

# WESTERN BLOT EBOOK

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400 450 500 550 600 650 700 750 800 850  
Wavelength (nm)

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# A WORD ON QUANTITATION

## MORE THAN JUST MEASUREMENT: WHY WESTERN BLOTS SHOULD ALWAYS BE QUANTITATIVE

### Why start a guidebook on Western blotting with a discussion on quantitation?

For many researchers new to Western blotting, the ease with which you can use an imager or scanner to quantify a band on a blot can sometimes lead to a scientist's worst nightmare—non-reproducible results and incorrect conclusions. Just because you can generate a number doesn't mean it's true. But it also doesn't mean that the number is never true.

### So can I get quantitative data from Western blotting? Should I care even if I don't want to quantify the bands on my blot?

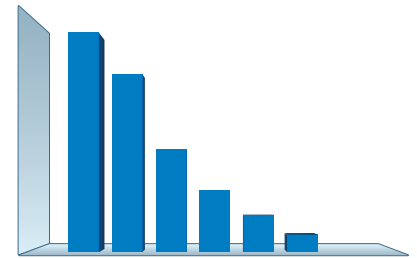
The answer to both questions is a resounding yes, and a review of what we mean when we use the word quantitative explains why.

### What does quantitative mean, really?

It can be surprisingly difficult to find a definition of the word quantitative that goes beyond a dictionary's entry of "can be measured." For scientists, it's a word that's used so often that the implication is everyone should know what it means. But different people use the word in different ways.

At Azure Biosystems, we embrace the definition put forward by the statistician Samuel S. Wilks<sup>1</sup>, who said that to quantify something you must measure it, and your measurements need to meet the following criteria:

- Your measurements are generated using a clearly defined process (the Western blot)
- The measurement process generates a reproducible outcome, i.e the measurements are precise<sup>2</sup>
- The measurement process generates a valid outcome that reflects the "true" measurement, i.e. the measurements are accurate<sup>3</sup>



The definition is a simple re-statement of good scientific measurement principles and helps remind us of what we need to achieve when we talk about generating quantitative Western blot data, i.e. Western blots that are reproducible and where we can verify measurement accuracy.

When looked at in this way you can see that these goals are clearly within reach for Western blotting if enough care is taken to reduce or control for variability during the Western blotting process. Furthermore, it should be clear that whether you want a numerical value for the intensity of a band or you merely want to know if the band is there, you still want results that are reproducible. Therefore, every Western blot should be quantitative.

### Making every Western blot quantitative

By focusing on best practices for Western blotting and how to choose appropriate experimental conditions, this guidebook provides an excellent starting point and resource for researchers interested in generating reliable, reproducible Western blots. Whether your final data is numbers on a graph or simply the image of the blot, we hope we've helped you make all your Western blots quantitative.

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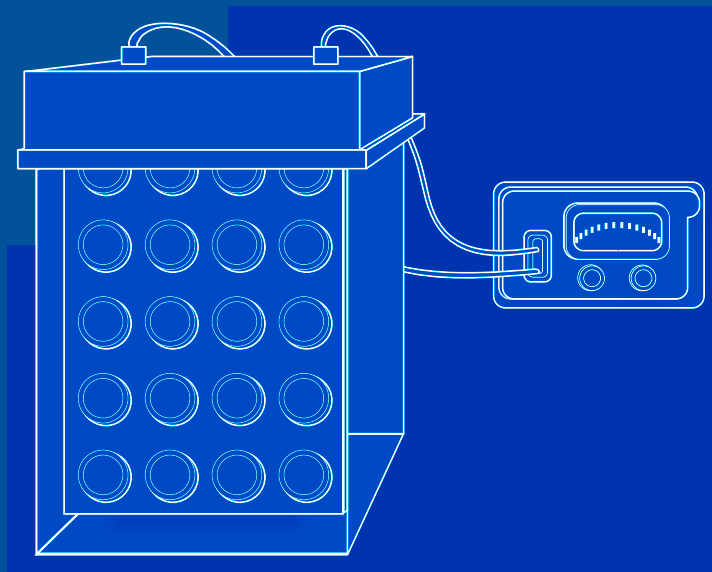
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# INTRODUCTION TO WESTERN BLOTTING



## 1.1. Background

Developed in the late 1970s and early 1980s, Western blotting is a widely used analytical technique that can identify one or more specific proteins in a sample containing a complex mixture of proteins<sup>1-3</sup>. The process originally consisted of gel electrophoresis to separate proteins by molecular weight, electrophoretic transfer to and immobilization of the proteins on a solid nitrocellulose membrane support, probing of the membrane with antibodies specific for the protein of interest, and detection of the bound antibody using radio-labeled staphylococcal Protein A followed by autoradiography for visualization.

Today, the Western blot continues to be a popular assay for analyzing protein expression and, in its current form, the first three steps are nearly identical to the original protocol. However, many technological advances in the ensuing years have increased the power of the approach. These advances include:

- Improvements in the detection process that enable highly sensitive detection of low-abundance proteins
- The use of safer, non-radioisotopic detection methods
- The development of quantitative assays
- The ability to detect multiple proteins simultaneously
- The use of sophisticated digital capture systems for easier detection and analysis.

And, like most useful methodologies, Western blotting technology continues to evolve.

To help scientists new to Western blotting understand the fundamentals of the technique, and to provide those already familiar with Western blotting with an overview of the current state-of-the-art technologies and techniques, we've developed this guidebook. In these pages we review current best practices for Western blotting as well as the underlying physics, chemistry, and biology of the method so that scientists can take full advantage of this long-used and still powerful technique.

## 1.2. Gel Electrophoresis

In the first step of a Western blot, proteins are physically separated from one another across a gel matrix in a process called gel electrophoresis (Figure 1.1). A protein sample is mixed with a loading buffer, loaded onto the gel, and then subjected to an electrical current which is applied to the gel/buffer system. The proteins, which are negatively charged under the experimental conditions, travel through the gel towards the positive electrode.

Depending on the type of gel and buffer system used, the distance a protein migrates through the gel matrix is governed primarily by the mass:charge ratio of the individual protein or simply the molecular weight of the protein.

Protein electrophoresis can be run under either native or denaturing conditions (Table 1.1). Denaturing conditions are suitable for most applications. However, if the three-dimensional structure of the protein needs to be retained, native electrophoresis conditions must be used.

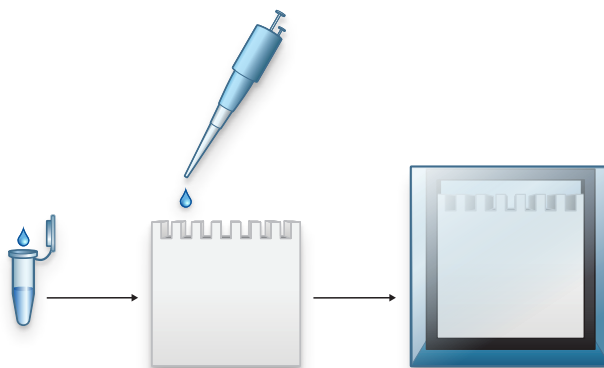


Figure 1.1. Polyacrylamide gel electrophoresis.

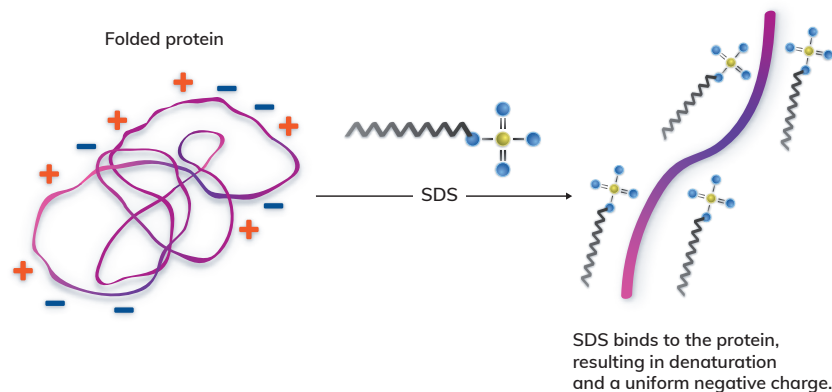
	Features	Uses	How To
Native	<ul style="list-style-type: none"> <li>Proteins retain three-dimensional structure and folding</li> <li>Proteins are separated by mass:charge ratio and cross-sectional area of proteins (Stokes radius)</li> </ul>	<ul style="list-style-type: none"> <li>Use to study protein complexes</li> <li>Use when detection antibody recognizes an epitope on the folded protein</li> </ul>	<ul style="list-style-type: none"> <li>Omit SDS from loading buffer, gel and running buffer</li> <li>Omit denaturing agent from sample buffer</li> <li>Do not heat sample prior to loading</li> </ul>
Denaturing	<ul style="list-style-type: none"> <li>Proteins are denatured using SDS (Figure 1.2) and heat</li> <li>Proteins are separated primarily by molecular weight</li> </ul>	<ul style="list-style-type: none"> <li>Use when native conditions are not required</li> </ul>	<ul style="list-style-type: none"> <li>Mix protein with sample buffer containing SDS and a denaturing agent</li> <li>Heat samples prior to loading</li> </ul>

**Table 1.1. Native vs. denaturing gel electrophoresis.**

### Gel Composition

The most commonly used protein electrophoresis approach is SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). SDS-PAGE gels are composed of a pH-buffered solution, a mixture of acrylamide and bis-acrylamide (the gel matrix components), and SDS. Ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) are used to catalyze polymerization of acrylamide monomers and incorporation of bis-acrylamide ensures crosslinking of individual strands of acrylamide polymers.

The resolving capability of the gel is determined by the gel pore size, which is governed by both the concentration of acrylamide as well as the concentration of the bis-acrylamide crosslinker. In general, higher percentage gels have a smaller pore size and are used to separate proteins with lower molecular weights. Lower percentage gels have a larger pore size and are used to separate higher molecular weight proteins. Table 1.2 illustrates the resolving power of gels with different percentages of acrylamide.



**Figure 1.2. Mechanism of SDS denaturation of proteins.**

MW Range, kDa	Gel Percentage
10–43	15
12–60	12
20–80	10
30–95	8
50–200	6

**Table 1.2. Resolving power of different concentrations of acrylamide.**

### Laemmli Discontinuous Gels

Discontinuous gels (also known as Laemmli gels) are among the most commonly used gel systems and can provide sharper, more defined bands than a continuous gel system<sup>4</sup>.

A discontinuous gel system consists of two stacked layers of gels, each with a different acrylamide concentration and pH, and a buffer that is at a different pH from both gels. The discontinuity between the pH of the two gels and the buffer alters the mobility of ions—specifically zwitterions—through the two gels, which in turn affects the mobility of the migrating proteins.

The top gel layer is called the stacking or focusing gel. The stacking gel contains a fixed low percentage of acrylamide and the lowest of the three pH levels. In a typical Tris-glycine buffer system, the pH for a stacking gel is 6.8, with a buffer at a pH 8.3. The purpose of the stacking gel is to concentrate all proteins into a single, tight band before they enter the lower portion of the gel, enhancing the sharpness and definition of individual protein bands in the resolving gel.

The lower gel, or resolving gel, contains the percentage of acrylamide needed to resolve the protein of interest. The pH of a typical resolving gel in a Tris-glycine buffer system is 8.8—more alkaline than both stacking gel and buffer. The purpose of the resolving gel is to separate proteins by size, and thus the composition of the gel (pH, percentage of acrylamide) is chosen to ensure that proteins move through the gel primarily based on molecular weight.

### Gradient Gels

The resolving power of a gel can also be improved by using a gradient of acrylamide that increases in concentration from the top of the gel to the bottom, thus creating a “gradient gel.” In a gradient gel, proteins progress through the gel until the pore size impedes further migration.

Gradient gels are a great choice when you want to use a single gel to resolve multiple proteins that span a wide range of molecular weights.

### Buffer Systems

In addition to varying the gel composition and setup, altering the electrophoresis buffer system can also optimize protein separation during gel electrophoresis (Table 1.3).

Standard SDS-PAGE gels use the alkaline buffer Tris-glycine, which provides adequate resolution for mid-size proteins. However, because the high alkalinity of the gel can lead to protein degradation and, thus, smearing of protein bands during longer run times, Tris-glycine is not ideal for resolving larger proteins (>150-200 kDa). Smaller proteins are also not easily resolved using Tris-glycine gels due to intermingling of SDS with the small molecular weight proteins in the stacking gel. This intermingling leads to fuzzy bands and reduced resolution of small (<10-15 kDa) proteins.

An alternative to Tris-glycine is the acidic Bis-Tris gel buffer that may be used in conjunction with two different running buffers: either MES (2-[N-morpholino] ethanesulfonic acid) buffer for small proteins or MOPS (3-[N-morpholino] propanesulfonic acid) buffer for mid-sized proteins. This system contains an additional reducing agent, sodium bisulfite, in the running buffer which works with the acidic Bis-Tris gel buffer to increase resolution and the sharpness of the protein bands.

Buffer System	pH	Advantages
Tris-glycine	Up to 9.5	Good for mid-ranged proteins; inexpensive
Tris-Bis	6.4	Sharp protein bands; two running buffer options for optimization based on MW
Tris-acetate	7.0	High resolution of large MW proteins
Tris-tricine	Up to 9.5	Tricine separates low MW proteins from free SDS

Table 1.3. Buffer systems for protein gel electrophoresis.

For resolving large molecular weight proteins, Tris-acetate gels are frequently used. The pH of Tris-acetate gels is close to 7.0, which supports protein stability during the long run times needed to adequately separate large molecular weight proteins. Tris-acetate can be used in both native and denaturing gels.

For resolving small proteins in the 1-10 kDa range, a Tris-tricine system is recommended. With a Tris-tricine gel and running buffer, free SDS can be separated from the low molecular weight proteins running near the leading edge of the gel front during electrophoresis.

### 1.3. Transfer to Membrane

After electrophoretic separation of proteins through the gel, the proteins are transferred to a solid membrane support for subsequent steps. Efficient transfer relies on the choice of membrane, the type of transfer apparatus used, and the composition of the transfer buffer.

#### Nitrocellulose Versus PVDF Membranes

Two types of membranes are commonly used for Western blots: nitrocellulose and polyvinylidene difluoride (PVDF). Both membrane types work well, but differences in experimental setup and sample composition will affect the choice of membrane. It should be noted that the signal intensity and background a sample produces on one type of membrane may be quite different on another type of membrane, even if the antibody and detection chemistry are the same.

Nitrocellulose membranes bind proteins strongly and nearly irreversibly. They are more cost effective than PVDF and were the first type of membrane used for Western blotting. Nitrocellulose is known for low background, particularly when using chemiluminescent detection (see below). Unlike PVDF, nitrocellulose does not require an activation step prior to use. However, nitrocellulose is fragile, making it difficult to manipulate the membrane, especially if the membrane is stored over a long period of time. Nitrocellulose also cannot be used with fluorescent detection methods as the membrane autofluoresces.

Like nitrocellulose, PVDF membranes have a strong affinity for proteins and are well-suited to Western blotting. They are more durable than nitrocellulose membranes making them popular for experiments that require multiple manipulations of the membrane. PVDF membranes are used for chemiluminescent and fluorescent detection and require incubation in methanol for activation prior to use. Low-fluorescence PVDF membranes provide the lowest fluorescent background and are recommended for fluorescent detection.

Conveniently cut, high-quality PVDF and nitrocellulose membranes are available from Azure Biosystems. To see these and all of our Western blotting reagents and consumables, visit [azurebiosystems.com/reagents](https://azurebiosystems.com/reagents).

#### Transfer Conditions

Efficient transfer of proteins relies on both the migration of proteins out of the gel and retention of proteins on the membrane. Like gel electrophoresis, the transfer step uses electricity to move negatively charged proteins towards the positively charged electrode. Transfer efficiency is affected by the type of transfer apparatus used, the individual protein, the transfer buffer, and the transfer conditions.

Learn more about how to choose between wet and semi-dry transfer methods on [page 32](#) in our Western Blot Tips and Guidelines chapter.

#### Wet or Semi-dry Transfer

Two types of transfer setups are popular for Western blotting, wet transfer (Figure 1.3 and Table 1.4) and semi-dry transfer (Figure 1.4 and Table 1.5).

**Wet Transfer**—In a wet transfer setup, a “transfer stack” is assembled, consisting of the gel and membrane surrounded on both sides by several layers of filter paper. The filter paper-gel-membrane-filter paper sandwich is surrounded by sponge-like fibrous pads on each side and this transfer stack placed into a cassette or holder.

Prior to assembly, the transfer stack components are pre-wetted with transfer buffer. After assembly, the transfer stack is submerged in a tank filled with transfer buffer, and an electrical current is passed through the buffer to transfer proteins from the gel to the membrane.

Protein transfer from gel to membrane can be optimized by altering the buffer system (see next section), transfer time, and transfer voltage.

The wet transfer method is used for most blotting applications and delivers highly consistent results. The method is especially useful for transferring proteins that span a wide range of molecular weights and, because of its consistency, wet transfer is recommended when quantitative analysis will be performed.

One of the disadvantages of wet transfer is the requirement for a large quantity of buffer for each transfer. Another disadvantage is heat generation, especially during longer transfers. Excess heat can lead to inconsistent transfer across the gel, protein denaturation, and even breakdown of the gel. Thus, keeping the transfer apparatus cool by using ice packs, a cooling circulator, and/or by performing the transfer in a cold room is highly recommended.

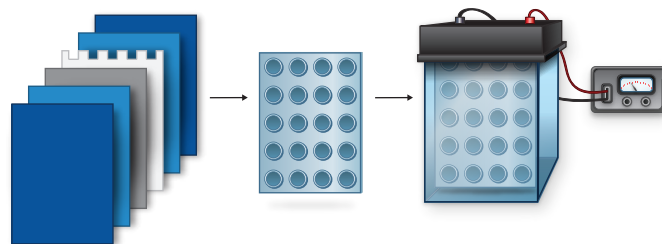


Figure 1.3. A typical wet transfer setup.

Advantages	Disadvantages
<ul style="list-style-type: none"> <li>Flexibility: <ul style="list-style-type: none"> <li>Transfer conditions are easily adjusted</li> <li>Multiple transfer buffer options enable more ways to optimize transfer</li> </ul> </li> <li>Supports transfer of a broad molecular weight range at one time</li> <li>Compatible with extended transfer times for large molecular-weight proteins</li> <li>Can be used for quantitative Westerns</li> </ul>	<ul style="list-style-type: none"> <li>Heating of buffer can interfere with transfer</li> <li>Cooling mechanism and/or cold room space required during transfer</li> <li>Large volumes of transfer buffer are required</li> </ul>

Table 1.4. Advantages and disadvantages of wet transfer.

**Semi-dry Transfer**—The semi-dry transfer setup uses a similar transfer stack to the wet transfer setup, but instead of getting submerged in a tank filled with buffer the transfer stack is placed directly between two electrode plates (Figure 1.4). Thus, this method avoids the large amounts of buffer needed in a wet transfer system (Table 1.5).

The big advantage of the semi-dry transfer setup is the fast transfer speed. Because the distance between the electrodes is minimized, a strong electrical field is generated which leads to rapid transfer.

Another advantage of this method is that two different transfer buffers can be used on each side of the transfer stack – one on the gel side designed to promote migration of proteins out of the gel and another on the membrane side that promotes retention of proteins on the membrane.

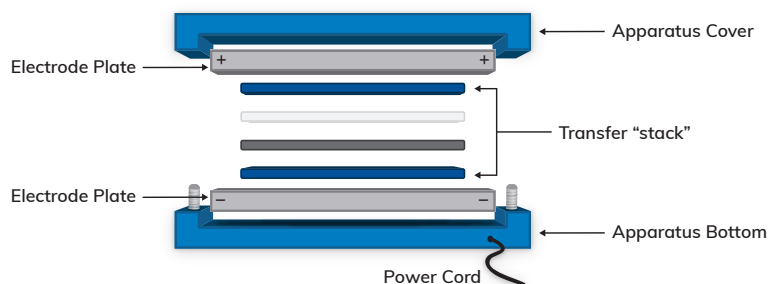


Figure 1.4. A typical semi-dry transfer setup.

However, the strong electrical field is also a disadvantage for this method. Low molecular weight proteins can be transported too far and move beyond the membrane. In addition, the longer transfer times needed for larger molecular weight proteins cannot be achieved because of the limited buffering capacity

Advantages	Disadvantages
<ul style="list-style-type: none"> <li>• Transfer is rapid</li> <li>• Can use discontinuous buffer system to optimize transfer of proteins</li> <li>• Little buffer is required</li> <li>• Easy to set up</li> <li>• Good for performing large numbers of blots analyzing the same protein</li> </ul>	<ul style="list-style-type: none"> <li>• High intensity field strength may cause low molecular weight proteins to migrate through membrane</li> <li>• Difficulty in transferring high (&gt;120 kDa) molecular weight proteins</li> <li>• Not recommended for quantitative Westerns</li> <li>• Gel can dry out</li> </ul>

**Table 1.5. Advantages and disadvantages of semi-dry transfer.**

### Transfer Buffers

Transfer buffers contain several components to promote protein transfer (Table 1.6). Buffer components are optimized based on the type of transfer system being used (wet or semi-dry), the type of gel employed, the choice of membrane, and the protein of interest.

**Buffer Properties**—Transfer buffers must have strong buffering capacities to maintain conductivity and pH during transfer. The buffer pH must be different from the isoelectric point (pI) of the protein-of-interest (the pH at which the protein has a net charge of zero) or the transfer will not occur.

The most common buffer choice is Tris at a pH of 8.1-8.5, which is higher than the pI of most proteins. Higher pH buffers such as CAPS or carbonate can also be used and are recommended when transferring high molecular weight or basic proteins.

For extremely basic proteins, acetic acid is the recommended transfer buffer.

**IMPORTANT:** When using acetic acid as a transfer buffer, proteins become positively charged and migrate towards the anode, requiring that the orientation of the transfer stack be reversed.

**Alcohol**—Methanol (and sometimes ethanol) is added to transfer buffers to counteract the effects of SDS, which reduces protein binding to nitrocellulose membranes. Alcohol removes SDS from proteins thereby promoting protein retention on the nitrocellulose membrane.

However, alcohol can also induce precipitation of proteins in the gel, and cause basic proteins to become positively charged or neutral which inhibits protein migration out of the gel. Thus, the amount of alcohol used in the transfer buffer should be optimized for the protein of interest.

Only high quality, analytical grade alcohol should be used when preparing transfer buffers.

Note that the addition of alcohol to the transfer buffer is only required if SDS and nitrocellulose membranes are used. If SDS is not used, such as for running a native protein gel, or when PVDF membranes are used, then alcohol is not needed in the transfer buffer.

**SDS**—Although SDS can inhibit binding of proteins to nitrocellulose membranes, it promotes elution of proteins out of the gel by increasing protein solubility. However, SDS increases the intensity of the transfer and may alter the antigenicity of some proteins. When used, the concentration of SDS in the buffer should be titrated for each protein of interest but should never exceed 0.05%.

**PVDF membranes should be used when SDS is included in the transfer buffer since SDS inhibits binding of proteins to nitrocellulose membranes.**



Component	Example	Use
Buffer	Tris, CAPS, Carbonate	Support conduction, maintain appropriate pH
Alcohol	Methanol, ethanol	Increase binding of proteins to membrane
Detergent	SDS	Promote migration of proteins out of gel
Tris-tricine	Up to 9.5	Tricine separates low MW proteins from free SDS

**Table 1.6. Transfer buffer components.**

**Discontinuous Transfer Buffers**—One of the advantages of a semi-dry transfer setup is the ability to use different transfer buffers on each side of the transfer stack, i.e. a discontinuous transfer buffer system. Discontinuous transfer buffer systems enable an additional level of transfer optimization—a buffer designed to promote protein elution from the gel can be used on the gel-side of the transfer stack and a different buffer designed to promote retention of proteins on the membrane can be used on the membrane-side of the transfer stack.

### Voltage and Time

Transfer times and voltage settings should be optimized for each transfer. While proteins generally transfer more rapidly at higher voltages, transfer efficiency is not always consistent. Insufficient current and/or time may result in incomplete transfer, while high current and/or lengthy transfer times may result in loss of proteins via transfer through the membrane without retention. Transfer condition guidelines are usually supplied with the transfer apparatus. However, these conditions can be adjusted based on the protein of interest.

In general, longer transfer times, with a lower current, are used in wet transfer while short transfer times with high current are used with semi-dry systems.

## 1.4. Membrane Blocking

While the antibodies used in Western blotting typically have a high affinity for a specific protein, they also tend to bind non-specifically and with low affinity to the Western blotting membrane. This non-specific binding can result in a high background signal when the blot is imaged, reducing detection sensitivity. To keep background signal as low as possible, the membrane is incubated in a blocking solution after transfer. The blocking solution works by binding to the non-specific antibody binding sites on the membrane, thus preventing antibody binding through occlusion.

Optimizing blocking conditions is important for obtaining high-quality Western blot data, especially when quantitative information is desired. Insufficient blocking can lead to high background, reducing the ability to detect a specific signal. In contrast, excessive blocking can mask legitimate epitopes, and some blocking agents can interfere with detection reagents. Several different types of blocking agents are available, and the blocking solution should be optimized for each antibody:antigen interaction.

### Blocking Buffer Components

#### Buffered Salt Solution

Blocking buffers contain a buffered salt solution compatible with the detecting antibody and method of detection. Generally, Tris- or phosphate-buffered saline (TBS; PBS) is used. However, PBS can interfere with alkaline phosphatase-based detection methods and phospho-specific antibodies. TBS should be the solution of choice when using these reagents.

#### Detergents

Low concentrations of mild, non-ionic detergents, such as Tween-20, make good blocking agents and can be added to the buffered salt solution. Detergents should be added just prior to use to prevent microbial growth, and care should be taken to make sure the detergent is completely solubilized to prevent artifacts, such as a nonuniform signal or background signal. Detergent should not be used as a blocking agent when fluorescent detection methods are used since some detergents autofluoresce and will cause high background signal.

### Protein Blocking Agents

Protein blocking agents are very common and highly efficient, as nitrocellulose and PVDF membranes have a high affinity for all proteins. However, some proteins can interfere with the antibody:antigen recognition process or inhibit detection methods. Thus, the best blocking agent should be determined empirically for each experiment.

**Non-fat Dry Milk**—Non-fat dry milk is one of the most popular blocking agents. It is economical, easy to prepare and contains a mixture of proteins that are efficient blockers. However, milk contains the abundant phospho-protein casein, which can interfere with phospho-specific antibodies. In addition, biotin is a component of milk that can inhibit detection methods that rely on streptavidin/avidin.

**Serum**—Whole serum, usually derived from horse or fetal calf, is another blocker that contains a mixture of proteins. Serum is less commonly used for Western blots than other blocking agents and contains immunoglobulins that can interact with primary or secondary antibodies leading to high background.

**Bovine Serum Albumin (BSA)**—The albumin protein isolated from cow serum is often used in place of milk, particularly when using phospho-specific antibodies. However, some preparations of BSA contain tyrosine-phosphorylated proteins that can interfere with the assay if anti-phosphotyrosine antibodies are used. BSA also cannot be used with anti-lectin antibodies as these antibodies will bind to the carbohydrates in BSA, causing high non-specific background.

**Other Single Proteins**—Other purified single proteins, such as casein protein, are available as blocking agents. The use of single proteins can help prevent cross-reactivity that can occur when milk or whole serum is used. Different proteins can be tested to optimize the blocking step.

### Protein-free Blocking Agents

Protein blockers can have cross-reactivity with primary antibodies, interfere with detection reagents and/or mask epitopes. Protein-free blocking agents have been developed to alleviate these problems. The water-soluble polymer polyvinylpyrrolidone, typically used as a blocking agent in Southern blots, can also be used for Western blots. Alternatively, protein-free blocking agents containing proprietary formulas are available from multiple companies.

### Blocking Conditions

Blocking efficiency is dependent on time and temperature. For most experiments, one hour of blocking at room temperature is sufficient. However, if lengthier blocking times are used (>2 hours) then blocking should be performed at 4°C to prevent microbial growth.

## 1.5. Membrane Incubation with Antibody

After blocking, the membrane is ready to be probed with antibody and unbound antibody washed away. The factors that influence probing and washing include whether a direct or an indirect detection method will be used, the quality and type of antibodies available, the number of antigens to be detected, the type of enzyme or tag that will be used for detection, and the incubation and wash conditions.

### Direct vs Indirect Detection

#### Direct Detection Method

In the direct detection method, the detection label (enzyme or tag) is conjugated directly to the primary antibody that recognizes the antigen of interest (Figure 1.5). This is the simplest form of detection requiring the fewest number of steps. While direct detection is fast and straightforward, it is often less sensitive than indirect detection as there are fewer steps in the process where signal amplification can occur. Also, adding a label directly to a primary antibody can be cost- and/or resource-prohibitive.

### Indirect Detection Method

The indirect detection method is more popular and often more sensitive than direct detection. With indirect detection, the antibody that recognizes the antigen of interest, called the primary antibody, is unlabeled (Figure 1.5). Detection happens through the addition of a labeled secondary antibody that recognizes a specific epitope on the primary antibody. This epitope is usually species-specific and is present in all antibodies generated in that species; for example several different primary antibodies generated in rabbits can all be recognized by the same anti-rabbit secondary antibody. Because of the broad specificity of the secondary antibody, one labeling reaction can result in detection of a wide range of primary antibodies, making this method highly cost-effective.

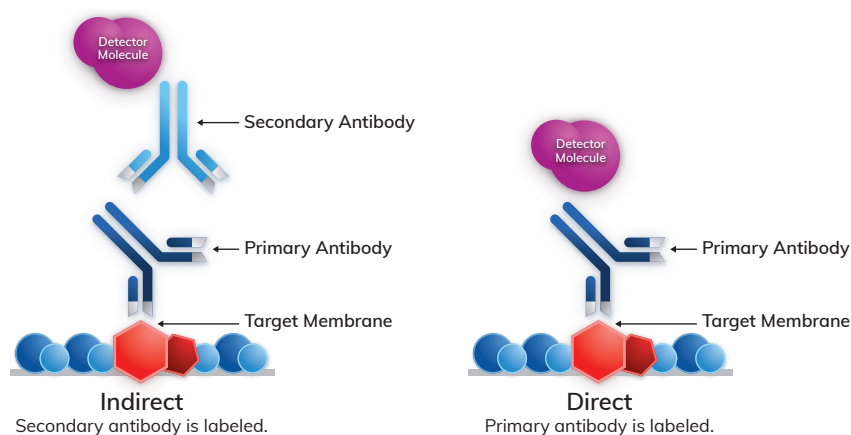


Figure 1.5. Indirect vs. direct detection.

The use of a secondary antibody for detection can increase sensitivity versus direct detection of the primary antibody when multiple secondary antibodies bind to a single primary antibody. Flexibility is also increased because the type of label used for detection can be changed easily through the use of a different secondary antibody.

Fluorescent secondary antibodies from Azure Biosystems deliver superior signal-to-noise ratios. Learn more at [azurebiosystems.com/antibodies](https://azurebiosystems.com/antibodies).

### Antibody Quality and Type

One of the major factors influencing the outcome of a Western blot is the quality of the antibody, especially the primary antibody. High-quality antibodies—antibodies that have high specificity, high binding affinity, and low background binding—increase the specificity and sensitivity of the assay. Both monoclonal and polyclonal primary antibodies can be used for a successful blot.

