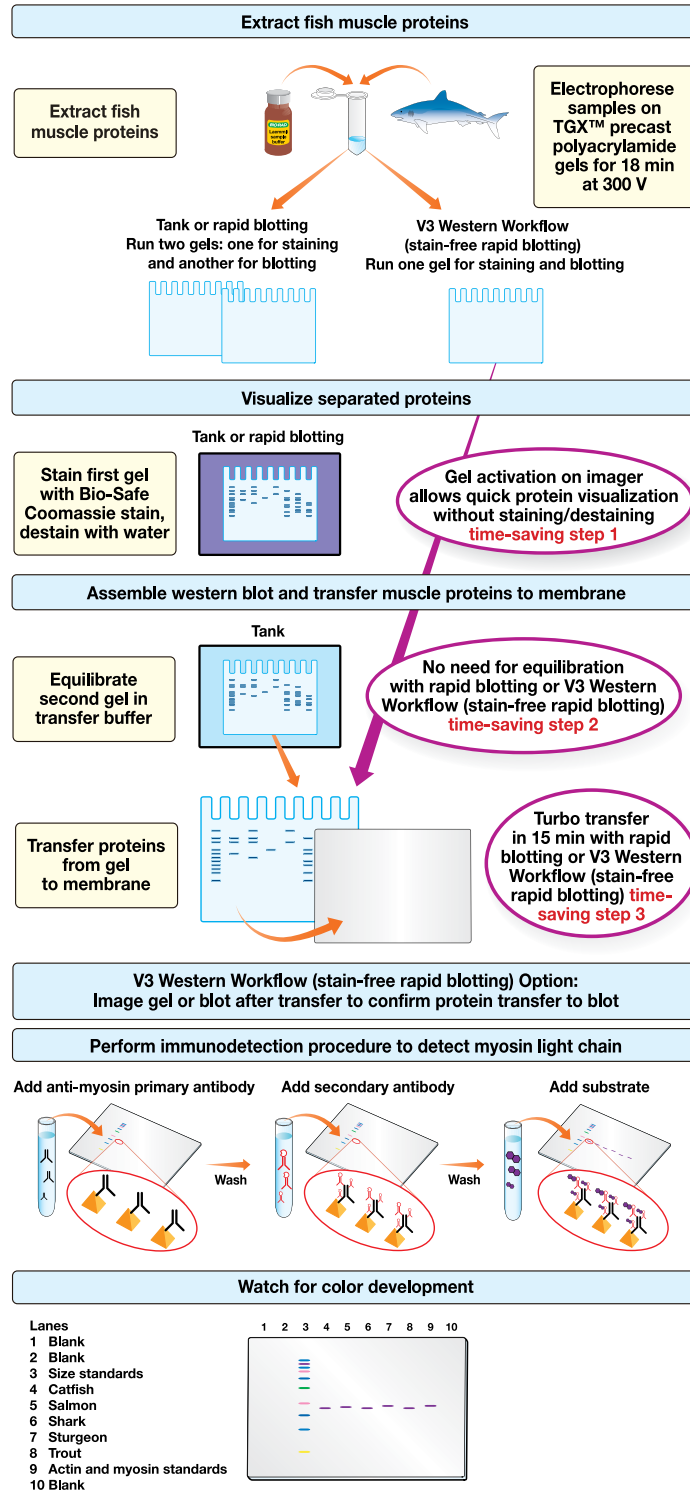


Fig. 6. Illustration of the western blotting workflow using traditional tank blotting, rapid blotting, or the most time-saving V3 Western Workflow (stain-free rapid blotting) protocol.

Workflow

Materials (sufficient for eight workstations, two to four students per workstation)

- Comparative Proteomics Kit I and II: Protein Profiler and Western Blot Module (1662850EDU)
- Fish samples, 1g each per workstation (obtained from a store, river, lake, or ocean)
- Scissors or knife to cut fish samples
- Mini-PROTEAN® TGX™ 4–20% precast polyacrylamide gels, 10 pack (4561093EDU)
- Distilled or deionized water (1 gallon)

Additional Required Items

- Mini-PROTEAN Tetra cell vertical electrophoresis chambers (1658004EDU)
- PowerPac™ Basic power supplies (1645050EDU)
- Digital dry bath, ambient to 100°C (1660562EDU)
- Rocking platform or shaker (1660709EDU)
- 2–20 µl adjustable-volume micropipets (1660506EDU)
- TBR-35 pipet tips, 2–200 µl, 1000 per box (2239347EDU)
- Prot/Elec pipet tips, 0.5–1000 µl, 1000 per box (2239917EDU)
- Trans-Blot® Turbo™ transfer system (1704150EDU)
- Trans-Blot Turbo mini nitrocellulose transfer packs, 10 pack (1704158EDU), or Trans-Blot Turbo midi nitrocellulose transfer packs, 10 pack (1704159EDU)
- Gel staining trays (1660477EDU)

Optional Accessories

- Gel Doc™ EZ imaging system (1708270EDU)
- White light sample tray (1708272EDU)
- Sample tray holder (1708276EDU)
- Pipet controller (1660490EDU)
- Gel cutter (1703760EDU) or Gel releasers (1653320EDU)
- Blot roller (1651279EDU)

Note: Detailed protocols describing all steps of electrophoresis as well as the traditional tank transfer method can be found in the Comparative Proteomics Kit I: Protein Profiler Module instruction manual (bulletin 10004530), available at www.explorer.bio-rad.com.

Note: Nitrocellulose is the recommended membrane for use with the following western blotting workflow. PVDF membranes will also work, but do require slightly longer transfer times to achieve equivalent signal intensity.

Reagent Preparation

1. Laemmli sample buffer: Add 0.3 g of DTT to 30 ml of Laemmli sample buffer. Swirl to resuspend. The final concentration of DTT will be 70 mM. Leftover solution should be stored at –20°C, as the DTT is labile. Prior to each use, warm the solution to room temperature to dissolve any SDS precipitates that form upon freezing.
2. Actin and myosin standard: Rehydrate actin and myosin standard by adding 500 µl Laemmli sample buffer to the vial of 500 µg lyophilized actin and myosin standard and incubate at room temperature for 20–30 minutes. Transfer the rehydrated actin and myosin sample to a screwcap tube labeled “AM” and heat for 5 min at 95°C. Store the rehydrated actin and myosin standard at –20°C for up to 12 months after heating.
3. Precision Plus Protein™ Kaleidoscope™ standards: Prior to each use, warm the solution to room temperature to dissolve any SDS precipitates that form upon freezing.
4. TGS running buffer: Mix 100 ml of 10x Tris-Glycine-SDS running buffer with 900 ml of distilled water. 1x TGS can be stored up to six months at room temperature.

