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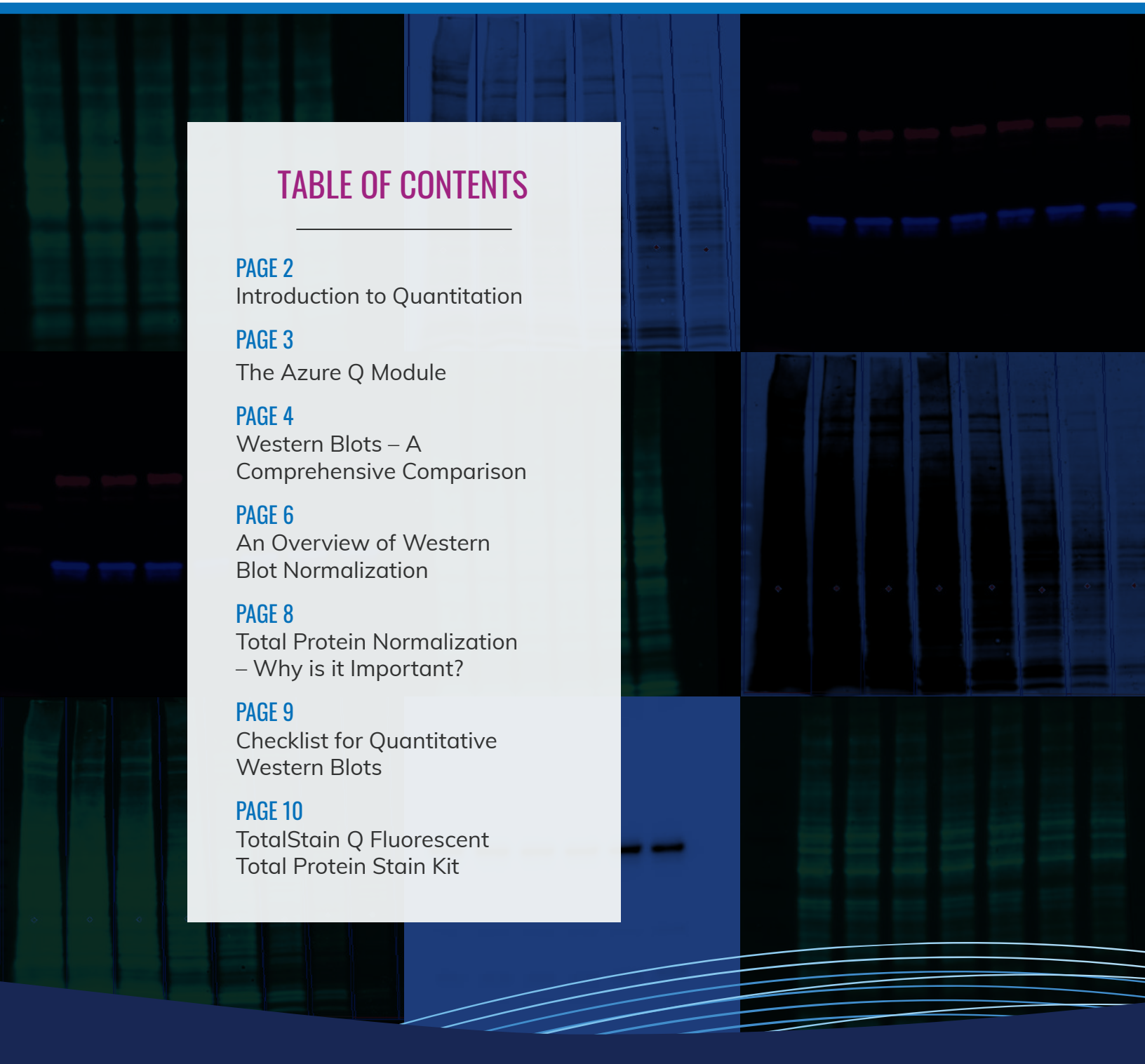
ULTIMATE GUIDE TO QUANTITATIVE WESTERN BLOTS



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INTRODUCTION TO 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.

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

THE AZURE Q MODULE

OPTIONAL GREEN FLUORESCENCE CHANNEL

Add **Total Protein Normalization** to your ECL or NIR Western Blots.
Reimagine what your imager can accomplish.