#### Polyclonal Versus Monoclonal Primary Antibodies

**Polyclonal Antibodies**—Polyclonal antibodies are made using an organism's immune response to the antigen of interest. They are isolated directly from the serum of immunized animals, typically rabbits, goats, donkeys, or sheep, and thus are a complex mix of all the antibodies generated by the animal. During the immune response, an individual B cell generates an antibody that recognizes a specific epitope on the antigen, with different B cells generating antibodies that recognize different epitopes. Thus, a "single" polyclonal antibody actually contains a mix of individual antibodies that recognize different regions of the same antigen. Because the individual antibodies recognize different sites on the antigen, more than one antibody can bind to the antigen at the same time, making polyclonal antibodies more sensitive than monoclonal antibodies. However, this wide range of binding specificities can sometimes lead to a higher background signal with polyclonal antibodies.

**Monoclonal Antibodies**—Monoclonal antibodies are collected from the supernatant of a hybridoma cell line—a cell line created by fusing a single antibody-synthesizing cell with a myeloma cell. These cell lines are usually of mouse or rat origin. Because a single antibody-producing cell, or clone, is used, the antibodies generated by the hybridoma cell line are identical to each other, and all recognize the same epitope on the antigen. Monoclonal antibodies are used when detecting specific forms of a protein, such as phosphorylation status, or when multiple primary antibodies are used at the same time. However, they can also be used for general detection of proteins. Monoclonal antibodies tend to give a less robust signal than polyclonal antibodies as there is only a single antibody binding to an individual protein.

### Secondary Antibodies

Multiple types of secondary antibodies can be used for Western blotting. The type of secondary antibody used depends on the species and class of the primary antibody, the detection method, and any other considerations that might warrant the use of a specialized secondary antibody.

**Species and Class Type of Primary Antibody**—While monoclonal antibodies are usually derived from mouse or rat cells, polyclonal antibodies can be isolated from a variety of animals. The secondary antibody must be specific for the species from which the primary antibody was derived. Therefore, anti-mouse or rat secondary antibodies must be used with a monoclonal antibody while anti-rabbit secondary antibodies should be employed when the primary antibody is isolated from rabbits.

Antibodies can be divided into different classes and subtypes (also called isotypes), depending on the type of heavy and light chain molecules that make up the antibody. The secondary antibody needs to be matched to the primary antibody class and/or subtype for appropriate recognition. Secondary antibodies can be purchased that recognize all classes (i.e. all mouse IgG antibodies) or a specific class, such as mouse IgG2a. Secondary antibodies that recognize all antibody classes are best used in situations where the class or isotype of the primary antibody is not known (such as when working with polyclonal antibodies). Secondary antibodies with a specific isotype are used when the isotype of the primary antibody is known, and can increase the specificity of the assay. Secondary antibodies with a specific isotype are also useful for detecting multiple proteins simultaneously as each secondary antibody can have a different label.

### Specialized Secondary Antibodies for Western Blots

**Anti H+L**—Anti heavy and light chain antibodies (H+L) are specific for both the heavy and light chain portions of the target protein. This type of secondary antibody is often used when the class of the primary antibody is unknown.

**Light-chain Specific**—Antibodies that recognize only the light chain portion of the antibody are typically used when Western blotting is being performed after an immunoprecipitation. The use of this antibody prevents recognition of the heavy chain of the precipitating antibody.

### Antibody Purification

Both primary and secondary antibodies can be purified to reduce background.

**Affinity Purification**—Affinity purification uses an affinity column—a column that contains the antibody's epitope immobilized onto a solid support—to remove antibodies that do not bind to the desired epitope. Affinity-purified antibodies have increased specificity due to the presence of fewer off-target binding antibodies.

**Pre-adsorption**—Pre-adsorption of antibodies can also be used to increase the specificity of binding and reduce cross-reactivity. For example, a rabbit anti-IgG antibody might be pre-adsorbed against mouse, rat, and goat IgG to prevent recognition of other species of IgG. Pre-adsorbed antibodies are highly specific and pre-adsorbed secondary antibodies are recommended for multiplex Western blotting, i.e. Western blots where multiple proteins are probed simultaneously.

### Antibody Conjugates

An important consideration in the choice of a primary (direct detection) or secondary (indirect detection) antibody is the type of label that is conjugated to the antibody. Detection antibodies can be labeled with enzymes (for chemiluminescent or fluorescent detection), fluorescent tags, or other small molecules that are used to amplify the signal (biotin).

**Enzymatic Labels**—Detection antibodies can be conjugated to enzymatic labels. The enzymes are then used to cleave products that produce light (chemiluminescence) or fluorescence for detection. The two most commonly used enzymes are alkaline phosphatase (AP) and horseradish peroxidase (HRP). Many products are available for use with enzymatic labels making them highly popular. Due to the kinetics of enzyme reactions, visualization of the signal must be timed appropriately to capture peak signal. The reaction can also saturate, limiting the dynamic range of protein concentrations that can be measured accurately.

**Fluorescent Labels**—Detection antibodies can also be labeled directly with a fluorescent tag, also called a fluorophore, and fluorescence measured using an imaging system. Upon excitation with light at the appropriate wavelength, the fluorophore emits light at a slightly longer wavelength. The emitted light is then captured and converted into a digital signal by an imager.

Unlike detection methods that rely on enzymes, the amount of light emitted from the fluorophore is consistent and directly proportional to the amount of protein on the membrane, making the assay truly quantitative. In addition, fluorescent labels are an excellent choice for multiplexing—simultaneous detection of multiple proteins in a single blot. For multiplexing, the different detection antibodies are labeled with fluorophores that emit light at distinct, spectrally separated wavelengths.

**Other Conjugates**—Biotin-labeled (biotinylated) detection antibodies can be used for sensitive detection of low-abundance proteins. This system relies on the strong binding interaction between biotin and fluorescently-labeled (or chemiluminescently-labeled) avidin/streptavidin. The detection antibody is typically conjugated to multiple biotin molecules, which results in a strong signal when multiple labeled avidin/streptavidin molecules bind to the biotin moiety.

## 1.6. Antibody Detection

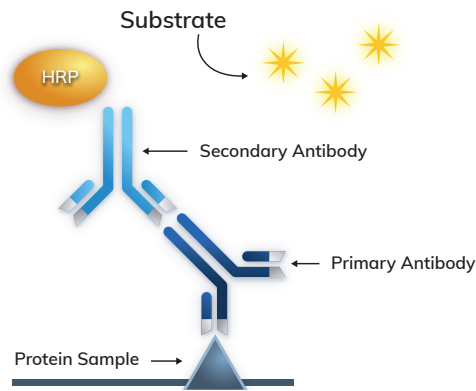
Antibody binding can be visualized using colorimetric, chemiluminescent, or fluorescent detection methods. This guide focuses on chemiluminescent and fluorescent detection. The choice of detection method should be made based on multiple factors including the desired sensitivity.

### Chemiluminescence

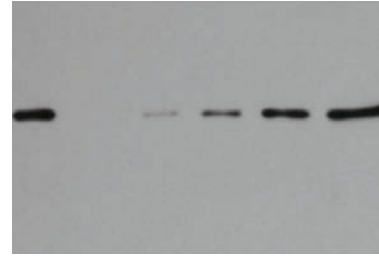
Chemiluminescence is a popular indirect detection method for Western blotting that relies on an enzyme-substrate reaction that emits light (Figure 1.6). Horseradish peroxidase (HRP) and alkaline phosphatase (AP) are two commonly used chemiluminescent enzymes, with the sensitivity of detection dependent on the choice of substrate—commercially available substrates for HRP can detect proteins in the femtogram range. Imaging of a chemiluminescent Western blot is historically done via exposure of the blot to x-ray film (Figure 1.7) and can also be done using a CCD-based imaging system.

Chemiluminescent detection is often used because it is specific, easy to perform, and highly sensitive—proteins can be detected at femtogram levels (Table 1.7). The technique is very good at answering the question, “Is my protein there or not?” however chemiluminescent detection is not very good at addressing questions such as, “How much of my protein is present relative to another protein? How much of my protein is in one sample compared to another sample? How do I control for sample loading inconsistencies?” We discuss the drawbacks to chemiluminescent detection in the next section.

Learn how to optimize your chemiluminescent Western blots on [page 35](#) in our Western Blot Tips and Guidelines Chapter.



**Figure 1.6. Chemiluminescent Western blotting—one signal, one protein.** In chemiluminescent detection, the antigen-primary antibody complex is bound by a secondary antibody conjugated to an enzyme, such as horseradish peroxidase (HRP). The enzyme catalyzes a reaction that generates light in the presence of a luminescent substrate, and the light can be detected either by exposure to x-ray film or by a CCD-based imaging system.



**Figure 1.7. A chemiluminescent Western blot detected on x-ray film.**

### Chemiluminescence Drawbacks

Unlike fluorescent tags, where one or more different proteins can be probed simultaneously using antibodies labeled with spectrally distinct fluorophores, chemiluminescent reactions emit light over a broad range of wavelengths. Thus, with chemiluminescent detection, emission wavelengths cannot be used to distinguish signals from different proteins. Instead, the proteins must be well-resolved electrophoretically.

For example, proteins with small differences in molecular weight, such as the same protein with and without a post-translational modification, tend to co-migrate during electrophoresis making them difficult to visualize simultaneously using chemiluminescence since the bands will most likely overlap.

Overlapping bands can also impact detection of normalization and loading controls. Unless these controls are well-resolved electrophoretically from the protein-of-interest, the blot must be either stripped and reprobed to detect the control, which renders the blot non-quantitative, or the controls must be placed on a separate blot, which is not a true loading control.

Furthermore, because chemiluminescence relies on an enzyme-substrate reaction, the amount of signal (emitted light) is subject to variations in reaction kinetics, variations which can be affected by reaction conditions, *i.e.* pH, temperature, substrate concentration, and enzyme concentration. This inherent variability makes chemiluminescence, at best, a semi-quantitative detection chemistry.

Finally, the traditional use of x-ray film as a method of visualization suffers from dynamic range limitations of the film that can often lead to signal saturation.

Advantages	Disadvantages
<ul style="list-style-type: none"> <li>• Sensitive</li> <li>• Easy, familiar chemistry</li> <li>• Compatible with film or digital imaging</li> </ul>	<ul style="list-style-type: none"> <li>• Semi-quantitative</li> <li>• Signal dependent on enzyme kinetics</li> <li>• Single protein only, loading controls require stripping and re-probing</li> </ul>

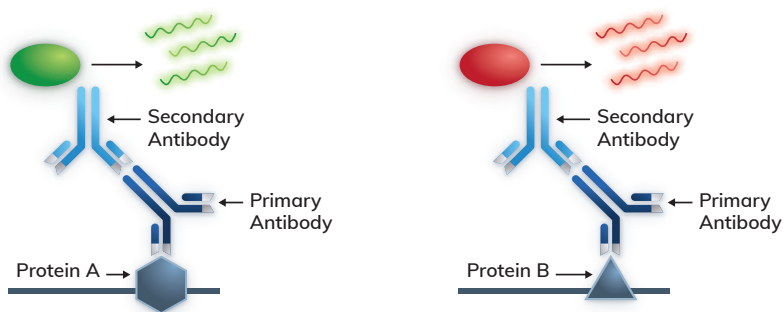
**Table 1.7. Chemiluminescent detection.**

## Fluorescence

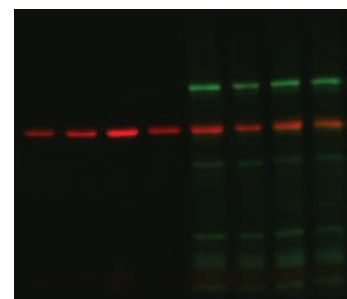
Fluorescent Western blotting uses secondary antibodies directly conjugated to fluorescent dyes. Unlike chemiluminescent Westerns, which are limited by the variable kinetics of the enzyme-substrate reaction, the amount of light emitted from fluorophores is highly consistent and directly proportional to the amount of protein on the membrane. This consistency means that fluorescent detection can provide a truly quantitative analysis of the proteins in question.

Fluorophores for fluorescent Western blotting can be chosen based on their specific excitation and emission spectra, enabling multiplexing (detection of multiple proteins simultaneously, Figure 1.8) for faster, more efficient studies. Thus, one of the biggest advantages of fluorescent detection versus chemiluminescence is the ability to use more than one antibody per assay. With multiplexing, normalization and loading controls can be imaged at the same time and on the same blot as the sample. In addition, the ability to use different fluorophores enables visualization of proteins that are not well-separated electrophoretically, for more convenient imaging of the same protein with and without post-translational modifications.

Find out how to improve your fluorescent Western blots on [page 36](#) in our Western Blot Tips and Guidelines chapter.



**Figure 1.8. Fluorescent Multiplex Western Blotting.** Multiplex detection is possible by using two or more fluorescent dyes and an instrument that can excite and detect the light from each dye.



**Figure 1.9. Multiplex fluorescent Western.**

Fluorescent Westerns are typically visualized using a digital imager rather than X-ray film (Figure 1.9). The newer generation of imaging systems often contain sophisticated cameras that exhibit a broader dynamic range than film, thus avoiding the signal saturation problems that limit the dynamic range of film. Finally, fluorescent dyes are relatively stable; blots can be archived and imaged months after the initial experiment as long as precautions are taken to avoid photo-bleaching of the fluorophores.

Learn more about doing multiplex fluorescent Western blots in our application notes:

- Three-color Western bBlots with AzureSpectra Antibodies ([page 66](#))
- Imaging Three-color Western Blots with the Azure 600 ([page 71](#))
- Increasing Assay Efficiency with Four-Color Detection ([page 85](#)).

## Fluorescence Drawbacks

While fluorescent detection is the best choice for quantitation and can greatly accelerate workflows for analyzing multiple proteins via Western blotting, the method has a few drawbacks (Table 1.8). Fluorescent detection can be less sensitive than chemiluminescent detection, depending on the protein being assayed. Reagents (e.g. bromophenol blue) and supplies (e.g. certain membranes) can autofluoresce leading to high background which reduces the limit of detection of the assay. When switching from a chemiluminescent assay, all primary and secondary antibodies need to be titrated individually to find the highest signal-to-noise ratio. Lastly, fluorescent Western blots are visualized using digital imagers rather than the x-ray film and developer paradigm established with chemiluminescent detection.



Advantages	Disadvantages
<ul style="list-style-type: none"> <li>• Multiplex capability</li> <li>• Increased quantitative accuracy</li> <li>• Fluorescent label stability allows blots to be stored and re-imaged later</li> </ul>	<ul style="list-style-type: none"> <li>• Can be less sensitive than chemiluminescence</li> <li>• Membranes auto-fluorescence can increase background</li> </ul>

**Table 1.8. Fluorescent detection.**

## Chemiluminescence vs Fluorescence

### Chemiluminescent and Fluorescent Westerns: Choose the Best Assay for Your Experiment

Both chemiluminescent detection and fluorescent detection are excellent methods and, when used together, can provide complementary information that enhances insight. Thus, a laboratory should not be a “chemiluminescent Western laboratory” or a “fluorescent Western laboratory” but a lab that uses the best assay for each experiment. Table 1.9 outlines when to use chemiluminescent detection and when fluorescent detection might be best.

Find out how to transition from chemiluminescent to fluorescent Western blots on [page 37](#) in our Western Blot Tips and Guidelines chapter.

Use Chemiluminescence to	Use Fluorescence to
<ul style="list-style-type: none"> <li>• Detect a single protein</li> <li>• Assay for presence/absence of a protein</li> <li>• Measure antibody responses</li> <li>• Follow protein purification</li> <li>• Detect low abundance proteins</li> </ul>	<ul style="list-style-type: none"> <li>• Detect multiple proteins simultaneously</li> <li>• Study posttranslational modifications</li> <li>• Have same-blot loading control</li> <li>• Have in-lane normalization</li> <li>• Perform quantitative Westerns</li> </ul>

**Table 1.9. Chemiluminescent detection vs. Fluorescent detection.**

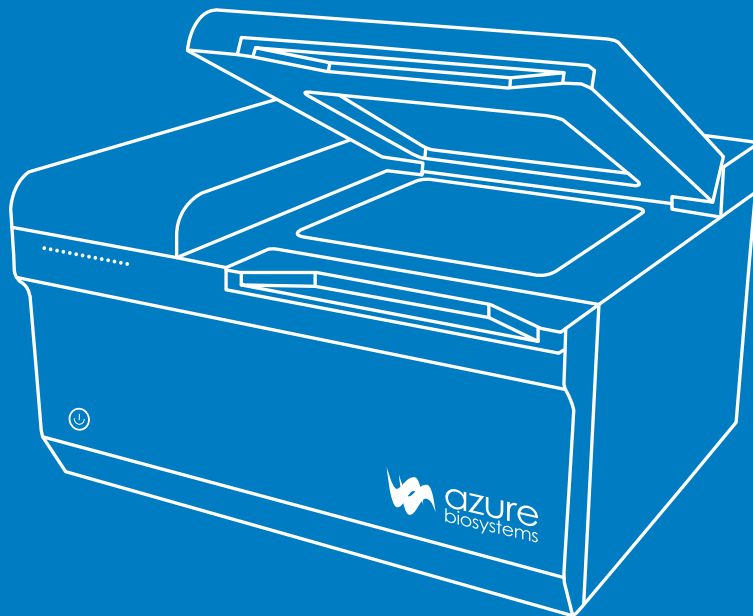
## 1.7. References

1. Renart J, Reiser J, Stark GR: Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. (1979) *PNAS* 76:3116-3120.
2. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. (1979) *PNAS* 76:4350-4354.
3. Burnette WN: Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. (1981) *Analytical Biochemistry* 112:195-203.
4. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. (1970) *Nature* 227 (5259): 680-685.



2

## IMAGING WESTERN BLOTS



## 2.1 Leaving the Darkroom – Moving to Digital Imaging for Better Quantitation

Digital imaging hardware and the associated quantification software are becoming ever more popular, surpassing film-based methods for gathering data. Compared to film, digital imaging offers a wider dynamic range, more accurate quantitation, and the ability to conduct multiplex imaging, all of which work together to greatly streamline workflows.

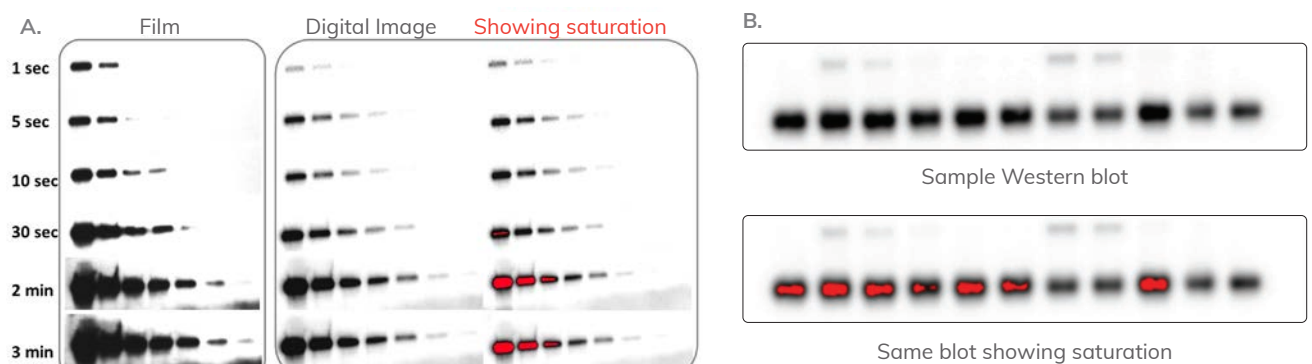
Here we discuss the key benefits of leaving the darkroom, and moving to digital imaging.

### Detection of Signal Saturation

When capturing data from your Western blot, through film or a digital imager, you are measuring the intensity of signal collected in a specific area of the blot. When the signal accumulation for a specific band on a Western blot reaches the saturation level, no further signal accumulation is possible. This means that if two bands on your blot are saturated, you have no way of knowing if they have the same signal level, or if their signal level is vastly different.

Using film, detection of saturation is essentially guesswork and it is easy to miss variations in protein levels or to underestimate the amount of protein present.

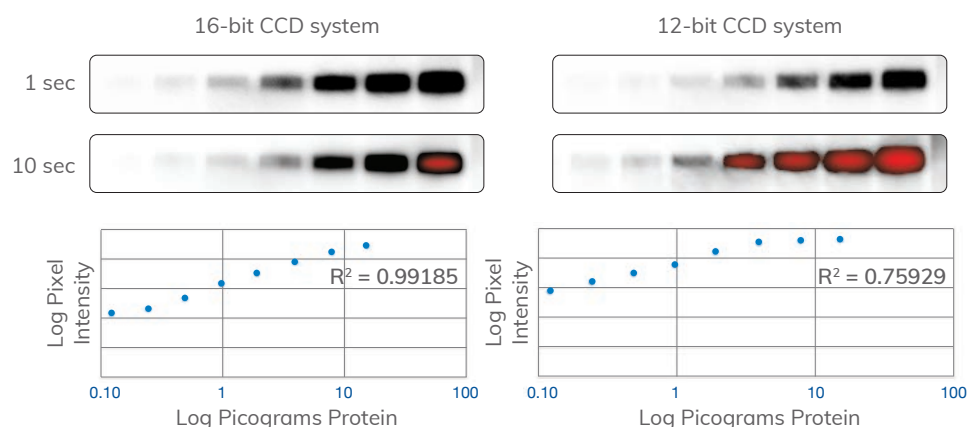
Digital imaging has two advantages. First, software for the quantification of digital imaging can notify the scientist when the acquired image has bands that are saturated. (Figure 2.1 A, B) Second, software for digital imaging can determine the optimal image time that prevents signal saturation of the most intense band. This feature can prevent over-saturation of the signal, making sure the data is within the linear range of the imaging system.



**Figure 2.1. A wider dynamic range and automatic saturation detection allow for improved quantification.** A. The same blot was imaged on both x-ray film and the Azure 600. The Azure 600 detects when saturation occurs and calculates an auto-exposure time to avoid saturated bands. B. The top blot is a sample western blot showing a variety of band intensities. Below is the same blot showing saturated bands, this blot should therefore be exposed for a shorter period of time for more accurate quantification, a process that is easy to do with today's digital imagers.

### Wide Dynamic Range

Dynamic range is the ratio of the maximum detectable signal to the background signal (i.e. noise). When comparing two different systems, the one with a wider dynamic range should provide faster workflows and more robust quantitation since both weak and strong signals can be measured during a single exposure. Without a wide dynamic range, strong signals will saturate before weak bands can be detected, requiring multiple exposures to visualize all bands and making quantitation either less reliable or even impossible (Figure 2.2).



**Figure 2.2. 16-bit Imaging for a Wide Dynamic Range.** A Western blot was imaged on both a 16-bit system and a 12-bit system. While the 10 second exposure appears similar on the different systems, the 12-bit system produces an image that is saturated and not suitable for analysis.

### High Resolution

Whether you're looking at a CCD camera-based system or a scanning imager, the resolution will determine the smallest feature size that you will be able to image. For CCD-based imagers, resolution is typically presented in megapixels (MPs), with a larger number of MPs providing a higher resolution. For scanning imagers resolution is provided in microns and indicates the smallest feature size you will be able to visualize. Higher resolution systems can facilitate imaging blots with poorly-resolved protein bands and can facilitate visualizing samples other than blots. For example, some high resolution scanning imagers can image individual cells within a tissue sample or microscope slide.

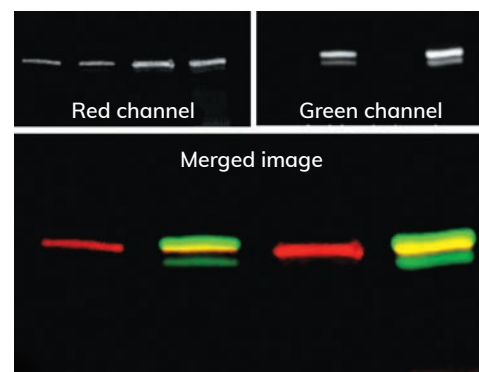
### Multiplex Assays

Film and chemiluminescent imaging limit the assay to one signal detected per Western blot. The most recent generation of digital imagers have introduced the possibility of multiplex analysis (Figure 2.3) to assays that were previously only able to analyze one protein at a time. Fluorescent antibodies spanning the visible and near infrared spectrum enable detection of multiple samples of interest within a single assay, making within-experiment controls possible, supporting the development of novel assays, and greatly improving and accelerating workflows.

### Image Digitization

To include a Western blot image in any publication, digitization of the image is required. With film, scanning of the film is required in order to analyze or quantitate the image. With digital imaging, the resulting image is immediately digitized and ready for quantitative analysis with software.

To summarize, digital imagers represent a significant improvement over traditional film imaging with increased sensitivity, dynamic range and image quality. Furthermore, they offer significant quality of life improvements such as automatic saturation detection and quantification, while also opening the door to the development of new assays through their multiplex capacity.



**Figure 2.3. Multiplex imaging significantly optimizes workflows and allows for new assay development.** Multiplexing on a single blot is a unique method available with digital imagers. Here, a western blot has been probed for both STAT1 (red channel) and phosphorylated STAT1 (green channel) using fluorescently conjugated secondary antibodies and imaged using infrared detection on the Azure 600.

## 2.2 Choosing a System – CCD Imagers, Scanners, and Hybrid CCD/Scanning Systems

Once a blot has been prepared the last step in the Western blotting workflow is image capture and analysis. Using the right imager and following best practices for data acquisition and analysis is just as important as choosing the right electrophoresis and blotting conditions when you want to generate reliable, high-quality Western blotting data. The choice of imaging system will depend on the type of studies being done, specifically the detection chemistry used and the sensitivity and resolution needed.

### Different Imaging Systems for Different Performance Needs

Today's digital imagers typically fall into one of two categories—CCD-based imagers and scanning systems. Choosing between the setups comes down to performance needs and whether you need the flexibility to support multiple imaging chemistries and applications. In both types of systems, one of the most important features to look for is dye flexibility. Not every fluorescent dye works with every imaging system, thus it's important to verify that the system has enough dye flexibility that it will work with the dyes used in your studies. This is especially true when you want to do multiplex fluorescent Western blots. For multiplexing, it is critical to look at systems with multiple excitation sources and multiple detection wavelengths. Additionally, you will want to make sure that the best light sources have been selected. For example, IR dyes are typically excited with lasers, in part because only lasers emit light close to the excitation peak of these types of dyes.

Here we discuss the key features to use for imaging system evaluation.

### CCD Imagers

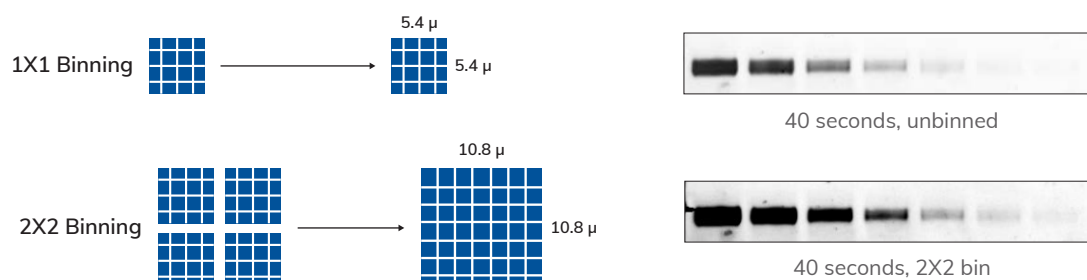
CCD imagers use a CCD camera for signal detection. The blot (or gel, or other sample being imaged) is placed into a chamber and, if necessary, illuminated using a light source. The emitted light is then captured and digitized by the sensor. With this type of system, because the entire sample is imaged at once, the uniformity of sample illumination and sample detection is critical. It is therefore important to consider the imaging field of view (FOV) in CCD platforms, the larger the FOV the more difficult it is to control illumination uniformity. Excitation uniformity is easier to control with a smaller field of view.

Imaging chemiluminescent Western blots is most effectively done using a CCD sensor. The CCD sensor samples the entire light spectrum simultaneously and efficiently, supporting the long integration times (from seconds to minutes) needed for sensitive detection. Like film exposures, CCD imaging allows the user to control the exposure time.

Azure Biosystems offers CCD imaging systems that combine affordability with performance. Learn more about our Azure Imaging Systems at [azurebiosystems.com/imaging-systems](https://azurebiosystems.com/imaging-systems).

### Key Features for Evaluation of CCD Imagers

**High Resolution, High Sensitivity**—One feature essential for digital imaging with a CCD camera is pixel binning. The advantages of pixel binning—higher sensitivity and a higher signal-to-noise ratio—are the result of combining neighboring pixels into a single larger pixel, or “super-pixel” (Figure 2.4). The larger size of the super pixel increases sensitivity by increasing the surface area available for photon detection without similarly increasing the noise (note that the lack of increased noise only applies to on-chip binning and is not a feature of binning performed computationally during data analysis). For example, a binning of 2x2 combines four adjacent pixels into a single super-pixel resulting in a four-fold increase in sensitivity to light, while keeping the noise the same as that of a single unbinned pixel. While the increased pixel size can greatly improve the sensitivity of the detector, the larger pixel size reduces the resolution. A binning of 1x1 uses the native pixel size—no pixels are combined—and thus takes advantage of the full resolution of the sensor.



**Figure 2.4. Pixel binning improves image sensitivity.** Pixel binning is a powerful technique for digital imaging that increases sensitivity by combining pixels to make a larger “super-pixel.” The super-pixel has a higher signal-to-noise ratio (SNR) than the unbinned pixel.

It is important to understand that with high levels of binning the final image will have significantly lower resolution, and that low resolution images may not be suitable for publication. Upscaling, a form of post capture image manipulation, can be used to rescue the lost resolution, but an upscaled image must not be used for quantitative purposes because pixels (data) is artificially added to the image and only raw data should be quantified.

**Wide Aperture for Chemiluminescent Detection**—Especially for chemiluminescence, the F stop is an important value to consider. The smaller the F stop, the wider the aperture, and the more light can be let in. Imagers with a small F stop can deliver more light to the sensor, reducing exposure times.

**Wide Range of Light Sources**—While chemiluminescent does not require a light source, fluorescent imaging does require both a specific light source and emission filter.

There are two main light sources used in fluorescent imaging: light emitting diodes (LEDs), and lasers. Historically, LEDs have been used because of their wavelength flexibility and low cost. But laser technology has advanced rapidly and now offers similar wavelength flexibility at a competitive price. Lasers are fundamentally different from and have several advantages over LEDs. Because lasers are monochromatic, they are highly effective at exciting fluorophores at a precise wavelength. In a multiplex experiment, precise excitation is critically important for specific excitation to avoid bleed through. LEDs, which are a broad band light source, can excite many more fluorophores but may also introduce cross excitation / bleed through if proper care is not taken in the experimental design.

## Scanning Systems

Scanning systems use laser light for sample excitation and either a photomultiplier tube (PMT) or avalanche photodiode (APD) to detect the emission signal. The sample is typically placed on a bed area and light excites the sample from the bottom. The emitted light can then be detected by a PMT or APD. Unlike CCD imagers, which image the blot or other sample all at once, scanning systems illuminate the sample one small section at a time as the sample is scanned. While scanning can take slightly longer than imaging with a camera-based system, the higher intensities possible with pinpoint rather than widely-distributed illumination can lead to greater sensitivity for more demanding studies. In addition, the ability to use PMTs and APDs, which are not compatible with a camera-based imaging approach, expands the range of detection chemistries and offers lower detection limits and overall better performance for visible and near infrared (NIR) fluorescence imaging.