5. Wash Buffer: 1x phosphate buffered saline (PBS) with 0.025% Tween 20.

Stock Reagent	Volume
Distilled water	1,346.25 ml
10x PBS	150 ml
10% Tween 20	3.75 ml
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TOTAL	1,500 ml

Notes: Wash buffer is used to wash membranes and rehydrate antibodies. Store at room temperature up to 2 weeks.

6. Blocking Solution: 5% dry blocker in wash buffer.

Stock Reagent	Volume
Distilled water	359 ml
10x PBS	40 ml
10% Tween 20	1 ml
Dry blocker	20 g (pack)
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TOTAL	400 ml

Notes: Blocking solution is 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.

7. Ready-to-use primary antibody (mouse anti-myosin antibody):
- Make a 200x stock solution of primary antibody by adding 0.5 ml of 1x PBS to the lyophilized anti-myosin light chain antibody, close the stopper, and shake to mix.
 - Mix 0.5 ml of 200x stock of anti-myosin antibody with 100 ml of blocker. Mix well. Use 10 ml per blot.
8. Ready-to-use secondary antibody (goat anti-mouse-HRP antibody):
- Make a 200x stock solution of secondary antibody by adding 0.5 ml of 1x PBS to the lyophilized goat anti-mouse-HRP (horseradish peroxidase) secondary antibody, close the stopper, and shake to mix.
 - Mix 0.5 ml of 200x secondary antibody with 100 ml of blocker. Mix well. Use 10 ml per blot.
9. HRP color detection reagent:

Stock Reagent	Volume
Distilled water	89.4 ml
10x PBS	10 ml
HRP reagent A	10 ml
HRP reagent B	0.6 ml
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TOTAL	110 ml

Notes: This reagent should be prepared within 1 hour of use. HRP color reagent is light sensitive and must be prepared and stored in a dark or foil-wrapped container. Ensure thorough reagent mixing.

Method: Rapid Blotting Workflow

A. Sample Preparation

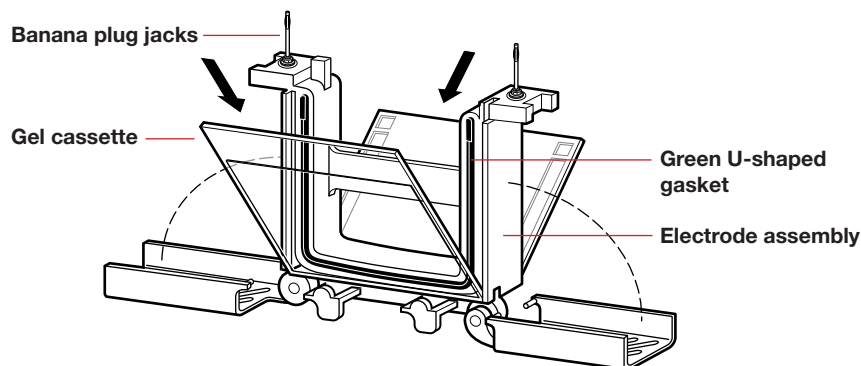
1. Label one 1.5 ml **fliptop** microtube for each of five fish samples. Also label one **screwcap** tube for each fish sample.
2. Add 250 μ l Bio-Rad Laemmli sample buffer with DTT to each labeled fliptop microtube.
3. Cut a piece of fish muscle about 0.25 x 0.25 x 0.25 cm (□) and transfer each piece into the appropriately labeled fliptop microtube. Try to make the pieces of muscle tissue the same size for each sample. Close the lids.
4. Flick the microtubes 15 times to agitate the tissue in the sample buffer.
5. Incubate for 5 min at room temperature.
6. Carefully transfer the buffer by pouring from **each fliptop** microtube into an appropriately labeled **screwcap** tube. Do not transfer the fish!

Note: It is not necessary to transfer all of the liquid to the screwcap tube, since only a few microliters are needed for gel loading. It is essential however, not to transfer any chunks of fish muscle to the screwcap tube.

7. Heat muscle extracts in screwcap tubes for 5 min at 95°C to denature the proteins in preparation for electrophoresis. Muscle extracts may be stored at room temperature while loading the gels for up to 3–4 hr. Alternatively, these samples may be stored for future use at –20°C for up to several weeks.

B. Gel Electrophoresis

1. Prepare a TGX™ gel cassette by removing the green tape from the bottom of the cassette, and removing the comb from the wells at the top of the cassette.
2. Assemble the gel with the short plate facing inward into the electrode assembly (contains the long banana plug jacks). Place a buffer dam or another TGX gel cassette on the opposite side of the electrode assembly.
3. Push both gels toward the middle of the electrode assembly, making sure that they are flush against the green U-shaped gaskets built into the clamping frame.



4. Lower the electrode assembly and gels into the Mini-PROTEAN® tank. Make sure that the red banana plug goes on the side of the tank with the red indicator.

5. Completely fill the inner chamber with 1x TGS electrophoresis buffer making sure the buffer covers the short plate (~150 ml). Flush the wells of the gels with 1x TGS buffer using a disposable plastic transfer pipet.
6. Fill the tank with 1x TGS buffer until the buffer reaches the 4 Gels line (see Caution note 2 below) on the tank, or to within 1 inch from the top of the tank.

Tip: If excessive foaming occurs upon filling the inner chamber and tank with 1x TGS buffer, a few quick sprays of ethanol from a spray bottle will quickly dissipate the foam caused by detergent in the running buffer, and will aid visibility for gel loading.