★ GREEN ★
CHEMI

Azure 300Q

Enjoy the sensitivity of chemiluminescence with the linearity and quantitative potential of fluorescent total protein stains such as Azure TotalStain Q.

Normalize signal in your chemiluminescent western blots with the addition of a fluorescent channel. Detect more than just housekeeping proteins for quantitative western blots on top of your traditional ECL detection.

★ GREEN ★
700nm & 800nm NEAR INFRARED
CHEMI

Azure 500Q

Break through the preconceived limits of two fluorescent channels with the Azure Q module.

Near-infrared lasers provide the highest signal to noise ratio with minimal crosstalk. The addition of a third fluorescent channel to the camera-based imager allows the simultaneous detection of housekeeping proteins or a total protein stain.

★ GREEN ★
700nm & 800nm NEAR INFRARED

Sapphire NIR-Q

Why limit your scanning capabilities to two channels?

With the Sapphire NIR-Q scanner, target two proteins of interest in the 700 and 800nm channels and add a third target protein OR total protein stain using the Q module (520nm).

★ FOR GREEN CHANNEL ★

TotalStain Q

TotalStain Q solves the issue of housekeeping proteins that lack linearity and normalization accuracy at high protein loads.

TotalStain Q is a total protein staining reagent, compatible with chemiluminescent or near infrared fluorescent Western Blot detection. The linear range between 1–50 µg of lysate makes it ideal for normalization of low expressed proteins that require high loads of protein to reach the sensitivity for the detection of the target.

WESTERN BLOTS – A COMPREHENSIVE COMPARISON

CHEMILUMINESCENCE OR FLUORESCENCE?

Chemiluminescence overview

Chemiluminescent Westerns are a popular indirect detection method for assessing protein expression, wherein an antibody that has conjugated to an enzyme reacts with chemiluminescent substrates to emit light that is then captured and archived on x-ray film or through digital imaging. Although only semi-quantitative, chemiluminescent Western blotting is a highly sensitive assay that can detect the presence or absence of a protein femtograms. Chemiluminescence is thus useful in detecting the induction of exogenous protein expression, confirming and following purification of a known protein, or for verification of antibodies during production, to list a few examples.

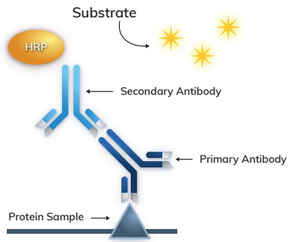
However, chemiluminescent Westerns can be difficult to perform. While the ultimate goal is to obtain a blot with a high signal-to-noise ratio, chemiluminescent Westerns can be plagued with high background, either in the form of an overall background that masks the signal from the protein of interest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

Fluorescence overview

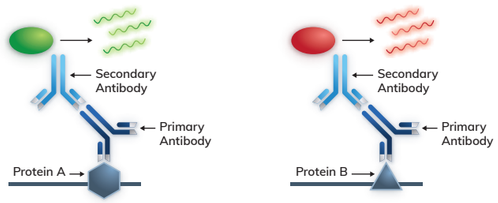
Fluorescent Western blots are the gold standard for quantitative Westerns, ideal for detecting multiple proteins simultaneously (multiplexing) and allowing in-lane normalization and detection of your protein of interest, as well as loading control. In addition, post-translational modifications can be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

In fluorescent Western blotting, the secondary antibody is directly conjugated to a dye that is excited by light, which is then 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.

Fluorescent Western Blotting allows for the detection in multiple channels simultaneously. For example, with the Azure Sapphire Biomolecular Imager RGBNIR image up to three proteins of interest and one total protein stain.



Primary antibodies probe for protein of interest. A secondary antibody is conjugated to horseradish peroxidase, an enzyme which enhances the light emitted from a chemiluminescent reaction.