Imaging fluorescent Western blots can also be done using a CCD sensor, but better performance can be obtained in a bed scanning system. Fluorescence imaging with a CCD imager is often less sensitive than chemiluminescence because fluorescence imaging does not enjoy the enzymatic signal amplification afforded by the horseradish peroxidase-coupled antibody. Fluorescence imaging with a laser scanner, however, can match or exceed chemiluminescence sensitivity because PMTs and APDs have an internal gain which serves to amplify the emission signal in a similar manner as the enzymatic amplification. Because CCD sensors cannot amplify the emission signal like a PMT or APD, laser scanners using a PMT and/or APD are the best choice for high sensitivity fluorescence imaging.



### Key Features for Evaluating of Scanning System

**Resolution**—Scanner resolution is important for application flexibility. Scanning systems enable fine tune control over the spatial resolution, allowing a user to select a resolution appropriate for a specific experiment. Most Western blots need only 100-200 $\mu$ m resolution, but some imaging application require much higher resolution to capture fine detail. For example, imaging stained tissue sections (IHC) may require 10 $\mu$ m resolution to visualize fine details in the tissue.

**Scan Speed**—Unlike CCD imagers that image the whole field of view at once, scanning systems scan the image line by line. The larger your scan area, the longer your scan will take. This is why it is important to select an imaging system with a fast scan speed, usually listed in cm/s. The faster the scan speed, the less time imaging your blot will take.

**Excitation Sources**—Scanner devices used for detecting fluorescence most commonly employ laser light for excitation. A laser is a collimated light source, meaning it produces a column of light such that nearly all the photons are travelling in the same direction/angle and nearly all the photons leaving the light source are effectively delivered to the endpoint (sample of interest/fluorophore).

By contrast LEDs, which have high divergence, produce a diffused pattern of excitation light where the light scatters/diffuses in many directions. When exciting a fluorophore, the more energy delivered to the fluorophore the greater the emission signal. Because lasers are a collimated light source and do not diverge like LEDs, lasers are much more effective at delivering excitation energy to the fluorophore and therefore produce a brighter emission signal yielding a lower limit of detection compared to LED excitation. This technological difference is critically important to achieving high quality images and data.

Another factor to consider is simply the number of light sources. The more lasers, the more dye flexibility. Furthermore, more lasers enable the detection of more fluorophores in a single sample – for example, a system with four unique lasers would enable the detection of four fluorophores from a single sample, increasing the multiplexing capacity, application flexibility, and throughput.

**Detector Technology and Light Collection**—Quantum efficiency and spectral response are important factors to review when evaluating scanning systems. It is also important to consider the number of detectors included in the scanning system. There are two main types of detectors used in scanning systems: Avalanche Photodiodes (APDs) and Photomultiplier Tubes (PMTs):

**PMTs**—PMTs belong to a class of vacuum tubes that convert photons into an electric signal. Photo multipliers have high internal gain, making them ideal for low light applications. The high internal gain is essential to high sensitivity fluorescence imaging.

Photomultiplier tubes operate using photoelectric effect and secondary emission. When light is incident on the photocathode, it emits electrons into the vacuum tube. These electrons are focused towards the electron multiplier (dynode), which multiplies the signal by secondary emission. The multiplied electrons are converted into an output signal by the anode (Figure 2.5).

PMTs are ideal for low light imaging, where internal gain is required in order to amplify the signal. This is a key reason why they are used for phosphor imaging and other high sensitivity blue light detection ie GFP detection.

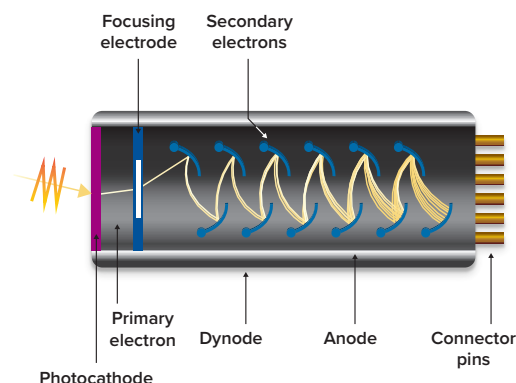


Figure 2.5. How a PMT works.

**APDs**—An APD is a highly sensitive semiconductor electronic device that exploits the photo electric effect to convert light to electricity (Figure 2.6). APDs are similar to PMTs in that they also amplify the emission signal resulting in enhanced sensitivity through signal amplification. However, APDs are quite different from PMTs. The most important difference between APDs and PMTs is their quantum efficiency.

APDs have a significantly higher quantum efficiency at longer wavelengths compared to PMTs, and because APDs can capture light more effectively than PMTs at longer wavelengths they are better suited for fluorescence imaging at wavelengths greater than 500nm . Combining the low autofluorescence associated with long wavelength fluorophores and the high quantum efficiency of APDs results in one of the most sensitive fluorescence detection systems. Although, bright blue dyes with a PMT detector can offer same or similar performance.

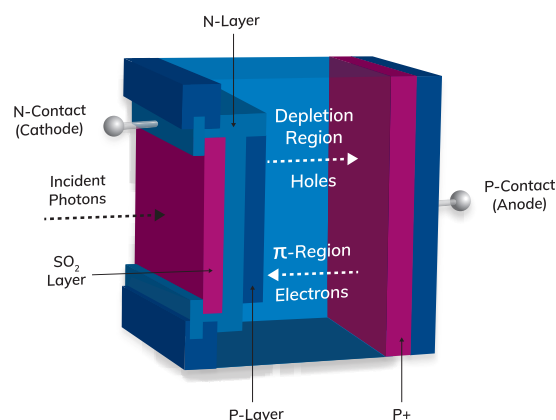


Figure 2.6. APD.

**Z-axis Flexibility**—While all scanners can scan in the both X and Y direction, not all systems can freely scan in the z- axis. Controlling the z-axis scan plane allows users to easily image a variety of samples. Membranes, gels, culture plates, and slides all have different optimal z-axis focal planes, so it is important to consider a system than can easily change the z-axis for optimal imaging of a variety of samples.

### Hybrid CCD/Scanning Systems

Hybrid CCD/scanning systems are a new class of imaging systems offering the best performance for a wide variety of chemistry and sample types. As discussed above, there are CCD systems which are ideal for chemiluminescence and gel imaging because they enable user control of the exposure time – short exposure for gel imaging and longer exposures for high sensitivity chemiluminescence imaging. However, because CCD detectors do not have a built in signal amplification mechanism they are not ideal for high sensitivity fluorescence imaging. Alternatively, laser scanners using PMTs and APDs can amplify fluorescence signals and provide a highly sensitivity fluorescence imaging system. Laser scanners however, do not allow for long exposures required for high sensitivity chemiluminescence detection, so laser scanners are not ideal for chemiluminescence imaging. Combining the advantages of a CCD imager for gels and chemi imaging with the high performance of laser scanning system using PMT and APD detectors for high sensitivity fluorescence offers the best of both worlds. Hybrid CCD/scanning systems enable high performance across multiple imaging modalities and a wide variety of sample types for high application flexibility – this is the ideal imaging system both chemiluminescent and fluorescent imaging.

Azure Biosystems offers a first-in-class hybrid CCD/scanning system, the Sapphire Biomolecular Imager. Learn more at [azurebiosystems.com/sapphire](https://azurebiosystems.com/sapphire).

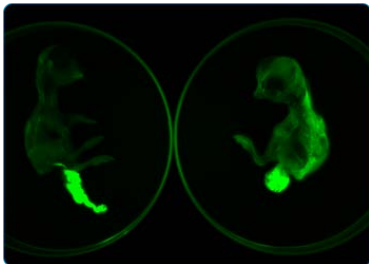
### CCD Imager vs Scanner vs Hybrid System: How to Choose

How to choose an imaging setup depends on the types of studies being done. For many labs, the performance of a CCD-based system covers most needs, especially for gels and chemiluminescent Western blotting. However, for labs that need high performance (i.e. sensitivity, resolution, dynamic range), to image large samples, and/or to use multiple imaging chemistries (phosphor imaging, chemiluminescence, and visible and NIR fluorescence) a scanning system may be a better choice.

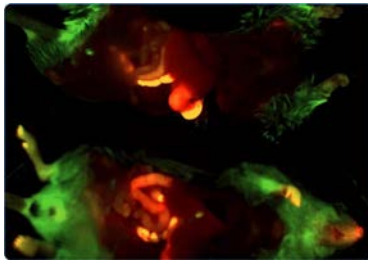
## 2.3. Imaging Beyond the Western Blot

Azure Biosystems offers CCD imagers and hybrid CCD/scanning systems that provide the value and versatility of imaging more than Western blots. Here are just a few examples of the different things you can image with our Azure Imaging System and Sapphire imagers.

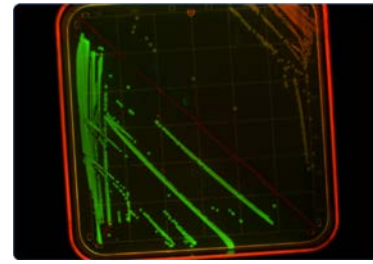
### Azure Imager System Images



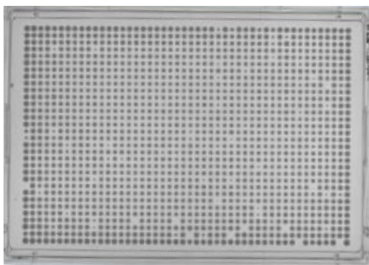
**Virulent Newcastle Virus Vs. "Normal" Newcastle Virus in chicken embryo imaged on Azure 600.** Images provided by Ray Izquierdo-Lara, Ana Chumbe, Katherine Calderon, Manolo Fernandez. FARVET SAC, Peru.



**Mouse imaging.** Acquisition was in RGB automatic mode – red channel was imaged at 704 ms and green channel for 703 ms, both at 60  $\mu$ m resolution. Imaged on Azure Imager System.



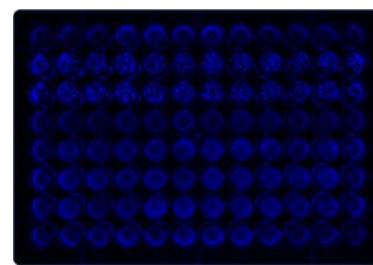
**An agar plate with *E. coli* expressing GFPmut3 (green) and mCherry (red).** The plate was imaged using red and green LEDs. Imaged on Azure Imager System.



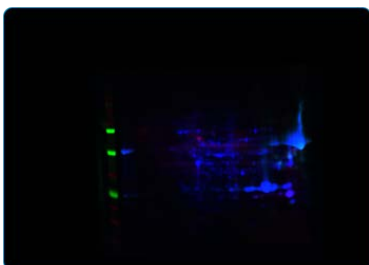
**Yeast colonies expressing GFP imaged in the Blue channel.** Imaged on Azure Imager System.



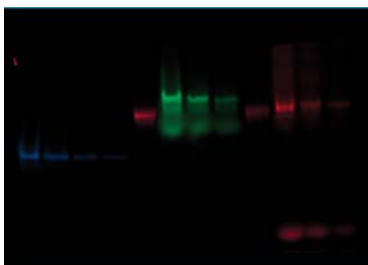
**Western blot using Cy5 and Cy3 of FtsZ1 and FtsZ2-1 from *Arabidopsis thaliana*.** Plant fluorescent Western. Imaged on Azure Imager System.



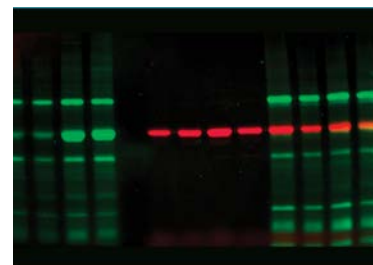
**GFP expressing cell monolayers with different protocols for fixation in different columns.** Imaged on Azure Imager System.



**2D Fluorescent Gel Imaged in Cy5/Cy3/Cy2.** Imaged on Azure Imager System.



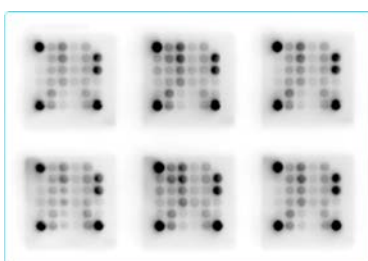
**Native Gel.** Imaged on Azure Imager System.



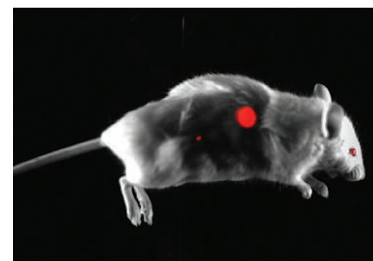
**TAMRA/GFP.** Imaged on Azure Imager System.



**GFP/mCherry.** Imaged on Azure Imager System.

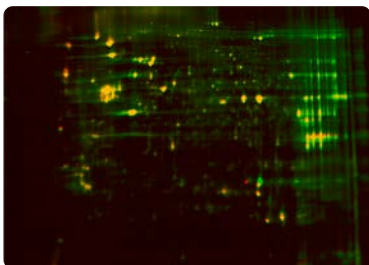


**Antibody Array.** Imaged on Azure Imager System.

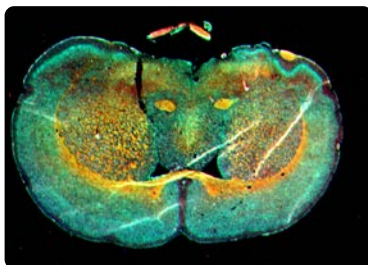


**Mouse with RFP-expressing subcutaneous tumor.**

## Sapphire Images



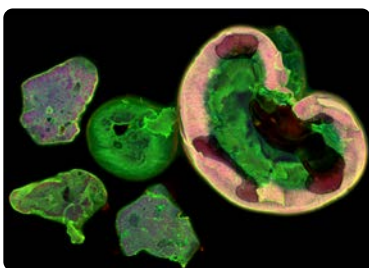
**In-Gel Fluorescence and 2D Gels.** Untreated and treated HeLa lysate were labeled with Cy3 and Cy5 respectively and simultaneously separated using IEF in the first dimension and SDS-PAGE in the second dimension then scanned at 100 $\mu$ m using the 520nm and 658nm lasers of the Sapphire Biomolecular Imager.



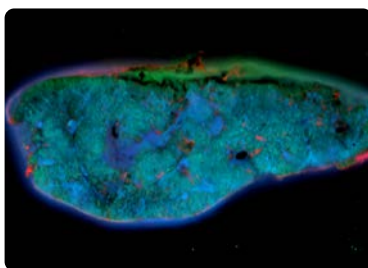
**Rat Brain.** Three color composite image of a rat brain scanned at 10 $\mu$ m on the Sapphire Biomolecular Imager.



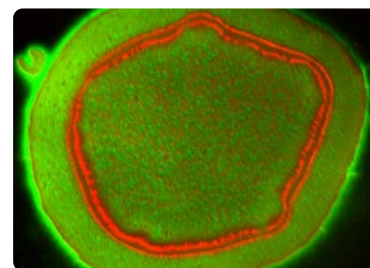
**Bee Head.** Composite image of a bee head scanned with the 488nm and 785nm lasers of the Sapphire Biomolecular Imager.



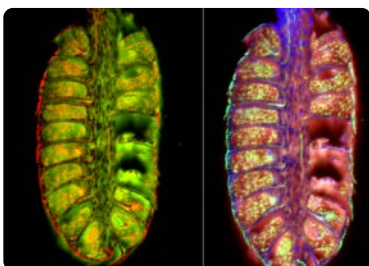
**Mixed Tissue.** Composite image of mixed tissue scanned at 20 $\mu$ m with the 658nm and 785nm lasers of the Sapphire Biomolecular Imager.



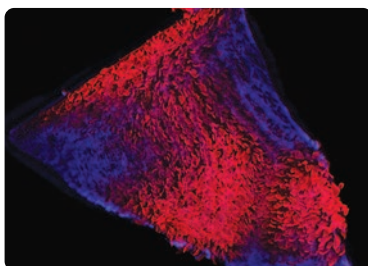
**Chicken Liver.** Coomassie stained chicken liver scanned at 10 $\mu$ m with the 488nm, 520nm and 658nm lasers of the Sapphire Biomolecular Imager.



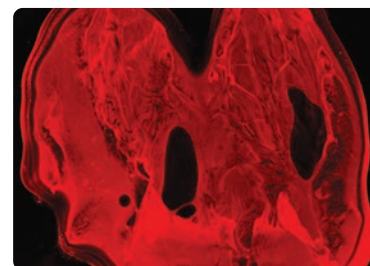
**Pelargonium Stem.** Two channel composite image of a Pelargonium Stem scanned at 10 $\mu$ m with the 658nm and 785nm lasers of the Sapphire Biomolecular Imager.



**Pine Cone.** Multichannel scanned image of a pine cone sliver, scanned with 488nm and 785nm lasers on the Sapphire Biomolecular Imager using the demo machine in Germany.



**Cow Stomach.**

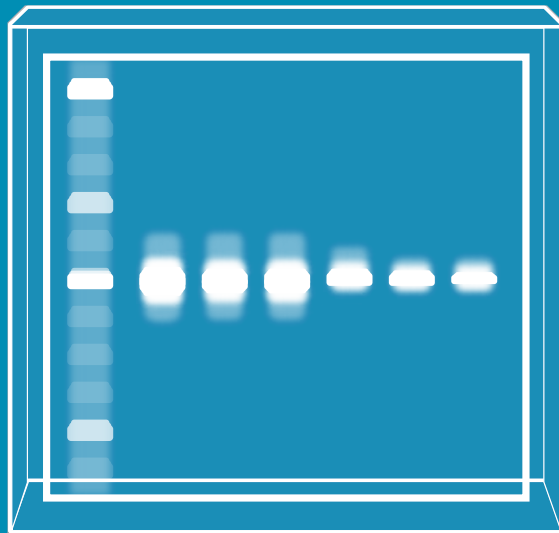


**Chicken Heart.**



3

## WESTERN BLOTTING TIPS AND GUIDELINES



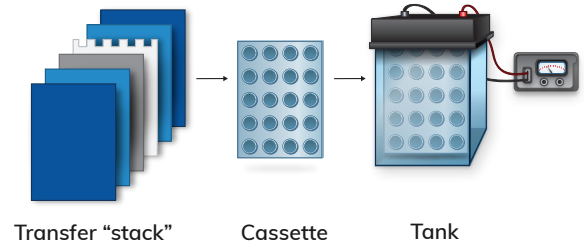
### 3.1. Tips for Successful Western Blot Transfer

Whether you choose to do a wet or dry transfer, there are many steps you can take to maximize transfer efficiency.

#### Wet Transfer

##### Benefits of Wet Transfer

Using a wet transfer apparatus, high intensity (1-2 hour) or lower intensity (overnight) transfers can be performed. This allows for optimizing transfer conditions for each individual protein. Transfer times can be shortened to prevent transfer of low molecular weight proteins through the membrane while longer transfer times can be used to promote complete migration of high molecular weight proteins out of the gel.



##### Drawbacks to Wet Transfer

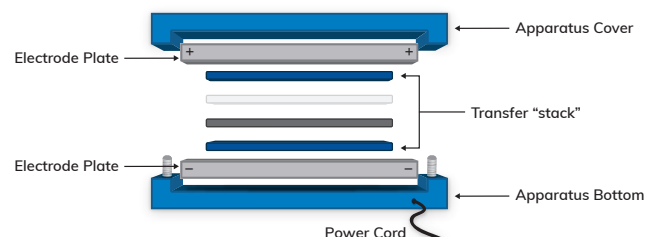
The transfer process generates heat, which can decrease the resistance of the transfer buffer resulting in inconsistent transfer across the gel. High heat can also result in breakdown of the gel itself. To prevent heating, transfer buffer should be pre-chilled prior to use. In addition, the transfer buffer should be kept cold during transfer. Long transfers are often performed in a cold room to aid in keeping the buffer cool. Additionally, self-contained ice blocks can be placed within the tank and are usually supplied with the apparatus. Alternatively, external-cooling mechanisms can be used to control heating.

Advantages	Disadvantages
<ul style="list-style-type: none"> <li>Flexibility:                             <ul style="list-style-type: none"> <li>Transfer conditions are easily adjusted</li> <li>Multiple transfer buffer options enable more ways to optimize transfer</li> </ul> </li> <li>Supports transfer of a broad molecular weight range at one time</li> <li>Compatible with extended transfer times for large molecular-weight proteins</li> <li>Can be used for quantitative Westerns</li> </ul>	<ul style="list-style-type: none"> <li>Heating of buffer can interfere with transfer</li> <li>Cooling mechanism and/or cold room space required during transfer</li> <li>Large volumes of transfer buffer are required</li> </ul>

#### Semi-Dry Transfer

##### Benefits of Semi-Dry Transfer

Semi-dry transfers are fast and easy and require little buffer. Transfer of more difficult to transfer proteins can be optimized by using a discontinuous buffer system, a feature unique to semi-dry transfer systems. In a discontinuous buffer system, the filter paper placed on the anode side of the stack is soaked in a different buffer from the filter paper placed on the cathode side of the stack. This can increase migration of the protein out of the gel while also promoting better retention on the membrane.





### Drawbacks to Semi-Dry Transfer

Semi-dry transfer systems have less flexibility and it can be difficult to transfer both high and low molecular weight proteins. Low molecular weight proteins can transfer through the membrane due to the high intensity blotting conditions while high molecular weight proteins may not efficiently transfer out of the gel due to decreased transfer times. However, transfer of high molecular weight proteins can be improved by using the discontinuous buffer system. Transfer times cannot be extended when using semi-dry transfer, as there is limited buffering capacity. In addition, the gel can dry out if insufficient buffer is used.

Advantages	Disadvantages
<ul style="list-style-type: none"> <li>• Transfer is rapid</li> <li>• Can use discontinuous buffer system to optimize transfer of proteins</li> <li>• Little buffer is required</li> <li>• Easy to set up</li> <li>• Good for performing large numbers of blots analyzing the same protein</li> </ul>	<ul style="list-style-type: none"> <li>• High intensity field strength may cause low molecular weight proteins to migrate through membrane</li> <li>• Difficulty in transferring high (&gt;120 kDa) molecular weight proteins</li> <li>• Not recommended for quantitative Westerns</li> <li>• Gel can dry out</li> </ul>

### Tips for Successful Transfers

- Take care when preparing transfer buffers; small inconsistencies can affect transfer
- Use high quality, reagent grade methanol when preparing buffers; impurities in methanol can decrease transfer efficiency
- Never dilute the transfer buffer
- Do not adjust pH of the transfer buffer
- For optimum transfer, do not reuse transfer buffer
- Make sure all equipment is clean
- Use a thinner gel (0.5-0.75 mm thickness)
- If using a PVDF membrane, pre-wet the membrane in 100% methanol prior to equilibration in transfer buffer
- Equilibrate transfer pads, filter paper, membrane and gel for at least 15 minutes in buffer(s)
- Remove all air bubbles and creases between each layer in the stack
- Ensure the stack is firmly held in place and pressure is applied evenly over the entire surface of the stack
- Ensure that electrodes are connected and free of debris and damage (plate electrodes)
- Make sure the current is applied correctly and proteins will migrate towards the membrane
- Use pre-stained molecular weight standards to help monitor transfer
- Stain the blot with a reversible total protein stain (e.g. Ponceau S) to check quality of transfer
- For difficult to transfer proteins, adjust methanol and SDS concentrations. SDS promotes migration of proteins out of the gel but can inhibit membrane binding. Methanol increases retention of proteins on the membrane but can hinder migration out of the gel.

### Wet Transfer Buffers

#### Towbin Transfer Buffer

- Standard wet transfer buffer
- SDS (0.025-0.1%) can be added to facilitate transfer of proteins

25 mM Tris, pH 8.3

192 mM glycine

20% methanol

+/- SDS

#### CAPS (3-[cyclohexylamino]-1 propane sulfonic acid) Buffer

- For blotting basic proteins
- For blotting prior to N-terminal sequencing

10 mM CAPS, pH 11

10% methanol

#### Dunn Carbonate Buffer

- For higher efficiency transfer of basic proteins
- Can be used to enhance ability of antibodies to recognize some antigenic sites

10 mM NaHCO<sub>3</sub>

3mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.9

20% methanol

### Semi-Dry Transfer Buffers

#### Bjerrum Schafer-Nielsen Buffers

- Standard semi-dry transfer buffer based on Towbin

48 mM Tris, pH 9.2

39 mM glycine

20% methanol

#### Discontinuous Tris-CAPS Buffer System

- Uses two different buffers to enhance transfer of proteins
- The filter paper assembled on the membrane side (anode) of the blot contains methanol
- The filter paper on the gel side (cathode) of the blot contains SDS

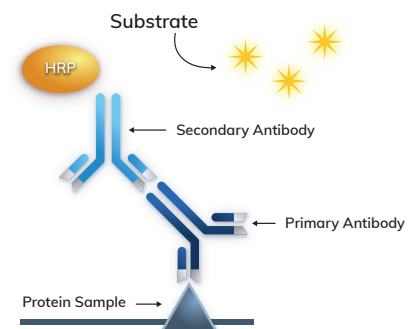
60 mM Tris, pH 9.6

40 mM CAPS

+ either 15% methanol or 0.1% SDS

## 3.2. How to Improve Your Chemiluminescent Western Blots

Chemiluminescent Westerns are popular assays for assessing protein expression. In this indirect detection method, chemiluminescent substrates emit light when reacted with an antibody conjugated to an enzyme. The emitted light is captured and archived on x-ray film (traditional), or through digital imaging. Chemiluminescent Western blotting is a highly sensitive assay and can detect femtograms of protein. Although it is only semi-quantitative, it is useful for detecting the presence or absence of



a protein. For example, chemiluminescence can be used to detect the induction of exogenous protein expression, to confirm and follow purification of a known protein, or for verification of antibodies during production.

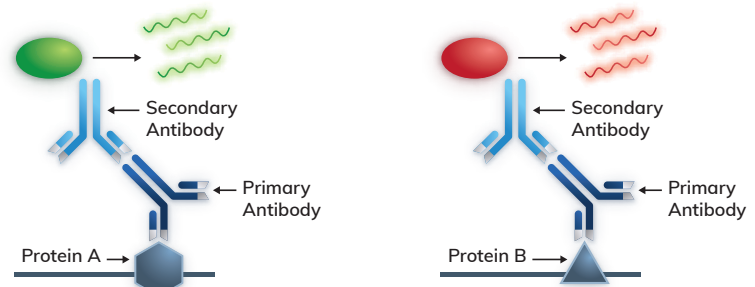
Chemiluminescent Westerns can be difficult to perform. The ultimate goal is to obtain a blot with a high signal-to-noise ratio. However, chemiluminescent Westerns can be plagued with high background, either in the form of an overall background that masks the signal from the protein of interest, or as bright dots and speckles and/or splotches scattered randomly over the blot. The increase in background noise can arise from a variety of factors. It can also be difficult to obtain a strong signal from the protein of interest.

### Tips for Improving Chemiluminescent Westerns

- Optimize the amount of protein to load on the gel. In general, 20-40 µg of total protein can be loaded without overloading the well. However, the total amount of protein should be optimized for each protein:antibody pair. Ideally, enough protein should be loaded to allow for easy capture of the signal without experiencing saturation.
- Choose the correct membrane. Nitrocellulose and PVDF membranes are commonly used, and each has advantages and disadvantages.
- Keep everything clean. Prevent background by thoroughly cleaning all equipment and trays prior to use. Only handle the gel and membrane with gloved hands. Keep trays covered during incubations.
- Ensure that all buffers are well mixed. Particulates in blocking and antibody incubation buffers will stick to the membrane and cause high background. Buffers can be filtered prior to use.
- If experiencing high background, use a larger volume of washing buffer and increase number and duration of washes.
- Try different blocking buffers. Some antibodies react with proteins in blocking buffers, causing a high background. Alternatively, some blocking buffers can mask the protein of interest, preventing detection. Non-fat dry milk and bovine serum albumin are the two most common protein blockers containing multiple proteins. Blocking buffers containing one protein can also be used. Protein-free buffers can be used when the primary antibody reacts with protein components in the buffer.
- Titrate both primary and secondary antibodies. Use a dot blot and checkerboard titration to determine the optimum primary and secondary antibody concentrations.
- Never dilute a horseradish peroxidase-conjugated secondary antibody in buffer with sodium azide. Sodium azide inhibits HRP activity.
- Use enough substrate. Make sure the blot is coated entirely with substrate to prevent local concentration differences.
- Try different substrates to increase sensitivity and signal duration. Different substrates are available with differing sensitivities for detecting high to moderate versus low abundance proteins. Different substrates also have different reaction rates and emit light for different durations of time. This can affect your ability to capture multiple exposures.
- The substrate may need to be equilibrated to room temperature before use to increase the enzyme activity.
- Use a digital imager rather than film. Digital imagers increase the linear dynamic range allowing easier detection of low abundance proteins while limiting saturation when detecting high abundance proteins.

### 3.3. How to Improve Your Fluorescent Western Blots

Fluorescent Western blots are the gold standard for quantitative Westerns. They are ideal for detecting multiple proteins simultaneously (multi-plexing), allowing in-lane normalization and detection of your protein of interest and loading control at the same time. In addition, post-translational modifications can be studied and quantitated easily.



In fluorescent Western blotting, the secondary antibody is directly conjugated to a dye, which is excited by light. The emitted light is detected by a digital imager and digitized for data analysis. Multiple proteins can be detected simultaneously by using secondary antibodies conjugated to different dyes with non-overlapping spectral emissions.