CAUTION:

1. **When running only 1 or 2 gels, DO NOT place the Companion Running Module in the tank. Doing so will cause excess heat generation and prevent proper electrophoretic separation.**
2. **When running the rapid electrophoresis protocol for TGX gels (300 V for 18 min), it is important to FILL the tank to the 4 Gels mark with 1x TGS electrophoresis buffer regardless of the number of gels being run.**
7. Heat fish samples and actin and myosin standard to 95°C for 5 min.
8. Load your gel:

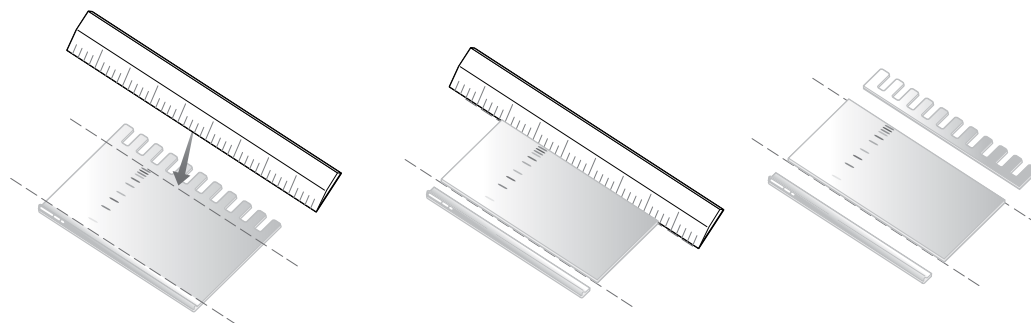
Lane	Volume	Sample
1 & 2	empty	empty
3	5 μ l	Precision Plus Protein™ Kaleidoscope™
4	3 μ l	fish sample 1
5	3 μ l	fish sample 2
6	3 μ l	fish sample 3
7	3 μ l	fish sample 4
8	3 μ l	fish sample 5
9	3 μ l	actin and myosin standard (AM)
10	empty	empty

9. Electrophorese for 18 min at 300 V in 1x TGS electrophoresis buffer.

Note: If unable to continue on to western blotting immediately, gels can be stored within their unopened plastic cassettes in a plastic zip bag containing a few ml of 1x TGS electrophoresis buffer at 4°C. Some diffusion of protein bands will occur, which may affect intensity of western blot results.

C. Western Blotting

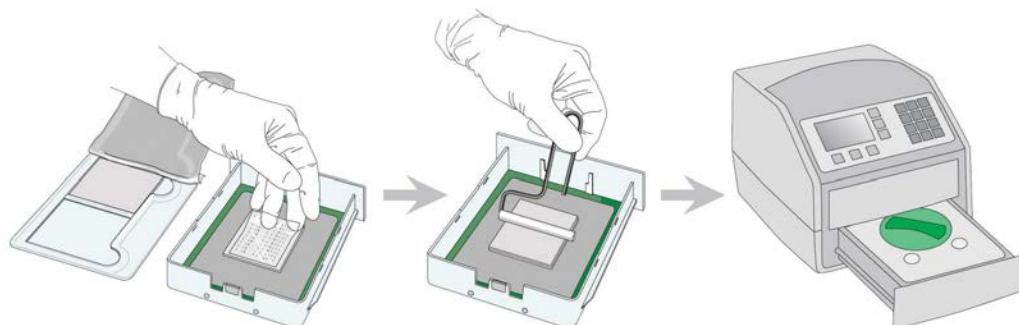
1. Using the green opening lever supplied with boxes of TGX gels, align the arrow cutout in the lever with the black arrows on the 4 sides of the TGX gel cassette. Wedge the teeth of the opening lever between the plates of the gel cassette, and crack the plates open using an upward or downward motion. Once the plates are cracked, slowly pry the plates apart. The gel will usually stick to one of the two plates.
2. Using the gel cutter, gel releaser, or a ruler, carefully cut away the wells and the very bottom of the gel.



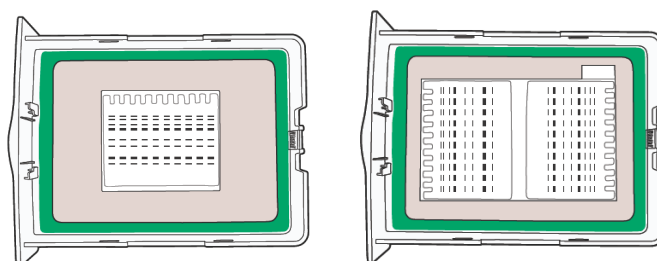
- To assemble the Trans-Blot® Turbo™ mini nitrocellulose transfer pack, open it by peeling away the top foil. Two pre-wet ion reservoir stacks marked “top (-)” and “bottom (+)” will be visible inside. Place the “bottom” stack, which contains the membrane, on the Trans-Blot Turbo tray with the membrane facing up.

Note: It is important to minimize handling of the membrane, especially with ungloved hands, which will leave protein-rich fingerprints on the blot! Use the very edges of the membrane to position the blot, and try to handle the membrane as little as possible.

- Using the blot roller, roll over the membrane to displace any air bubbles trapped between the stack and the tray.
- Place the gel on top of the membrane, followed by the “top” ion reservoir stack. Use the blot roller again to remove any air bubbles in the transfer sandwich.
- Place the tray cover on top of the transfer sandwich, press down firmly, and turn the dial clockwise to lock it in place. Insert the locked tray into the Trans-Blot Turbo unit.



Note suggested gel positioning below when running one or two gels per tray. (Wells are shown only to indicate orientation — you will have removed the wells from your gels by this point.) When running two gels per tray, tops of the gels should face outward, and one midi nitrocellulose transfer pack, rather than two mini nitrocellulose transfer packs, can be used to transfer the two gels.



7. Set up transfer conditions on the Trans-Blot Turbo unit following the instructions outlined in the instrument manual, and run the protein transfers.
Conditions for one mini gel sandwich per tray – 1.3 A, 25 V, 15 min
Conditions for two mini gel sandwiches per tray – 2.5 A, 25 V, 15 min
8. Once transfer is complete, and while wearing gloves, disassemble the gel sandwich, taking care to handle only the outer edges of the membrane. Peel back the membrane from the corner of the gel slowly and check for the presence of the prestained standards on the membrane. If the prestained standards have transferred to the membrane, remove the membrane from the sandwich entirely.
9. If performing immunodetection immediately, place the membrane in a tray with 25 ml of blocking solution. Incubate the membrane in blocking solution for 10 min at room temperature.