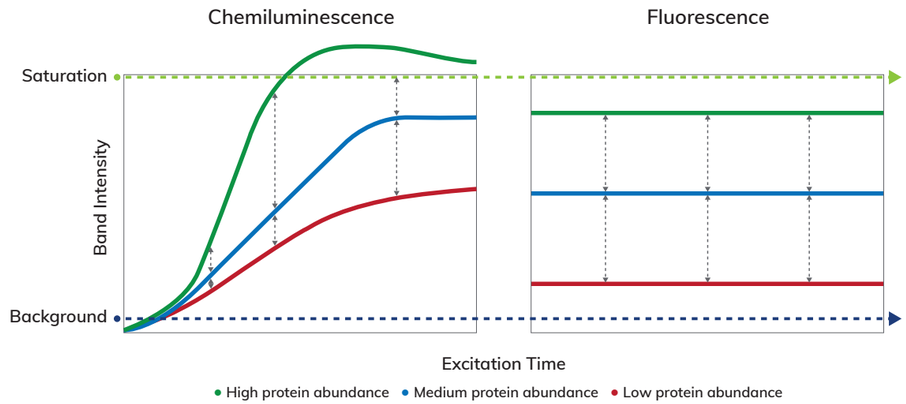
Two primary antibodies can probe for two separate proteins of interest. Secondary antibodies are conjugated to fluorescent labels known as fluorophores. These fluorophores have distinct excitation and emission spectra.

The ability to identify up to four distinct proteins of interest reduces the time spent stripping and reprobing. The direct correlation between signal intensity and protein abundance makes fluorescent western blotting perfect for quantitative analysis of post-translational modifications.

Which is right for you?

Although the process of immunoblotting has been around for over 40 years, innovations in digital imaging have led to advances in both chemiluminescent and fluorescent western blots. Chemiluminescence has been established and integrated in many labs globally due to its traditional use with film and high sensitivity. However, the kinetic and enzymatic nature of this reaction promotes saturation in highly abundant samples, making a chemiluminescent blot non-quantitative as a signal will not accurately represent protein levels. Additionally, the reaction time of chemiluminescence is limited by the quality of substrate and only allows probing for a single protein of interest. There is increased cost associated with stripping a chemiluminescent blot and reprobing to perform semi-quantitative analysis.

Fluorescent western blotting has been rapidly adopted due to its capacity for multiplex detection and cost-efficiency. LED and laser-based excitation of fluorescent probes increases sensitivity and reduces cross-excitation between probes of adjacent wavelengths. The ability to identify up to four distinct proteins of interest reduces the time spent stripping and reprobing. The direct correlation between signal intensity and protein abundance makes fluorescent western blotting perfect for quantitative analysis of post-translational modifications.



Chemiluminescent vs. fluorescent signal. Due to the enzymatic nature of a chemiluminescent reaction, signal/band intensity increases greatly over time. This can be problematic for highly abundant proteins that saturate the detector. Since fluorophores are bound directly to the secondary fluorescent conjugate, the signal intensity is correlated directly to protein abundance.

Sources

1. https://www.azurebiosystems.com/wp-content/uploads/2016/04/Improve-Chemi-Blots-app-note_3.pdf
2. https://www.azurebiosystems.com/wp-content/uploads/2016/04/Improve-Fluorescent-Blots-app-note_3.pdf

AN OVERVIEW OF 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[1,2] leading to inaccurate measurements of relative protein abundance. Instead, quantitative Western blotting experts[1,2] and the journals they publish in[4] are recommending a new gold standard for normalization—normalizing to total protein detected in each lane, preferably by staining on the membrane.

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.[1,2] 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.[1,3] 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.[2,3]

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.[1] 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.

Western Blot Normalization: Housekeeping Protein vs Total Protein

Housekeeping protein	Total protein
BENEFITS	
• Familiar, commonly used	• Large linear dynamic range • Low variability • Consistant across sample types • No change with experimental conditions
CHALLENGES	
• Narrow linear dynamic range • Abundance can vary with experimental conditions • Abundance may not be consistent between sample types • High variability • Must ensure housekeeping protein physically resolved from protein of interest on gel	• Must ensure total protein stain used is compatible with antibody binding and detection method

DRAWBACKS TO HOUSEKEEPING PROTEINS FOR NORMALIZATION:

Inconsistent expression across samples and conditions

Easily affected by experimental treatment and conditions

High abundance and narrow linear range compared to protein of interest

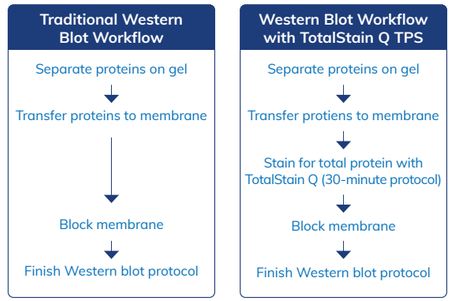
Difficulties with multiplex analysis such as phosphorylation PTMs

Interference with protein of interest and difficulty with visual identification

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.[1-4] Many total protein stains are available that can be used to stain gels and membranes.[1] Total protein stains provide a larger dynamic range and demonstrate lower variability and cleaner data than housekeeping proteins.[1,2]

Total protein normalization can be much faster than using a housekeeping protein, especially for chemiluminescent blots because the staining step takes less time that stripping and reprobing the blot. Ideally, total protein staining is conducted on the membrane, either before or after immunodetection.[2] With some stains such as TotalStain Q 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 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.

> "It is not uncommon to "correct" Western blot signals for protein loading by normalizing to [housekeeping proteins present in the same replicate], e.g.: β-actin, α-tubulin, transferrin, GAPDH, HPRT1. The problem with this approach is that a linear relationship between signal intensity and the mass or volume of sample loaded must be confirmed for every antigen... It is typically better to normalize Western blots using total protein loading as the denominator [given its wider linear range]."[4]
>
> —Journal of Biological Chemistry Transparency is the Key to Quality

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 TotalStain Q Fluorescent Total Protein Stain for total protein normalization of fluorescent Western blots in the Accurate Western Blot Normalization With TotalStain Q 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.

TOTAL PROTEIN NORMALIZATION – WHY IS IT IMPORTANT?

If you love Western blotting as much as we do, you'll have noticed a growing trend toward using total protein staining rather than housekeeping proteins to normalize for load. But what does total protein normalization (TPN) involve and what advantages does it offer? Read on to find out and get your hands on a free sample of TotalStain Q – our newest reagent for total protein staining.

What is TPN?

For many years, researchers have used housekeeping proteins such as actin, tubulin or GAPDH to normalize Western blot loading. These are usually detected alongside the target of interest – either in parallel or by stripping and re-probing the blot – before being used to determine the relative target abundance in different samples. This is achieved by dividing the signal intensity of the target by that of the housekeeping protein in each lane.

Although TPN is based on a similar concept, it uses the entire protein content of each sample for normalization instead of relying on only a single housekeeping protein. You can see an example of total protein staining in the figure below, where we've used TotalStain Q to detect total protein in six identical samples of HeLa lysate before immunodetecting pSTAT3.

What are the advantages of TPN?

As well as providing a quick visual confirmation that protein transfer is even across the membrane before researchers move ahead with labor-intensive immunostaining protocols, TPN delivers more accurate quantification than using individual housekeeping proteins.

One reason for this is that TPN accounts for lane-to-lane variations inherent in Western blotting. TPN also overcomes the problem of experimental treatments influencing housekeeping protein expression, since the entire protein population of the sample is considerably less susceptible to change than a single housekeeping protein.

Additionally, because total protein stains are linear over a wider dynamic range than individual housekeeping proteins, TPN allows for the detection of both low abundance target proteins and highly expressed housekeeping proteins with less risk of over-saturation.

When should I use TPN?

Anyone performing Western blotting can benefit from TPN. Moreover, with TPN fast becoming the new standard required by journals for Western blot quantification, there has never been a better time to switch from using housekeeping proteins for normalization.

BENEFITS OF TOTAL PROTEIN NORMALIZATION:

Avoid lane-to-lane variation inherent to Western Blotting

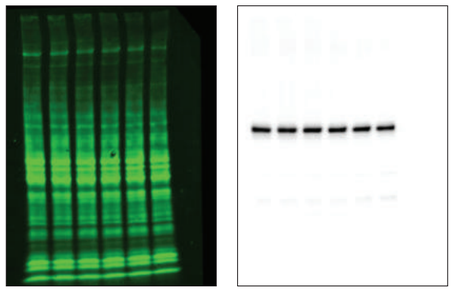
Less susceptible to experimental treatment than single proteins

Linear over a wider dynamic range

How can I get started with TPN?

