Western Blotting

The Complete Guide to Stain-Free Western Blotting

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BIO-RAD

Welcome

In this eBook, you will learn about the benefits of the proprietary Stain-Free technology from Bio-Rad Laboratories, Inc., get guidance for each step of the workflow, and discover Bio-Rad resources to ensure you are efficient and successful in western blotting.

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The Bio-Rad Western Blotting Workflow

Western blotting is a powerful technique to separate and detect proteins, estimate their quantities, and determine their molecular weights. Proteins of interest are extracted from cells or tissues and processed in a multistep western blotting workflow. Western blots can also be used in a variety of other applications, such as characterization of protein-protein interactions, posttranslational modifications, and antibodies.

The Bio-Rad Western Blotting Workflow consists of six steps, each critical to producing high-quality data:

1. Sample preparation
2. Electrophoresis
3. Transfer
4. Immunodetection
5. Image acquisition
6. Image analysis

In this guide, you will learn how Stain-Free technology fits into the western blotting workflow, and how this technology can improve your western blotting workflow and results.

What Is Stain-Free Imaging Technology?

Stain-Free technology uses a polyacrylamide gel containing a proprietary trihalo compound that makes proteins fluoresce directly in the gel after a short UV photoactivation (Figure 1). This trihalo compound covalently binds to tryptophan residues, enhancing their fluorescence when exposed to UV light. As a result, you can immediately visualize proteins in gels or on blots. Stain-Free technology enables the detection of proteins at levels as low as 10–25 ng. A Bio-Rad Stain-Free–enabled imager is required.

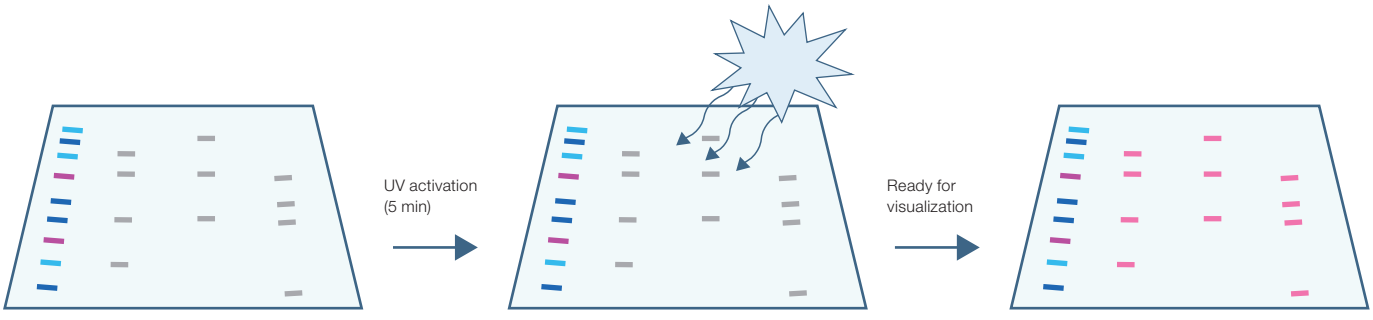
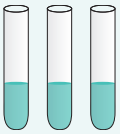
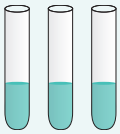
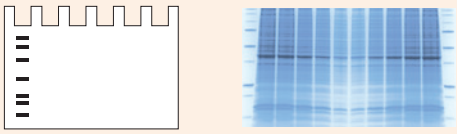
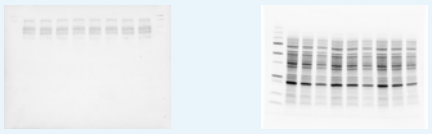
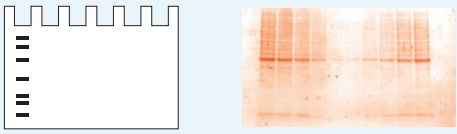
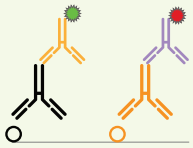
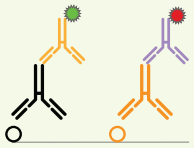
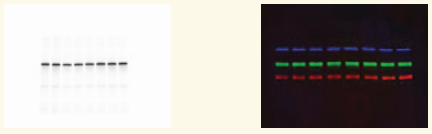
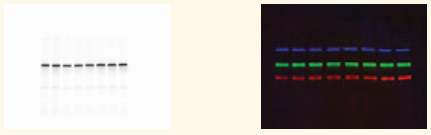
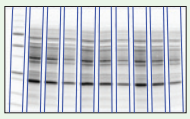


Fig. 1. Stain-Free gel activation. Activate Stain-Free gels with UV light and visualize total protein in minutes.

