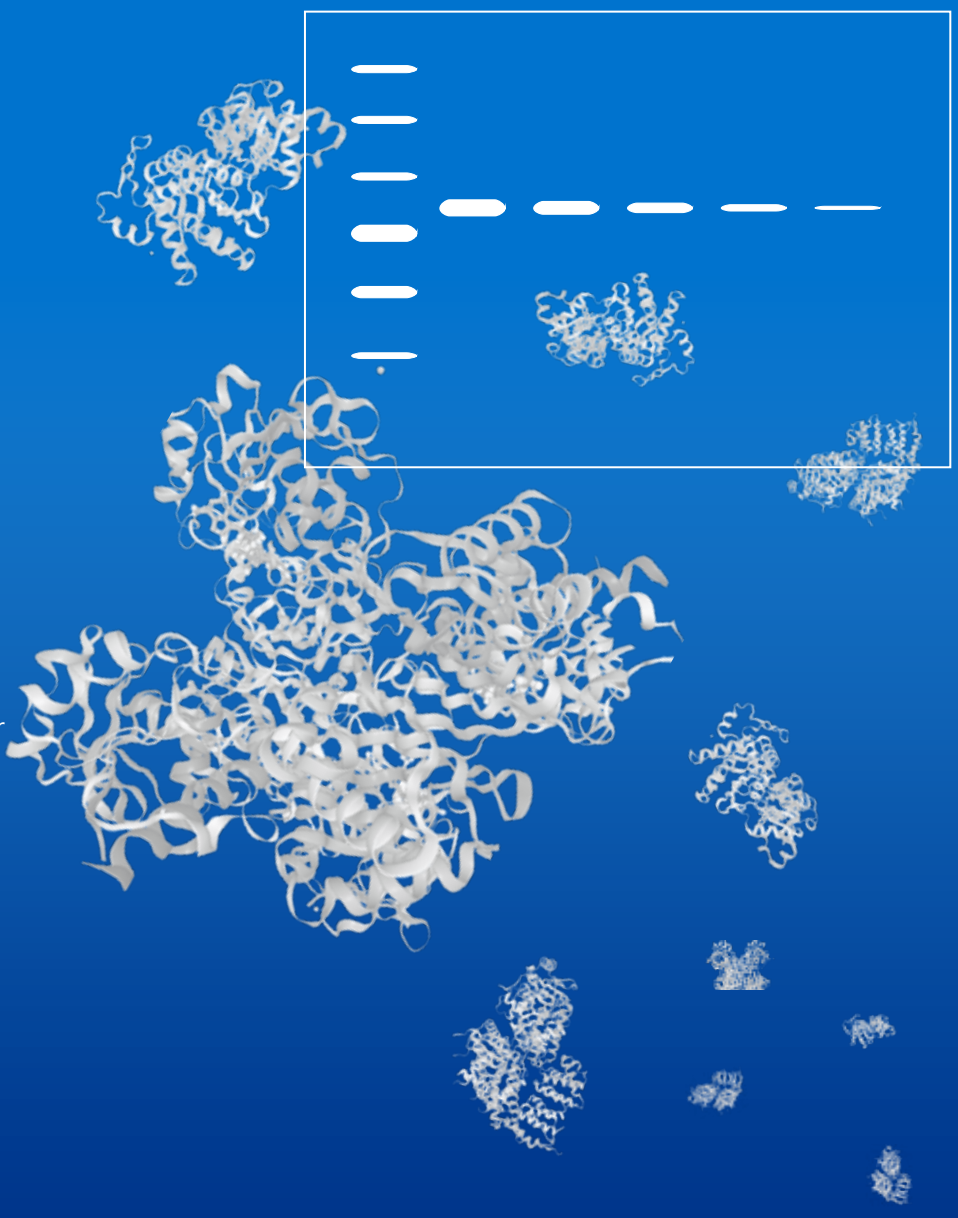
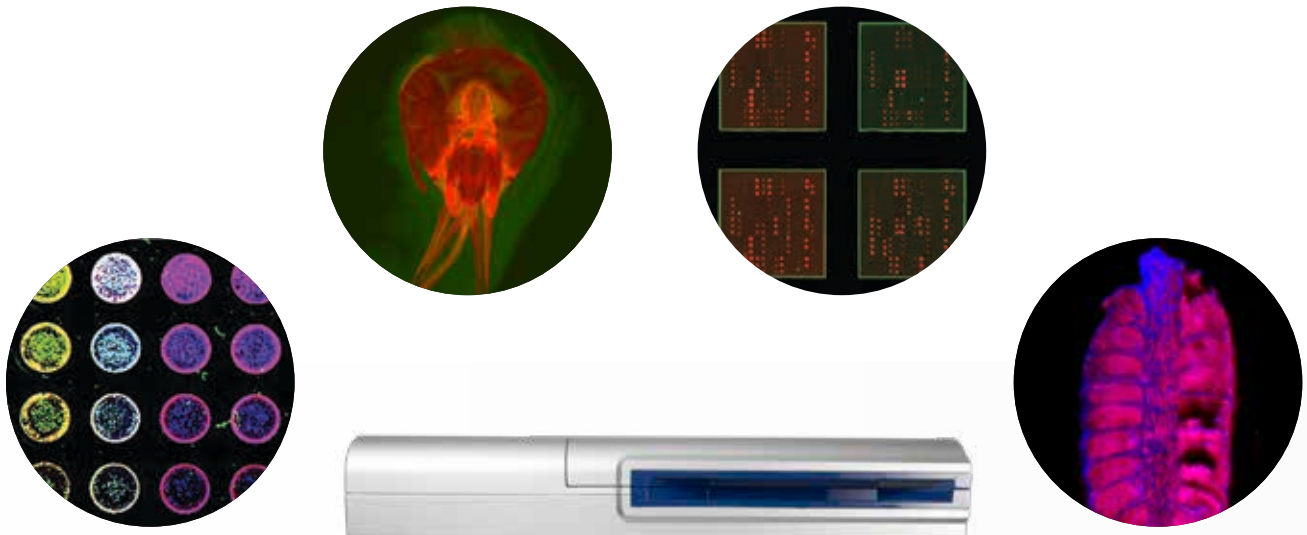
Western Blotting for Proteomics Labs



Can your imager do *this*?