Whether you're new to TPN, or you've already introduced total protein staining into your Western blotting workflow, you should consider giving TotalStain Q a try.

TotalStain Q adds just a short dye-and-wash step to your protocol between protein transfer and blocking buffer incubation and is compatible with subsequent Western blot detection using enhanced chemiluminescence or Near Infrared (NIR) fluorescence. Provided your imaging system has a green (Cy3) channel or a Safe-Dyes channel, there's absolutely nothing stopping you from making TPN part of your Western blot routine.



TotalStain Q is a rapid fluorescent stain for quantifying total protein. HeLa lysate 5µg replicates imaged using TotalStain Q (left – Cy3 channel, green) and pSTAT3 (right – enhanced chemiluminescence, grayscale).

Contact us today for a FREE SAMPLE and we guarantee you'll never look back.

CHECKLIST FOR QUANTITATIVE WESTERN BLOTS

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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- NIR fluorescence
- Chemiluminescence
- Phosphorimaging

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TotalStain Q Total Protein Stain

Easily stain total protein for the most accurate western blot normalization.

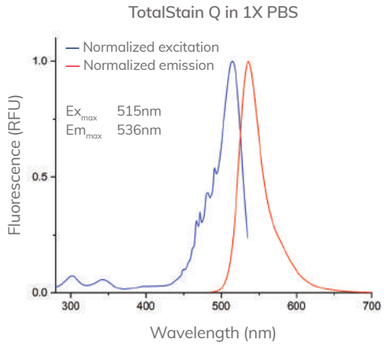
▶ Azure Catalog Number AC2225

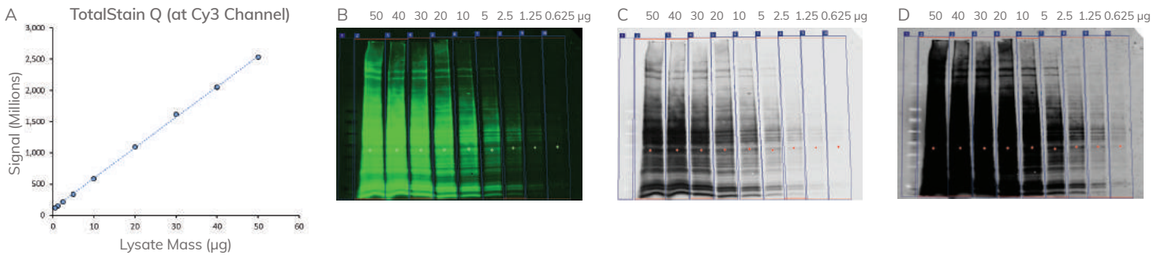
TOTALSTAIN Q FLUORESCENT TOTAL PROTEIN STAIN KIT

QUANTITATIVE AND RAPID FLUORESCENT REAGENT FOR TOTAL PROTEIN NORMALIZATION OF WESTERN BLOTS

A linear total protein stain even for high loads of cells lysates

The quantitative immunodetection of low abundant targets sometimes requires to load big amounts of protein in the gel. Highly expressed housekeeping proteins used for normalization lack proportionality in these conditions. TotalStain Q is a fluorescent total protein stain with linearity up to 50 µg of lysate, making it ideal for normalization of low expressed proteins. Kits for PVDF and nitrocellulose membranes.

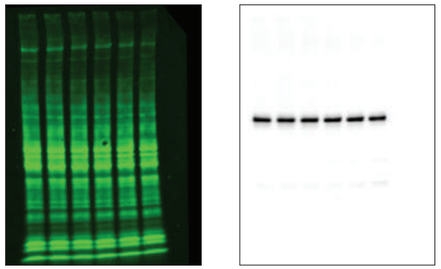




HeLa lysate dilution series 50µg – 0.625µg imaged in Cy3 channel. (A, B) Color image showing the broad Linear Range from 1-50µg; R^2=0.9950. (C, D) Grayscale images at different contrast to display the full range of quantification.