Note: If immunodetection cannot be performed directly following western blotting, wet blots may be stored in a plastic zip bag, or for ease of handling, several blots may be placed between two plastic transparency sheets and then inserted into a plastic zip bag with a few ml of the transfer buffer from the Trans-Blot Turbo tray, and stored at 4°C or in a freezer until immunodetection can be performed.

Note: It is important that the membrane not dry out until the entire immunodetection procedure has been completed. If the membrane is allowed to dry, detection of the western blotting signal will be substantially decreased.

D. Immunodetection

1. Pour off the blocking solution.
2. Add 10 ml anti-myosin primary antibody to the tray and place on the rocker for 10 min at room temperature, or overnight at 4°C. Longer incubation times will result in more intense bands. Adjust the rocker to a faster setting if necessary to ensure the antibody solution is constantly washing over the membrane.
3. Pour off the anti-myosin primary antibody.
4. Rinse the membrane in approximately 50 ml of wash buffer and pour off.
5. Add another 50 ml of wash buffer to the membrane and place on the rocker for 3 min. Longer wash times will not harm the experiment. Reduce the rocker speed if splashing occurs.
6. Pour off the wash buffer.
7. Add 10 ml secondary antibody to the tray and place on the rocker for 10 min. Adjust the rocker to a faster setting if necessary to ensure the antibody solution is constantly washing over the membrane.
8. Pour off the secondary antibody solution.
9. Rinse the membrane in approximately 50 ml of wash buffer and pour off.
10. Add another 50 ml wash buffer to the membrane and place on the rocker for 3 min.
11. Pour off the wash buffer.

12. Add 10 ml HRP color detection reagent, place on the rocker, and allow at least 10 min at room temperature for bands to develop. It is preferable to allow at least 30 min for color development. If necessary, the blot can incubate in HRP color detection reagent overnight at room temperature.
13. Once the bands have developed, discard the detection reagent and rinse the membrane twice in 50 ml of distilled water. Image the blots on a Gel Doc™ EZ system using the white sample tray. Alternatively, blots can be imaged on a color scanner between two clear plastic transparency sheets so that the scanner remains dry.

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

**Alternative Method:
V3 Western Workflow™**
(Stain-Free Rapid Blotting)

Additional Required Items

- Mini-PROTEAN® TGX Stain-Free™ 4–20% gels, 10 pack (4568093EDU)
- Gel Doc EZ imaging system (1708270EDU)
- Stain-free sample tray (1708274EDU)

A. Sample Preparation

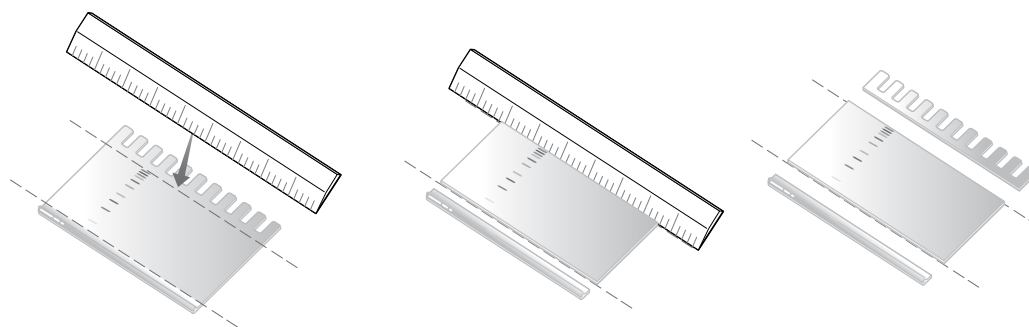
1. Follow instructions for the rapid blotting protocol.

B. Gel Electrophoresis

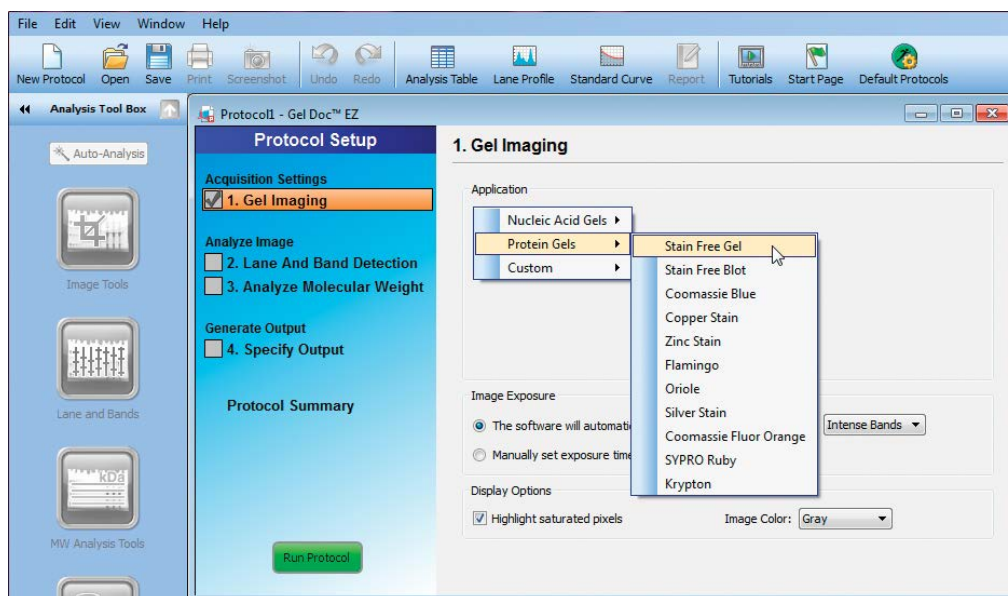
1. Follow instructions for the rapid blotting protocol.