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At Azure Biosystems, we develop easy-to-use, high-performance imaging systems and high-quality reagents optimized for generating quantitative data from western blots. From our innovative, first-in-class hybrid laser scanner-imager, the Sapphire Biomolecular Imager, to our Radiance-Q ECL substrate which has an exceptionally wide dynamic range and long half-life, we provide performance in places other companies simply overlook. Here are just a few of these excellent products. See our full portfolio of western blotting instruments and reagents by visiting **vwr.com/azure**.

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Our workhorse western blot and gel imager for detecting RGB fluorescence, NIR fluorescence, and chemiluminescence.

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	RGB	NIR	Chemi	Blue/White/UV
c600®	✓	✓	✓	✓
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c400®	✓		✓	✓
c300®			✓	✓
c280™			✓	✓
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Only c200, c300, c400, and c500 are upgradable.

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01 Quantitation

Like all assays, the quality and quantitativeness of western blot data is only as good as the weakest, most variable link in the measurement process. No matter the quality, accuracy, and precision of everything else, the step where accuracy and reproducibility are the poorest will be the step that defines and limits how quantitative your western blot will be. Which is why we've created this guide. In these pages, we provide practical, hands-on recommendations on how to obtain high-quality, quantitative western blot data so that you can draw solid conclusions and generate insights you trust.

01.1 Defining quantitative

Before discussing how to get quantitative data from a western blot, it's useful to define what we mean by "quantitative." For a measurement to be quantitative, it must meet the following criteria:

- The measurement is generated using a defined process, *i.e.* the western blot
- The process generates a reproducible outcome, *i.e.* is precise
- The measurement reflects a "true" outcome, *i.e.* is accurate

Laying out the definition of "quantitative" in this way provides a guidepost for the steps we can take to ensure that western blot data is quantitative—the western blot process needs to be well-defined and the steps followed as consistently as possible, the data need to be reproducible, and the data should be consistent with data collected using an orthogonal method such as mass spectrometry.

In the rest of this section we focus on how to meet these criteria, highlighting where paying special attention to consistency or performing reagent validations and controls can make a measurable impact on the reliability and reproducibility of your western blot data.

> "Just because we can put numbers on an image does not imply that we should—a quantified biomolecule should relate directly to the true quantity of that biomolecule if it is to be meaningful."[1]

Additional definitions

QUANTITATIVE VS. QUALITATIVE

A ***qualitative*** Western blot shows the presence or absence of a protein of interest but provides no information on relative protein abundance. A ***quantitative*** Western blot is used to detect specific proteins and measure relative protein abundance.

PRECISION

How close measurements are to each other

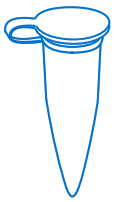
ACCURACY

How close a measurement is to the "true" measurement

Precise but not accurate

Accurate **and** precise

Accurate but not precise



01.2 Process samples the same way

Consistent sample preparation and handling is an important first step for generating quantitative westerns. Even small differences in incubation times, buffer composition, or temperatures (variable room temperature, freezing at -20°C vs. holding on ice), can noticeably detract from the reproducibility of your western blot data[2]. Once you establish a reliable protocol for cell or tissue lysis and sample handling, follow the procedure as closely as possible when preparing samples that will be compared to each other.

01.3 Validate your antibodies

Journals want to see that an antibody does, in fact, detect the protein of interest, and it's just good experimental technique to validate antibodies in your own lab using your own samples and techniques. This is especially important when using an antibody that you have never published with before.

"Quantitation of Western blots is not always required but it can be fraught with traps for the unwary investigator and often sparks lively debate among scientists."[4]

Simply verifying that your primary antibody detects a protein at the expected molecular weight is not enough. It's important to show that binding is specific to the target protein, preferably by more than one method. An excellent set of recommendations has been published by the International Working Group for Antibody Validation, which lists five distinct methods, or pillars, for validating antibodies[3]. Four of these pillars are appropriate for validating antibodies used in Western blotting, and we've summarized those here.

- **Genetic validation:** Demonstrate the specificity of your antibody by showing that signal is eliminated or reduced when protein expression is reduced, either by knockout of the gene or elimination of the target protein's translation via RNAi.
- **Orthogonal validation:** Demonstrate that measurement of protein abundance using your antibody correlates strongly with the measurement of protein abundance using an orthogonal method such as mass spectrometry.

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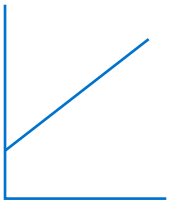
- **Independent antibody**: Demonstrate that the measurement of protein abundance using your antibody correlates strongly with the measurement of protein abundance using a second, already validated antibody that recognizes a different, non-overlapping epitope on the same target protein.
- **Tagged protein expression** (this is a specific variation of the independent antibody method): Express an epitope-tagged version of the target protein and demonstrate that measurement of protein abundance using your antibody correlates with measurement of protein abundance using a validated antibody that recognizes the epitope tag.

Note that antibody validation is application-specific—an antibody validated for ELISAs is not validated for western blotting (and vice versa) as binding occurs under distinctly different conditions.

01.4 Validate signal linearity and optimize dynamic range



In order to obtain an accurate measurement from a western blot, you need to ensure that the detected signal is proportional to the amount of protein present—in other words, that detection is in the linear range. However, there are multiple steps in the western blotting process that can lead to signal saturation, necessitating validation of signal linearty at each of these steps. Note that many of these steps can also be optimized to maximize the dynamic range of the signal.

Verify that the amount of sample used is in the linear range

To obtain the most robust quantitative western blot data, we recommend generating a standard curve that covers the full range of sample amounts you will assess, testing multiple replicates for each amount (Figure 1). A plot of signal intensity versus sample amount will indicate how much protein you can load and still be confident that your signal is in the linear range.