Chemiluminescent western blots

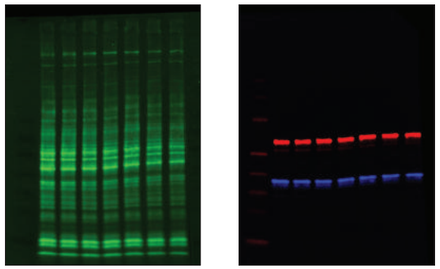
TotalStain Q is compatible with chemiluminescent western blots



HeLa lysate 5µg replicates. (Left) TotalStain Q imaged in Cy3 channel (Green). (Right) pSTAT3 chemiluminescent detection (Grayscale).

NIR fluorescent western blots

TotalStain Q is compatible with two-color NIR fluorescent western blots



HeLa lysate 5µg replicates. (Left) TotalStain Q imaged in Cy3 channel (Green). (Right) hnRNP K detection in 700nm channel (Red); GAPDH detection in 800nm channel (Blue).

Total protein normalization is the new standard required by journals for western blot quantification. Housekeeping proteins (HKPs) are widely used as an internal reference for western blot normalization. They are often highly expressed, lose proportionality at high protein loads, and only represent one portion of your whole sample. For this reason, using HKPs is an unreliable method of calculating normalization ratios.

TotalStain Q accurately accounts for lane-to-lane variations inherent in western blotting. With linearity up to 50 µg of lysate, TotalStain Q is ideal for the normalization of lowly expressed proteins that require high sample loads. Kits are available for both PVDF and nitrocellulose membranes.

TotalStain Q is compatible with any instrument capable of green channel excitation (including the Azure Imager & Sapphire Q modules). Combine TotalStain Q with your downstream near-infrared or chemiluminescent imaging protocol for quantitative analysis, without any destaining step required. This two component, room temperature stable fluorescent stain makes total protein normalization both easy and efficient.

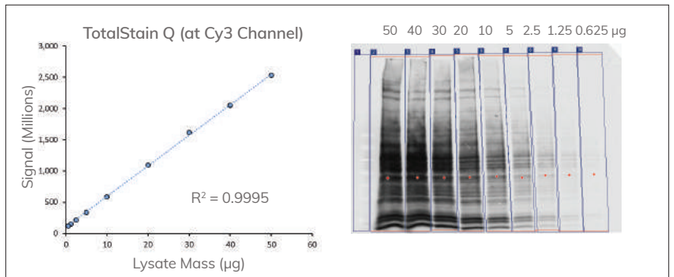


Image showing the broad linear range from 1–50µg; R^2 = 0.9995.

KEY FEATURES

Linearity—Total protein signal remains linear over a range of 1–50 µg of lysate, making it ideal for most common samples in the lab.

Convenience—Simple stain-and-wash 20 minute protocol post-transfer and pre-blocking. No destaining required.

Versatility—Compatible with ECL and NIR fluorescent immunodetection on PVDF in all Azure Imaging Systems. Also used for transfer efficiency verification.

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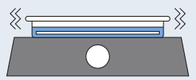
TOTALSTAIN Q FLUORESCENT TOTAL PROTEIN STAIN KIT CONVENIENT WORKFLOW

Takes 20 minutes from start to finish

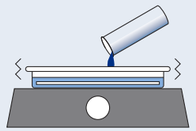
1



Rinse

After transfer 5-minute membrane rinse in water (10mL, gentle agitation)

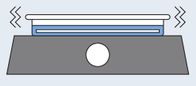
2



Stain

5-minute TotalStain Q Staining Solution membrane incubation (10 mL, gentle agitation)

3



Wash

3 washing steps of the membrane of 3 minute each one in Working Washing Solution (10mL, gentle agitation)

4

Imaging

Image acquisition using digital imaging system at Cy3 Channel or Safe Dyes Channel

5

Immunodetection

Continue with Blocking Step of Western Blot after rinsing the membrane with water. No destaining required (if the PVDF membrane is dry, pre-wet it with 100% methanol, then incubate in water for 5 minutes)

The Sapphire™ Biomolecular Imager

Yes! The Sapphire can!

FLUORESCENCE | CHEMILUMINESCENCE
PHOSPHOR IMAGING | DENSITOMETRY

One Imager.
Multiple Capabilities.
Countless Applications.

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