Although similar to chemiluminescent Westerns, there are additional factors that must be taken into consideration when performing fluorescent Westerns:

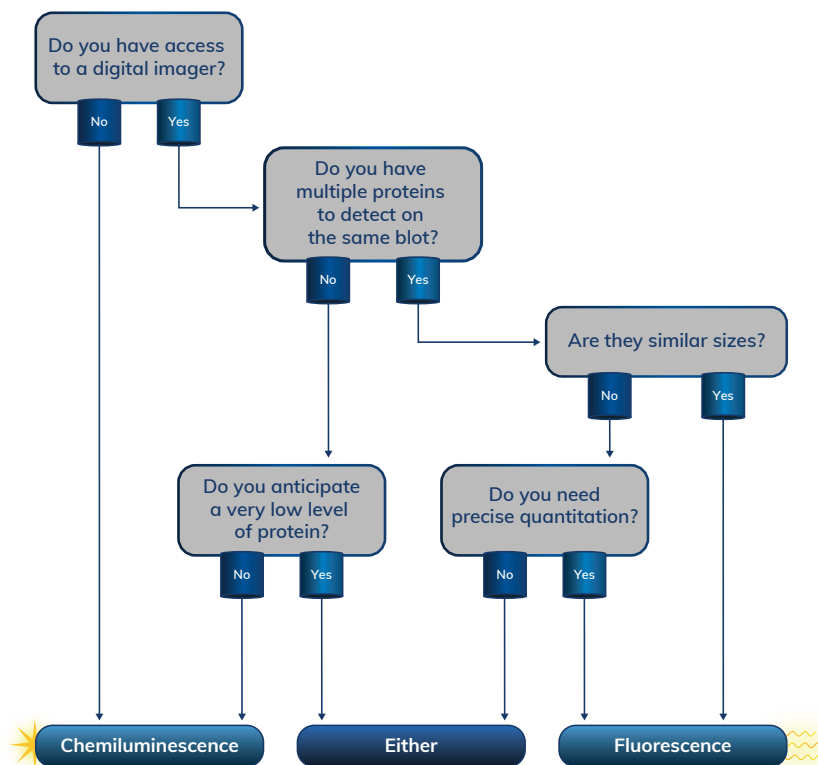
- Titrate both primary and secondary antibodies. Use a dot blot and checkerboard titration to determine the optimum primary and secondary antibody concentrations.
  - When multi-plexing, optimize detection of each target separately prior to simultaneous detection.
  - Primary antibodies may need to be increased 2-5x compared to concentrations used in chemiluminescent Westerns.
  - Secondary antibodies may also need to be increased (1:5000 is a recommended starting dilution).
- Use a PVDF membrane with low autofluorescence. Nitrocellulose and some PVDF membranes can autofluoresce causing high background.
- Avoid inks and dyes that can fluoresce. Use a pencil to mark the blot. Common dyes such as bromophenol blue and Coomassie autofluoresce.
- Keep everything clean. Prevent background by thoroughly cleaning all equipment and trays prior to use. Only handle the gel and membrane with gloved hands. Use powder-free gloves. Keep trays covered during incubations.
- If you are using fluorescent molecular weight markers, skip a lane before loading samples. This will prevent the signal from the molecular weight markers from bleeding into sample lanes.
- Work with fluorescent antibodies on the bench top, but store stocks in the dark.
- When multiplexing, use primary antibodies made in different species and secondary antibodies that are highly cross-adsorbed to prevent cross recognition.
- Avoid spectral overlap when multiplexing. Choose fluorophores that have optically distinct spectra.
- To increase the specific signal, always detect the strongest target in the blue channel, the middle in the green channel and the weakest in the red channel.
- When archiving blots, store them in the dark.

### 3.4. Transition from Chemiluminescent to Fluorescent Western Blots

#### Bring Fluorescent Western Blotting to Your Lab. It's Easier Than You Think.

Chemiluminescence is the most familiar method of detection for Western blotting and offers great sensitivity. However, many scientific questions and experimental designs require the additional information provided by fluorescent Western blotting; this includes precise quantitation and visualization of similarly sized proteins within the same sample.

Once you make the decision to move to fluorescent Western blotting, what comes next? First, you can check if fluorescent Western blotting is right for your experiment using the flow chart in the figure below. Then, refer to the tips and advice provided in this document to get started with your first fluorescent Western blot.



Chemiluminescent or Fluorescent Western Blotting.

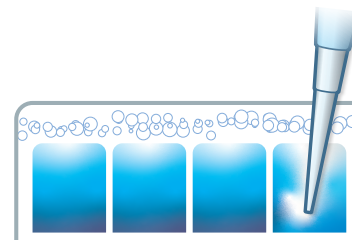
#### Before You Run the Gel

##### Dilute Ladder

Some pre-stained protein ladders fluoresce strongly and can interfere with detection of proteins expressed at a low level. Try diluting the ladder 1 to 10 before loading.

##### Optimize Sample Concentration

As with any new assay, quality results are dependent on first optimizing your assay for the samples to be used. Sample load volume may need to be adjusted for the best possible results. When first switching from chemiluminescent to fluorescent Westerns, try a slightly higher sample load volume to confirm that your assay is working. Once you have a positive result, you can lower the sample load volume and push for more sensitive results.

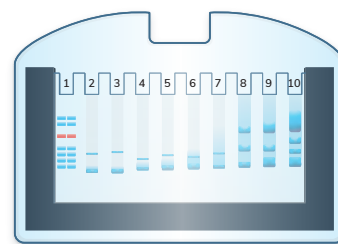


Make considerations for the protein ladder, sample concentrations, and loading dye.

### While Running the Gel

#### Be Wary of Loading Dye

Many loading dyes can fluoresce. To prevent this from interfering with the signal on a fluorescent blot, run the dye front off the gel or cut it off before transfer.



Before setting up the transfer, make sure to trim off the loading dye if possible to prevent it from interfering with your data.

### Transferring to the Membrane

#### PVDF Membranes

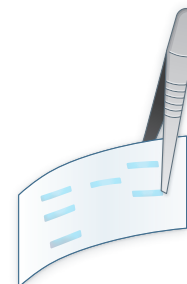
For the best signal-to-noise ratio, use a membrane with minimal autofluorescence such as Azure's low fluorescence PVDF membranes.

#### Membrane Handling

Any contamination to the membrane will be obvious in a fluorescent Western blot. Always use forceps when handling membranes.

#### Products

- Pre Cut PVDF (AC2108 – AC2109)



Use PVDF membranes and handle only with forceps.

### Probing the Membrane

#### Blocking

As with chemiluminescent blots, it is important to completely block the membrane to avoid nonspecific binding of antibodies and to reduce background. Azure Protein Free Blocking Buffer and Azure Fluorescent Blot Blocking Buffer are formulated to not only reduce background, but also to stabilize the fluorescent dyes of AzureSpectra secondary antibodies for enhanced signal detection.

#### Optimize Antibodies

Antibody concentration may need to be optimized and adjusted for the best possible results. When first switching from chemiluminescent to fluorescent Westerns, use the manufacturer's recommendations for antibody dilutions.

#### Incubation Trays

Fluorescent secondaries can become quenched when exposed to bright light for long periods of time. When incubating your sample with light sensitive antibodies, cover your blot with an opaque material (such as with Azure's opaque incubation trays) to protect from quenching.

#### Products

- Azure Protein Free Blot Blocking Buffer (AC2112)
- Azure Fluorescent Blot Blocking Buffer (AC2190)
- Fluorescent secondary antibodies (AC2128 – AC2139, AC2156 – AC2171)
- Opaque Incubation Trays (AC2120 – AC2123)

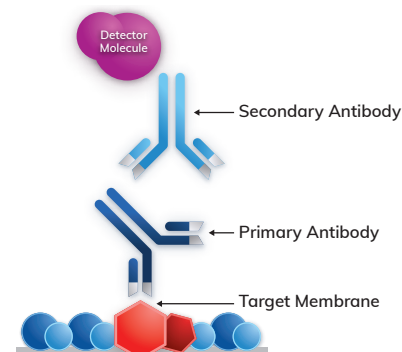


Diagram of the antigen-antibody and antibody-antibody interactions in Western blotting detection.



Tip: use opaque containers to protect the light-sensitive fluorophores during incubation.

## Washing

### Stringent Wash

Because the fluorescent dyes can adhere to the membrane, washing is extremely important to reduce background. Make sure to use a stringent wash, especially when working with near-infrared fluorophores. Azure Fluorescent Blot Wash Buffer is specially formulated for use with fluorescent Western blots.

### Wash Volume

The volume and duration of washing is also important to rid the membrane of any free dye and antibody. We recommend two quick rinses in 25mL wash buffer followed by three 5 minute washes in 25mL wash buffer.

### Final Rinse

Any detergent in the wash buffer can also fluoresce, so rinsing in PBS or TBS after the final washing step is essential to lower background signal.

### Products

- Azure Blot Washing Buffer (AC2113)
- Azure Fluorescent Blot Washing Buffer (AC2145)



To prevent background signals and get the clearest data, follow the recommended steps such as thorough washes after antibody incubation.

## Imaging

### Reduce Background Contamination

When many people use the same imager, the insides and trays can become contaminated. Background Quenching Sheets absorb background fluorescence to improve signal-to-noise ratios.

### Products

- Quenching sheets (AC2144, AC2147)



To prevent background fluorescence from interfering with your signal, use Azure's quenching sheets.

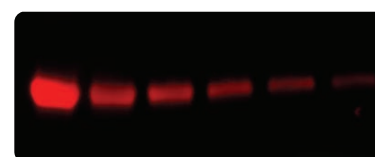
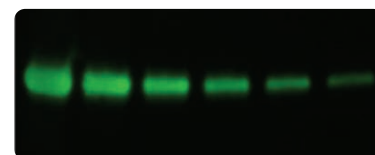
## Want some help getting started?

A demo kit is a great way to try out fluorescent Western blotting in your own lab with your own samples and primary antibodies. It contains everything you need to perform single color fluorescent Western blotting:

- PVDF Membrane
- Fluorescent Blot Wash Buffer
- Fluorescent Blot Blocking Buffer
- Secondary Antibody
- 2 Quenching Sheets

### AzureSpectra Demo Kits

- Goat  $\alpha$ -Mouse 700 (AC2172)
- Goat  $\alpha$ -Rabbit 700 (AC2173)
- Goat  $\alpha$ -Mouse 800 (AC2174)
- Goat  $\alpha$ -Rabbit 800 (AC2175)
- Goat  $\alpha$ -Mouse 650 (AC2176)
- Goat  $\alpha$ -Rabbit 650 (AC2177)
- Goat  $\alpha$ -Mouse 550 (AC2178)
- Goat  $\alpha$ -Rabbit 550 (AC2179)



### 3.5. Western Blot Normalization

#### Quantitative Westerns: What is the Best Way to Normalize Your Western blot?

Far from being an “is-it-there-or-not” technique, modern digital detection instruments can make Western blotting reproducible and quantitative. By working within the linear dynamic range of your detection method and normalizing the data to control for variations in protein load and membrane transfer, you can get truly quantitative results. But what is the best way to normalize a Western blot? In the past, the gold standard normalization method was to use a housekeeping protein based on the assumption that the levels of these proteins are fairly consistent across experimental conditions and cell lines. However more recent studies have shown that this assumption is not always true<sup>1,2</sup> leading to inaccurate measurements of relative protein abundance. Instead, quantitative Western blotting experts<sup>1,2</sup> and the journals they publish in<sup>4</sup> are recommending a new gold standard for normalization—normalizing to total protein detected in each lane, preferably by staining on the membrane.

#### Western Blot Normalization: Housekeeping Protein vs Total Protein

Normalization method	Housekeeping protein	Total protein
<b>Benefits</b>	<ul style="list-style-type: none"> <li>Familiar, commonly used</li> </ul>	<ul style="list-style-type: none"> <li>Large linear dynamic range</li> <li>Low variability</li> <li>Consistent across sample types</li> <li>No change with experimental conditions</li> </ul>
<b>Challenges</b>	<ul style="list-style-type: none"> <li>Narrow linear dynamic range</li> <li>Abundance can vary with experimental conditions</li> <li>Abundance may not be consistent between sample types</li> <li>High variability</li> <li>Must ensure housekeeping protein physically resolved from protein of interest on gel</li> </ul>	<ul style="list-style-type: none"> <li>Must ensure total protein stain used is compatible with antibody binding and detection method</li> </ul>

#### Using Housekeeping Proteins for Normalization

The most significant drawback of using housekeeping proteins is that their levels may not be consistent across samples and conditions.<sup>1,2</sup> It is possible to use a housekeeping protein for normalization, but you must first spend the time and effort to validate your choice, and may need to examine multiple potential standards before you find one that is truly expressed at the same level across all of your samples and does not change across your experimental conditions.

A second significant challenge associated with housekeeping proteins is their high abundance.<sup>1,3</sup> If the housekeeping protein is present at a very high level in your sample, this limits the amount of sample you can load on the gel because you will need to keep the housekeeping protein within the linear range of detection and not saturate the signal for the housekeeping protein. This is particularly problematic if the protein of interest is not similarly highly expressed because the two proteins will not be within the same linear range of detection.<sup>2,3</sup>

A third challenge to consider if you're doing multiplex Western blots—such as comparing phosphorylated and non-phosphorylated forms of the same protein—is the complexity of generating primary and secondary antibodies from non-overlapping species.

Finally, it is always possible that detecting the housekeeping protein could interfere with detection of the protein of interest.<sup>1</sup> Ideally, the housekeeping protein should be a different size than the protein of interest so the two proteins are spatially resolved on the blot. This becomes increasingly difficult when an experiment examines multiple proteins of interest on the same blot.



### Using Total Protein Staining for Normalization

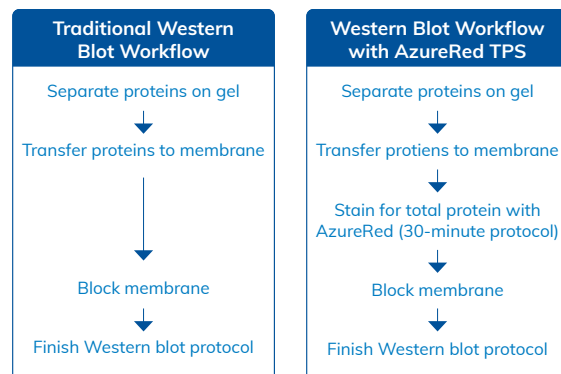
With total protein normalization, instead of trying to find a protein that can represent the total amount of sample that transferred to the membrane, total protein is measured on the membrane directly and this value is used as the denominator when normalizing.<sup>1-4</sup> Many total protein stains are available that can be used to stain gels and membranes.<sup>1</sup> Total protein stains provide a larger dynamic range and demonstrate lower variability and cleaner data than housekeeping proteins.<sup>1,2</sup>

Total protein normalization can be much faster than using a housekeeping protein, especially for chemiluminescent blots because the staining step takes less time than stripping and reprobing the blot. Ideally, total protein staining is conducted on the membrane, either before or after immunodetection.<sup>2</sup>

With some stains such as AzureRed Fluorescent Total Protein Stain, it is possible to stain the blot before immunodetection and then to image total protein simultaneously with the protein(s) of interest. With this simplest of workflows, images for the protein(s) of interest and total protein are automatically aligned, avoiding the need to resize and align images captured at different times.

The analysis workflow after image capture is essentially unchanged compared to using a housekeeping protein; the signal density for the entire lane or a large portion of the lane is used for normalization instead of the density for a single band.

Staining the membrane with a total protein stain provides an added quality control benefit, allowing verification that membrane transfer was complete and free of artifacts.



### Looking for More Information About Western Blot Normalization?

Please see the Western Blot Normalization application note to learn more about the different methods of Western blot normalization and to help decide which normalization approach is best for your blot. Read more about using AzureRed Fluorescent Total Protein Stain for total protein normalization of fluorescent Western blots in the Accurate Western Blot Normalization With AzureRed Fluorescent Protein Stain application note.

### References

1. Moritz CP. Tubulin or not tubulin: heading toward total protein staining as loading control in Western blots. *Proteomics*. 2017;17:1600189.
2. Thacker JS et al. Total protein or high-abundance protein: which offers the best loading control for Western blotting? *Anal Biochem*. 2016;496:76-78.
3. McDonough AA et al. Considerations when quantitating protein abundance by immunoblot. *Am J Cell Physiol*. 2015;308(6):C426-C433.
4. Fosang AJ, Colbran RJ. Transparency is the key to quality. *J Biol Chem*. 2015;209(50):29692-29694.

## 3.6. Troubleshooting

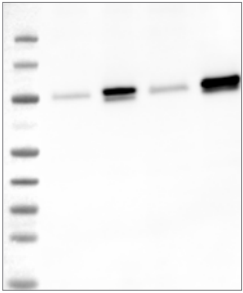
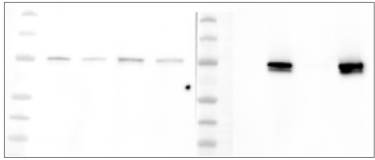

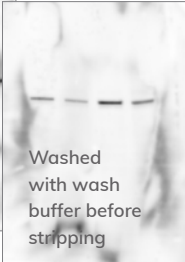
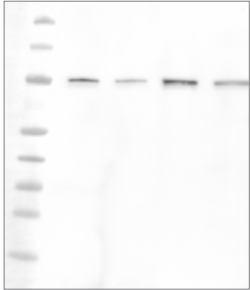
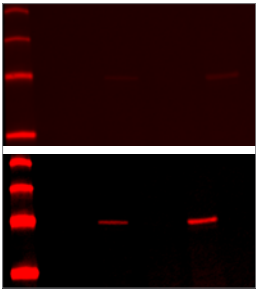
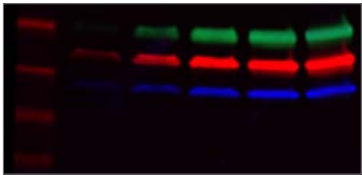
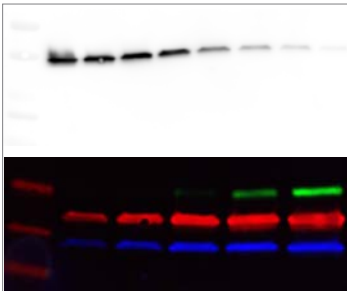

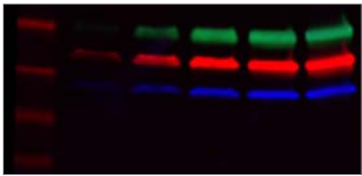
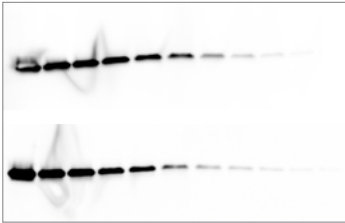

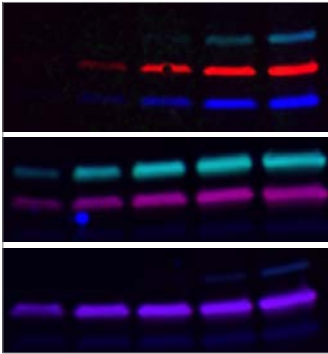
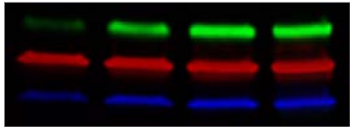
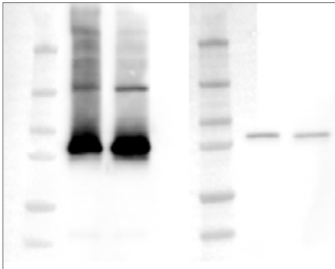
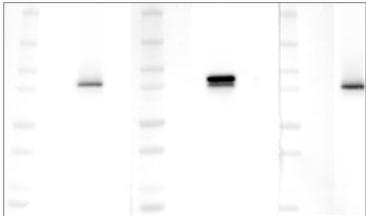
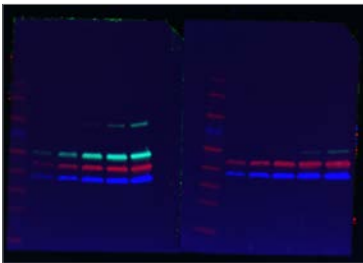
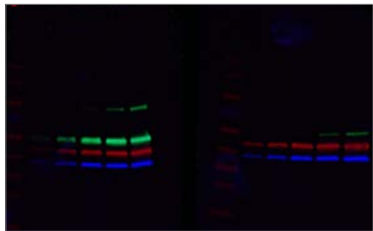
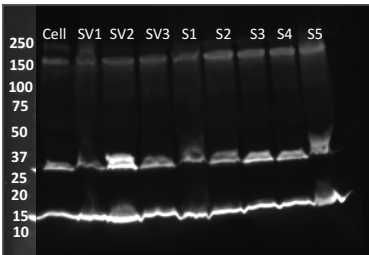
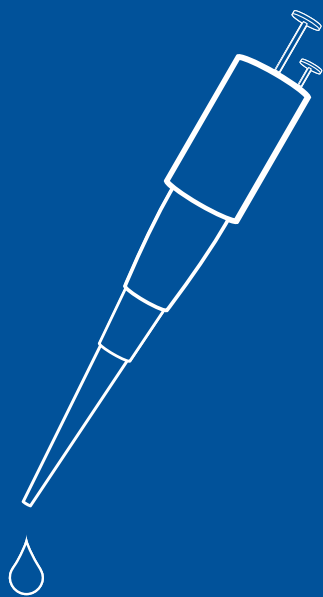
Image	The problem	The fix	Resulting images
	Membrane not fully stripped	<ul style="list-style-type: none"> <li>• Ensure adequate buffer volume and stripping time</li> </ul>	
 	Insufficient or incorrect wash prior to stripping	<ul style="list-style-type: none"> <li>• Rinse blots with ultra pure water for at least 5 minutes before stripping</li> <li>• Do not wash with wash buffer</li> </ul>	
	Marker too bright	<ul style="list-style-type: none"> <li>• Cover the marker before imaging</li> <li>• Allow blot to dry before imaging</li> <li>• Dilute marker before loading</li> </ul>	
 	Bubbles in transfer	<ul style="list-style-type: none"> <li>• Press and smooth filter paper after covering membrane to remove bubbles</li> <li>• Ensure adequate transfer buffer is present in cartridge</li> </ul>	

Image	The problem	The fix	Resulting images
	Insufficient or uneven transfer pressure	<ul style="list-style-type: none"> <li>• Transfer set up should be very tight</li> <li>• Place gel and membrane in the middle of the cassette</li> </ul>	
	Antibody cross-reactivity	<ul style="list-style-type: none"> <li>• Avoid antibodies from species that are too "close" (ex. rat and mouse)</li> <li>• Avoid secondaries for the animal in which another secondary was created (ie. If using one goat anti-rabbit, avoid using a donkey anti-goat in the same assay)</li> <li>• Block in between antibodies that might cross-react</li> </ul>	
	Poor quality antibody	<ul style="list-style-type: none"> <li>• Screen antibodies</li> <li>• Other causes: <ul style="list-style-type: none"> <li>• Non-specific binding</li> <li>• Too much antibody</li> <li>• Insufficient blocking</li> </ul> </li> </ul>	
	High background	<ul style="list-style-type: none"> <li>• Use low-fluorescence membrane</li> <li>• Use correct washing protocol and wash buffer</li> <li>• After washing rinse with water</li> <li>• Do not use excessive amounts of antibody</li> <li>• Dry the membrane before imaging</li> </ul>	
	Cellular debris in samples	<ul style="list-style-type: none"> <li>• Sonicate and filter</li> <li>• For DNA in samples, shear samples with syringe or add DNase</li> </ul>	



4

# PROTOCOLS



## 4.1. HRP/Chemiluminescent Blot Detection with Radiance Substrate

### Long Protocol for Catalog Numbers

- AC2100—Radiance Sample, sufficient for 140 cm<sup>2</sup> membrane
- AC2101—Radiance, 150 ml, sufficient for 1500 cm<sup>2</sup> membrane

### Additional Materials Required

- Electrophoresis apparatus and buffers for SDS-PAGE
- Tank and buffers for electrophoretic transfer of proteins from gel to membrane
- Nitrocellulose or PVDF membrane, cut to size of gel. All membrane products available from Azure (see the Related Products section) are compatible with Radiance.
- Washing buffer (PBS-T or TBS-T). For best results, use Azure Fluorescent Blot Washing Buffer (see the Related Products section)
- Blocking buffer
- Primary antibody compatible with your application
- Secondary antibody, conjugated to Horseradish peroxidase (HRP) corresponding to your primary antibody (see the Related Products section)
- CCD-based detection system, or film (see the Related Products section)

### Background

Radiance chemiluminescent horseradish peroxidase (HRP) substrate is specially formulated for CCD imaging and to provide excellent performance with Azure Imaging Systems. Radiance produces a strong, long-lasting signal, which, combined with very low background levels, allows for long exposure times enabling the detection of lowabundance proteins. Additionally, the signal from Radiance is linear with respect to protein amount over a broad range of concentrations, displaying no substrate depletion at high protein loads, allowing the user to take full advantage of the linear range of the CCD detection method. Radiance is also compatible with X-ray film detection, though the limited dynamic range of film will make resulting data less quantitative.

Since 1988, enhanced chemiluminescence or ECL (1) has become one of the most common detection methods in Western blotting (2). In this method, the secondary antibody is conjugated to the enzyme Horseradish peroxidase (1,2). Once bound to the membrane, the secondary antibody is detected by incubating the blot with a solution containing an HRP substrate that generates a light-emitting product after reaction with HRP (Figures 4.1, 4.2). The chemiluminescent signal can be detected by exposing the blot to X-ray film, or by imaging with a CCD camera. Radiance is an enhanced chemiluminescent substrate specially developed for CCD imaging. Radiance produces a bright signal with very low background for extremely high sensitivity and a detection limit of attomoles of protein. Additionally, the Radiance signal is long lasting, which combined with the low background, allows long-term exposures to detect low-abundance proteins.

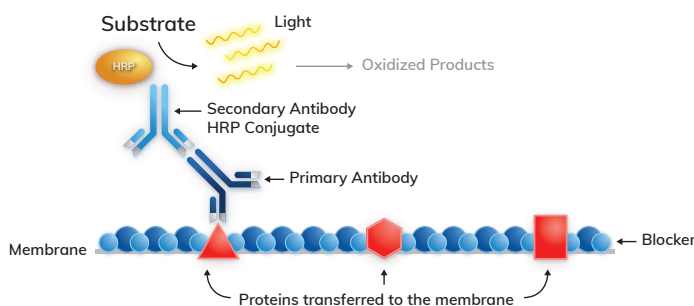


Figure 4.1. The principle of chemiluminescent Western blotting.

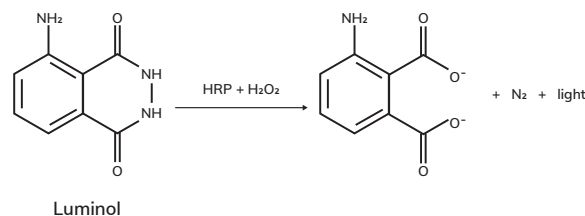
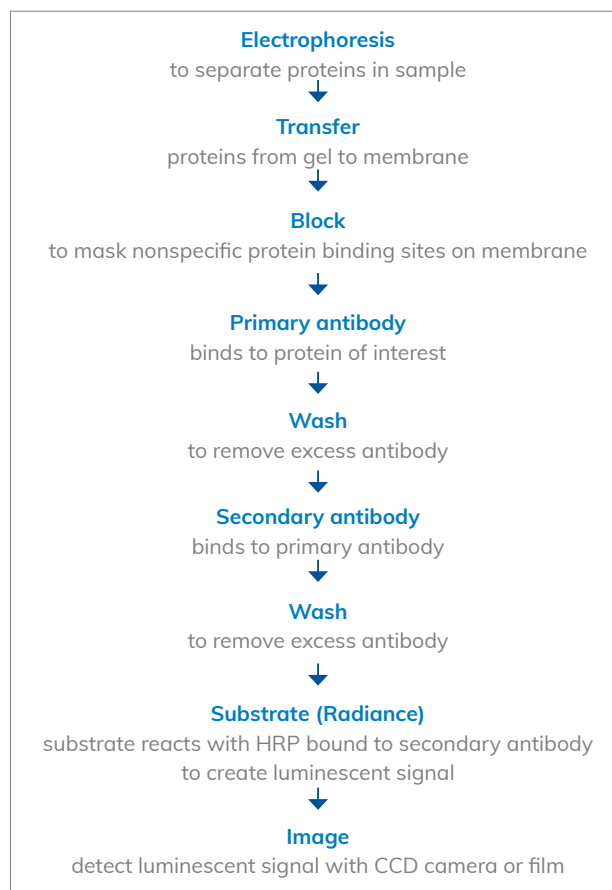


Figure 4.2. Chemiluminescence of luminol.

## Overview of the Protocol for Chemiluminescent Western Blots



### Quick Protocol

For additional information, see the detailed protocol which follows.

Step	User Notes
1. Prepare your protein blot	
2. Block membrane for 1 hour at room temperature (RT)	
3. Incubate blot with primary antibody for one hour at RT with gentle agitation	
4. Wash blot: <ul style="list-style-type: none"> <li>• 1 x quickly</li> <li>• 1 x 15 min, with 1 ml/cm<sup>2</sup> membrane</li> <li>• 3 x 5 min, with at least 0.5 ml/cm<sup>2</sup> membrane each time</li> </ul>	
5. Incubate blot with secondary antibody for one hour at RT with gentle agitation	
6. Wash blot: <ul style="list-style-type: none"> <li>• 3 x 5 min, with at least 0.5 ml/cm<sup>2</sup> membrane each time</li> </ul>	
7. Mix Radiance components 1:1 and place 0.1 ml/cm <sup>2</sup> on blot for 2 minutes	
8. Drain excess reagent	
9. Cover damp blot with plastic wrap and image by CCD camera or exposure to X-ray film	

## Detailed Protocol

Step	Notes
<b>1. Prepare a protein blot</b>	
1.1. Separate the protein sample(s) via electrophoresis	<ul style="list-style-type: none"> <li>Any electrophoresis system and buffer, such as Laemmli, is compatible with Radiance.</li> <li>A wet or tank transfer method is preferred, though semi-dry methods should also be compatible. We have found that the buffer system developed by Bolt et al (3) works well.</li> </ul>
1.2. Transfer proteins to membrane	<ul style="list-style-type: none"> <li>Both nitrocellulose and PVDF membranes are compatible with Radiance.</li> <li>If using PVDF, first wet membrane with a 1 min incubation in 100% MeOH followed by water for ~5 min and then transfer buffer for 5-10 min.</li> <li>For slot blot applications, nitrocellulose is much more convenient than PVDF because it is more difficult to avoid bubbles with PVDF.</li> </ul>
<b>2. Block membrane</b>	
<ul style="list-style-type: none"> <li>Incubate the blot in a blocking buffer with gentle agitation for 1 hour at room temperature (RT). Use 0.2 to 0.5 ml of blocking buffer per cm<sup>2</sup> of blot to provide adequate blocking.</li> </ul>	<ul style="list-style-type: none"> <li>Blocking masks non-specific protein binding sites on the membrane, reducing background and increasing the specificity of binding of the primary antibody to the protein of interest.</li> <li>The optimal blocking buffer will depend in part on the nature of the antigen of interest, and on the quality of the primary antibody. Common blocking agents including non-fat dry milk have been found to be compatible with Radiance.</li> <li>10 to 20 ml is usually sufficient for a typical 7 x 9 cm mini-blot.</li> </ul>
<b>3. Incubate blot with primary antibody</b>	
<ul style="list-style-type: none"> <li>Dilute primary antibody in blocking buffer.</li> <li>Incubate blot with primary antibody solution for 1 hour at RT with gentle agitation.</li> </ul>	<ul style="list-style-type: none"> <li>Optimal primary antibody dilutions must be determined empirically.</li> <li>For CCD imaging, we recommend primary antibody dilutions from 1:1000 to 1:10,000. A good initial dilution is 1:5000.</li> <li>If the blot will be imaged on film, use 2-5x less primary antibody than for CCD imaging. For example, if 1:1000 dilution of the primary antibody was optimal for CCD detection, 1:5000 is suitable for film detection.</li> <li>Antibody can be added to a dish and placed on a shaker, or a smaller volume (5-10 ml) can be used by sealing the blot into a bag and placing it on a rotary platform.</li> </ul>
<b>4. Wash blot to remove excess primary antibody</b>	
<ul style="list-style-type: none"> <li>1 x quickly</li> <li>1 x 15 min, with 0.7 ml/cm membrane</li> <li>3 x 5 min, with at least 0.3 ml/cm<sup>2</sup> membrane each time.</li> </ul>	<ul style="list-style-type: none"> <li>For best results, use Azure Fluorescent Blot Washing Buffer (AC2113) which is optimized for chemiluminescent as well as fluorescent blots. PBS-T or TBS-T are also compatible with Radiance.</li> <li>We recommend washing or rocking blots in a clean dish on a shaker to provide gentle agitation.</li> <li>For example, a standard 7x9 membrane requires: ~50 ml of washing solution for the 15 min wash; and ~20 ml of washing solution for 5 min washes.</li> </ul>
<b>5. Incubate blot with secondary antibody</b>	
<ul style="list-style-type: none"> <li>Dilute secondary antibody in blocking buffer.</li> <li>Incubate blot with secondary antibody solution for 1 hour at RT with gentle agitation.</li> </ul>	<ul style="list-style-type: none"> <li>Optimal secondary antibody dilutions must be determined empirically.</li> <li>We recommend secondary antibody dilutions of 1:5,000 to 1:20,000. A good initial dilution is 1:10,000.</li> <li>If the blot will be imaged on film, use 2-5x less secondary antibody than for CCD imaging. 1:50,000 dilution is a good starting point for film detection.</li> <li>See also notes for step 3.</li> </ul>
<b>6. Wash blot to remove excess secondary antibody</b>	
<ul style="list-style-type: none"> <li>3 x 5 min, with at least 0.3 ml/cm<sup>2</sup> membrane each time.</li> </ul>	<ul style="list-style-type: none"> <li>See notes for step 4.</li> </ul>



## 7. Incubate blot with Radiance

- Mix components 1 and 2 in a 1:1 ratio in sufficient amounts to obtain at least 0.1 ml/cm<sup>2</sup> of the blot and add to the blot.
- It is better to prepare the working solution just before use. However, mixed reagent is stable for several hours at RT.
- Allow substrate to react with blot for 2 minutes.
- Be careful not to touch or put pressure on the blot as this can result in non-specific background.
- Use only plastic forceps, not metal; metal forceps damage the blocked surface, creating new adsorption sites. Also, traces of metal may act as a catalyst for non-enzymatic substrate oxidation, resulting in very high background.
- The minimal amount of working reagent is 0.1 ml/cm<sup>2</sup>. For example, for a 7 x 9 cm blot, this minimal volume is 7x9x0.1=6.3 ml.
- If using the minimal amount of working reagent, incubation may be done without agitation. Make sure the membrane surface is level so adequate reagent is held by surface tension.
- Incubation may also be done with gentle agitation in a tray just slightly larger than the membrane. Increase the reagent volume as necessary to ensure the membrane is adequately covered with reagent.