Stain-Free Workflow Compared to Traditional Western Blotting

The Stain-Free workflow takes only 5 hours to complete, versus 16 hours for traditional western blotting. In addition to saving time, Stain-Free technology also allows you to assess transfer efficiency and collect other information quickly and easily during the western blotting process.

Stain-Free Western Blotting	Advantages of the Stain-Free Workflow	Traditional Western Blotting
Sample Preparation		**Sample Preparation**
	–	
Electrophoresis (and Imaging)		**Electrophoresis (and Imaging)**
	TGX Stain-Free Gels can be run in as little as 16 minutes	
Transfer (and Imaging)		**Transfer (and Imaging)**
	Trans-Blot™ Turbo Transfer System transfers in 7 min No stain necessary Verify transfer immediately	
Immunodetection		**Immunodetection**
	Verify transfer before adding antibody, saving time and cost	
Image Acquisition		**Image Acquisition**
	–	
Image Analysis		**Image Analysis**
	TPN signal is a good loading control — no stripping and reprobing needed	

The Advantages of Stain-Free Technology

Stain-Free Technology Provides More Sensitivity than Coomassie Blue Stains

Stain-Free gel data are comparable to data from gels stained with traditional dyes, with a few exceptions. In general, the sensitivity of Stain-Free technology is equal to that of Coomassie Brilliant Blue (CBB) for proteins containing low-to-medium levels of tryptophan. However, for proteins with higher tryptophan content, Stain-Free technology provides much higher sensitivity than CBB (Figure 2).

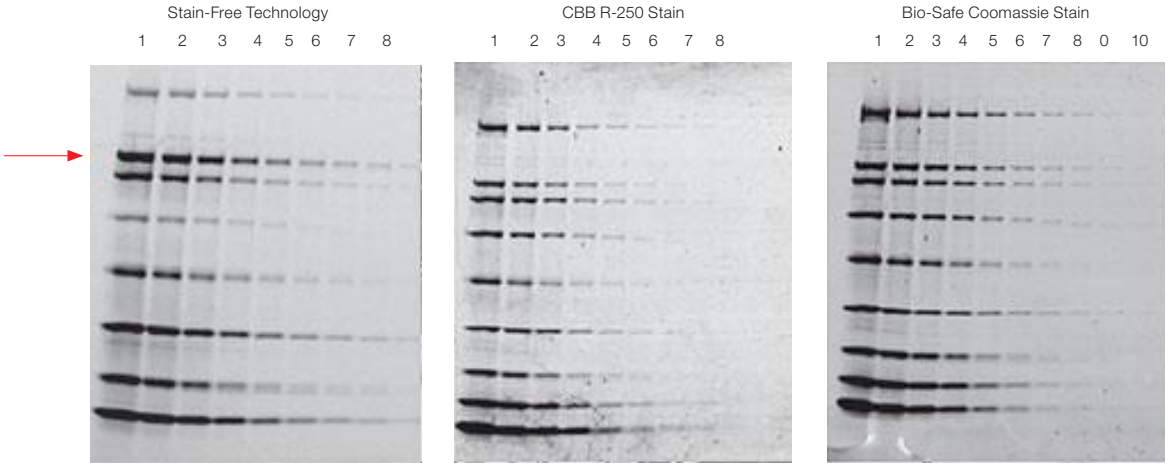


Fig. 2. Comparison of a Stain-Free gel image with Coomassie Brilliant Blue (CBB) R-250– and Bio-Safe Coomassie (G-250)–stained gel images. Serial 1:2 dilutions of broad range unstained molecular weight standards were separated on a 4–20% Criterion Stain-Free Tris-HCl Gel. The gel was imaged with a Stain-Free–enabled imager, then stained with Coomassie stains and imaged on a densitometer. Arrow indicates ß-galactosidase.