Note that the linear range of your system should be determined for each antibody-protein pair.

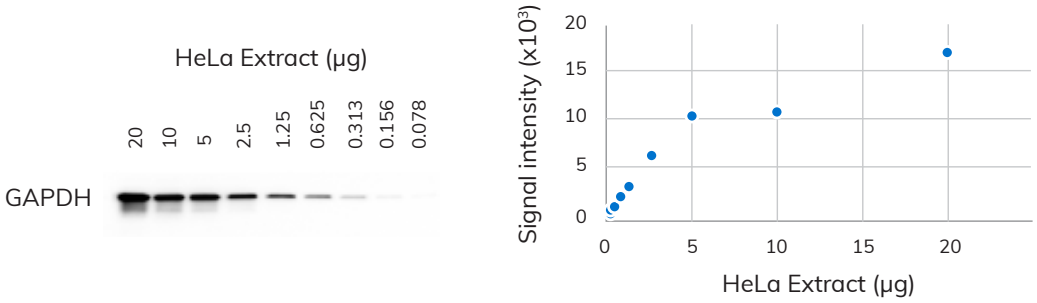


Figure 1. Determining linear range of sample amount. The linear range for this sample lies between 0.078 and 5 µg.

▶ IMPORTANT!

For the most reliable western blot quantitation, we recommend imaging all samples to be compared on the same blot, using the same exposure time, and without stripping and reprobing. While normalization can be used to bridge the different conditions, there's always the risk of introducing variation that impacts both accuracy and precision, such as non-uniform stripping of the blot.

Check how transfer conditions affect signal linearity

The amount of protein a membrane can hold can saturate. We recommend testing several transfer times and choosing the shortest time that leads to a linear signal. If the signal remains saturated at all transfer times tested, it is likely that a different step is contributing to signal saturation. In this situation you can continue optimizing other parameters and then return to transfer time and verify that membrane capacity is not saturated.

▸ DID YOU KNOW?

Today's digital imagers and scanners can detect chemiluminescent signal over a much wider dynamic range than film, and the most powerful scanners can provide signal detection at the same sensitivity as film.

Titrate your primary antibody

We recommend testing different antibody dilutions against the full range of target protein amounts you expect to detect. This will ensure that your assay is sensitive enough to detect the lowest amount of target protein, and has a wide enough dynamic range that detection of your highest amount of protein is still in the linear range (Figure 2).

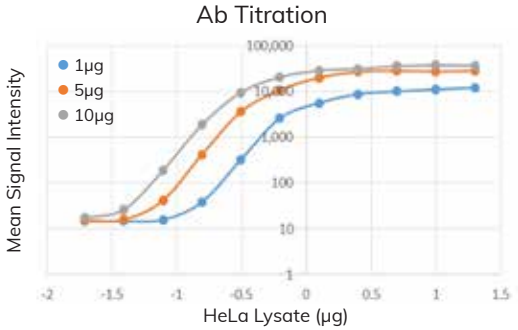


Figure 2. Titrating primary antibody to find the optimum dilution. Three different amounts of primary antibody were added to 10 mL of Azure Chemiluminescent blocking buffer while the other western blot conditions were held constant. The lowest amount of antibody that gives the widest dynamic range is 5 µL.

Optimize image acquisition time

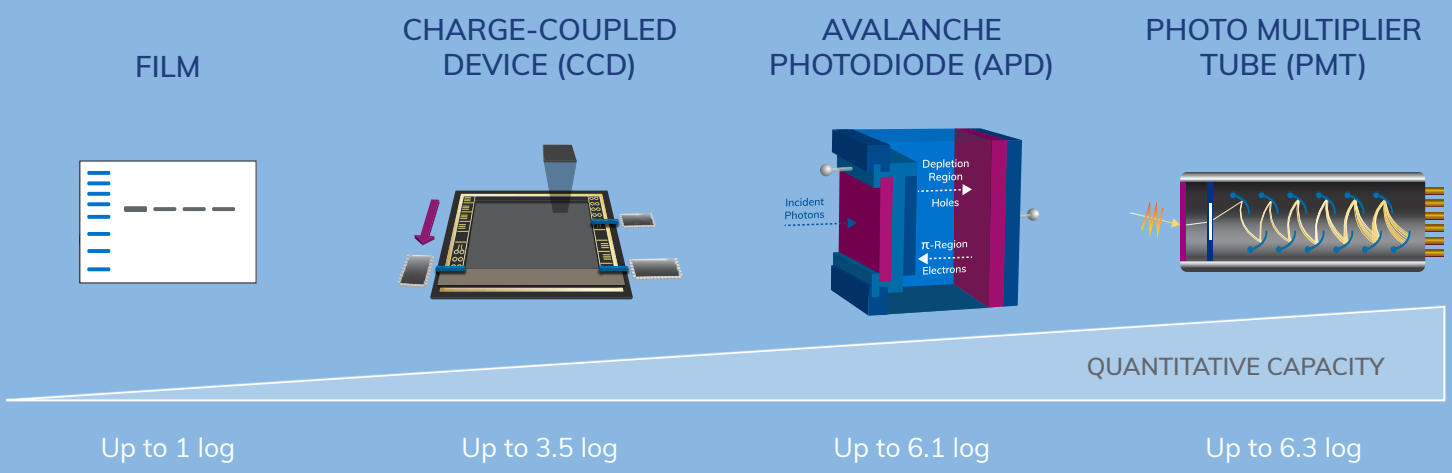
The last step to assess for linearity is the exposure time of the blot to the detection system, whether that's film or a digital imager or scanner. While exposure times need to be long enough to detect the lowest amount of protein, the exposure should be short enough that the highest amount of protein detected is still in the linear range.