## 8. Drain excess reagent

- Remove excess substrate via capillary action by touching a KimWipe® or other absorbent material to the edge of the blot.

## 9. Image blot

- While blot is damp, cover with transparent plastic wrap and either place blot in CCD imager, or expose blot to film.
- We recommend trying three exposures; 30 sec, 2 min, and 5 min.
- The blot can be imaged and re-imaged for several hours; 70% of the initial signal will remain after 60 minutes, and substantial signal will remain after 8-10 hours.

## Troubleshooting & FAQ

Western blotting can require substantial optimization due to the multiple steps involved. The correct amount of protein to load on the gel and the best dilutions of primary and secondary antibodies must be determined empirically. Some common questions are addressed below:

Problem	Possible Solutions
High background	<ul style="list-style-type: none"> <li>• Reduce primary antibody concentration by increasing the dilution factor.</li> <li>• Try a different blocking buffer.</li> <li>• Try a shorter exposure time.</li> <li>• Increase washing time.</li> </ul>
No or low signal	<ul style="list-style-type: none"> <li>• Check that correct primary antibody used.</li> <li>• Check that secondary antibody recognizes primary (for example if the primary is a rabbit antibody, that the secondary is goat-anti-rabbit).</li> </ul>
White spots within bands	<ul style="list-style-type: none"> <li>• Improve transfer, making sure to remove any bubbles between the gel and the membrane.</li> </ul>
Speckled background	<ul style="list-style-type: none"> <li>• Filter secondary antibody.</li> <li>• Filter blocking and washing buffers.</li> <li>• Ensure that the laboratory environment is clean, to minimize dust, debris or any other particles that might come in contact with the blot. Cover the dish during incubation or washing steps.</li> <li>• Use non-powdered gloves, or switch to a different kind of gloves. We recommend powder-free nitrile gloves or polyethylene gloves.</li> </ul>

## References

1. Thorpe GH, Kricka LJ, Moseley SB, Whitehead TP, Phenols as enhancers of the chemiluminescent horseradish peroxidase-luminol-hydrogen peroxide reaction: application in luminescence-monitored enzyme immunoassays. *Clin Chem*. 1985 Aug; 31(8): 1335-41.
2. Leong MM, Fox GR., Enhancement of luminol-based immunodot and Western blotting assays by iodophenol. *Anal Biochem*. 1988 Jul; 172(1): 145-50.
3. Bolt M.W., Mahoney P.A., High-efficiency blotting of proteins of diverse sizes following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal Biochem*. 1997 May 1; 247(2): 185-192.

## Related Products

Catalog Number	Product	Size
AC2113	Azure Fluorescent Blot Washing Buffer	500 ml
AC2114	Goat-anti-rabbit HRP-conjugated secondary antibody	500 µl
AC2115	Goat-anti-mouse HRP-conjugated secondary antibody	500 µl
AC2105	Low Fluorescence Western Membrane (PVDF) 7x9 cm	10 sheets
AC2106	Nitrocellulose Transfer Membrane 0.45 µm 7x9 cm	10 sheets
AC2107	Nitrocellulose Transfer Membrane 0.22 µm 7x9 cm	10 sheets

## 4.2. Fluorescent Western Blot Protocol with AzureSpectra Reagents

Quantitative, multi-color and near-IR fluorescent Western blotting kits.

### Long Protocol for Catalog Numbers

- AC2193—AzureSpectra rb650/ms550 Kit
- AC2191—AzureSpectra IR rb700/ms800 Kit
- AC2192—AzureSpectra IR ms700/rb800 Kit

#### All kits

- Low-fluorescence PVDF transfer membrane 9x7 cm. 10 each
- Background Quenching Sheets, 10 each
- Azure Fluorescent Blot Blocking Buffer, 1x ready-to-use solution, 300 ml
- Azure Fluorescent Blot Washing Buffer, 10x concentrate, 250 ml

#### For AC2193

- Goat-anti-rabbit IgG 650, 40 µl
- Goat-anti-mouse IgG 550, 40 µl

#### For AC2191

- Goat-anti-rabbit IgG IR700, 40 µl
- Goat-anti-mouse IgG IR800, 40 µl

#### For AC2192

- Goat-anti-mouse IgG IR700, 40 µl
- Goat-anti-rabbit IgG IR800, 40 µl

### Shipping and Storage Conditions

Before opening, the kit may be stored at +4°C. AzureSpectra fluorescent secondary antibody conjugates may be stored at +4°C for up to a month. For longer term storage, store antibody conjugates at -20°C. Gently mix and briefly spin the tubes before taking aliquots.

Azure Fluorescent Blot Blocking Buffer must be stored at +4°C. Azure Fluorescent Blot Washing Solution can be stored between 4°C and 25°C. Do not dilute excessive amounts of the concentrates to the final working concentration. Prepare only as much as you need for each assay. Store PVDF membranes at ambient temperature in a sealed bag protected from light and moisture. Store background quenching sheets in a sealed bag at ambient temperature.

### Additional Materials Required

- Primary antibodies: mouse and/or rabbit IgG.
- Electrophoresis apparatus, power supply, gels and buffers for standard Laemmli SDS-PAGE.
- Electro-blotting apparatus and transfer buffer (see Reference 4).
- Methanol.
- 1x PBS or TBS without Tween.
- Forceps with flat smooth tips. Plastic (non-metal) forceps are strongly recommended.
- Powder-free gloves compatible with fluorescent applications. Polyethylene gloves are strongly recommended.
- Incubation and washing trays with smooth interiors such as those provided by Azure (product numbers AC2120, AC2123, AC2150 and AC2153).
- Rotary or rocking platform – a rocking platform is preferred as mixing with a rocking action typically generates more uniform background than orbital shaking.
- AzureSpectra rb650/ms550 Kit (AC2193): Fluorescent imager compatible with dyes excitable in green (530nm – 560nm) and red (600nm – 650nm) light. Further details are discussed in the “Imaging” section.

- AzureSpectra IR rb700/ms800 and ms700/rb800 Kits (AC2191 and AC2192): an imager compatible with dyes excitable in far-red (630nm – 700nm) and near infrared (750nm – 800nm) light. Further details are discussed in the “Imaging” section.

### Introduction to Multi-Color Fluorescent Western Blotting

Western blotting provides a means to assay the presence and the expression level of a protein of interest in a complex mixture. Proteins are separated electrophoretically, transferred to a membrane substrate, and the protein of interest is detected with specific antibodies<sup>(1, 2)</sup>. The antibody specific to the protein of interest can be directly labeled, for example, using radioactivity, or can be detected by the use of a labeled secondary antibody. Frequently, a secondary antibody conjugated to horseradish peroxidase (HRP) is used, and the location of the protein is detected via chemiluminescence<sup>(3)</sup>.

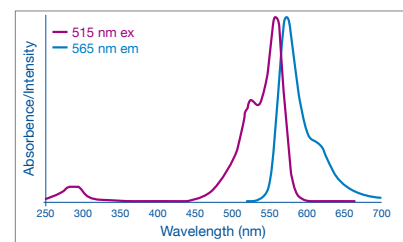
When using chemiluminescence, only one protein can be detected per blot. Assaying for a second protein requires stripping and re-probing the blot, a time consuming procedure. Imaging a Western blot using fluorescence allows for multiple proteins to be assayed on one blot by using secondary antibodies labeled with fluorophores having unique excitation and emission spectra<sup>(3)</sup>.

The AzureSpectra rb650/ms550 Kit and AzureSpectra IR rb700/ms800 and ms700/rb800 Kits provide the means to assay two proteins on a single Western blot. Each kit contains two secondary antibody conjugates, each pre-labeled with a different fluorescent reporter. Fluorescent reporters utilized in the AzureSpectra rb650/ms550 Kit are excitable in the visible fluorescence range. The excitation and emission spectra of the AzureSpectra 550 and 650 conjugates are shown in Figures 4.3 and 4.4. Fluorescent reporters used in the AzureSpectra rb700/ms800 and ms700/rb800 Kits are excitable by far-red and near-infrared light. The excitation and emission spectra of the AzureSpectra NIR conjugates are shown in Figures 4.5 and 4.6.

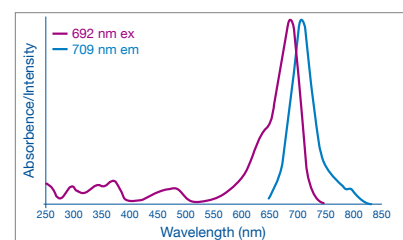
Until recently fluorescent reporters were not widely used for Western blotting applications for various reasons. Membranes typically used for Western blotting had high levels of autofluorescence, appropriate imaging instruments were not readily available, and the time required to acquire an image of a typical blot was relatively long. The most important factor that prevented the use of some fluorescent detector molecules in Western blotting applications was that these proteins need to remain hydrated to maintain their high levels of fluorescence. Western blot membranes can dry quickly and lose the water necessary to sustain fluorescence, and, unfortunately, re-hydrating the membrane does not restore the fluorescence of certain detector fluorophores.

Today, all the above problems can be easily addressed. PVDF membranes with low autofluorescence have become available, and there are several choices of fast and high resolution fluorescent imaging instruments including laser-based scanners and LED-based gel documentation imagers. The most difficult problem is to preserve the hydration of fluorescent detector molecules long enough to be able to image the blot within a reasonable period of time.

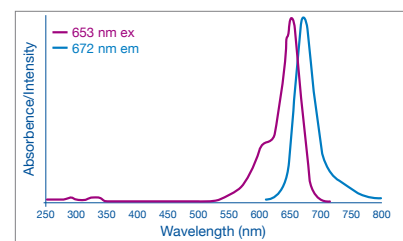
We have developed the Azure Protein Free Blocking Buffer and Azure Fluorescent Blot Blocking Buffer to address this problem. The blocking solution provided with the kit contains a component that provides an efficient blocking of the PVDF membrane from non-specific protein binding. At the same time,



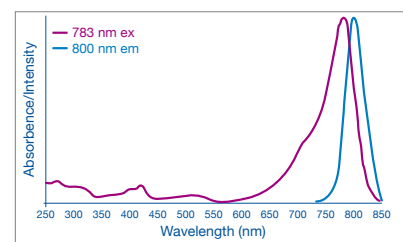
**Figure 4.3.** Absorption and emission spectra of AzureSpectra 550 goat-anti-mouse IgG antibody conjugate (AC2159)



**Figure 4.4.** Absorption and emission spectra of AzureSpectra 700 IgG antibody conjugate (AC2128 and AC2129)



**Figure 4.5.** Absorption and emission spectra of AzureSpectra 650 goat-anti-rabbit IgG antibody conjugate (AC2165)

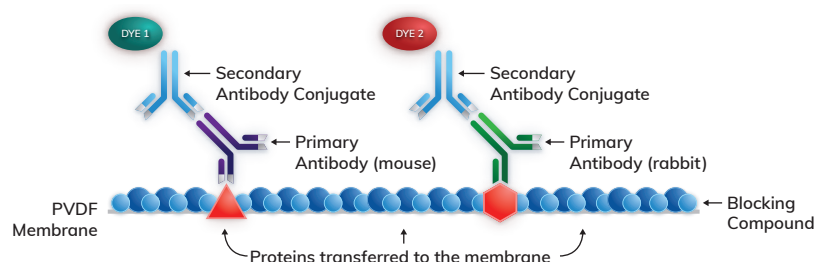


**Figure 4.6.** Absorption and emission spectra of AzureSpectra 800 IgG antibody conjugate (AC2135 and AC2136)

it preserves the hydrated environment in the membrane sufficiently enough to protect the hydration of the proteins and stabilize their fluorescence. This stabilization effect preserves most of the fluorescent intensity even after the membrane appears completely dry. The remaining fluorescence is only 20 to 40% lower than its peak value and is sufficient to reliably detect sub-nanogram amounts of a protein of interest. When using high quality primary antibodies and optimized assay conditions, single picogram detection can routinely be achieved.

The secondary antibody conjugates provided in the AzureSpectra rb650/ms550 Western Kit and AzureSpectra IR rb700/ms800 and ms700/rb800 Kits are labeled anti-mouse IgG and anti-rabbit IgG conjugates. Therefore, the AzureSpectra kits are appropriate for experiments incorporating most readily available primary antibodies, raised in mouse and/or rabbit.

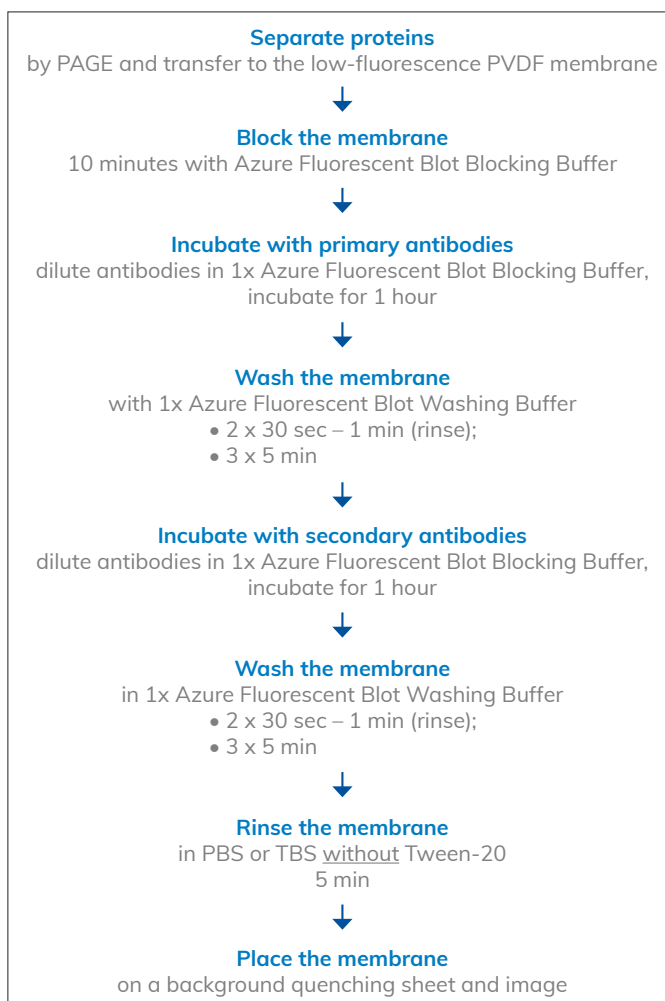
A schematic representation of the two-color Western blot is shown in Fig. 4.7.



**Figure 4.7. Schematic diagram of the two-color assay utilized in the AzureSpectra Western Kits.**

## Overview of the Protocol for Fluorescent Western Blots

### Quick Protocol



For additional information, see the detailed protocol which follows.

## Detailed Protocol

Step	User Notes
1. Prepare your protein blot.	
2. Block membrane for 10 minutes at room temperature (RT).	
3. Incubate blot with primary antibody for one hour at RT with gentle agitation.	
4. Wash blot with 1x washing buffer: <ul style="list-style-type: none"> <li>• 2 x quickly</li> <li>• 3 x 5 min with 25 ml each time.</li> </ul>	
5. Incubate blot with secondary antibody for one hour at RT with gentle agitation.	
6. Wash blot with 1x washing buffer: <ul style="list-style-type: none"> <li>• 3 x 5 min with 25 ml wash buffer each time</li> <li>• 1 x 5 min with 20-50 ml PBS or TBS without detergent.</li> </ul>	
7. Place blot on background quenching sheet and drain excess liquid.	
8. Image using CCD camera.	

## Imaging

Step	Notes
<b>1. Protein separation and transfer</b>	
<ol style="list-style-type: none"><li>1.1. Separate protein samples by polyacrylamide gel electrophoresis using a mini-sized gel (i.e. 8 x 10 cm).</li><li>1.2. Cut a small notch from one corner of the membrane to help properly identify the orientation and the surface side of the membrane that will contain the transferred proteins.</li><li>1.3. Pre-wet the membrane in methanol for a few moments until the membrane is completely wet.</li><li>1.4. Transfer the wet membrane from methanol to purified water (Milli-Q quality or distilled) and incubate on a rocker or shaker for at least 5 minutes. Make sure that the membrane is fully immersed in water and does not float on the water's surface.</li><li>1.5. Transfer the membrane from water to transfer buffer, and incubate for 5 minutes.</li><li>1.6. Assemble the transfer sandwich as required for your transfer apparatus.</li><li>1.7 Perform the transfer overnight at 15 V, or for 1 to 2 hours at 70 V with an ice pack.</li></ol>	<ul style="list-style-type: none"><li>• A semi-dry transfer does not work well for fluorescent applications as it typically generates relatively high and non-uniform background, especially in the channel excited with green light. We currently suggest using a tank transfer method for fluorescent applications.</li><li>• Increasing the transfer time, especially if performed at 70 V, causes an increase in background. Increasing voltage beyond 70 V to shorten the transfer time also causes an increased and non-uniform background, especially in the channel excited with red light.</li><li>• For a fast transfer method that works well for fluorescent blots, we recommend Azure Transfer Buffer (available from Azure, product number AC2127). When using Azure Transfer Buffer, the transfer should be carried out for 15 to 30 minutes at 12 V/cm.</li></ul>
<b>2. Membrane blocking and binding of the primary antibodies</b>	
<ol style="list-style-type: none"><li>2.1. Once the transfer is completed (step 1.7), remove the membrane from the sandwich and place it into a tray with Milli-Q or distilled water for 5 minutes.</li></ol>	<ul style="list-style-type: none"><li>• All incubations and washes are performed on a rocker or shaker with gentle agitation at room temperature. A rocker is preferred over a shaker. In our experience, a rocking-action mixing generates more uniform background than orbital-shaker mixing.</li></ul>

- 2.2. Transfer 10 ml of 1x Azure Fluorescent Blot Blocking Buffer to an incubation tray. Make sure that the tray is of an appropriate size for your membrane.
  - 2.3. Transfer the membrane from water into Azure Fluorescent Blot Blocking Buffer and block the membrane for 10 minutes with gentle agitation.
  - 2.4. While the membrane is in the Azure Fluorescent Blot Blocking Buffer, prepare the incubation mixture containing your primary antibody or an appropriate mixture of primary antibodies diluted in 10 ml 1 x Azure Fluorescent Blot Blocking Buffer. Gently mix the incubation solution by inverting the tube several times. Do NOT vortex.
  - 2.5. After the blocking is completed (step 2.3), remove the solution from the tray and add the solution of primary antibodies to the membrane.
  - 2.6. Incubate the membrane with the solution of primary antibodies for 1 hour at room temperature with gentle agitation.
- Incubation of the membrane in water prior to blocking improves retention of transferred proteins.
  - It is very important that the side of the membrane containing transferred proteins is facing up during all incubation and washing steps.
  - The optimum dilution factor for primary antibodies must be determined experimentally by performing a titration experiment. A good starting point is the dilution factor suggested by the antibody supplier. In our experience, the optimal concentrations of primary antibodies are often lower, and in many cases significantly lower than those suggested for common colorimetric Western blotting procedures. Higher concentrations of primary antibodies often do not result in any higher specific fluorescent signals. Instead, higher concentrations of primary antibodies cause high background and appearance of many non-specific bands.

### 3. Washing the membrane

- 3.1. About 10 minutes before the end of the incubation step, prepare 300 ml of 1x Azure Fluorescent Blot Washing Buffer. Mix 30 ml of the Azure Fluorescent Blot Washing Buffer concentrate provided in the kit with 270 ml of purified water (Milli-Q quality or distilled). This amount is sufficient to complete the experiment.
  - 3.2. Add 25 ml of the Azure Fluorescent Blot Washing Buffer to a new tray.
  - 3.3. After the incubation (step 2.6) is completed, transfer the membrane from the incubation tray into the tray with the Azure Fluorescent Blot Washing Buffer prepared in step 3.2. Use clean forceps to transfer the membrane. Make sure that the orientation notch is in the same position so that the side of the membrane containing the transferred proteins is facing up.
  - 3.4. Rinse the membrane twice for 30 seconds to 1 minute each time with gentle agitation.
  - 3.5. Remove the Azure Fluorescent Blot Washing Buffer completely or transfer the membrane into a new clean tray.
  - 3.6. Wash the membrane 3 more times for 5 minutes each with gentle agitation, replacing with 25 ml of fresh Azure Fluorescent Blot Washing Buffer each time.
- Rinsing step 3.4 is more efficient if done manually instead of using a rocker or shaker. Make sure that the membrane is rinsed uniformly to remove the majority of the excess primary antibody incubation solution. However, all agitations must be done gently to prevent any scratching or damage to the surface of the membrane. Scratches or other damages to the blocked surface of the membrane create active sites that can adsorb fluorescent secondary antibody conjugates and cause fluorescent artifacts and/or high background.

### 4. Probing the membrane with the AzureSpectra secondary antibody fluorescent conjugates

- 4.1. During the last washing step, prepare the mixture of secondary antibody fluorescent conjugates. Transfer 10 ml of the Azure Fluorescent Blot Blocking Buffer into a new 15 ml tube. Add 4 µl each of the labeled conjugates provided in the kit (dilution factor 1:2500). If using an AzureSpectra NIR Kit, 1 µl each of the IR700- and IR800-labeled conjugates provided in the kit (dilution factor 1:10,000) may be used.
  - 4.2. After the last washing step is completed, remove the Azure Fluorescent Blot Washing Buffer and add the secondary conjugate mix to the tray with the membrane.
  - 4.3. Incubate the membrane with the secondary antibody conjugate mixture for 1 hour at room temperature with gentle agitation.
- When diluting the secondary antibody conjugates, gently mix by inverting the tube several times. Do NOT vortex.



## 5. Final washing

- 5.1. Remove the incubation solution and wash the membrane as in step 3.4–3.7 using Azure Fluorescent Blot Washing Buffer with gentle agitation.
  - 5.2. Remove the Azure Fluorescent Blot Washing Buffer and add 20–50 ml of PBS or TBS that does NOT contain Tween-20 or any other detergents and wash the membrane for 5 minutes with gentle agitation.
  - 5.3. Remove the membrane from the solution with clean forceps and transfer onto a black background quenching sheet and allow the excess liquid to run off the membrane by holding the plastic sheet with the membrane vertically.
- The purpose of step 5.2 is to remove from the membrane Tween-20 that is present in Azure Fluorescent Blot Washing Buffer. Removal of Tween-20 stabilizes the structure of the AzureSpectra conjugates and preserves the fluorescence when the membrane dries.
  - The membrane will not fall off the plastic sheet as it adheres to it while still wet. Do not use any wipes or tissue to remove the excess liquid as this may increase the background and introduce random fluorescent artifacts. Minor liquid excess does not affect the results.

The membrane can be imaged immediately after the final wash step. For blots conducted with AzureSpectra rb650/ms550 Western Kit, the highest sensitivity is achieved when the membrane is dry. This typically takes between 15 and 30 minutes and the membrane changes its appearance from translucent to uniform white. After this moment the fluorescence remains unchanged or increases slightly within 30 minutes to 1 hour depending on the ambient temperature and relative humidity. After two hours the fluorescence may decrease 20 to 40% depending on its initial intensity and on how well Tween-20 was removed from the blot during the last washing step. Blots conducted with AzureSpectra IR rb700/ms800 and ms700/rb800 Kits can be imaged wet or dry.

Review the absorption and emission spectra of the AzureSpectra conjugates included in your kit (Fig. 1 through 4) to choose appropriate setting of your imaging instrument. In most cases the settings appropriate for imaging Cy3 and Cy5 fluorescent dyes will work well for imaging the AzureSpectra 550 and 650 conjugates, and settings appropriate for imaging IR680 and IR800 fluorescent dyes will work well for the AzureSpectra IR700 and IR800 conjugates respectively.

## Troubleshooting & FAQ

Western blotting can require substantial optimization due to the multiple steps involved. The correct amount of protein to load on the gel and the best dilutions of primary and secondary antibodies must be determined empirically. Some common questions are addressed below:

## References

Question	Answer
What are your recommendations for primary antibody dilution?	Follow the manufacturer's recommendations for the antibody dilution. Typical antibody dilutions for primary antibodies range from 1:250 to 1:5000. High concentrations of primary and secondary antibodies can result in high background levels on the blot.
Can we use nitrocellulose membranes?	Nitrocellulose membranes have very high autofluorescence that makes it impossible or very difficult to detect low amounts of proteins. Furthermore, only special "low fluorescence" PVDF membranes are suitable for high sensitivity fluorescent Western blotting applications. In our experience, the best performing type of membrane is the one provided with the kit. If you require a different size membrane, you can use Immobilon-FL from Millipore (Catalog No. IPFL00010).
What kinds of transfer methods are acceptable for use with the kit?	We currently suggest using a standard tank transfer method for fluorescent Western blotting applications. Semi-dry transfer systems do not work well for fluorescent applications as they typically generate relatively high and non-uniform background, especially in the channel excited with green light. Azure's Azure Transfer Buffer can be used for a quick wet transfer.
Can we dry the blot and image [n] days after we do the Western assay?	The best performance for the blot is attained within 2 hours of drying it. In order to preserve the fluorescence on the dried blot when using AzureSpectra rb650/ms550 Western Kit, it is important that Tween-20 is completely removed. See step 5.2 for details. If necessary, step 5.2 may be repeated one more time. The membrane can be dried after this point. However, lower protein signal levels are expected with prolonged storage.

Can we use the blocking solution and/or wash solution we typically use with our current blotting system?	We advise against using alternate blocking or washing solutions. Alternate components may result in a higher background or reduced sensitivity. When the membrane is dried after using other blocking solutions, the fluorescent intensity and stability of fluorophores may be reduced to a greater extent. If you intend to use your blocker, we suggest using Azure Protein Free Blocking Buffer or Azure Fluorescent Blot Blocking Buffer for membrane blocking (step 2.4), and using your specific blocker to prepare the primary antibody incubation solution (step 2.5).
We do not detect signal on the blot.	Check to see if the transfer was successful by using a protein standard; if this is positive, check the imaging system to confirm the correct excitation and emission settings. Typically, imaging channels optimized for Cy3 and Cy5 dyes work well with the AzureSpectra 550 and 650 conjugates and settings for IR680 and IR800 dyes for AzureSpectra 700 and 800 conjugates. If you are trying to detect small amounts of a target protein, try to increase the concentration of your primary antibody first. If this is unsuccessful, also increase the concentration of the secondary conjugates.
Our lab generally uses Tris/Phosphate based buffers; will this work with the kit?	We suggest using the Blocking and Washing solutions supplied with the kit to guarantee best performance. However if you require different buffer conditions, test a small blot before using larger quantities of kit components. However, if you do not use the Azure Protein Free Blocking Buffer or Azure Fluorescent Blot Blocking Buffer reagent, you may lose the benefits of its protective effect on the fluorescence stability when your membrane dries.

1. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A*. 1979; 76 (9): 4350-4354.
2. Burnette WN. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem*. 1981; 112(2):195-203.
3. Patton, Wayne F. A thousand points of light: The application of fluorescence detection technologies to two-dimensional gel electrophoresis and proteomics. *Electrophoresis*. 2000; 21: 1123-1144.

### 4.3. Azure HRP Stripping Buffer

Stripping buffer for chemiluminescent Western blots.

#### Short Protocol for Catalog Number

- AC2154—HRP Stripping Buffer

#### Storage Information

Azure HRP Stripping Buffer is stable at room temperature (4°C-25°C) for at least one year.

#### Warnings and Precautions

- Azure HRP Stripping Buffer is for research use only.
- Always wear gloves when handling membranes and reagents.
- Refer to MSDS for additional safety information.
- The product is guaranteed to be free of manufacturer defects, and to function as described when the provided protocol is followed by properly trained personnel.

#### Short Protocol

Step	User Notes
1. Rinse blot with 20 mL water at room temperature for 5 minutes with agitation.	
2. Strip blot using 20 mL HRP Stripping Buffer at room temperature for 5 minutes with agitation. Ensure blot is completely covered in stripping buffer.	
3. Wash blot with 1x PBST or 1x TBST for 5 minutes with agitation. Repeat twice for a total of 3 washes.	
4. Block membrane and perform antibody incubations.	

#### Tips

- Azure HRP Stripping Buffer is compatible with both nitrocellulose and PVDF membranes.
- Because stripping blots may reduce signal intensity, probe for the lowest expressed antigen first, for best results.

## 4.4. AzureRed Fluorescent Protein Stain

Fluorescent total protein stain for gels and blots.

### Protocol for Catalog Number

- AC2124—AzureRed Fluorescent Protein Stain

### Storage Information

Store AzureRed Dye in a freezer at -15 °C to -30 °C in the original brown bottle provided and protect from light. The AzureRed Powder A and Powder B are stable at room temperature for one year.

### Warnings and Precautions

- AzureRed total protein stain is for research use only.
- Always wear gloves when handling membranes and reagents.
- Refer to MSDS for additional safety information.
- The product is guaranteed to be free of manufacturer defect, and to function as described when the enclosed protocol is followed by properly trained personnel. Please see the Warranty section for more information.

### Kit Contents (AC2124)

AzureRed total protein stain, 5 ml

- AzureRed Powder A 4 packets, 10.1 g each
- AzureRed Powder B 23.4 g
- AzureRed Dye 5 ml

AzureRed Stain, 5 ml, kit is sufficient for staining twenty SDS-PAGE mini-gels (8 cm x 11 cm), four full-sized 2D gels (17 cm x 17 cm), or forty mini-blots (9 cm x 7 cm).