C. Western Blotting

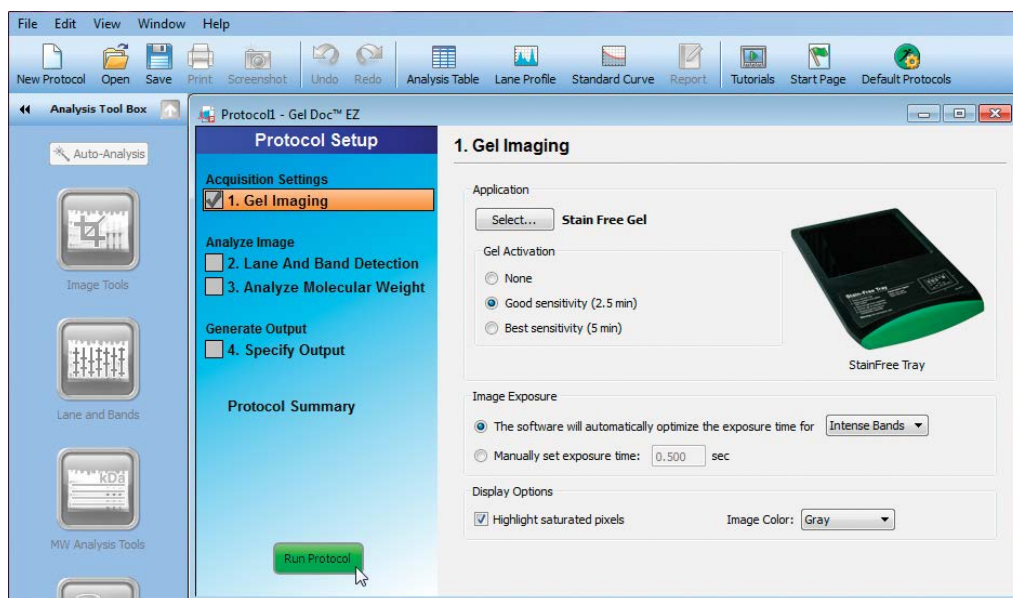
1. Using the green opening lever supplied with boxes of TGX Stain-Free gels, align the arrow cutout in the lever with the black arrows on the 4 sides of the TGX gel cassette. Use the teeth on the opening lever to wedge between the plates of the stain-free gel cassette, and crack the plates open using an upward or downward motion. Once the plates are cracked, slowly pry the plates apart. The gel will usually stick to one of the two plates.
2. Using the gel cutter, gel releaser, or a ruler carefully cut away the wells and the very bottom of the gel.



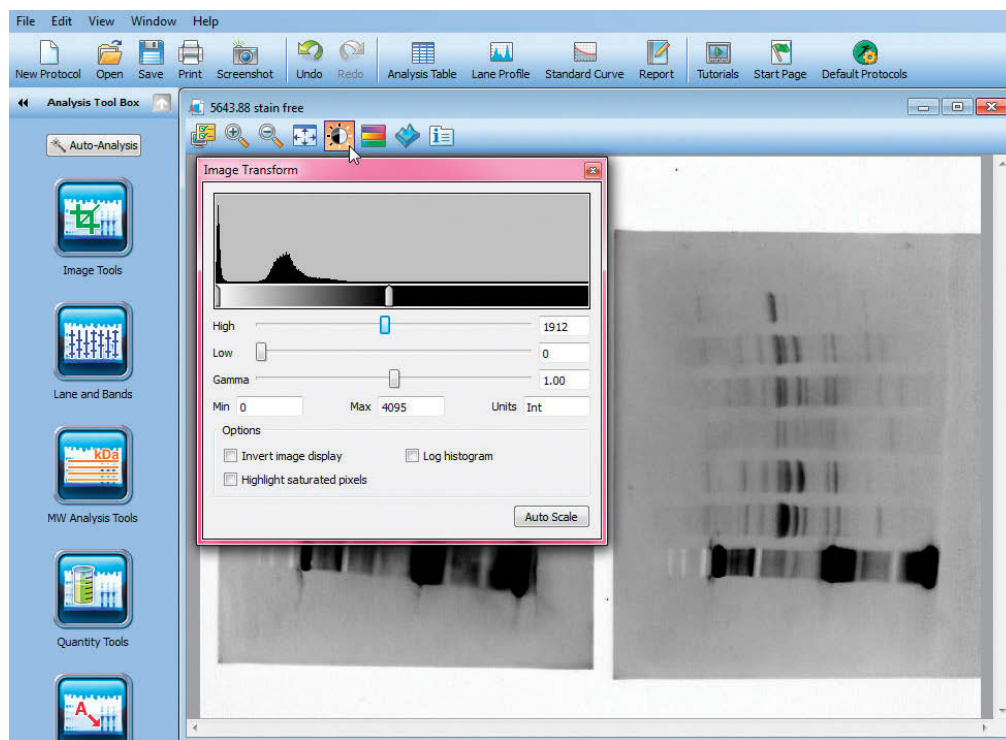
- To image the stain-free gel on the Gel Doc EZ imager, spray the stain-free tray with water to allow ease of gel manipulation. Place the gel on the stain-free tray and double click the icon for Image Lab™ software on the computer connected to the imager. Follow the prompts on the start page under Gel Imaging Application: Select > Protein Gels > Stain-Free Gel.



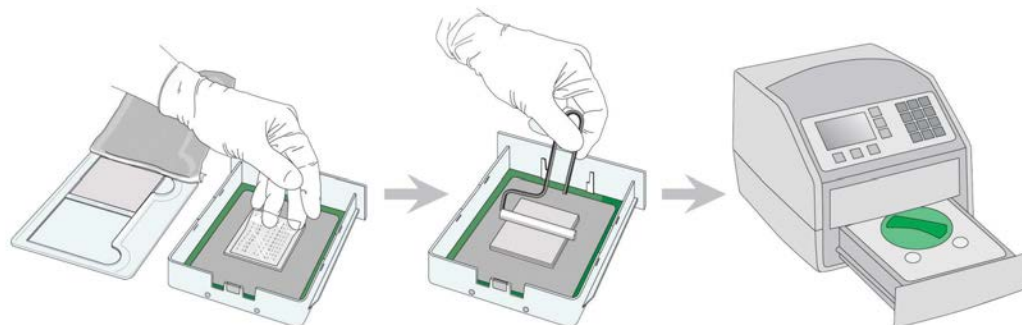
- Choose the activation time based on the detection sensitivity you prefer and run the Stain-Free imaging protocol. We recommend using a gel activation time of 2.5 minutes or less to minimize the chances of altering any epitopes within your samples.