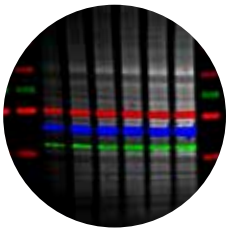
Choose the right substrate for quantitative chemiluminescent blots

Chemiluminescence ***can*** be quantitative with the proper controls and the right ECL substrate. Choose a substrate with a wide dynamic range, sensitive detection, and a long half-life for the most reliable and reproducible quantitation.

Dynamic range is affected by the detection technology

Film is typically linear over a range of up-to 1 log, a fairly narrow dynamic range. In contrast, Photomultiplier tubes can deliver linear detection over approximately 6.3 logs.





01.5 Normalize to total protein

Normalization is critical for reliable western blot quantitation—by finding a feature that is expected to remain invariant across samples and conditions you can control for variations in sample preparation, loading, and transfer, and generate a relative measurement of your target protein to that invariant feature.

One widely-used method has been to normalize the signal of your target protein to that of a housekeeping protein such as GAPDH, tubulin, or actin, which was assumed to have stable and invariant levels of expression. A series of recent studies have shown how flawed this assumption can be, and now many western blotting experts and scientific journals, such as The Journal of Biological Chemistry, are recommending that western blots are either normalized to total protein instead of a housekeeping protein, or that the protein used for normalization is validated as having stable expression using the same cells and conditions as the test samples[2,4,5,6].

Choosing a total protein stain (TPS)

A recent study has uncovered marked molecular heterogeneity in different HeLa cell isolates at the genome, transcriptome, and proteome levels, highlighting the hazards of normalizing to a specific protein versus total protein[7].

There are different total protein stains (TPS) to choose from and the TPS you choose will affect the complexity of the total protein normalization (TPN) workflow (for a nice overview of TPS stains, see Mortiz[5]). Important considerations when choosing a TPS include:

- Dynamic range
- Detection limits
- Visualization method
- Staining time
- Visualization time
- Consistency across tissues and experimental conditions
- Compatibility with antibody-based detection

For the simplest TPN workflows, we recommend using a fluorescent stain that will enable simultaneous detection of the TPS and target protein (or proteins), although this will require a fluorescent detector with two or more channels. Some TPSs are also compatible with chemiluminescent detection.

01.6 Use consistent western blotting protocols

Carefully and stringently following an established protocol helps reduce variability and improve measurement precision for robust quantitative western blots. Not only should you reduce variability by using the same reagents or implementing lot-to-lot controls, keeping your incubation and wash times the same will also help ensure consistent and reliable results.

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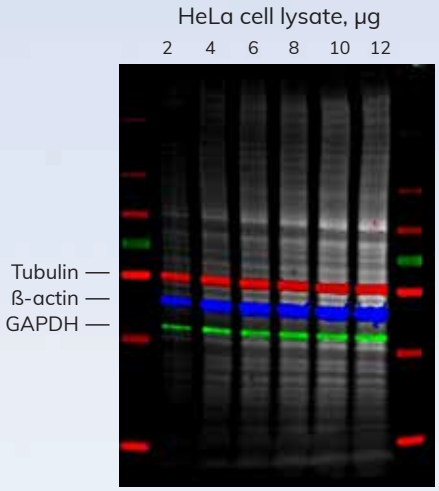
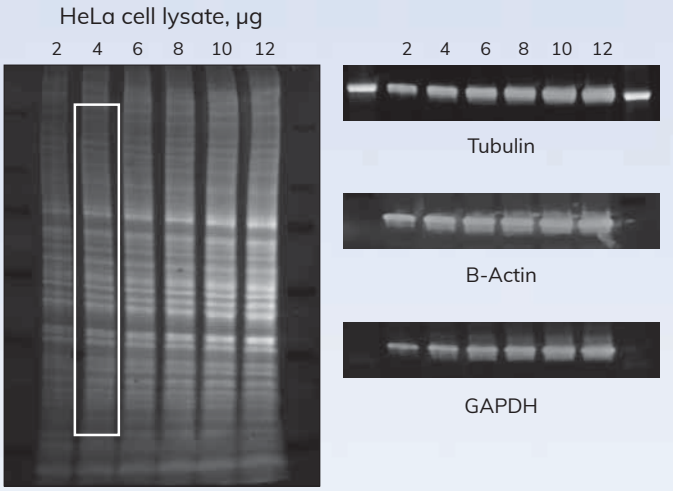
Total Protein Normalization with AzureRed Total Protein Stain

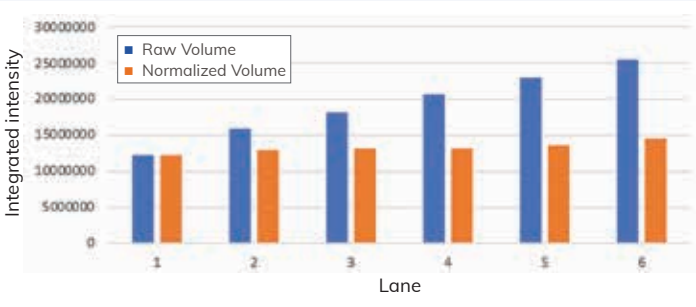
Normalizing to total protein can be just as straightforward as normalizing to a housekeeping protein. In this example, we demonstrate TPN using AzureRed Total Protein Stain to normalize signal from three different proteins found in HeLa cell lysate—tubulin, ß-actin, and GAPDH.



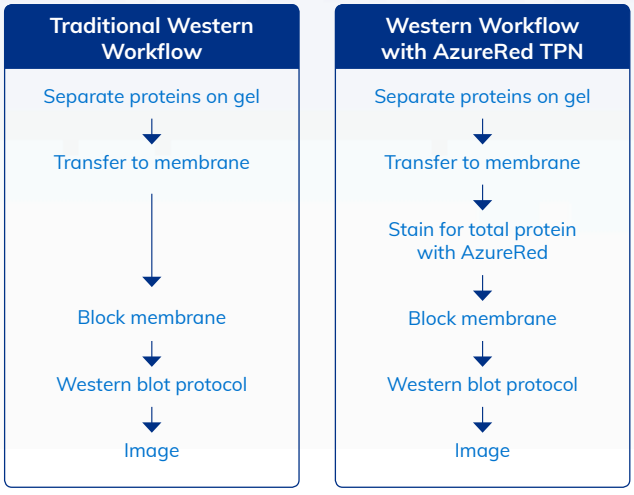
A. Four-color detection of a blot with increasing amounts of HeLa cell lysate. Tubulin is in red, actin is in blue, GAPDH is in green, and AzureRed/total protein is in white.