The limit of detection for the Stain-Free gels is 8–28 ng, similar to that of silver stains (0.6–1.2 ng), while Coomassie Brilliant Blue R-250 Staining Solution can detect protein amounts of at least 35–50 ng. Some fluorescent stains detect proteins below the 1 or 0.5 ng limit. Stain-Free gels have more reproducible data with smaller coefficients of variation compared to CBB or silver stains (McDonald et al. 2008, McDonald 2009).

Visit **bio-rad.com/Stain-Free** to learn more about Stain-Free technology.

The Dynamic Range of Stain-Free Technology

The linear dynamic range for protein quantitation is the range where the signal intensity on a blot proportionally increases with the increase in protein load. Ideally, the protein load should fall within the quantitative linear dynamic range of the antibody used for its detection (Taylor and Posch 2014). Stain-Free gels provide a predictable linear dynamic range of 10–80 µg of total protein load from cell or tissue lysates at a higher range of protein load (Figure 3A) and from 1–20 µg at a lower range (Figure 3B) (Taylor et al. 2013, Hammond et al. 2013).

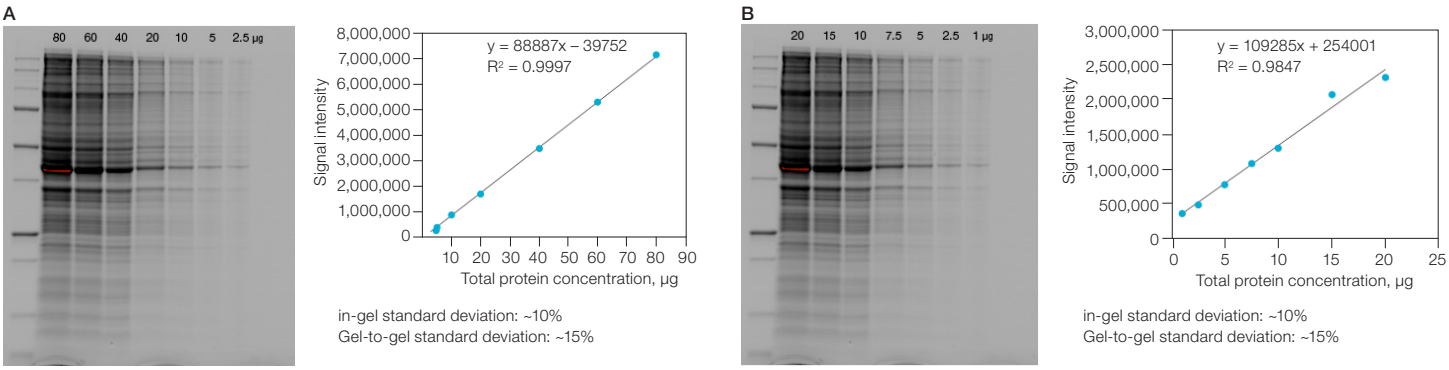


Fig. 3. Linear dynamic range provided by Stain-Free technology for total protein measurements. A, HeLa cell lysate dilutions from 80 to 2.5 µg total protein; **B**, HeLa cell lysate dilutions from 20 to 1 µg total protein.

Verify Your Protein Transfer in Minutes with No Staining

The complete transfer of proteins to the membrane is critical in western blotting. Traditional verification methods like Ponceau S, SYPRO Ruby, and other blot stains are more time-consuming than the Stain-Free method. Verifying protein transfer from gels to membranes using a Stain-Free–enabled imager can be accomplished in as little as two minutes. Unlike with traditional dye-based techniques, the observed intensity of the bands does not depend on the duration of staining or destaining (Figure 4).