### Shipping and Storage Conditions

Product may be shipped refrigerated or frozen on blue ice or dry ice. Shipping at ambient temperature (below 27°C) is acceptable if the total dispatch time is no longer than 5 days. Upon receipt, store AzureRed Dye in a freezer at -15 °C to -30 °C in the original brown bottle provided and protect from light. AzureRed Powder A and Powder B may be stored at room temperature in a dry location.

### Additional Materials Required

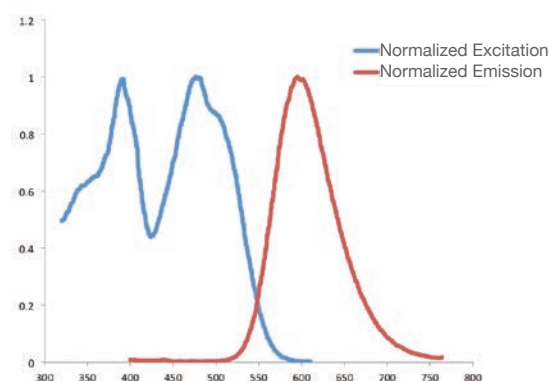
- High-purity water (distilled, Milli Q, or equivalent)
- 100% ethanol
- Staining tray
- Shaking or rocking platform

### About AzureRed

AzureRed is based on epicocconone, a small, naturally occurring fluorescent compound<sup>1</sup> that reversibly binds to lysine, arginine, and histidine residues in proteins and peptides to yield an intensely redfluorescent product.<sup>2</sup> This unique mechanism provides sensitive quantification of proteins in 1D and 2D gels of all chemistries, on both PVDF and nitrocellulose blots<sup>3-5</sup> and provides unparalleled compatibility with Mass Spectrometry.<sup>6-8</sup>

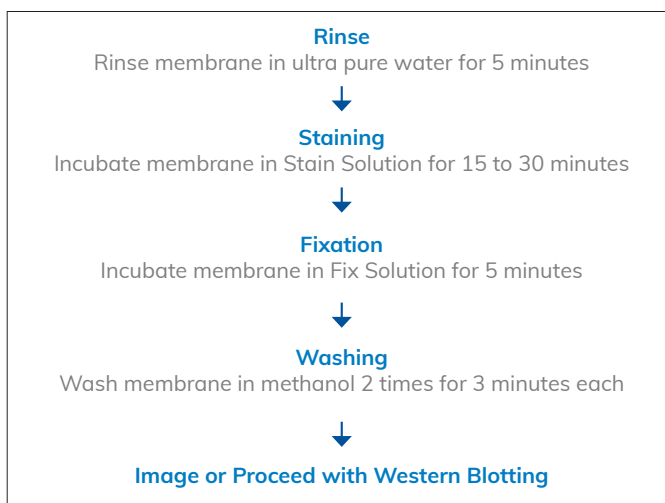
### Excitation and Emission Spectra

The excitation and emission spectra of AzureRed can be seen in Figure 4.8.



**Figure 4.8. Excitation and Emission Spectra of AzureRed Dye.**

## Overview of AzureRed Membrane Staining Protocol



### Preparation of Solutions

Before staining, prepare Fix, Stain, and Wash solutions as described below. These solutions are stable for up to 1 year when stored at room temperature. Precipitates or dust present in the solutions will result in speckling on gels. If observed, filter solutions before use. The amount of reagents in each packet of AzureRed Powder A or B is sufficient to prepare 1 L of solution. Do not split the packets. Once a packet is opened, the entire contents should be used. For preparation of larger volumes, use more than one packet.

- **Fix Solution**—Add contents of one AzureRed Powder A packet (10.1 g) to 850 ml of high-purity water in a 1 L bottle. Mix until dissolved. Add 150 ml 100% ethanol and mix thoroughly.
- **Stain Buffer**—Add contents of one AzureRed Powder B packet (23.4 g) to 1 L of high-purity water in a 1 L bottle. Mix until completely dissolved.
- **Wash Solution**—Mix 850 ml high-purity water and 150 ml 100% ethanol in a 1 L bottle.

Gel Dimensions	Solution				
	Fix	Stain		Wash	Fix
		Stain Buffer	AzureRed Dye		
8 cm x 11 cm x 1 mm (mini-gels)	100 ml	50 ml	250 µL	100 ml	100 ml
13.3 cm x 8.7 cm x 1 mm (small format 2D gels)	200 ml	100 ml	500 µL	200 ml	200 ml
17 cm x 17 cm x 1 mm	500 ml	250 ml	1.25 ml	500 ml	500 ml
17 cm x 17 cm x 1.5 mm	500 ml	250 ml	1.25 ml	500 ml	500 ml
15 cm x 19 cm x 1 mm	500 ml	250 ml	1.25 ml	500 ml	500 ml
15 cm x 19 cm x 1.5 mm	500 ml	250 ml	1.25 ml	500 ml	500 ml
20 cm x 25 cm x 1 mm	750 ml	375 ml	1.875 ml	750 ml	750 ml
20 cm x 25 cm x 1.5 mm	750 ml	375 ml	1.875 ml	750 ml	750 ml

**Table 4.1. Volumes of Solutions For Different Gel Sizes.**

Azure Imaging System	Recommended Imaging Channel
150, 200, 280, 300, 500	UV365
400, 600	Cy3
Sapphire Biomolecular Imager – RGB, RGBNIR	520

**Table 4.2. AzureRed is imageable with both UV and green light.** However, best sensitivity is achieved when detected with green light.

## Detailed Protocol, Blot Staining

Step	Notes
<b>1. Washing</b>	
<ul style="list-style-type: none"> <li>Following transfer, wash blot for 5 min in water.</li> <li>Proceed to PVDF (2) or Nitrocellulose (3) protocol.</li> </ul>	<ul style="list-style-type: none"> <li>For best results, run the buffer front off the base of the gel during electrophoresis prior to transfer.</li> <li>Do not allow membrane to dry during staining.</li> <li>For all steps, use 50 ml for small blots, 400 ml for large.</li> </ul>
<b>2. PVDF Protocol</b>	
<b>2a. Staining</b>	
<ul style="list-style-type: none"> <li>Place blot protein side down in Stain Solution.</li> <li>Stain blot with gentle rocking for 15–30 min.</li> </ul>	<ul style="list-style-type: none"> <li>Prepare Stain Solution: Allow AzureRed Dye to warm to room temperature. Mix thoroughly. For small blots, dilute 125 µl AzureRed Dye in 50 ml Stain Buffer. Mix well.</li> <li>For large blots, dilute 1 ml of AzureRed Dye in 400 ml Stain Buffer. Mix well.</li> </ul>
<b>2b. Acidification</b>	
<ul style="list-style-type: none"> <li>Place blot in Fix Solution and incubate with gentle rocking for 5 min.</li> </ul>	<ul style="list-style-type: none"> <li>Blot will appear green.</li> </ul>
<b>2c. Wash</b>	
<ul style="list-style-type: none"> <li>Rinse blot 3 times with 100% ethanol for 2–3 min each, until green background on blot has been completely removed.</li> </ul>	<ul style="list-style-type: none"> <li>Methanol may used instead of ethanol.</li> </ul>
<b>2d. Drying</b>	
<ul style="list-style-type: none"> <li>Hang blot from a peg or dry on wire mesh to allow blot to dry evenly.</li> <li>Allow blot to dry completely before imaging.</li> </ul>	<ul style="list-style-type: none"> <li>If using in a multiplex Western blot, do not dry membrane after staining. Upon completion of the washing step, proceed directly to blocking the membrane.</li> </ul>
<b>3. Nitrocellulose Protocol</b>	
<b>3a. Staining</b>	
<ul style="list-style-type: none"> <li>Place blot protein side down in Stain Solution.</li> <li>Stain blot with gentle rocking for 15–30 min.</li> </ul>	<ul style="list-style-type: none"> <li>Prepare Stain Solution: Allow AzureRed Dye to warm to room temperature. Mix thoroughly. For small blots, dilute 125 µl AzureRed Dye in 50 ml Stain Buffer. Mix well.</li> <li>For large blots, dilute 1 ml of AzureRed Dye in 400 ml Stain Buffer. Mix well.</li> </ul>
<b>3b. Acidification</b>	
<ul style="list-style-type: none"> <li>Place blot in Fix Solution and incubate with gentle rocking for 5 min.</li> </ul>	<ul style="list-style-type: none"> <li>Blot will appear green.</li> </ul>
<b>3c. Washing</b>	
<ul style="list-style-type: none"> <li>Wash blot 1 time in Wash Solution for 5 min with gentle rocking.</li> <li>Wash blot 2 times in high-purity water for 5 min with gentle rocking.</li> </ul>	

### 3d. Drying

- Allow blot to dry completely before imaging.
- If using in a multiplex Western blot, do not dry membrane after staining. Upon completion of the washing step, proceed directly to blocking the membrane.

## Detailed Protocol, Gel Staining

Step	Notes
<b>1. Fixation</b>	
<ul style="list-style-type: none"> <li>• Fix gel in Fix Solution for a minimum of 1 hr with gentle rocking.</li> <li>• For correct volumes at each step, refer to Table 4.1.</li> </ul>	<ul style="list-style-type: none"> <li>• For gels thicker than 1 mm or backed gels, increase the fixation time to 1.5 hr.</li> <li>• The fixation time can be extended to overnight.</li> </ul>
<b>2. Staining</b>	
<ul style="list-style-type: none"> <li>• Prepare the Stain Solution immediately prior to staining.</li> <li>• Remove gel from Fix Solution and place in Stain Solution, minimizing carryover of the fixing solution.</li> <li>• Stain gel for 1 hour with gentle rocking.</li> </ul>	<ul style="list-style-type: none"> <li>• To prepare Stain Solution: Allow AzureRed Dye to warm to room temperature. Mix thoroughly, then dilute 1 part AzureRed Dye in 200 parts Stain Buffer. Mix well. Refer to Table 4.1 for volumes of solutions used for different gel sizes.</li> <li>• Stain Solution will degrade over time. Prepare only as much as is needed and use immediately.</li> <li>• Increase staining time to 1.5 hours for gels 1.5 mm thick or backed gels. Extending the staining time to 2 hours will not affect results.</li> <li>• DO NOT stain for longer than 2 hours.</li> </ul>
<b>3. Washing</b>	
<ul style="list-style-type: none"> <li>• Remove gel from Stain Solution, rinse with high-purity water, and wash in Wash Solution for 30 min with gentle rocking.</li> </ul>	<ul style="list-style-type: none"> <li>• For 1.5 mm thick gels, or gels with high background fluorescence, increase washing time to 45 min.</li> </ul>
<b>4. Acidification</b>	
<ul style="list-style-type: none"> <li>• Remove gel from Wash Solution and place in Fix Solution.</li> <li>• Incubate in Fix Solution for 30 min with gentle rocking.</li> </ul>	<ul style="list-style-type: none"> <li>• This step can be repeated or extended to overnight to reduce background staining.</li> <li>• If performing this step overnight, protect the gel from light.</li> </ul>
<b>5. Imaging</b>	
<ul style="list-style-type: none"> <li>• Detect fluorescence at 610 nm using standard fluorescence scanners and CCD camera systems. For recommended imaging settings, refer to Table 4.2.</li> </ul>	<ul style="list-style-type: none"> <li>• Compatible excitation sources include green light and UV light.</li> <li>• Detect fluorescence using a 610 nm band pass or 560 nm long pass filter.</li> </ul>

## Destaining

AzureRed staining is reversible and the stain may be removed for subsequent analysis such as Western blotting.

1. **To destain while minimizing protein loss**—Wash blot overnight in 50 mM ammonium carbonate.
2. **To rapidly destain PVDF membranes**—Wash blot with 50% acetonitrile containing 30 mM ammonium carbonate for 15 min.
3. **To rapidly destain nitrocellulose membranes**—Wash blot with 50% ethanol or methanol containing 50 mM ammonium carbonate for 15 min.
4. **To rapidly destain protein gels**—Wash blot with 50% ethanol or methanol containing 50 mM ammonium carbonate for 15 min to 1 hour.

## Storage

Gels may be stored at 4 °C in 1% citric acid and protected from light. For extended storage (up to 6 months), add AzureRed Dye to the storage solution at 1:200. Prior to imaging, rinse gels 2 x 15 min in Wash Solution. Incubating in Fix Solution for 15 minutes can reduce background.

Blots may be stored dry, in the dark, at room temperature.

## Troubleshooting & FAQ

Problem	Possible Solutions
High background	<ul style="list-style-type: none"> <li>• Handle gels with clean non-powdered gloves, and avoid contamination with dust.</li> <li>• Ensure concentrated AzureRed Dye was brought to room temperature and thoroughly mixed prior to dilution.</li> <li>• Ensure stain was thoroughly mixed into Stain Buffer before adding to gel.</li> <li>• Stain only one gel per tray.</li> <li>• Use high-purity water (distilled, Milli-Q, or equivalent).</li> </ul>
No or low signal	<ul style="list-style-type: none"> <li>• Check pH during staining step; pH should be between 9.5 and 10.5. Carry-over acid from the fixation step can result in poor staining.</li> <li>• Stain may fade with long exposure times and associated heating on CCD-based instruments.</li> <li>• Ensure stain concentrate was brought to room temperature and mixed thoroughly before dilution.</li> <li>• Staining for over 2 hours in alkaline conditions destabilizes proteins, and leads to diffusion of protein bands from the gel matrix.</li> </ul>
Negative staining	<ul style="list-style-type: none"> <li>• Use high-quality SDS in the preparation and running of the gel.</li> <li>• Extend fixation time to overnight.</li> <li>• Use correct volumes of Fix and Wash Solutions.</li> <li>• Extend washing time.</li> </ul>
Speckled background	<ul style="list-style-type: none"> <li>• Filter buffers to remove dust or precipitates.</li> <li>• Protect gel from airborne particles.</li> </ul>

## References

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# 5

## APPLICATION NOTES



# Three-Color Western Blots with AzureSpectra Antibodies

## Visualize Three Proteins at Once – with AzureSpectra labeled antibodies

Classic western blotting techniques using chemiluminescent detection are typically useful for detecting one protein at a time. To evaluate multiple proteins of interest using chemiluminescence, stripping and re-probing the blot or splitting the samples into separate assays is usually required.

Visualizing multiple proteins of interest within the same sample simultaneously – or multiplexing – is possible with fluorescent antibodies. Antibodies carrying different fluorophores (with non-overlapping spectra) can be detected simultaneously with the right imaging system. With a digital imager like the Azure 600 you can visualize three proteins at once.

Why would you need to visualize multiple proteins at once? Convenience is an obvious benefit to multiplexing, as loading controls are often used for Western blot data. By using different fluorescently labeled secondary antibodies for the protein of interest along with the loading control, clear and quantifiable results are ready to be analyzed immediately, even if the proteins are close in molecular weight. Additionally, quantitation is increasingly desirable when publishing protein expression data. What if you have two different proteins of interest, such as phosphorylated and non-phosphorylated version of a protein? In order to include a loading control for accurate comparison of different lanes, a third color would be necessary. Conveniently, 3-color western blotting is possible and easy with AzureSpectra labeled antibodies and the Azure 600 system.

The Azure 600 imaging system is designed for multicolor fluorescent Western blotting, with two nearinfrared (NIR) and 3 visible fluorescent channels. This application note demonstrates a procedure to obtain three color Western Blots using the Azure 600 and Azure's broad selection of fluorescently labeled secondary antibodies. In addition to pre-labeled antibodies, Azure also offers an Antibody Labeling Kit that provides everything you need to

fluorescently label your primary or secondary antibodies. The non-overlapping emission spectra of the fluorophores in each of the available dyes means you have the opportunity to easily perform multiplexing assays.

## Materials and Methods

### Run and transfer gel

HeLa cell lysates were prepared and electrophoresed on a 4-15% Mini-Protean TGX gel, using 1, 2, 5, 10, and 20 µg of lysate for each lane, respectively. After electrophoresis and separation, proteins were transferred to a low fluorescence PVDF membrane using Azure transfer buffer.

### Western blotting

After transfer, the membrane was blocked for 10 minutes then probed with 5 µg of mouse anti-GAPDH, 5 µg of rabbit anti-beta-actin, and 5 µg of rat anti-tubulin for 1 hour. The blot was rinsed twice and then washed three times for 5 min each with 25 mL of Azure IR wash buffer. Next, the blot was incubated for 1 hour with the labeled secondary antibodies: 2 µg of goat anti-mouse-550, 4 µg goat anti-rabbit-700, and 4 µg goat anti-rat-800. After incubation, the blot was washed as before in Azure IR wash buffer followed by a 5 min rinse in 25 mL of PBS.

Step	Materials	Part number
Run Gel	SDS Polyacrylamide Gel	
Transfer	PVDF Membrane	AC2105
	Azure Transfer Buffer	AC2127
Block Blot	AzureSpectra Fluorescent Blot Blocking Buffer	AC2190
Probe Blot	Primary Antibodies	Specific for proteins of interest
	Azure IR Wash Buffer	AC2145
	Goat-anti-rat 800	AC2138
	Goat-anti-rabbit 700	AC2128
	Goat-anti-mouse 550	AC2159
	PBS	

Table 1. Materials and product numbers.

Image Western blot

After rinsing in PBS, the blot was allowed to dry before imaging on the Azure Biosystems 600 digital imager.

Results and Conclusions

The Azure 600 and AzureSpectra antibodies allow for quick and easy detection of three different proteins in the same samples on the same western blot. Accurate quantitative analysis of your fluorescent western blot can be performed immediately after imaging.

Figure 2 shows the digital image of the western blot labeled with three different primary antibodies (anti-tubulin, anti-beta actin, and anti-GAPDH) and fluorescently labeled secondary antibodies. The individual channels are shown in grayscale and all three are merged in the three-color image (upper left). The ability to visualize three proteins at once increases the potential information that can be obtained from individual western blot experiments. With more information, better quantitative analysis can be performed – strengthening your data and your science.

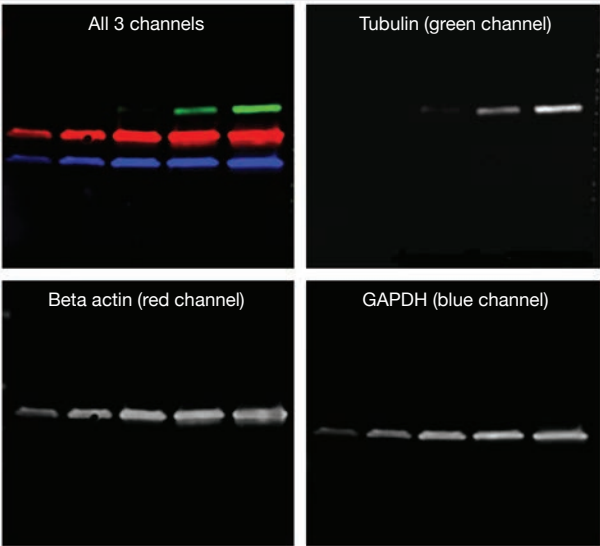


Figure 2. Digital image of 3-color western blot using Azure Biosystems 600 imager. Lanes (from left to right) loaded with 1, 2, 5, 10, 20 µg HeLa cell lysate. Probed for tubulin (top), beta actin (middle) and GAPDH (bottom). The following settings were used: Light sources 6/7/4; Exposure time 1 s/13 s 204 ms/677 ms; Filter positions 6/7/4; Aperture 6400; Focus 5000/5250/5000; bin level 1x1.

Primary Antibody	Secondary Antibody	Excitation/ Emission	Azure Imaging Channel
Rat anti-Tubulin	AzureSpectra Goat-anti-rat 800	785 nm/ 815 nm	IR800
Rabbit anti-beta-actin	AzureSpectra Goat-anti-rabbit 700	660 nm/ 720 nm	IR700
Mouse anti-GAPDH	AzureSpectra Goat-anti-mouse 550	551 nm/ 565 nm	Cy3

# Phosphorylated protein detection is more efficient by fluorescent Western blot

## Introduction

The detection of phosphorylated proteins usually requires showing both nonphosphorylated and phosphorylated species. For traditional chemiluminescent methods, the negligible size difference between the phosphorylated and nonphosphorylated protein of interest requires probing with a phospho-specific antibody, then stripping, and then re-probing with the protein-specific antibody. Along with potential negative effects on data quality, the process of stripping and re-probing can take a considerable amount of time and reagents.

The drawbacks from the traditional chemiluminescent method of detection can be overcome by using fluorescent secondary antibodies. Fluorescent secondary antibodies bearing two different fluorophores (with non-overlapping spectra) allow for multiplexing; this means both phosphorylated and non-phosphorylated species can be detected simultaneously.<sup>1</sup>

The ability to multiplex not only makes IR detection the faster way to visualize phosphorylated and non-phosphorylated versions of a protein of interest, but it also makes quantitation more accurate. Stripping a blot is not always even or completely effective; moreover, the process of stripping can sometimes take off some of the original protein.<sup>2</sup> Quantitation of proteins before and after stripping is less accurate than if no stripping were involved. Thus, a protocol that visualizes modified and unmodified proteins at the same time offers improved accuracy as well as convenience of speed (see Table 1 for comparison).

## Methods

### Multiplexing protocol: Probing for two proteins of the same weight

Untreated and IFN $\alpha$ -treated HeLa lysates were loaded on an SDS polyacrylamide gel and resolved by electrophoresis. The proteins were transferred to a PVDF membrane using Azure Transfer Buffer. The membrane was split and one membrane was probed using a

chemiluminescent detection protocol while the other membrane was probed using a protocol with fluorescent antibodies as detailed below.

### Chemiluminescent detection

After transfer, the first blot was probed for phospho-STAT1. First the membrane was blocked for 30 minutes before incubation with 4  $\mu$ g rabbit anti-phospho-STAT1 for 1 hour. The membrane was then rinsed twice with 25 mL Azure Fluorescent Wash Buffer (AFWB) followed by three 5 minute washes with AFWB. Next, it was incubated with 3  $\mu$ g anti-rabbit-HRP for 1 hour, followed by a wash as before with AFWB. After washing, the blot was incubated with 10 mL of the chemiluminescent substrate Radiance for 2 minutes. The blot was imaged directly by the Azure Imaging System.

Time Investment for Western Blots		
Protocol steps	Chemi Time (min)	Fluorescent Time (min)
Blocking	30	10
Primary incubation	60	60
Wash	15	15
Secondary incubation	60	60
Wash	15	15
Add substrate	2	–
PBS rinse or Water rinse	–	5
	5	–
Stripping	5	
Wash	15	
Blocking again	30	
Primary incubation	60	
Wash	15	
Secondary incubation	60	
Wash	15	
Add substrate	2	
<b>TOTAL TIME</b>	<b>6 hrs 29 min</b>	<b>2 hr 45 min</b>

Table 1. Time required for each western blot protocol.

After imaging the blot, the probed membrane was stripped: first, the membrane was rinsed in high purity water for 5 minutes; second, the membrane was placed in Azure HRP Stripping Buffer for 5 minutes. Following stripping, the membrane was washed with 25 mL AFWB, 2 times fast and then 3 times for 5 minutes each.

To probe for STAT1, the membrane was blocked again for 30 minutes and then incubated with 4 µg mouse anti-STAT1 for 1 hour. The membrane was then rinsed twice with 25 mL AFWB followed by three 5 minute washes with AFWB. Next it was incubated with 3 µg anti-rabbit-HRP for 1 hour, followed by a wash with AFWB in a similar manner as before. After washing, the blot was incubated with 10 mL Radiance for 2 minutes. The blot was imaged again by the Azure Imaging System.

### Using fluorescent antibodies and Infrared (IR) detection

After transfer, the membrane was blocked for 10 minutes at room temperature. To probe for the proteins of interest, the membrane was incubated with 4 µg rabbit anti-phospho-STAT1 and 4 µg mouse anti-STAT1 for 1 hr. The membrane was then rinsed twice with 25 mL Azure IR Fluorescent Wash Buffer (AIWB) followed by three 5 minutes washes with AIWB. Following the wash, the membrane was incubated with 4 µg of anti-rabbit-800 and 4 µg anti-mouse-700 for 1 hour. The probed membrane was washed with AIWB in the same manner as before. After the AIWB wash, the blot was rinsed with 25 mL of PBS for 5 minutes before imaging. The blot was imaged with the Azure Imaging System, using the filters IR-800 (green) and IR-700 (red).

## Results and Conclusions

In this note, two corresponding western blot membranes were subjected to either chemiluminescent or IR detection for analysis of phosphorylated STAT1. HeLa cells were either treated with IFNα to induce phosphorylation of STAT1 or left untreated, and then protein lysates were harvested and resolved by gel electrophoresis followed by transfer to a membrane for western blotting. Each blot was probed for STAT1 and phospho-STAT1 using the same primary antibodies, but secondary antibodies that were either HRP-conjugated (Figure 1) or fluorescently labeled (Figure 2) were used depending on the detection method.

The comparisons presented here aim to demonstrate the improved convenience and accuracy of fluorescence detection as compared to traditional chemiluminescent methods. The main differences between methods include secondary antibodies used and the number of steps involved. The extra steps in chemiluminescent detection methods include stripping the membrane, which leaves the possibility for uneven removal of antibodies and possible loss of target proteins. Additionally, chemiluminescent detection required approximately 6.5 hours of time to attain data of both unmodified and phosphorylated STAT1. With fluorescent secondary antibodies, STAT1 and phospho-STAT1 could be imaged simultaneously with different IR filters in the digital imager in less than 3 hours (see Table 1). Both protocols can provide quality results, so it's important to choose the protocol that best fits your needs.<sup>1,2</sup>



Figure 1. Chemiluminescent western blot of STAT1 and phospho-STAT1. The blot was first probed with anti-phospho-STAT1 and imaged. After stripping, the blot was re-probed with anti-STAT1 and imaged. Lanes: Ladder, 1) 10 µg untreated HeLa lysate, 2) 10 µg IFNα-treated, 3) 20 µg of untreated, 4) 20 µg IFNα-treated.

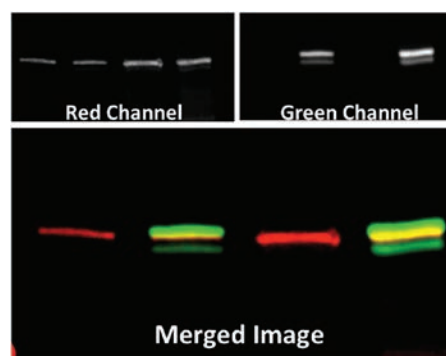


Figure 2. Fluorescent western blot of STAT1 and phospho-STAT1. The blot was probed with anti-phospho-STAT1 and anti-STAT1 followed by fluorescent secondary antibodies, and then imaged on Azure Imaging System. Top right is the green channel, using IR-800; top left is the image of the red channel, using IR-700. Bottom image is both channels merged. Lanes are the same as in Figure 1.

## References

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Products and Reagents	Product Number
Low Fluorescence PVDF Membrane	AC2105
Azure Transfer Buffer	AC2127
Azure Fluorescent Wash Buffer	AC2113
Azure IR Fluorescent Wash Buffer	AC2145
Goat-Anti Rabbit HRP Secondary Antibody	AC2114
Goat-Anti Mouse HRP Secondary Antibody	AC2115
AzureSpectra 700 Goat-anti-mouse Secondary Antibody	AC2129
AzureSpectra 800 Goat-anti-rabbit Secondary Antibody	AC2134
Radiance Chemiluminescent Substrate	AC2101
Azure HRP Stripping Buffer	AC2154

# Imaging Three-color Western Blots with the Azure 600

Multicolor detection is a powerful application of fluorescent Western blotting. Two or more proteins can be detected on one blot using antibodies labeled with different fluorophores (Figure 1). The ability to simultaneously assay multiple proteins on a Western blot represents a significant advantage of fluorescent detection over chemiluminescent detection, because chemiluminescent detection requires stripping and re-probing a blot or using duplicate blots to detect two proteins, approaches that can introduce errors and variation.

Measuring two or more proteins is important for quantitative Western experiments. With multicolor detection, the amount of a protein of interest can be normalized to that of a housekeeping protein, allowing levels of the protein of interest to be compared between samples. Also, with multicolor fluorescent detection, proteins that are similar in size and not well separated spatially on a gel, such as phosphorylated and non-phosphorylated isoforms of a protein, can be measured at the same time.

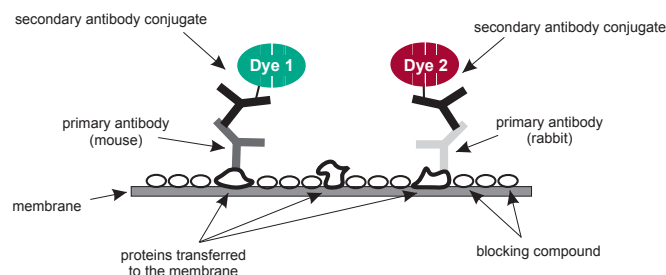


Figure 1

The Azure 600 imaging system is designed for multicolor fluorescent Western blotting, with two near-infrared (NIR) and 3 visible fluorescent channels. This application note demonstrates a procedure to obtain three-color fluorescent blots using WesternDot® reagents. WesternDot® reagents are secondary antibodies conjugated to Qdot® nanocrystals enhanced with VIVID® technology. The WesternDot® reagents WesternDot® 585, WesternDot® 655, and WesternDot® 800 comprise an ideal combination for 3-color Western blotting experiments because these fluorophores have well-resolved, non-

overlapping emission spectra (Figure 2). All WesternDot® reagents can be excited with blue or UV light (Figure 2), so imaging each protein independently on a multiplex fluorescent blot requires an instrument such as the Azure 600 that has emission filters specific for the emission of each WesternDot® reagent.

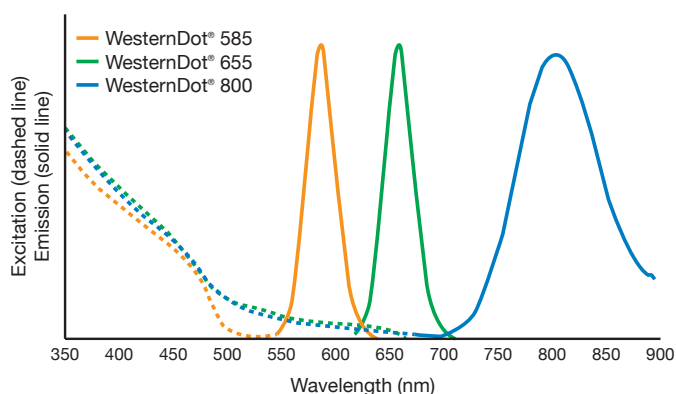


Figure 2

## Methods

Approximately 10 µg each of cell lysates from cells induced to express EGFR or phosphorylated-AKT were mixed and loaded on a Novex® Bolt™ 4-12% Bis-Tris Plus 10-well gel. After electrophoretic separation, proteins were transferred to a nitrocellulose membrane using the iBlot® 2 Gel Transfer Device. Western blot processing was carried out with an iBind™ Western Device using the iBind™ FD Solution standard protocol.