B. Individual channels of the same blot. To calculate the total protein signal, simply draw a box around the entire lane and normalize your signal-of-interest to the total protein signal as usual.



C. Quantitation of the tubulin signal normalized to total protein (orange) shows how TPN can correct for loading differences.



D. The AzureRed TPS Workflow adds only an extra incubation step to the western blot protocol.

Radiance Plus

See bands that other substrates miss and quantify even low-intensity signal thanks to Radiance Plus's high signal-to-noise ratio and wide dynamic range. The most sensitive HRP substrate available from Azure, Radiance Plus delivers attomole sensitivity and a long-lasting signal.

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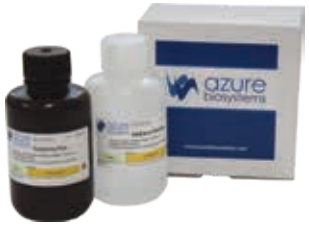

Keep your blots consistent with
AzureSpectra Fluorescent Western Blotting Kits

Get everything you need for generating consistent western blots in one convenient and easy-to-use kit. We handle the quality control of the reagents so you can stay focused on your science.

- VWR Catalog Number 10147-374
- VWR Catalog Number 10147-376
- VWR Catalog Number 10147-378

References

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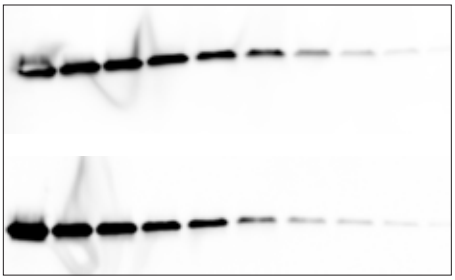
02 Troubleshooting

While some view Western blotting as an art, you don't need to be a scientific Seurat to generate consistently good Westerns. Attention to the little details can help. In this troubleshooting guide, we review some common Western blotting problems, their causes, and ways to overcome them so that you can turn your Western blot woes into Western blot wows.

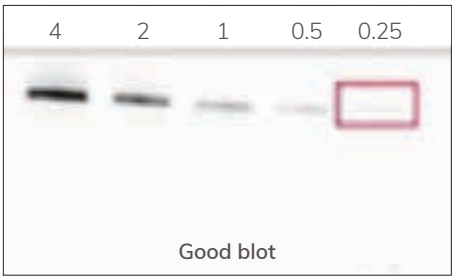
02.1 General gel and blotting issues

PROBLEM: Swirls around bands

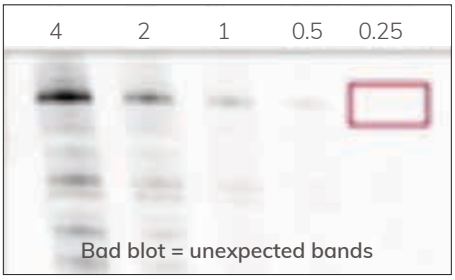


CAUSE	SOLUTION
Uneven protein transfer from the gel to the blotting membrane.	When assembling the blot, ensure that the transfer stack fits tightly together. You may need to change the sponge pads to improve the tightness of the fit.

PROBLEM: Non-specific bands present



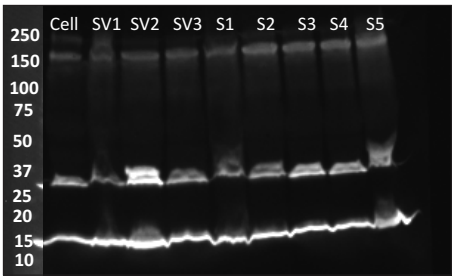
Good blot



Bad blot = unexpected bands

CAUSE	SOLUTION
Incomplete blocking.	Change your blocking buffer. Many commonly used blocking buffers can mask the epitopes on your target, making them difficult to detect. Others may outright fail to block non-specific binding to irrelevant lysate proteins. Using an engineered blocking buffer instead of milk can reduce non-specific binding, while enhancing antibody-antigen interactions.
Primary antibody has low-specificity for your protein-of-interest.	Run additional purification steps on your primary antibody or generate new antibody.
Using too high of a concentration of primary antibody can reduce its specificity, leading to the appearance of off-target bands.	Increase the dilution of your primary antibody and increase the incubation time, if necessary, to offset any reduction in signal. Performing the primary antibody incubation step at 4°C can help decrease non-specific binding of your antibody.

PROBLEM: Bands are wavy, smiley, or generally not straight



CAUSE	SOLUTION
You might have cellular debris and/or DNA in your sample.	Change how you prepare your sample by: • Adding or increasing sonication • Heating for a longer time • Spinning down your sample to pellet debris
Your loading buffer might not be well buffered or have too much salt.	Try preparing fresh loading buffer, use a different type of buffer, or reduce the salt concentration of your sample buffer through dilution, dialysis/micro-dialysis, or chromatographically.
You may be running your gel too fast, causing the gel to heat up, degrading the polymer.	Set an appropriate maximum current and keep the voltage low.
Your gel running buffers might not provide enough buffering capacity.	Select the right buffer for your sample.

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

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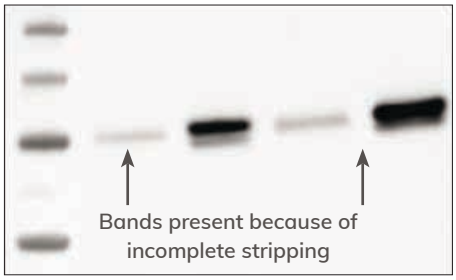
PROBLEM: Bands remain after stripping blot



CAUSE

Incomplete stripping before reprobing.