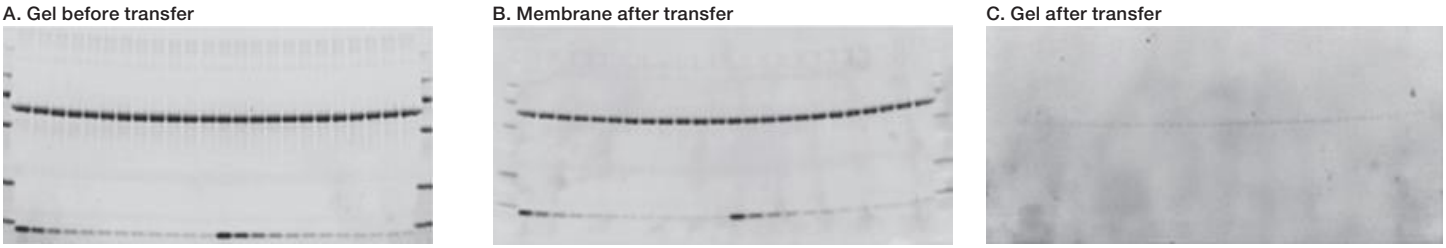


Fig. 4. Rapidly assess transfer using Stain-Free technology. Images were taken of the gel before (**A**) and after (**B**) transfer and of the membrane after transfer (**C**) using the Criterion Stain-Free Gel Imaging System. Serial 1:2 dilutions of hemoglobin (starting quantity, 80 ng), with 1.8 µg of bovine serum albumin (BSA)/lane as a carrier (top band), were electrophoretically separated on a 4–20% Criterion Stain-Free Tris-HCl Gel, 26-Well.

Stain-Free Technology Is Compatible with Downstream Applications

Proteins that have passed through the Stain-Free workflow are compatible with many downstream applications such as western blotting, mass spectrometry, and chromatography (Figure 5). The Coomassie dye–based method does not allow the same gel to be used for transfer during western blotting or for mass spectrometry. This compatibility streamlines the workflow and enables total protein normalization and quantitation.

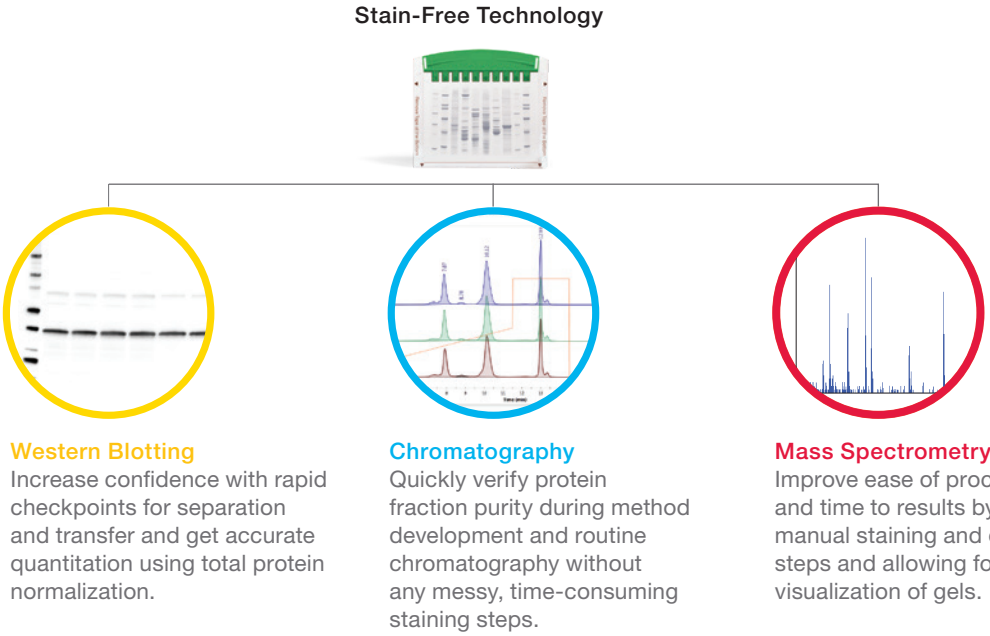


Fig. 5. Compatibility of Stain-Free technology with common downstream applications.

Summary of Stain-Free Technology Advantages

- Offers more sensitivity and better dynamic range than Coomassie Blue stains
- Performs quality assessments at each step without the need for staining and destaining
- Increases accuracy and produces reliable quantitative results via total protein normalization
- Is compatible with downstream applications
- Visualizes gels minutes after electrophoresis

Watch a video about the benefits of Stain-Free technology:

The Stain-Free Western Blotting Workflow

Sample Preparation

Maintain the same sample preparation workflow with Stain-Free imaging technology.
Use a protein quantitation assay to determine the concentration of your sample.