The membrane was probed with 2 mls of iBind™ FD solution containing the three primary antibodies: mouse anti-EGFR (diluted 1:200), rabbit anti-phospho-AKT (diluted 1:400), and chicken anti-GAPDH (diluted 1:400). Secondary antibodies used were WesternDot® 585 goat anti-mouse, WesternDot® 800 goat anti-rabbit, and WesternDot® 655 goat anti-chicken (Table 1). All secondary antibodies were diluted 1:100 in iBind™ FD Solution.

The blot was imaged with four imaging systems, as shown in Table 2.



Results

The same three-color blot was imaged with four different imaging systems. The acquired images are shown in Figure 3. The Odyssey instrument (Figure 3a) does not have the capability to detect visible fluorescent dyes, so only one protein, phosphorylated AKT detected in the IR800 channel, is visible. The Typhoon instrument (Figure 3b) has settings for detection of Qdot® 800 and Qdot® 655, and a merged image of both channels is shown. Only GAPDH, detected at 655 nm, is visible; no signal from phosphorylated AKT is seen. The LAS-1000 instrument (Figure 3c) has a blue light source capable of exciting all three WesternDot® reagents, but only one emission filter so all fluorescent signals are detected in a single image. EGFR (detected at 585 nm) and GAPDH are visible, but phosphorylated AKT is not.

Of the four imaging systems used, only the Azure 600 (Figure 3d) detected all three WesternDot® conjugates. The 600 has default settings for common fluorescent dyes and can also be manually configured. To image the WesternDot® conjugates custom settings were used; the blot was excited using the settings for imaging Cy2 (460 nm), and detected using three different emission filters (Table 2). All three channels are merged in the image shown (Figure 3d), with WesternDot® 655 shown in blue, WesternDot® 585 in red, and WesternDot® 800 in green.

The ability to detect three proteins on a single blot dramatically increases the amount of information that can be obtained from a Western blot experiment. For example, two proteins and a loading control can be detected

Primary antibody	Secondary antibody	Excitation/Emission
Mouse anti-EGFR	WesternDot® 585 goat anti-mouse	405-485 nm/585 nm
Rabbit anti-phospho-AKT	WesternDot® 800 goat anti-rabbit	405-485 nm/800 nm
Chicken anti-GAPDH	WesternDot® 655 goat anti-chicken	405-485 nm/655 nm

Table 1: Protein targets, primary antibodies, and secondary antibodies

Imaging system (manufacturer)	EGFR (WesternDot® 585)	GAPDH (WesternDot® 655)	Phospho-AKT (WesternDot® 800)
Odyssey (LI-COR)	NA	NA	800 channel
Typhoon™ FLA 9000 (GE Healthcare)	NA	Qdot® 655, PMT 1000	Qdot® 800, PMT 1000
LAS-1000 (Fujifilm Life Science)	Blue-light excitation 3-second exposure	Blue-light excitation 3-second exposure	Blue-light excitation 3-second exposure
600 (Azure)	Excite Cy2/ detect orange filter	Excite Cy2/ detect red filter	Excite Cy2/ detect 800 filter

Table 2: Imaging systems and settings used for each target protein

simultaneously, allowing quantitative comparisons to be made between proteins and between samples. WesternDot® reagents are available with a variety of species specificities, for flexibility in designing three-color experiments. With the Azure 600 and WesternDot® reagents, three-color Western blots are at your fingertips.

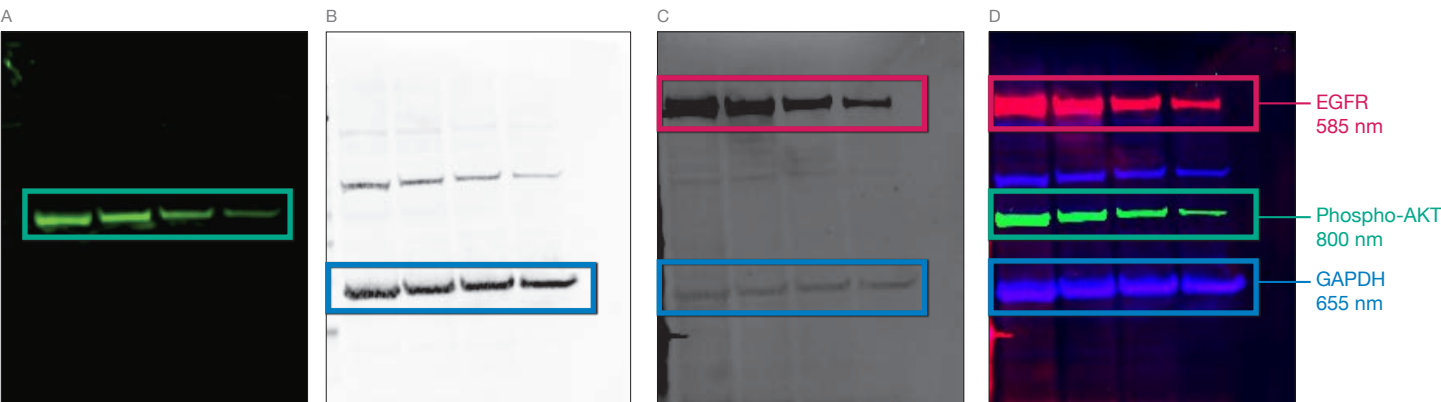


Figure 3. Images of the same three-color Western blot, detected with WesternDot® reagents WesternDot® 585, WesternDot® 655, and WesternDot® 800, collected using four different imaging systems. a. LI-COR Odyssey; b. GE Healthcare Typhoon 9000; c. FUJIFILM LAS-1000; d. Azure 600.

# Superior electrophoresis results with Lonza reagents and the Azure Imaging Systems

## Introduction

Lonza, a leader in electrophoresis reagents, and Azure Biosystems, manufacturer of gel doc and Western blot imaging systems, have teamed up to demonstrate how to easily obtain excellent DNA and protein gel electrophoresis data. Lonza provides a complete line of reagents for nucleotide and protein electrophoresis including precast gels, buffers, stains, and markers for fast and reliable electrophoresis. Using precast gels saves time and allows for reproducible results due to tight quality control. The Azure Imaging System family of digital imaging systems from Azure Biosystems offers a range of instruments to suit imaging needs from gel documentation to quantitative imaging of multicolor fluorescent Western blots. Each instrument is capable of imaging commonly used DNA and protein stains, having illumination sources that include both white light and dual-UV transilluminators, as well as epi white and blue light. The Azure Imaging System instruments include user-friendly interfaces to make capturing the best possible image a breeze.

This application note demonstrates the high-sensitivity and quality results that can be obtained combining the expertise of these two companies for DNA and protein electrophoresis and analysis.

## Fast nucleotide electrophoresis and sensitive detection with the FlashGel™ System and Azure Imaging System

The FlashGel™ System from Lonza consists of precast agarose gel cassettes that are run in an accompanying dock. Electrophoresis does not require preparation of running buffer or a separate staining step. Each gel contains a proprietary stain that is 5 to 20 times more sensitive than ethidium bromide and that can be imaged using either blue light or UV light with the same settings and filters that would be used with ethidium bromide. The Azure Biosystems Imaging System instruments

offer multiple options for imaging the FlashGel™ dye; UV transillumination, UV transillumination with a blue conversion screen that essentially converts the light source to a blue transilluminator, and epi blue light.

Figure 1 shows the excellent separation, sensitivity, and image quality obtained imaging a double tier FlashGel™ with the Azure Imaging System, using the UV transilluminator and blue conversion screen. The image was captured using a 10 second exposure. Fragments ranging from 50 bp to 1500 bp in size were well resolved on a 2.2% agarose gel in less than 5 minutes. High reproducibility is seen across the gel and across both tiers and the image demonstrates uniform intensity across the gel with excellent signal-to-noise. For samples loaded and imaging specifics, please see experimental details at the end of the application note.

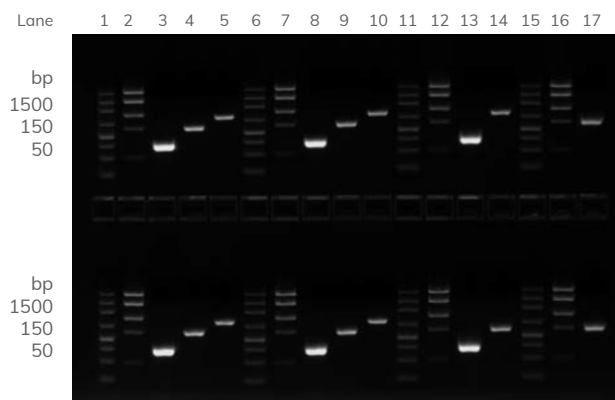


Figure 1. FlashGel™ imaged with the Azure Imaging System, using UV transilluminator with blue conversion screen.

Figure 2 compares the limit of detection achieved when imaging a FlashGel™ System with the Azure Imaging System imaging system using either UV transillumination with a blue light conversion screen (Figure 2A), UV transillumination (Figure 2B), or epi blue light (Figure 2C). Though imaging with the blue conversion screen provides the optimal balance of sensitivity and low background, in every image a band containing less than 0.2 ng of nucleic acid

is easily detected. For details of samples loaded and imaging settings, please see experimental details at the end of the note.

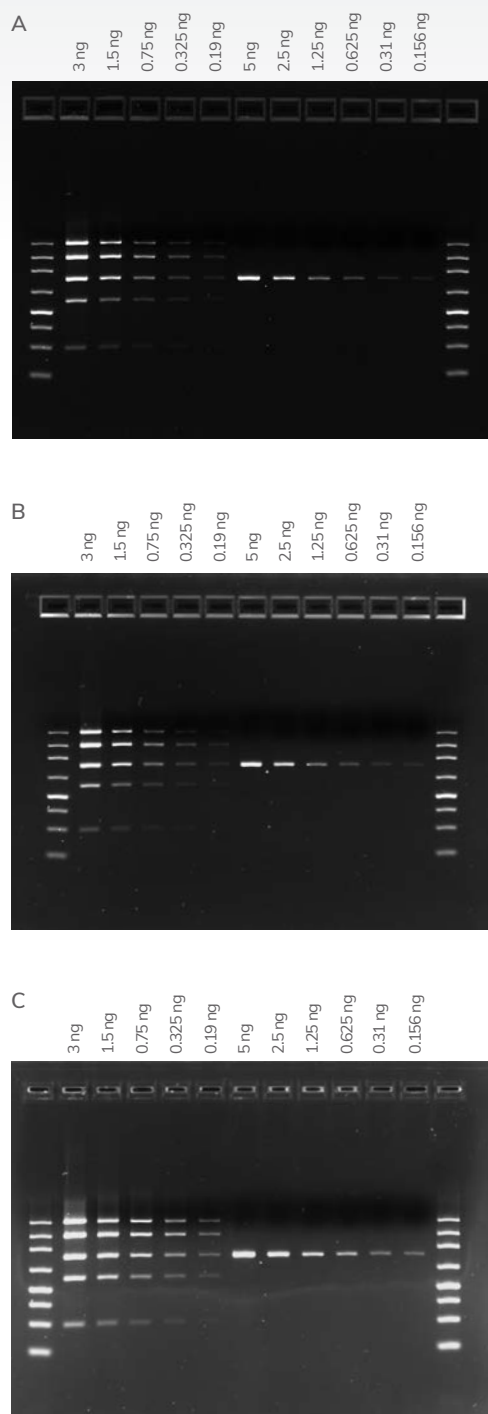


Figure 2. Comparison of sensitivity obtained imaging a FlashGel™ with the Azure Imaging System using either (A) UV transilluminator with blue conversion screen, (B) UV transilluminator (UV 302), or (C) epi blue light.

## Accurate electrophoresis and sensitive detection of larger-format Latitude™ gels with the Azure Imaging System

Latitude™ gels are precast agarose gels containing ethidium bromide. The gels are precision cast for high accuracy and reproducibility. Figure 3 shows a midi 1% SeaKem™ LE Plus gel imaged using an Azure Imaging System and UV transilluminator. The large imaging area of the Azure Imaging System instrument is well able to handle the larger-format gel (10x15 cm) and the even lighting and exposure reveal the highly reproducible electrophoresis of samples both across the entire gel and between the top and bottom set of wells. For details of samples loaded and imaging settings, please see experimental details at the end of the note.

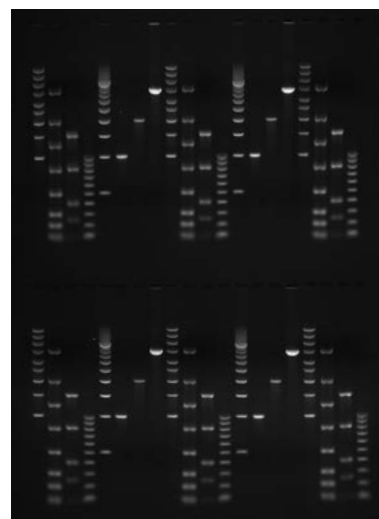


Figure 3. Imaging a Latitude™ Midigel with the Azure Imaging System.

## Azure Imaging System imaging of protein gels stained with ProSieve™ EX Safe Stain

For routine protein electrophoresis, PAGER™ Gold precast Tris-glycine gels provide sharp resolution in a convenient, easy-to-use package. Figure 4 shows a PAGER™ Gold gel stained with Lonza's ProSieve™ EX Safe Stain and imaged using white light transillumination on the Azure Imaging System. The image demonstrates high sensitivity and excellent signal-to-noise. For details of samples loaded and imaging settings, please see experimental details at the end of the note.

Figure 5 demonstrates the limit of detection for a PAGER™ Gold gel stained with ProSieve™ EX Safe Stain. A series of two-fold serial dilutions of carbonic anhydrase were loaded on the gel. A band containing less than 10 ng of protein is easily visualized in the image.

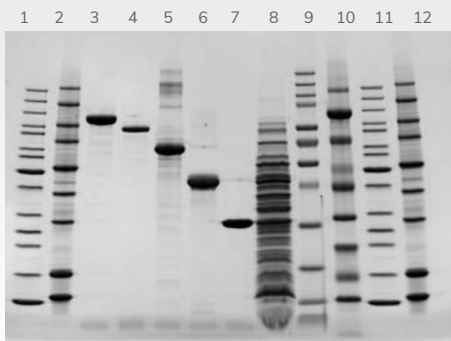


Figure 4. PAGEr™ Gold protein gel stained with ProSieve™ EX Safe Stain and imaged using the white light transilluminator of the Azure Imaging System.

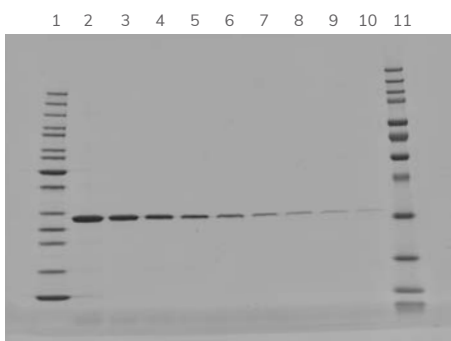


Figure 5. High sensitivity of ProSieve™ EX Safe Stain-stained gel imaged with the Azure Imaging System.

## Maximize convenience without sacrificing quality

The data presented demonstrate the high-quality results that result from combining the convenience of Lonza's precast gels and other reagents with the flexible, high-resolution imaging of the Azure Imaging Systems. The Azure Imaging Systems provide light sources and filters compatible with all commonly used nucleotide and protein stains, including the proprietary stain included in Lonza's nucleotide FlashGel™ as well as Lonza's ProSieve™ EX Safe Stain for protein. The large imaging area of the Azure Imaging System instruments allows imaging of both small and large gels and the user-friendly interface makes image capture a breeze. To learn more about Lonza electrophoresis products please visit the Lonza website and to learn more about the Azure Imaging Systems please visit [www.azurebiosystems.com](http://www.azurebiosystems.com).

## Experimental details

### Figure 1

DNA samples were separated on a 2.2% 16+1 well

double tier FlashGel™ DNA Cassette (Lonza product #57032). The samples loaded were as follows: lane 1, 4  $\mu$ L 50-1500 bp FlashGel™ Marker (Lonza product #57033); lane 2, 4  $\mu$ L FlashGel™ QuantLadder (Lonza product #50475); lane 3, 5  $\mu$ L 150 bp DNA Fragment; lane 4, 5  $\mu$ L 250 bp DNA fragment; lane 5, 5  $\mu$ L 350 bp DNA fragment. The samples were then repeated across the gel. DNA fragments were diluted in 1X FlashGel™ Sample Buffer (Lonza). The gel was run for 4.5 minutes at 275 V. Imaging was conducted with an Azure Imaging System using the UV transilluminator at 302 nm with blue light conversion screen (Azure SKU AC1025). Exposure was 10 seconds.

### Figure 2

DNA samples were separated on a 2.2% 12+1 well single tier FlashGel™ DNA Cassette (Lonza product #57031). The samples loaded were as follows: lane 1, 50-1500 bp DNA markers (Lonza product #57032); lanes 2-6, serial two-fold dilutions of FlashGel™ QuantLadder (Lonza product #50475) starting with 3 ng of the smallest (100 bp) band in lane 2; lanes 7-12, serial two-fold dilutions of a 400 bp DNA fragment starting with 5 ng in lane 7; lane 13, 50-1500 bp DNA marker. The gel was run for 8 minutes at 275 V. Imaging with UV transillumination and blue conversion screen (Azure SKU AC1025) was conducted using a 10 second exposure. Imaging with UV transillumination (302 nm) was conducted using the AutoExposure function of the Azure Imaging System. Imaging with epi-blue light was conducted with an exposure time of 2 seconds.

### Figure 3

DNA samples were separated on a 1% SeaKem LE Plus gel containing ethidium bromide (Lonza product #57230). Samples loaded were as follows: lane 1, 4  $\mu$ L 1-10 kb DNA Marker (Lonza product #50471); lane 2, 8  $\mu$ L FlashGel™ 100-4000 bp DNA Marker (Lonza product #50473); lane 3, 8  $\mu$ L FlashGel™ QuantLadder (Lonza product #50475); lane 4, SimplyLoad™ 100 bp DNA ladder (Lonza product #50327); lane 5, SimplyLoad™ 500 bp DNA ladder (Lonza product #50329); lane 6, 1 kb DNA fragment (BioVentures); lane 7, 2 kb DNA fragment (BioVentures); lane 8, 4 kb DNA fragment (BioVentures). The samples were then repeated across the gel. The gel was set up in an Owl B2 chamber with 1X TBE (with 0.5  $\mu$ g/mL EtBr). The gel was run at 115 V (approximately 5 V/cm) for 85 minutes. The gel was imaged using UV transillumination at 302 nm. Exposure time was 6 seconds.

## Figure 4

Protein samples were separated on a 4-20% PAGEr™ Gold gel (Lonza product #58105). Samples loaded were as follows: lane 1, 5 µL PageRuler Marker (Thermo Fisher); lane 2, 4 µL ProSieve™ Unstained Marker (Lonza); lane 3, 1 µg β-galactosidase; lane 4, 2.5 µg Phosphorylase B; lane 5, 2 µg BSA; lane 6, 2 µg ovalbumin; lane 7, 2 µg carbonic anhydrase; lane 8, 4.5 µL E. coli LE 392 lysate; lane 9, 5 µL ProSieve™ QuadColor™ Protein Marker (Lonza product #193837); lane 10, 6.5 µL ProSieve™ Color Marker (Lonza); lane 11, 5 µL PageRuler Marker (Thermo Fisher); lane 12, 3 µL ProSieve™ Unstained Marker (Lonza). The gel was run for 40 minutes at 200 V. After electrophoresis, gel was placed immediately into ProSieve™ EX Safe Stain (Lonza product #00201455), heated in a microwave for 55 seconds, then stained with shaking for 20 minutes. The gel was then destained by pouring off the stain, adding water, and warming in a microwave with a piece of paper towel to absorb excess stain. The gel was imaged on the Azure Imaging System using the White Light Table (Azure SKU AC1029) and default settings for aperture and focal height.

## Figure 5

Protein samples were separated on a 4-20% PAGEr™ Gold gel (Lonza product #58105). Samples loaded were as follows: lane 1, PageRuler Marker (Thermo Fisher); lanes 2-10, two-fold serial dilutions of carbonic anhydrase starting with 2 µg of protein in lane 2; lane 11, ProSieve™ QuadColor™ Protein Marker (Lonza product #193837). The gel was run for 40 minutes at 200 V. After electrophoresis, the gel was stained, destained, and imaged as described for Figure 4.



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# Lonza

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# DNA Dye Detection Limits using Azure Imager Systems

## Introduction

Gel-based detection of DNA was first described in 1972 by Aaij and Borst<sup>1</sup> and has gone on to become a cornerstone technique of molecular-biology. With the development of increasingly advanced and sensitive DNA technologies, reliable, accurate quantification of low concentration DNA products is becoming increasingly desirable.

The traditional method for gel-based DNA detection is to add ethidium bromide (EtBr) during gel casting. However, the use of EtBr is falling out of favor due to the mutagenic characteristics of EtBr arising from its intercalation of double stranded DNA which may affect DNA replication or transcription.

Additionally, although EtBr is not classified as hazardous waste at low concentrations many organizations treat it as such, often at great expense. Whilst staining of gels post-run with EtBr can reduce the amount of hazardous waste generated, it does not address issues arising from exposure of workers to a mutagen. Because of this a variety of non-mutagenic DNA dyes have been developed; however, unless they are capable of matching, or improving on the sensitivity of EtBr then their use is unlikely to gain traction. With the rise and advancement of digital imaging technology and techniques, the use of non-mutagenic DNA dyes ("safe" stains) has increased in popularity do to their combined ease of use and dynamic range. Therefore, we assessed the limit of detection (LoD) for various DNA dyes using Azure Imagers from Azure Biosystems.

## Methods

### Sample Preparation

New England Biolabs Quick Load 1kb DNA Ladder, which features known concentrations of DNA for each band, was serially diluted 1:1 in water and 6X gel loading dye (See Table 1 for dilutions).

## Gel Casting and Running

0.8% agarose gels in 1x TAE were cast, 10  $\mu$ L of each dilution was loaded and gels were run in 1x TAE for 120 to 150 minutes at 70V. One gel was cast with EtBr at a dilution of 1:10,000, all other gels were cast without dye and post-stained. Post-staining was performed with a 1:10,000 dilution of either EtBr, EZ-Vision<sup>®</sup> Bluelight, SYBR<sup>®</sup> Gold, SYBR<sup>®</sup> Green or SYBR<sup>®</sup> Safe in TAE buffer for 30 minutes.

## Gel Imaging

Following staining, gels were imaged on both the Azure 200 and 600 imagers. Gels stained with EtBr were imaged using the UV transilluminator (302 nm), all other gels were imaged using Epi-Blue lights with an orange filter. In all instances images were captured using auto-exposure settings. No difference in sensitivity was detected between images generated by the Azure 200 or 600 confirming that any limit of detection differences are dye dependent.

Band	4kb	3kb	2kb
Lane	Mass (pg) in 10 $\mu$ L		
1	4125	15625	6000
2	2063	7813	3000
3	1031	3906	1500
4	516	1953	750
5	258	977	375
6	129	488	188
7	64	244	94
8	32	122	47

**Table 1.** DNA ladder dilutions.



## Results and Conclusion

In this note the sensitivity of various DNA dyes was assessed. A 1kb DNA ladder of known concentration was serially diluted and samples were resolved by agarose gel electrophoresis. One gel was cast with EtBr with all others post-stained. Sample gel images displaying the 2kb band are shown (Figure 1) along with a summary of DNA dye LoDs (Table 2).

Briefly, post-staining of gels with EtBr did not provide the same LoD as casting gels with EtBr in situ. All alternative DNA dyes displayed a similar LoD to EtBr post-stained when observing larger DNA fragments. Whilst EtBr displayed an enhanced LoD, when detecting smaller fragments, compared to EZ-Vision® Bluelight, SYBR® Gold and SYBR® Green; SYBR® Safe demonstrated an identical LoD.

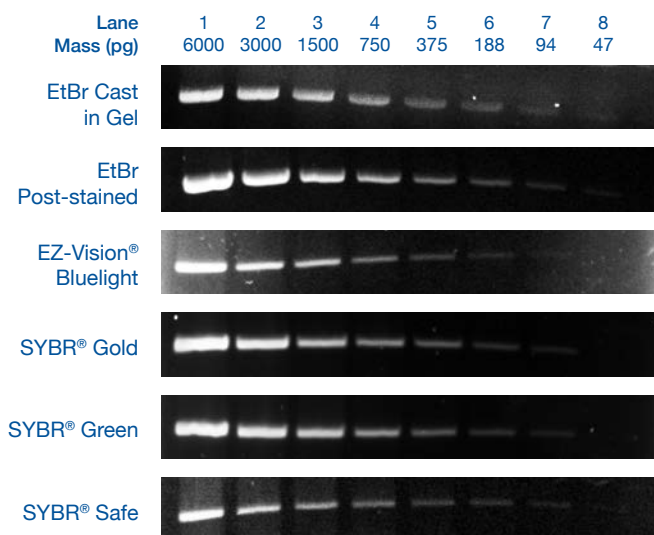
These results suggest that labs looking to reduce worker exposure to mutagenic EtBr whilst also reducing the amounts of hazardous waste produced do not have to accept a DNA dye with a lower limit of detection.

## Reference

1. Aaij, C. & Borst, P. The gel electrophoresis of DNA. *Biochim Biophys Acta* 269, 192-200 (1972).

Band	4kb	3kb	2kb
Stain			
Limit of Detection (pg)			
EtBr Cast in Gel	32	122	47
EtBr post-stained	64	244	94
EZ-Vision® Bluelight	32	244	188
SYBR® Gold	32	122	94
SYBR® Green	32	244	94
SYBR® Safe	32	122	47

**Table 2.** Summary of DNA dye LoDs. Gray shading indicates highest limit of detection.



**Figure 1.** Samples gel images. Representative blots showing 2kb band of DNA ladder.

# Imaging Viral Load in Chicken Embryos

## Introduction

Newcastle Disease Virus (NDV) is a contagious disease, capable of transmission to humans, and with a high risk of a severe epizootic in birds, particularly domestic poultry which are typically reared at high density.

While the effects of NDV are mild in humans, its effect in avian hosts are more pronounced and vary based on the age and species of the host, as well as the strain of the virus. Three strains of NDV, with differing levels of virulence, have been well characterized in chicken eggs. Strains of intermediary virulence are known to infect primarily the umbilical tissue of the chicken embryo, whilst more virulent strains infect other tissues indiscriminately.

As such many studies have been performed to investigate the impacts of the varying strains along with the mechanisms of viral load in domestic chickens. However, such studies have often been limited in their ability to accurately and rapidly assess viral load in vivo. With the development of sensitive high-resolution imaging systems, such as the Azure 600, it is now possible to quickly and easily image fluorescently tagged NDV in chicken embryos, and, to some extent, quantify the viral load in various embryonic tissues.

## Methods

Chicken embryos were infected at E10 and incubated for a further 72 hours. Embryos were infected with either 100-1000 PFU of virulent or non-virulent recombinant NDV tagged with eGFP, and compared with a non-infected control. The embryos were placed on petri dishes and imaged with the Azure 600 using Epi Blue imaging mode with an automated capture.

## Results and Conclusions

Figure 1 shows an embryo infected with a non-virulent form of the virus next to an uninfected embryo (negative control). The infected embryo shows the non-virulent strain of the virus confined primarily in the umbilical tissue.

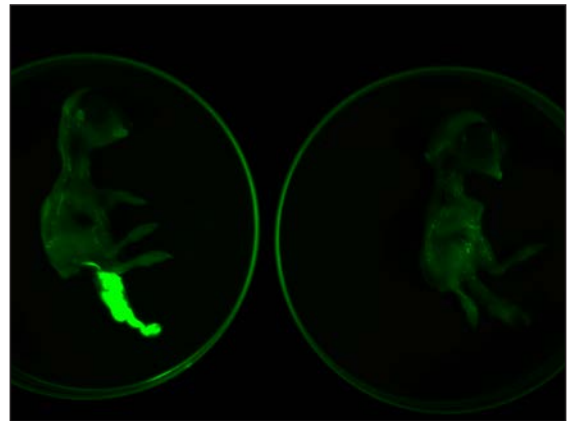


Figure 1. NDV eGFP No-VIR (left) and Negative Control (right).

Figure 2 shows the same non-virulent virus-infected embryo imaged alongside an embryo infected with a more virulent strain of NDV. The virulent form of the virus infects the embryo more indiscriminately, and the virus is detected throughout the embryo.

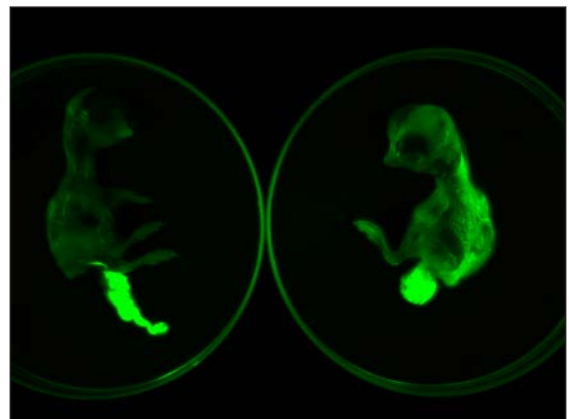


Figure 2. NDV eGFP No-VIR (left) and NDV eGFP VIR (right).



Figure 3 shows the extent of the virulent infection compared to the negative control, uninfected embryo.

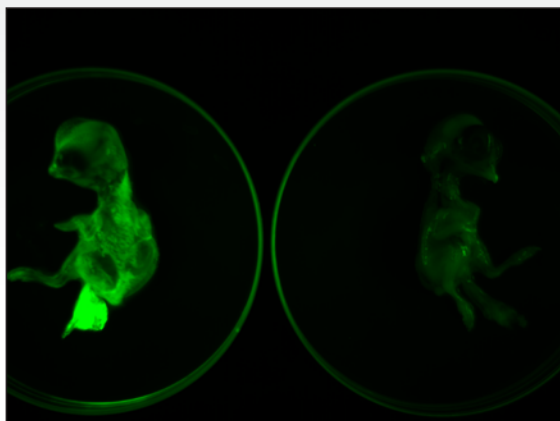


Figure 3. NDV eGFP VIR (left) and Negative Control (right).

## Conclusions

The utility of fluorescent imagers like the Azure Biosystems 600 extends far beyond Western blotting, including a large number of applications that utilize fluorescence excitation. This application note showcases one such application – imaging a fluorescently tagged virus in animal models allowing for the generation of high-resolution quantifiable images.

## Acknowledgements

Thank you to Ray Izquierdo-Lara, Ana Chumbe, Katherine Calderon, Manolo Fernandez. FARVET SAC, Peru, for providing the images.

# Phosphor Imaging with the Sapphire Biomolecular Imager

## Introduction

While fluorescent and chemiluminescent imaging techniques and their detectors are becoming more sensitive and selective, photostimulated luminescence from phosphor storage plates remains a key application for many researchers, especially for applications such as electrophoretic mobility shift assays (EMSA), enzyme assays, and in vivo imaging.

Traditional phosphor imagers are often single function and require a large footprint. The Azure Biosystems Sapphire Biomolecular Imager offers the ability to scan storage phosphor screens for filmless autoradiography, with exceptional dynamic range and image quality thanks to the use of laser excitation and photon multiplier tube (PMT) detection. The compact Sapphire Biomolecular Imager also incorporates exceptional visible fluorescent and near infra-red scanning along with true chemiluminescent and white light imaging making it a true lab workhorse.