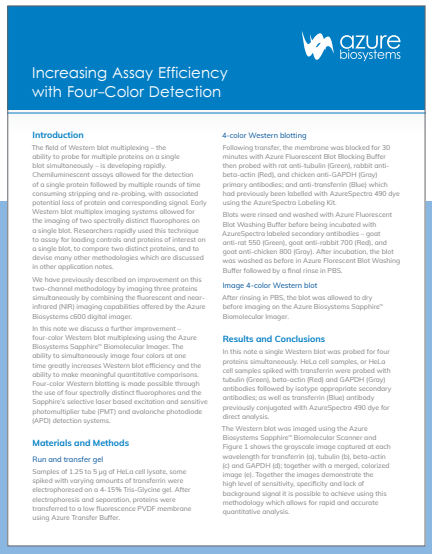
SOLUTION

Optimize your stripping conditions by either increasing the length of time your blot is incubated in the stripping buffer or changing buffers.

You also need to ensure that the blot is completely submerged in the stripping buffer and has sufficient agitation to move the buffer completely over the top of the blot.

In addition, some protein-antibody pairs are more resistant to stripping than others, so the conditions that work for one study might not work for another.

Another solution to consider is multiplexing detection by using fluorescently-labeled secondary antibodies. With fluorescent antibodies, you can detect as many as four different signals depending on your system.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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 c600 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, colorized 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.

Learn more about running multiplex fluorescent Westerns in the application note, **"Increasing Assay Efficiency with Four Color Detection."**

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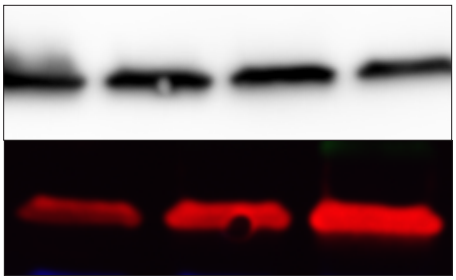
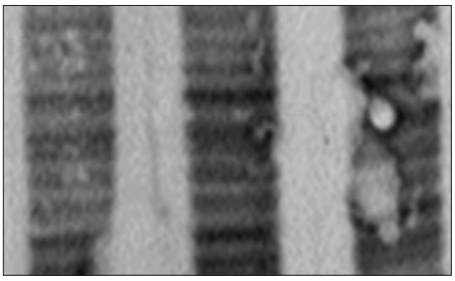
PROBLEM: Bubbles or empty spaces in your bands





CAUSE

Air trapped between the blotting membrane and the gel.

SOLUTION

When assembling your blot, take care to completely remove any gaps or air trapped between the blotting membrane and gel. You can use your gloved hand, a roller, or a sufficiently long glass or plastic pipet to gently roll out any air caught between the membrane and the gel.

Insufficient contact between the primary antibody and the blot. If there is not enough primary antibody incubation solution to completely cover the blot and/or if the primary antibody solution does not move across the surface of the blot, regions of the blot may remain unexposed to the antibody.

Increase the volume of primary antibody incubation solution and verify that the solution covers all parts of the membrane during the incubation step. While you should use a small incubation tray to reduce antibody waste, the tray should large enough that the membrane can move around.

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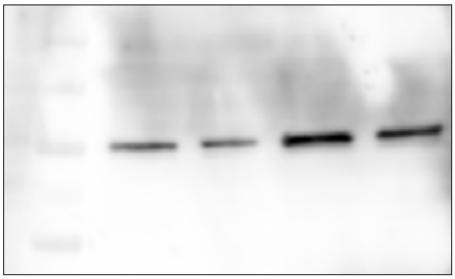
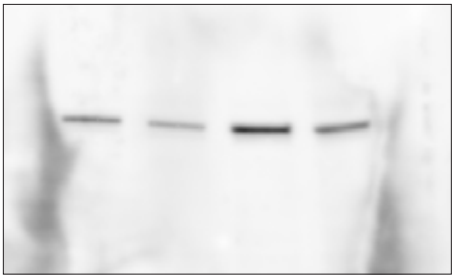
02.2 Chemiluminescent western blot issues

PROBLEM: Weak chemiluminescent bands

CAUSE	SOLUTION
Not enough sample.	Increase the amount of sample or antibody used. However, increasing reagents is not always possible, such as when you have a limited availability. There are additional ways to further optimize detection: • Choose a substrate with the right sensitivity. Some HRP substrates can deliver better signal without increasing background, especially if you've already optimized blocking and wash buffers. • Choose a substrate that isn't time sensitive so you can increase detection time. Some HRP substrates deliver signal that remains linear over hours instead of minutes, enabling more accurate quantitation.

PROBLEM: High background on a chemiluminescent western blot





CAUSE	SOLUTION
Insufficient washing.	Increase the number of washes or the length of the washing time. Additionally, increasing the amount of detergent or using a stronger detergent, such as SDS or NP-40, can further reduce background noise.
Incomplete blocking.	Change your blocking buffer. Many commonly used blocking buffers can mask the epitopes on your target, making them difficult to detect. Others may outright fail to block non-specific binding to irrelevant lysate proteins. Using an engineered blocking buffer instead of milk can reduce non-specific binding, while enhancing antibody-antigen interactions.

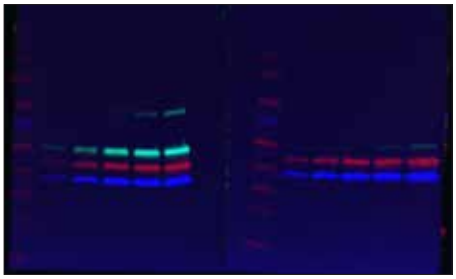
continued...