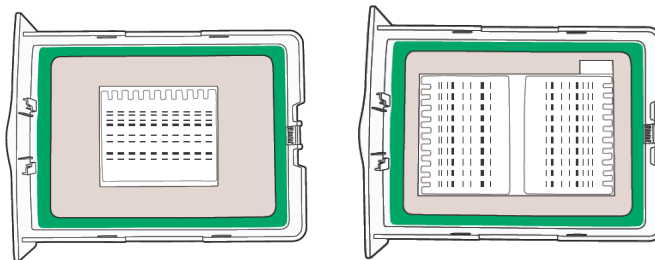
- After gel activation, Image Lab software will automatically optimize the exposure time for intense or faint bands, as selected by the user in the screen above (Image Exposure buttons). To lighten or darken the image post-exposure, click on the Image Transform button (black and white sun symbol) and adjust the toggle bars for high, low, or gamma as desired.



6. Once the gel is imaged and the image file is saved, make note of the exposure time used to generate the image. This will be your manually set exposure time if you wish to image the post-transfer gel or the blotted membrane to check for successful protein transfer. Image Lab will have recorded the previous exposure time and it will be visible in the Protocol Setup window next to the “Manually set exposure time” line. Record exposure time here: _____ sec.
7. To assemble the Trans-Blot® Turbo™ mini nitrocellulose transfer pack, open it by peeling away the top foil. Two pre-wet ion reservoir stacks denoted “top (-)” and “bottom (+)” will be visible inside. Place the “bottom” stack, which contains the membrane, on the Trans-Blot Turbo tray with the membrane facing up.
8. Using the blot roller, roll over the membrane to displace any air bubbles trapped between the stack and the tray.
9. Place the gel on top of the membrane, followed by the “top” ion reservoir stack. Use the blot roller again to remove any air bubbles in the transfer sandwich.
10. Place the tray cover on top of the transfer sandwich, press down firmly, and turn the dial clockwise to lock it in place. Insert the locked tray into the Trans-Blot Turbo unit.



Note suggested gel positioning below when running one versus two gels per tray. When running two gels per tray wells should face outward on both gels, and one midi nitrocellulose transfer pack can be used to transfer the two gels instead of two mini nitrocellulose transfer packs. Wells are shown only to indicate gel orientation. You will have removed the wells from your gels by this point.



- Set up transfer conditions on the Trans-Blot Turbo unit following the instructions outlined in the instrument manual and run the protein transfers.

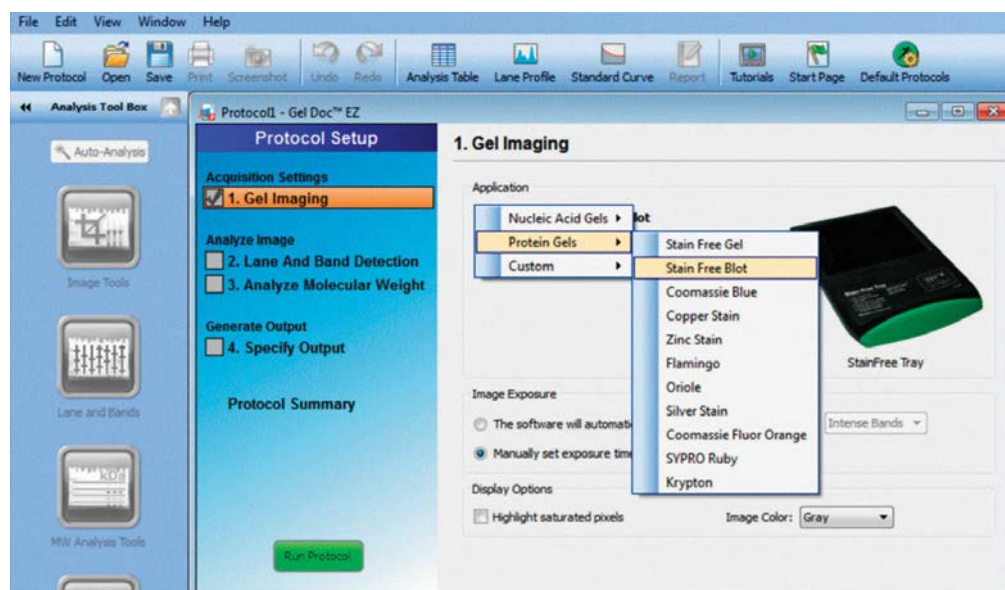
Conditions for one mini gel sandwich per tray – 1.3 A, 25 V, 15 minutes.

Conditions for two mini gel sandwiches per tray – 2.5 A, 25 V, 15 minutes.

- Once transfer is complete, and while wearing gloves, disassemble the gel sandwich taking care to handle only the outer edges of the membrane. Peel back the membrane from the corner of the gel slowly and check for the presence of the prestained standards on the membrane. If the prestained standards have transferred to the membrane, remove the membrane from the sandwich entirely.

Note: It is important to minimize handling of the membrane, especially with ungloved hands which will result in protein-rich fingerprints on the blot! Use the very edges of the membrane to position the blot and try to handle the membrane as little as possible.

- Repeat the stain-free imaging steps for the post-transfer gel and/or the blotted membrane, taking care to choose the appropriate application from the menu (Stain-Free Gel or Stain-Free Blot). Click the option for manually setting the exposure time, and enter the time recorded in step 6 in order to have this exposure time match the exposure time of the pre-transfer gel.





14. If performing immunodetection immediately following imaging, place the membrane in a tray with 25 ml of blocking solution. Incubate the membrane in blocking solution for 10 minutes at room temperature.

Note: If immunodetection cannot be performed directly following western blotting, wet blots may be stored in a plastic zip bag, or for ease of handling, several blots may be placed between two plastic transparency sheets and then inserted into a plastic zip bag with a few ml of the transfer buffer from the Trans-Blot Turbo tray, and stored at 4°C or in a freezer until immunodetection can be performed.

Note: It is important that the membrane not dry out until the entire immunodetection procedure has been completed. If the membrane is allowed to dry, detection of the western blotting signal will be substantially decreased.