Electrophoresis, Activation, and Imaging

Immediately activate your gel to check transfer efficiency with Stain-Free technology.

Electrophoresis is performed the same way as with standard western blotting techniques. The main difference is that after running the gel, you can activate and image it immediately on a Bio-Rad Stain-Free–compatible imager (Figure 6). If proceeding to western transfer, activate the gel for 45 sec prior to imaging. If gel evaluation is the desired endpoint, activate for 5 min prior to imaging.

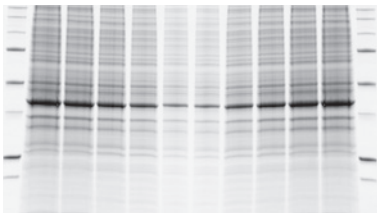


Fig. 6. Example Stain-Free gel image. Gel activation can be performed in as little as 45 seconds, allowing you to perform a quick check prior to western transfer.

Transfer

After activating your Stain-Free gel, transfer proteins to a membrane following your typical western blotting transfer protocol.

Once the transfer is complete, capture another gel image to evaluate the transfer process. When imaging, ensure a fair comparison by using the same exposure time and contrast settings you used to image the gel prior to transfer (Figure 7). To finish evaluating the transfer, image the Stain-Free blot and check all three images. If the transfer is complete, then proceed to immunodetection.

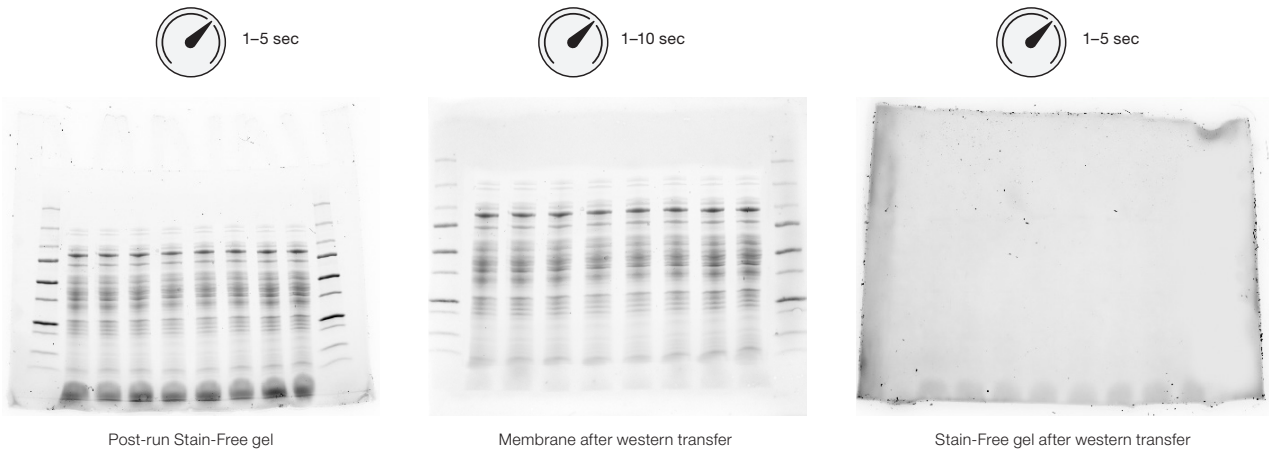


Fig. 7. Stain-Free technology makes it easy to check that western transfer is complete. Left, pre-transfer gel image; center, post-transfer membrane image; right, post-transfer gel image. Because activation is nonreversible, it does not need to be performed again after western transfer. Gels and membranes can be imaged in seconds.