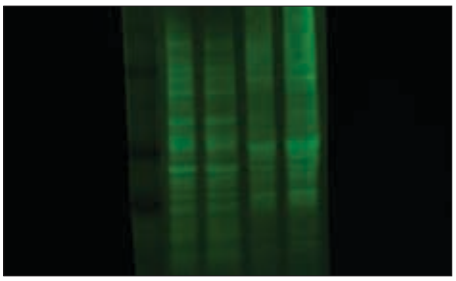
PROBLEM: High background on a chemiluminescent western blot, *continued*

CAUSE	SOLUTION
Using too high of a concentration of primary antibody can reduce its specificity, leading to general binding to the membrane.	Increase the dilution of your primary antibody and increase the incubation time, if necessary, to offset any reduction in signal. Performing the primary antibody incubation step at 4°C can help decrease non-specific binding of your antibody.

02.3 Fluorescent western blot issues

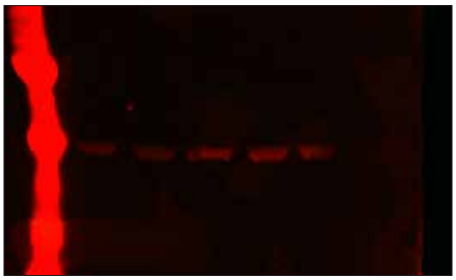
PROBLEM: High background on a fluorescent western blot





CAUSE	SOLUTION
Nitrocellulose membranes and some types of PVDF membranes can autofluoresce, leading to a high background signal.	Switch to a low-fluorescence PVDF membrane.
Imaging a wet membrane.	Dry the membrane with methanol before imaging. When the blot dries there is less autofluorescence from the membrane and the specific signal from the fluorophore becomes brighter. Drying the blot with methanol amplifies this effect by removing more water than air-drying alone.
Using too high of a concentration of primary antibody can reduce its specificity, leading to general binding to the membrane.	Increase the dilution of your primary antibody and increase the incubation time, if necessary, to offset any reduction in signal. Performing the primary antibody incubation step at 4°C can help decrease non-specific binding of your antibody.
Insufficient washing.	Increase the number of washes or the length of the washing time. Additionally, increasing the amount of detergent or using a stronger detergent, such as SDS or NP-40, can further reduce background noise.

PROBLEM: Marker is too bright



CAUSE

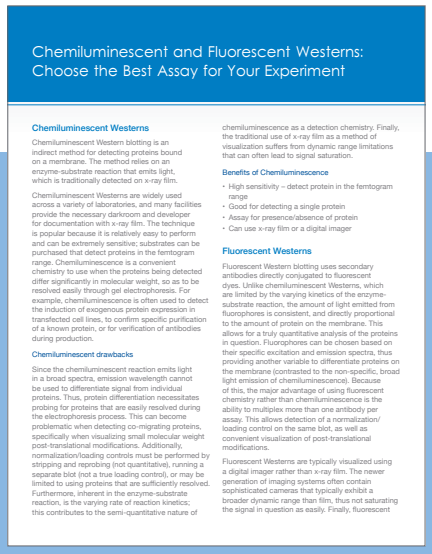
Loaded too much marker.

SOLUTION

Increase the dilution of your marker.

Most molecular weight ladders autofluoresce in the IR 700 channel, which is convenient for determining the molecular weight of bands in a fluorescent western blot. However, if you're used to running chemiluminescent Western blots, you may be overloading your marker lane—we recommend running approximately one-tenth the amount of molecular weight marker on a fluorescent Western blot compared to the amount you would run on a chemiluminescent Western blot. If you can see strong bands on your membrane after transfer, you have probably loaded too much.

You can also try covering the lane with the strong bands. Sometimes, it's too late, and you can't go back in time and load less ladder. In this case, we recommend covering up the bands with a non-fluorescent, clean, opaque material.



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

Chemiluminescent Westerns

Chemiluminescent Western blotting is an indirect method for detecting proteins bound on a membrane. The method relies on an enzyme-substrate reaction that emits light, which is traditionally detected on x-ray film.

Chemiluminescent Westerns are widely used across a variety of laboratories, and many facilities provide the necessary darkroom and developer for documentation with x-ray film. The technique is popular because it is relatively easy to perform and can be extremely sensitive; substrates can be purchased that detect proteins in the femtogram range. Chemiluminescence is a convenient chemistry to use when the proteins being detected differ significantly in molecular weight, so as to be resolved easily through gel electrophoresis. For example, chemiluminescence is often used to detect the induction of exogenous protein expression in transfected cell lines, to confirm specific purification of a known protein, or for verification of antibodies during production.

Chemiluminescent drawbacks

Since the chemiluminescent reaction emits light in a broad spectra, emission wavelength cannot be used to differentiate signal from individual proteins. Thus, protein differentiation necessitates probing for proteins that are easily resolved during the electrophoresis process. This can become problematic when detecting co-migrating proteins, specifically when visualizing small molecular weight post-translational modifications. Additionally, normalization/loading controls must be performed by stripping and reprobing (not quantitative), running a separate blot (not a true loading control), or may be limited to using proteins that are sufficiently resolved. Furthermore, inherent in the enzyme-substrate reaction, is the varying rate of reaction kinetics; this contributes to the semi-quantitative nature of chemiluminescence as a detection chemistry. Finally, the traditional use of x-ray film as a method of visualization suffers from dynamic range limitations that can often lead to signal saturation.

Benefits of Chemiluminescence

- High sensitivity – detect protein in the femtogram range
- Good for detecting a single protein
- Assay for presence/absence of protein
- Can use x-ray film or a digital imager

Fluorescent Westerns

Fluorescent Western blotting uses secondary antibodies directly conjugated to fluorescent dyes. Unlike chemiluminescent Westerns, which are limited by the varying kinetics of the enzyme-substrate reaction, the amount of light emitted from fluorophores is consistent, and directly proportional to the amount of protein on the membrane. This allows for a truly quantitative analysis of the proteins in question. Fluorophores can be chosen based on their specific excitation and emission spectra, thus providing another variable to differentiate proteins on the membrane (contrasted to the non-specific, broad light emission of chemiluminescence). Because of this, the major advantage of using fluorescent chemistry rather than chemiluminescence is the ability to multiplex more than one antibody per assay. This allows detection of a normalization/loading control on the same blot, as well as convenient visualization of post-translational modifications.