D. Immunodetection

1. Follow instructions for the rapid blotting protocol.

Expected Results

An example of how the membrane should appear after blotting is shown in Figure 7. 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 in glossary) recognized by the antibody, thus making antibody binding specificity an indicator of protein homology.

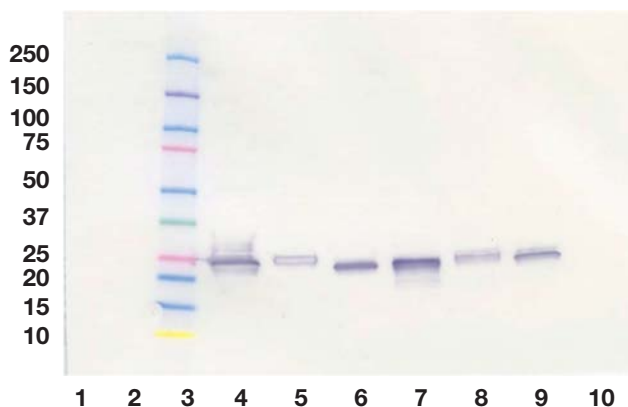


Fig. 7. Immunoblot to detect myosin light chain protein. Lanes 1 and 2, empty; lane 3, Precision Plus Protein Kaleidoscope standards; lane 4, catfish; lane 5, salmon; lane 6, shark; lane 7, sturgeon; lane 8, trout; lane 9, actin and myosin standard; lane 10, empty. A purple/gray band corresponding to myosin light chain is visible in all protein samples following HRP colorimetric detection. Sizes (in kD) of the Kaleidoscope prestained standards are denoted on the right.

The stain-free gel option allows total protein visualization (and quantitation using the Image Lab™ software provided with the Gel Doc™ EZ system) without the added cost expenditure of running a second, identical protein gel and the time expenditure of performing the Coomassie Blue staining and destaining process. Additionally, the post-transfer gel or the membrane can be imaged following blotting to verify that successful protein transfer occurred. Figure 8 depicts an example set of images from an experiment using a TGX Stain-Free™ gel.

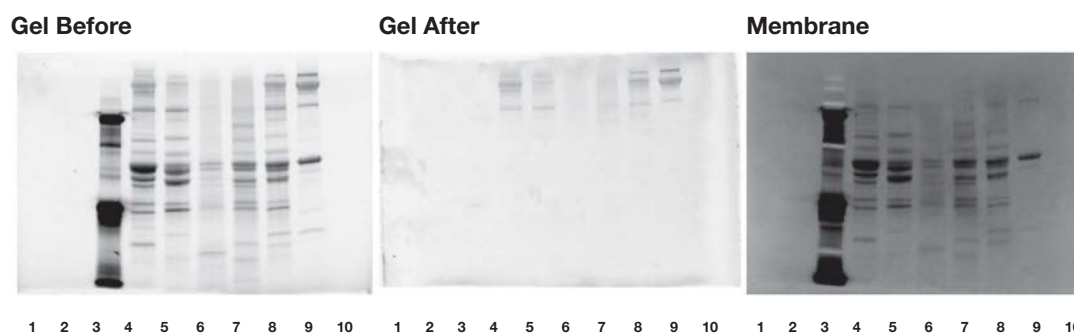
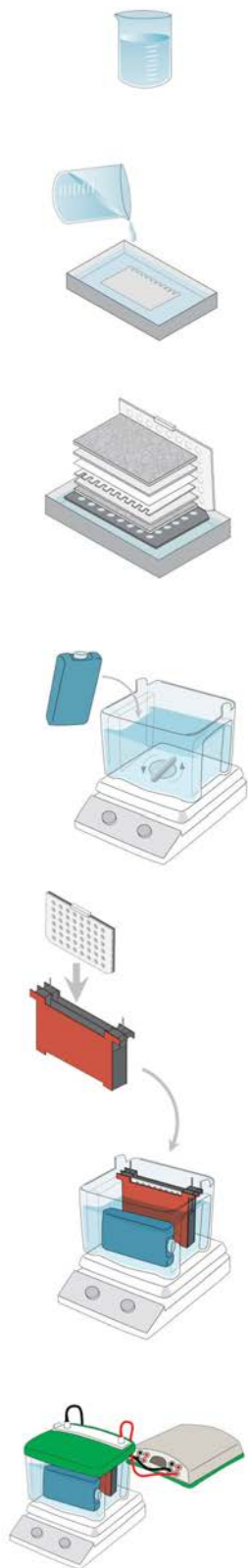


Fig. 8. Stain-free imaging of total fish protein samples in the gel before and after western transfer, and on the nitrocellulose membrane after western transfer. Lanes 1 and 2, empty; lane 3, Precision Plus Protein Kaleidoscope standards; lane 4, catfish; lane 5, salmon; lane 6, shark; lane 7, sturgeon; lane 8, trout; lane 9, actin and myosin standard; lane 10, empty. Gel activation was performed at the "Good Sensitivity" (2.5 min) setting on the Gel Doc EZ imaging system. Proteins are visible on the gel prior to transfer, and are visible on the membrane after transfer.

Tank Blotting Procedure

1. Prepare transfer buffer sufficient for the transfer tank and for equilibration of gels and membrane.
2. Equilibrate gels and membranes in transfer buffer.
3. Place membrane and gel between buffer-soaked filter paper.
4. Place the transfer tank on a magnetic stir plate and fill the tank halfway with transfer buffer.
5. Add a stir bar and begin stirring. If needed, begin cooling the transfer tank with an ice pack.
6. Insert the gel holder cassette into the blotting module latch side up, with the black side of the cassette facing the black side of the blotting module. Repeat with additional cassettes if needed. Place blotting module with cassettes in the tank.
7. Add transfer buffer to the tank until the buffer level reaches the top line.
8. Place the lid on top of the tank, making sure that the color-coded cables on the lid are attached to the proper electrodes.
9. Connect the cables to the power supply, making sure to match the colors on the cables to those on the power supply inputs.
10. Upon completion of the run, remove the cassettes and disassemble the gel and membrane sandwich.