Immunodetection

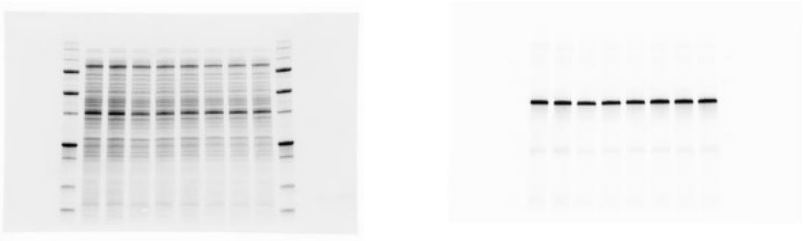
No change to your immunodetection workflow is necessary with Stain-Free imaging technology.
Both chemiluminescence and fluorescence detection methods are compatible.

Image Acquisition

Once your blot is ready for image acquisition, use a Bio-Rad Stain-Free–enabled imager to acquire images of the Stain-Free blot.

Stain-Free Gel Imaging of Chemiluminescence Western Blots

For chemiluminescence detection, select Blots in Applications, then the Chemi option. Acquire a blot image on the Stain-Free–enabled imager just before applying the enhanced chemiluminescence (ECL) reagent to achieve the most accurate representation of the total protein present (Figure 8).



Stain-Free blot | Chemiluminescent blot

Fig. 8. Examples of Stain-Free and chemiluminescence blot images.

Stain-Free Imaging of Fluorescence Western Blots

For multiplex western blot applications, use the red, far red (FR), and near infrared (NIR) channels for detecting proteins of interest and reserve the blue channel for total protein detection using Stain-Free technology (Figure 9).

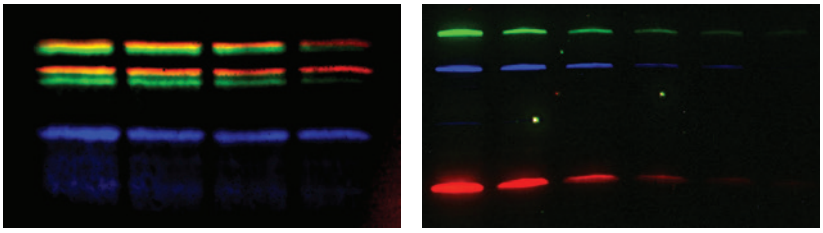


Fig. 9. Examples of multiplex western blots using 800 nm, 650 nm, and rhodamine channels.

Image Analysis

Perform image analysis for a Stain-Free blot the same way you would for traditional western blotting using Image Lab Software.

Volume Box Tools and Lane and Band Tools are two common approaches for quantitating western blots. Use Total Protein Normalization to equalize your quantitative western blot.

Meet the Bio-Rad Western Blotting Imagers

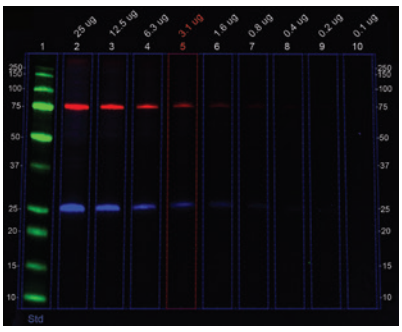
	ChemiDoc MP Imaging System	ChemiDoc Imaging System	GelDoc Go Gel Imaging System
Features	**High-end imaging system for the best fluorescence and chemiluminescence detection** ▪ Sensitive detection of RGB and FR/NIR secondary antibodies with low fluorescence background ▪ Chemiluminescence sensitivity matching X-ray film ▪ Easy-to-use software, perfect for multi-group use ▪ Multiplex fluorescence detection ▪ ChemiDoc MP Installation/Operational Qualification (IQ/OQ) Kit available	**Chemiluminescence sensitivity matching X-ray film** ▪ Easy-to-use software perfect for multi-group use ▪ Upgradable to ChemiDoc MP System for multiplex fluorescence detection ▪ ChemiDoc IQ/OQ Kit available	**Best for smaller research labs doing routine gel documentation** ▪ High-resolution personal imaging ▪ Easy-to-use software with minimal setup time ▪ Large imaging area with a compact footprint ▪ IQ/OQ tools and software for managing user accounts and permissions

Visit **bio-rad.com/ChemiDoc** to learn more about ChemiDoc Systems.