Fluorescent Westerns are typically visualized using a digital imager rather than x-ray film. The newer generation of imaging systems often contain sophisticated cameras that typically exhibit a broader dynamic range than film, thus not saturating the signal in question as easily. Finally, fluorescent

Learn about choosing fluorescent or chemiluminescent detection for your western blots in our application note, "**Chemiluminescent and Fluorescent Westerns: Choose the Best Assay for Your Experiment.**"

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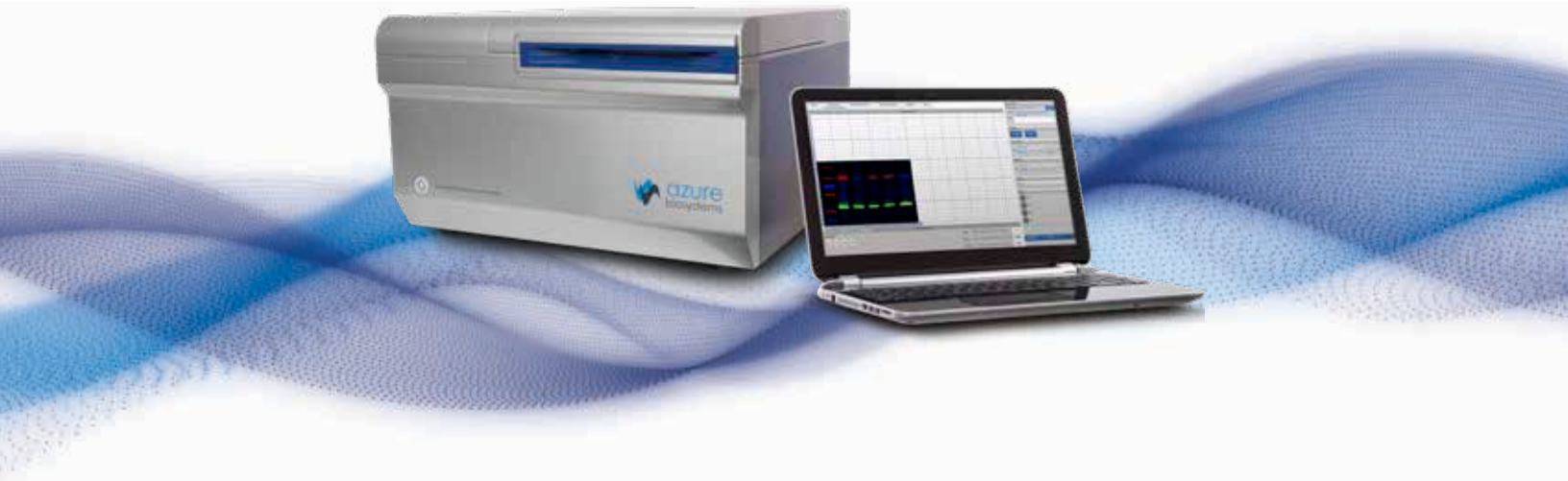
REDEFINING more



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QUANTITATIVE WESTERN BLOT • ELECTROPHORETIC MOBILITY ASSAY
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STAIN-FREE WESTERNS • **PHOSPHOR IMAGING** • TISSUE SECTION IMAGING

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03 Q-Checklist

Getting quantitative data from your western blot takes more than just a fluorescent or chemiluminescent secondary and a digital imager. You need to consider your sample prep, your normalization method, and the linearity of your signal. To help keep your quantitative western blot workflows on track, we've created this checklist to ensure your western blot quantitation is as accurate as it can be.

Consistency is the key to quantitative western blots

- ☐ Have you treated your samples consistently, ensuring cells and tissues are thoroughly lysed, immediately placed on ice, and/or been treated with appropriate reagents such as protease inhibitors?
- ☐ Have you loaded similar amounts of total protein in each lane (within a factor of 2- to 5-fold)?
- ☐ Are you using a robust and consistent western blotting protocol (reduce variability by using the same reagents for each blot or implementing lot-to-lot controls, and keeping your transfer conditions and incubation and wash times the same)?
- ☐ Are you normalizing to total protein?
- ☐ Have you validated the specificity of your antibodies?
- ☐ Have you verified that your system is linear? The linearity of your signal can be affected by multiple factors. Here are steps you can take to ensure linearity and improve the accuracy of your quantitation:
  - ☐ Are the highest and lowest amounts of your protein-of-interest in the linear range (note that you need to verify linearity with each protein-antibody pair)?
  - ☐ Are you saturating your membrane?
  - ☐ Have you optimized your primary and secondary antibody dilutions? Titrating your antibody against your protein-of-interest can reveal an antibody dilution that delivers the widest dynamic range.
  - ☐ Have you optimized your image acquisition time to ensure you are in the linear range of your imager?

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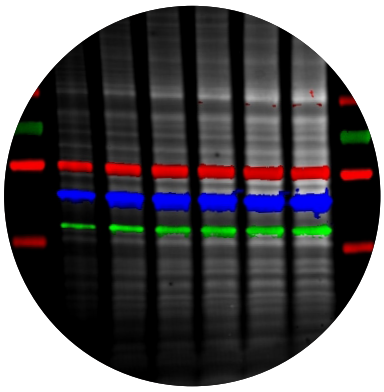
Western Blot Quick Quiz

What's the best, most efficient way to do total protein normalization of NIR blots?

~~A.~~ Probe for two proteins, image, strip the blot, restain for total protein, and re-image

~~B.~~ Run two blots

Q. With Sapphire™. Run one blot, probe for two proteins, stain for total protein, and image



Use the **Sapphire NIR-Q** to probe up to 2 proteins and the **Sapphire RGBNIR** to probe up to 3 proteins.

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