## Materials and Methods

### Sample Preparation

An American Radiolabeled Chemicals  $^{14}\text{C}$  standard with slices from 1000 to  $0.004\ \mu\text{Ci/g}$  was exposed to a BAS-MS storage phosphor screen ( $0.9\ \text{DPM/mm}^2/\text{hr}$  sensitivity) for 3 hours.

### Storage Phosphor Plate Imaging

Following exposure, the storage phosphor screen was imaged using the Phosphor Imaging module of the Sapphire Biomolecular Imager with standard excitation and detection settings. Samples were quantified and limits of detection (LOD) and dynamic range (DR) calculated.

## Results and Conclusions

The Sapphire Biomolecular Imager captures high quality images with a large dynamic range from standard storage phosphor screens (Figure 1). Following sample quantification using AzureSpot analysis software, the LOD for American Radiolabeled Chemicals  $^{14}\text{C}$  standard after a 3-hour exposure was determined to be  $0.036\ \mu\text{Ci/g}$ . Furthermore, a highly linear dynamic range from 1000 to  $0.036\ \mu\text{Ci/g}$  was observed ( $r^2 = 0.99$  [Figure 2]).

To conclude, the Sapphire Biomolecular Imager is capable of imaging storage phosphor screens producing high quality images for later quantification; while also offering the ability to rapidly image fluorescent, chemiluminescent and NIR samples all within a single compact footprint.



Figure 1. American Radiolabeled Chemicals Carbon-14 standard exposed to Storage Phosphor Screen for three hours then imaged on the Sapphire Biomolecular Imager.

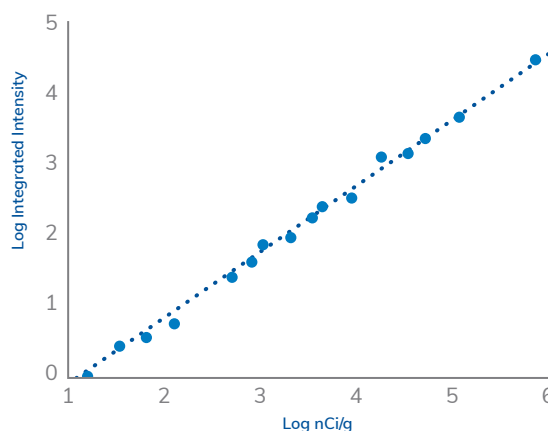


Figure 2. Sample:  $^{14}\text{C}$  autoradiographic standard; Imaging: Phosphor; LOD:  $0.036\ \mu\text{Ci/g}$ ; DR: 5.4 orders of magnitude; Linearity:  $R^2=0.99$

Step	Product	Part number
Sample preparation	American Radiolabeled Chemicals – <sup>14</sup> C standard	ARC0146F
Storage phosphor plate	BAS-IP-MS – Storage phosphor screen	IS1011
	Cassette for Phosphor Imaging	IS1012
Phosphor plate imaging	Azure Biosystems – Sapphire Biomolecular Imager with Phosphor Imaging Upgrade	IS1002, IS1004

Table 1. Materials and product numbers.

# Detecting Proteins In-Situ with In-Cell Western Blotting

## Introduction

Western blotting was first described by three groups in 1979. Since then numerous refinements to the basic technique, reagents used and imaging technologies have massively broadened the usage of Western blotting, making it a cornerstone protocol in life sciences today.

However, the general workflow of a Western blot has remained the same throughout this time. Samples are isolated and protein is extracted and standardized. Proteins are then separated by gel electrophoresis, transferred and probed with primary and secondary antibodies before being visualized. With numerous steps, Western blotting as a protocol requires a large investment in equipment, training, reagents and, perhaps most importantly, time.

A standard Western blot protocol takes two days to work from sample to image. Therefore, when analyzing a large number of samples or performing optimizations, it is easy to see how Western Blotting can quickly become a bottleneck in your research workflow.

Enter in-cell Western blotting – a radical rethinking of the standard protocol coupling the ability to accurately quantify intracellular proteins from Western blotting with the repeatability, quick turnaround and high-throughput of an ELISA. Cells are grown on sterile plates,

before being fixed and permeabilized *in situ*. Subsequent labelling is performed as normal with a specific primary antibody followed by an isotype specific fluorescent conjugated secondary antibody. Conjugates in the near-infrared (NIR) spectrum are commonly used to reduce auto-fluorescence and noise typically associated with tissue culture plastics.

The Azure Biosystems Sapphire™ Biomolecular Scanner is specifically designed to allow for visualization in two NIR channels (658 nm and 785 nm) as well as visible fluorescent wavelengths (488 nm and 520 nm), allowing for multiplex imaging within wells. This multiplex capacity allows for the assessment of multiple proteins of interest in a single well which is useful for looking at total and phosphorylated versions of a protein or to detect an in-well standard to allow for quantification across a plate.

With a laser based excitation system using photomultiplier tube (PMT) and avalanche photodiode (APD) detectors, signal selectivity, sensitivity and speed of imaging achievable by the Sapphire make it an ideal choice for in-cell Western blotting.

## Materials and Methods

### Culture cells

HeLa cells were serially diluted and seeded into a sterile 96 well tissue culture plate at a volume of 0.2 mL per well, and grown until approximately 80% confluent. All wells were fixed and permeabilized using 100% methanol for 15 minutes at room temperature.

### In-cell Western blotting

Following fixation and permeabilization, cells were rinsed in PBS, blocked with 1% fish gelatin in PBS for one hour at room temperature, then probed for alpha-tubulin and beta-actin overnight at 4°C. Samples were washed three times with PBS prior to incubation with AzureSpectra 550 and AzureSpectra 800 conjugated secondary antibodies for 60 minutes.

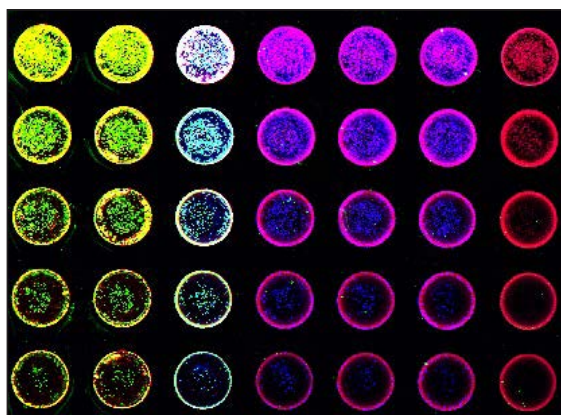


Figure 1. Representative image of an in-cell Western blot.

Secondary antibodies were incubated along with RedDot™ 1 Nuclear Stain to normalize for total cell number. Plates were washed as previously described before imaging.

### Imaging

After washing the plate was imaged on the Azure Biosystems Sapphire™ Biomolecular Scanner using the Plate focus setting.

## Results and Conclusions

In this note we demonstrate the ability to quantify intracellular proteins *in situ* using the Azure Biosystems Sapphire™ Biomolecular Scanner. HeLa cells were grown in a 96-well plate probed with alpha-tubulin and beta-actin antibodies followed by isotype appropriate secondary antibodies conjugated with AzureSpectra 550 and AzureSpectra 800 respectively. All wells were incubated with RedDot™ 1 Nuclear Stain to normalize for total cell number. Images were acquired using an Azure Biosystems Sapphire™ Biomolecular Scanner and are displayed in Figure 2. The representative image shown demonstrates the high level of sensitivity and specificity achievable.

Performing in cell Western blotting on 96 well plates allows for accurate measurement of intracellular proteins expression *in situ*; and provides a high throughput methodology to assess multiple stimulations, end-points, proteins of interest and replicates on a single plate. By using NIR antibodies and the Azure Biosystems Sapphire™ Biomolecular Scanner the potential for in well multiplex analysis also exists offering further improvements to throughput.

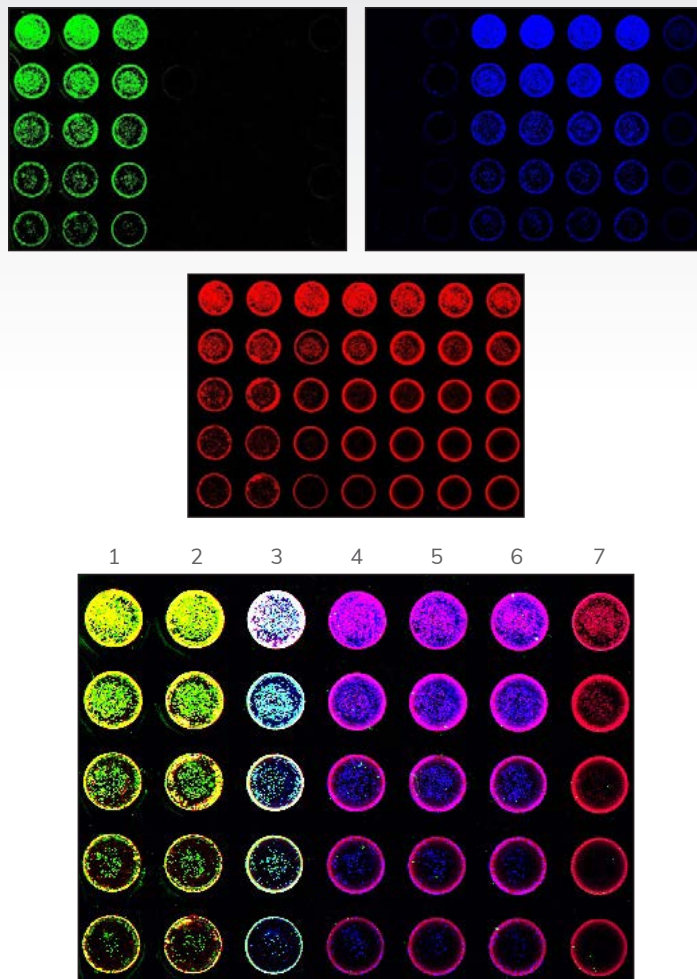


Figure 2. A serial dilution of HeLa cells were seeded into a 96-well plate, cultured, fixed and permeabilized. A) Columns 1-3 were probed for beta-Actin using AzureSpectra 550 (green). B) Columns 3-6 were probed for Tubulin using AzureSpectra 800 (blue). C) The entire plate was stained with RedDot1 Nuclear Stain as a normalization control (red). D) The individual channels were scanned simultaneously then combined into a single composite image using the Sapphire Capture Software.

# Increasing Assay Efficiency with Four-Color Detection

## Introduction

The field of Western blot multiplexing – the ability to probe for multiple proteins on a single blot simultaneously – is developing rapidly. Chemiluminescent assays allowed for the detection of a single protein followed by multiple rounds of time consuming stripping and re-probing, with associated potential loss of protein and corresponding signal. Early Western blot multiplex imaging systems allowed for the imaging of two spectrally distinct fluorophores on a single blot. Researchers rapidly used this technique to assay for loading controls and proteins of interest on a single blot, to compare two distinct proteins, and to devise many other methodologies which are discussed in other application notes.

We have previously described an improvement on this two-channel methodology by imaging three proteins simultaneously by combining the fluorescent and near-infrared (NIR) imaging capabilities offered by the Azure Biosystems 600 digital imager.

In this note we discuss a further improvement – four-color Western blot multiplexing using the Azure Biosystems Sapphire™ Biomolecular Imager. The ability to simultaneously image four colors at one time greatly increases Western blot efficiency and the ability to make meaningful quantitative comparisons. Four-color Western blotting is made possible through the use of four spectrally distinct fluorophores and the Sapphire's selective laser based excitation and sensitive photomultiplier tube (PMT) and avalanche photodiode (APD) detection systems.

## Materials and Methods

### Run and transfer gel

Samples of 1.25 to 5 µg of HeLa cell lysate, some spiked with varying amounts of transferrin were electrophoresed on a 4-15% Tris-Glycine gel. After electrophoresis and separation, proteins were transferred to a low fluorescence PVDF membrane using Azure Transfer Buffer.

### 4-color Western blotting

Following transfer, the membrane was blocked for 30 minutes with Azure Fluorescent Blot Blocking Buffer then probed with rat anti-tubulin (Green), rabbit anti-beta-actin (Red), and chicken anti-GAPDH (Gray) primary antibodies; and anti-transferrin (Blue) which had previously been labelled with AzureSpectra 490 dye using the AzureSpectra Labeling Kit.

Blots were rinsed and washed with Azure Fluorescent Blot Washing Buffer before being incubated with AzureSpectra labeled secondary antibodies – goat anti-rat 550 (Green), goat anti-rabbit 700 (Red), and goat anti-chicken 800 (Gray). After incubation, the blot was washed as before in Azure Florescent Blot Washing Buffer followed by a final rinse in PBS.

### Image 4-color Western blot

After rinsing in PBS, the blot was allowed to dry before imaging on the Azure Biosystems Sapphire™ Biomolecular Imager.

## Results and Conclusions

In this note a single Western blot was probed for four proteins simultaneously. HeLa cell samples, or HeLa cell samples spiked with transferrin were probed with tubulin (Green), beta-actin (Red) and GAPDH (Gray) antibodies followed by isotype appropriate secondary antibodies; as well as transferrin (Blue) antibody previously conjugated with AzureSpectra 490 dye for direct analysis.

The Western blot was imaged using the Azure Biosystems Sapphire™ Biomolecular Scanner and Figure 1 shows the grayscale image captured at each wavelength for transferrin (a), tubulin (b), beta-actin (c) and GAPDH (d); together with a merged, colored image (e). Together the images demonstrate the high level of sensitivity, specificity and lack of background signal it is possible to achieve using this methodology which allows for rapid and accurate quantitative analysis.

The ability to image four proteins on a single blot greatly increases the efficiency of Western blotting, saving time and precious samples and allows for better quantitative analysis. The methodology also allows for the use of novel assays such as investigations of protein:protein interactions or on-blot total vs phosphorylated protein assays.

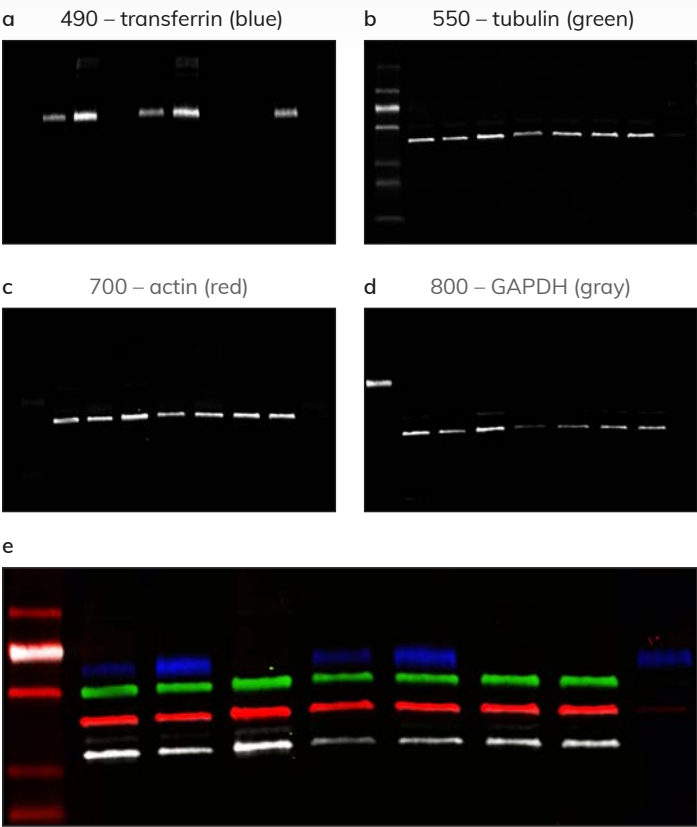


Figure 1. Digital images of 4-color Western blot, captured using Azure Biosystems Sapphire™ Biomolecular Imager. Transferrin (a), tubulin (b), actin (c) and GAPDH (d) were probed on a single blot using distinct fluorescent and near-infrared targeting antibodies. Images were captured using the Azure Biosystems Sapphire™ Biomolecular Imager at the specified wavelengths and merged into the four-color multiplex image (e). Sensitive and specific detection of all four proteins was possible with no evidence of background auto-fluorescence or bleed between channels.

Step	Product	Part number
Electrophoresis & transfer	4-15% Tris-Glycine gel	N/A
	PVDF Membrane	Azure, AC2105
	Azure Transfer Buffer	Azure, AC2127
Blocking & antibody labelling	AzureSpectra Fluorescent Blot Blocking Buffer	Azure, AC2190
	AzureSpectra Labeling Kit – 490	Azure, AC2185
Probe Blot	Primary Antibodies	Per protein of interest
	AzureSpectra Goat-anti-rat-550	Azure, AC2162
	AzureSpectra Goat-anti-rabbit-700	Azure, AC2128
	AzureSpectra Goat-anti-chicken-800	Azure, AC2137
	PBS	N/A

Table 1. Material and product numbers.



# Accurate Western Blot Normalization with AzureRed Fluorescent Protein Stain

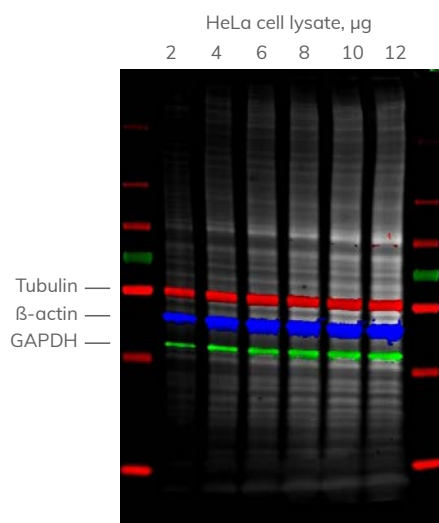
## Introduction

An important component of quantitative Western blotting is normalization of the band intensity obtained from the target protein against a reference whose intensity should only vary proportionally to the quantity of material in the sample. Traditionally, “housekeeping proteins” – proteins thought to be consistently expressed across different sample types – are used as such a reference. However, in some cases, housekeeping proteins may not be expressed consistently between samples. Studies have emerged showing that experimental conditions may alter the expression of some commonly used housekeeping proteins.<sup>1</sup> In other situations, housekeeping proteins may not be present in the sample at a level that is similar enough to the protein of interest to fall within the linear range of detection.<sup>2-3</sup>

An increasingly popular alternative normalization method that avoids many of the drawbacks of relying on housekeeping proteins is “total protein normalization” or TPN. In this method, the volume of the protein of interest is compared to the total amount of protein loaded in the lane. TPN presents advantages over using a single protein as a standard including not being susceptible to unexpected changes in the amount of the standard protein between samples. Instead of looking at the intensity of one protein, the total density for each lane is measured. Because TPN technology eliminates many of the issues related to signal normalization, publications are increasingly requiring the use of total protein signal for normalization.<sup>4</sup>

AzureRed Fluorescent Protein Stain is a quantitative total-protein stain for gels and blots that is compatible with downstream protein analysis, including Western blotting. AzureRed is a perfect choice for staining applications, including post-transfer staining to confirm uniform protein transfer from gel to membrane, and staining quantitative Western blots as part of a TPN protocol.

In this application note, we demonstrate that AzureRed Fluorescent Protein Stain is ideally suited for TPN because of its wide, linear dynamic range and sensitivity. Additionally, it is fully compatible with standard Western blot workflows, which we demonstrate by normalizing a tricolor fluorescent Western blot (three different proteins detected) to total protein using AzureRed stain.



**Figure 1.** AzureRed is imaged simultaneously with three proteins of interest. The gel was loaded with dilutions of HeLa cell lysate. After transfer, the blot was stained with AzureRed and then probed for tubulin,  $\beta$ -actin, and GAPDH without a destaining step. The blot was scanned with each of the four lasers of the Sapphire Biomolecular Imager. In this overlay of the four channels, total protein (AzureRed stain) is shown in gray; tubulin in red,  $\beta$ -actin in blue, and GAPDH in green.

## Methods

Samples of HeLa cell lysate were separated by polyacrylamide gel electrophoresis and proteins transferred from the gel to a low-fluorescence PVDF membrane (Azure Biosystems product AC2127; AC2105). After protein transfer, the blot was stained with AzureRed Fluorescent Protein Stain (Azure Biosystems product AC2124), according to the PVDF staining protocol (available at [www.azurebiosystems.com](http://www.azurebiosystems.com)).



Following transfer, the blot was washed briefly in water then stained in AzureRed stain solution according to the protocol. After the 30-minute staining procedure, the blot was blocked using Azure Fluorescent Blot Blocking Buffer (Azure Biosystems product AC2190) and incubated with primary antibodies for Western blot detection (Table 1).

The blot was imaged using the Sapphire Biomolecular Imager by scanning the blot with all four laser simultaneously. The three target proteins were detected with the lasers as shown in Table 1, while AzureRed staining was detected using the 520nm laser.

Images were analyzed using AzureSpot software.

Target	Primary antibody	Secondary antibody	Detected using laser
Tubulin	Rat anti-tubulin	Goat anti-rat AzureSpectra 700	658nm
β-actin	Rabbit anti-β-actin	Goat anti-rabbit AzureSpectra 490	488nm
GAPDH	Chicken anti-GAPDH	Goat anti-chicken AzureSpectra 800	784nm

Figure 1. Overview of a Western blotting protocol using indirect antigen detection.

## Results

Scanning the tricolor Western blot stained with AzureRed Fluorescent Protein Stain on the Sapphire Biomolecular Imager results in a four-channel composite image (Figure 1). All four channels are imaged and aligned as part of image capture, removing the need to align the images at a later point, and not adding any additional imaging steps in the workflow. The composite image with an overlay of the four resulting images allows easy comparison of the three proteins with each other and with total protein load.

Viewing the single channel images allows visual inspection of the dynamic range of AzureRed and the three proteins (Figure 1A-D). The signal intensity for total protein in each lane (for AzureRed stain) or band (for each of the three proteins detected) was quantified using AzureSpot software. Figure 2E shows the relationship between each signal and the amount of protein loaded on the gel. The quantitative nature of AzureRed stain is observed in the excellent correlation of signal to amount of protein loaded ( $R^2 = 0.994$ ). This correlation was superior to that of any of the three individual proteins (Figure 2E), each of which is frequently used as a housekeeping protein.

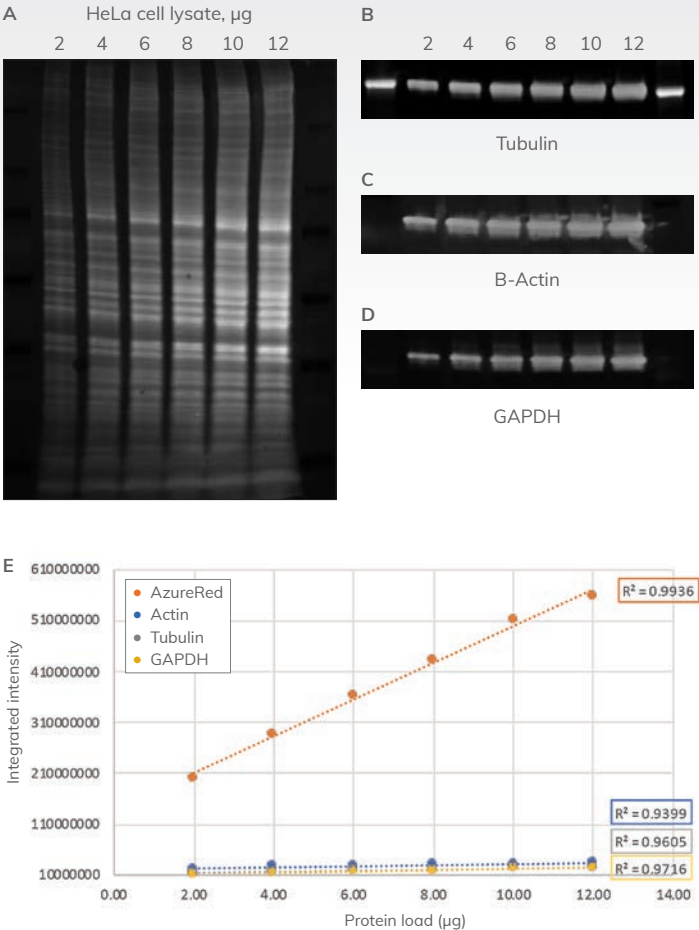


Figure 2. AzureRed has a wider linear dynamic range compared to common housekeeping proteins. Single color channel images for A) AureRed staining for total protein B) Tubulin C) B-actin and D) GAPDH. The superior dynamic range of the AzureRed signal to the actual amount of protein loaded is displayed in image (A). The signals from the total protein and the housekeeping proteins were quantified using the AzureSpot software, and the resulting values are plotted against the amount of protein loaded for each sample (E). AzureRed showed superior correlation and a much broader dynamic range than the common housekeeping proteins.

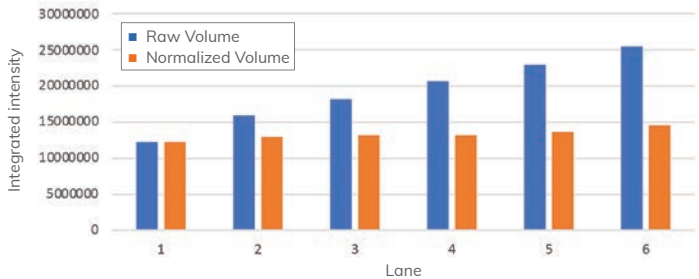


Figure 3. Tubulin signal normalized to total protein as assessed using AzureRed Fluorescent Protein Stain. The tubulin band was quantified using AzureSpot software. Raw tubulin band volume (blue bars) and band volume normalized to total protein (orange bars) are shown. Normalizing the signal volume using AzureRed corrects for any errors in sample loading or differences in transfer across the membrane.

The use of the AzureRed signal for total protein as a standard to normalize protein signal is shown in Figure 3. Normalizing the tubulin signal for each lane, by the total protein signal for the same lane, results in excellent agreement across samples, with a coefficient of variation of normalized values of only 4%.

## Conclusion

AzureRed Fluorescent Protein Stain is an ideal choice for quantitative fluorescent Western blotting. The wide, linear dynamic range ensures that AzureRed acts as an effective standard to account for variations in protein load. A key feature of AzureRed is the simplicity of fitting it into your existing workflow (Figure 4).

AzureRed dye can be detected simultaneously alongside commonly used fluorescent probes without interference, allowing multiple proteins to be assessed on a single blot. Using the quantitative AzureRed signal for total protein normalization of multicolor blots makes it possible to quantify multiple proteins on a single blot without stripping or destaining. The protocol is quick, and imaging occurs simultaneously with the proteins of interest.

Learn more about AzureRed Fluorescent Protein Stain at [www.azurebiosystems.com](http://www.azurebiosystems.com).

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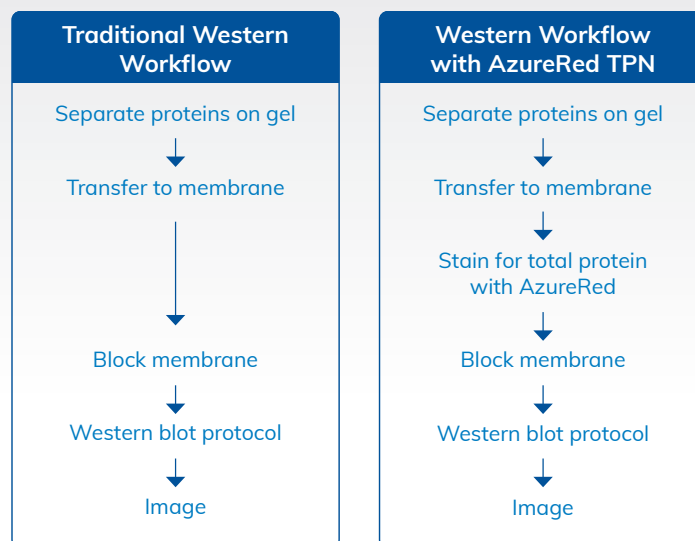


Figure 4. Total protein normalization with AzureRed fits easily into any Western blotting workflow.

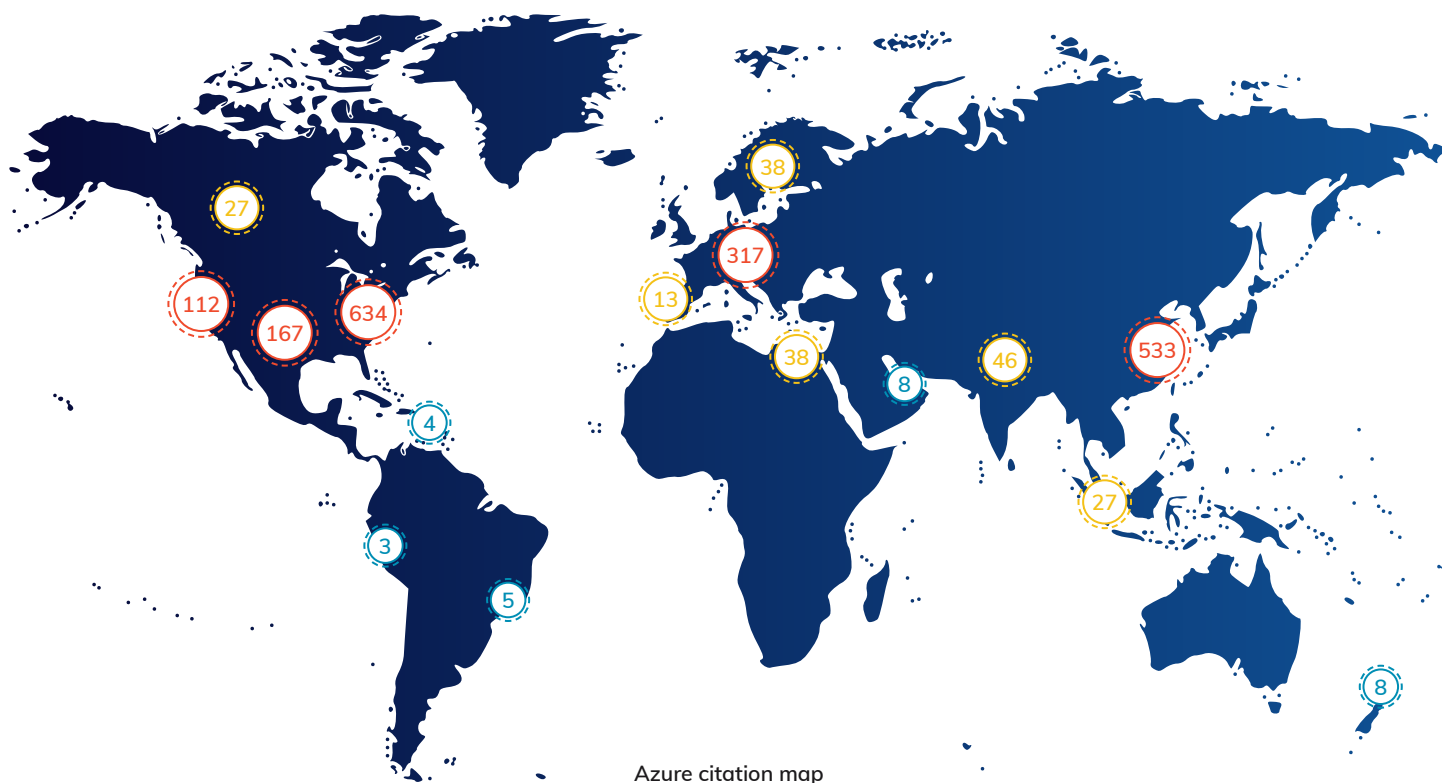


# 6

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