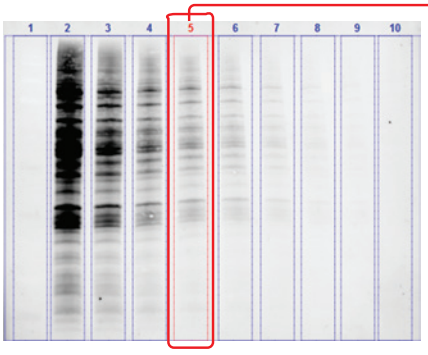
Visit **bio-rad.com/GelDoc** to learn more about the GelDoc Go System.

Analyze with Certainty on a PC or Mac

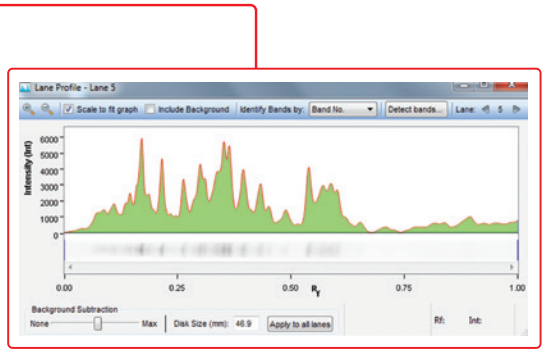
Automated lane and band detection, molecular weight determination, and normalization are just a few clicks away with Image Lab Software.



Annotation to make interpretation and publication easy.



Stain-Free imaging allows for total protein normalization.



Lane profiling of total protein signal helps remove background and refine band detection.

Visit **bio-rad.com/ImageLab** to learn more about Image Lab Software.

Benefits of Total Protein Normalization (TPN)

Reliable assessment of changes in target protein expression levels requires measuring both the target protein and the loading control protein in their linear dynamic ranges for immunodetection. Traditionally, housekeeping genes like actin, β-tubulin, or GAPDH have served as loading controls and in data normalization (Figure 10). Unfortunately, housekeeping proteins (HKPs) are usually highly expressed, whereas target proteins are often expressed in low abundance. In the HKP approach, large amounts of cell lysates may need to be loaded to detect target proteins, which can result in the overloading of HKPs and yield oversaturated reference bands out of their linear range.