**Trans-Blot Turbo Blotting Procedure**

1. After gel electrophoresis, open the transfer pack that matches your gel (mini or midi) and place the anode stack on the cassette base. Place single mini or midi stacks in the middle of the cassette base; two mini gels can be placed on a midi stack with each gel bottom facing the center. Use the blot roller to remove any air trapped between the pad and membrane. No equilibration is required.
2. Place the gel on the anode stack (which includes the membrane) and the cathode stack on the gel. Roll to remove trapped air.
3. Place the lid on the cassette and lock it into place by turning the green knob clockwise. Ensure the locking pins fully engage their locking slots.
4. Turn the instrument on and slide the cassette into either cassette bay. If using two cassettes, each must be using the same size transfer pack.
5. Start the transfer. With the cassette inserted into the instrument, press TURBO and select the gel type. Press A:RUN to start the top tray, B:RUN for the bottom tray. Select LIST to select a preprogrammed protocol or NEW to create and run a new protocol.
6. At the end of the run, RUN COMPLETE appears on the screen. Remove the cassette from the instrument and unlock the lid. (Caution: the cassettes may be warm.) Remove the membrane from the transfer sandwich and discard the remaining transfer pack materials.

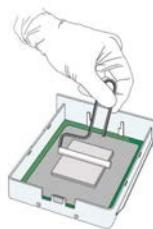


Fig. 9. Illustration of the steps required for western transfer using traditional tank blotting procedure compared to the Trans-Blot® Turbo™ blotting procedure.

Conclusions

The V3 Western Workflow™ (stain-free rapid blotting) streamlines the ability to immediately visualize, verify, and validate protein blotting results at various steps along the way. Protein separation after electrophoresis can be visualized with stain-free gel technology, protein transfer to the membrane can be verified prior to immunodetection, and western blot results are easily validated upon imaging of the processed membrane. In addition to the convenience of monitoring the progress of the experiment in real time, the ability to achieve excellent protein separation under rapid electrophoresis conditions using TGX™ gels, as well as the ability to complete protein transfer to membranes in 15 minutes with the Trans-Blot® Turbo™, both yield significant time savings in the classroom. A comparison of the steps involved in the traditional tank blotting procedure versus the Trans-Blot Turbo procedure is shown in Figure 9.

The numerous combinations of time-saving steps in the rapid blotting protocol and the V3 Western Workflow (stain-free rapid blotting) allow customization and optimization of experimental design to best fit classroom needs on an individual basis. Using less than one third of the time required for traditional tank blotting, the V3 Western Workflow (stain-free rapid blotting) allows the same results to be obtained in significantly less time, allowing more time for discussion, interpretation, and inquiry-based follow-up.

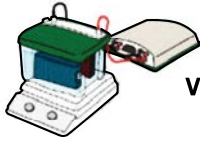


Hands-on time expenditure					
		VS		+	
	Tank blotting		Rapid blotting (staining required)		V3 Western Workflow (stain-free rapid blotting)
Protein extraction	15		15		15
Electrophoresis	18		18		18
Protein visualization	180		180		< 3
Protein transfer					
Equilibration	15		0		0
Setup	30		5		5
Transfer	30–150		15		15
Immunoblotting	45		45		45
Color detection			all 10 min–overnight		
Total Hands-On Time	343–463		288		111
Time Savings	—		≥ 55		≥ 232

Fig. 10. Summary of the time savings gained through use of the V3 Western Workflow.

For further classroom extension activities, including creation of phylogenetic trees based upon data from the Protein Profiler and Western Blot Modules used here, please refer to the complete instruction manuals for these Modules (bulletins 10004530 and 10004531), available at www.explorer.bio-rad.com.

Glossary

Actin	A major muscle protein organized into thin filaments.
Amino acids	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.
Antigen	Any agent that provokes an acquired immune response, and is bound specifically by either antibodies or T cells.
Antibody	Immunoglobulin protein formed in response to a challenge of the immune system by a foreign agent. Antibodies bind to specific proteins.
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.
Charge Density	The ratio of charge to mass of a protein.
Conjugate	A substance formed by the covalent bonding of two types of molecules, such as horseradish peroxidase linked or “conjugated” to an antibody.
Dalton	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.
Disulfide bond	The S-S bond between amino acids.
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.
Electrophoresis	The migration of charged molecules in an electric field toward the electrode with the opposite charge.
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 a 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.
Enzyme-linked immunosorbent assay (ELISA)	An immunological assay that involves binding protein samples to multi-well polystyrene plates, and uses antibodies to probe for the presence of specific proteins in the sample. This assay is frequently used in disease diagnosis.
Epitope	The part of a foreign organism or its proteins that is being recognized by the immune system and targeted by antibodies, T cells, or both – also called an 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.

Gel electrophoresis	Technique used to separate molecules that carry electric charges. The molecules separate from each other according to the different rates at which they migrate through an electric field set up in a gel soaked in a chemical solution.
Horseradish peroxidase (HRP)	An enzyme frequently used to label secondary antibodies. HRP oxidizes substrates such as 4CN for colorimetric detection.
Immunodetection	Any of a number of immunological assays that employ antibodies to detect the presence of a specific antigen, including western blotting, ELISA, and immunohistochemistry.
Immunohistochemistry	An immunological assay that uses antibodies to detect the presence of specific antigens in individual cells of a tissue sample.
Kilodalton (kD)	1,000 daltons.
Myosin	A major muscle protein organized into thick filaments.
PAGE	Polyacrylamide gel electrophoresis.
Peptide	A molecule comprised of two or more amino acids.
Phylogeny	The evolutionary relationship of species based on lineage and history of descent.
Ponceau S	A dye used to prepare a stain for reversible detection of protein bands on nitrocellulose or PVDF membranes after western blotting. It frequently requires extensive washing with water to completely remove the stain from membranes.
Posttranslational modification	Alterations of proteins after they are synthesized by the cell, such as phosphorylation, glycosylation, or cleavage.
Profile	A distinct pattern of bands on a protein gel, used to identify a sample or species.
Primary antibody	In an immunoassay, the primary antibody binds a specific antigen, conferring specificity to the assay.
Protein	A functional assembly of one or more polypeptides.
Proteomics	The study of proteins and their functions.
SDS	Sodium dodecyl sulfate.
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.
Substrate	The target molecule for an enzyme.
Western	See Blot.

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