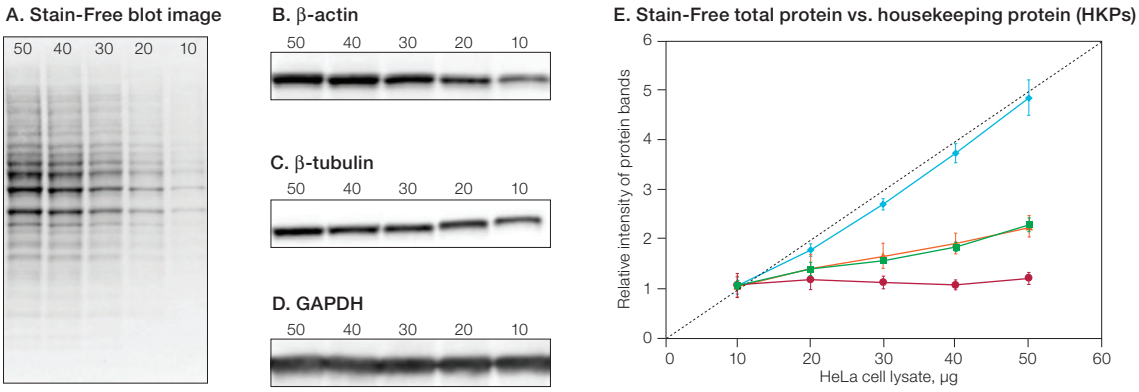


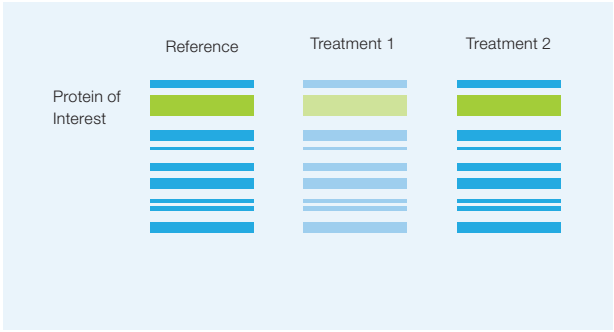
Fig. 10. Stain-Free assessment of several housekeeping proteins provides a linearity comparison of Stain-Free total protein measurement and immunodetection of three HKPs in 10–50 µg of HeLa cell lysate. A–D, representative images of a Stain-Free blot (**A**) and chemiluminescence blots for β-actin (**B**), β-tubulin (**C**), and GAPDH (**D**). Lane labels correspond to total protein load in µg. **E**, relative signal intensity for total protein (assessed via Stain-Free imaging) and housekeeping proteins. GAPDH (—●—) signal intensity remains relatively constant over assessed concentrations. Although the β-actin (—▲—) and β-tubulin (—■—) signals appear linear, the densitometric ratio was far below the predicted quantitative response of actual loading, whereas the Stain-Free signal (—◆—) correlated to the expected result (- - - -).

Furthermore, HKP expression levels may not be constant but instead may vary with different experimental treatments and other factors. Stain-Free technology eliminates these issues by quantifying total proteins on the same blot as the proteins of interest. This method of data normalization, called total protein normalization (TPN), provides more accurate protein quantitation and western blotting results than traditional HKP approaches.

In this method, the total density for each lane is measured from a blot, and a lane profile is obtained. Specialized software from Stain-Free–enabled imagers can interpret the data from the lanes in three dimensions so that the same lane profile data can be viewed as a three-dimensional peak. The background is adjusted in such a way that the total background is subtracted from the sum of the density of all the bands in each lane.

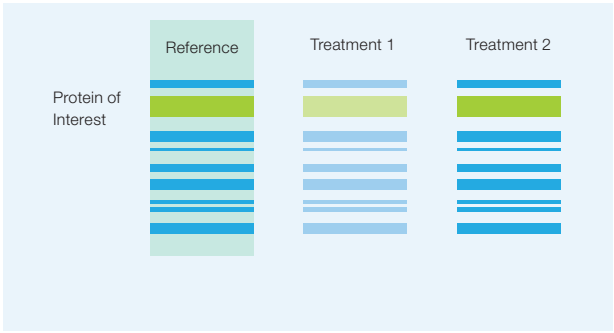
How to Apply TPN: Step by Step

1



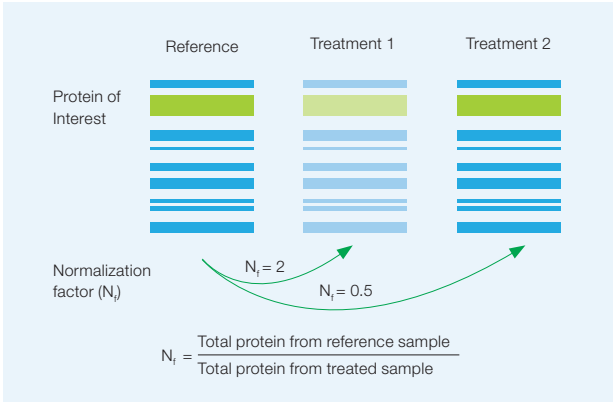
- Quantify the signals from the protein of interest and the total protein signal in each lane
- Use software packages such as Image Lab Software to access tools for TPN

2



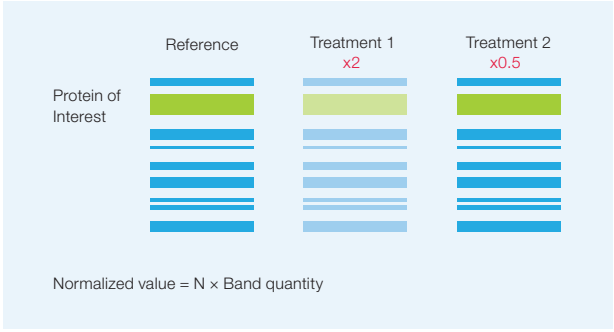
- Choose a reference lane for the blot
- Use any lane as the reference, since the normalization factor will be applied to all other lanes as the ratio relative to the reference lane

3



- For each lane, determine the normalization factor by dividing the signal intensity of the reference lane by total protein of the sample

4



- Apply the normalization factor to the protein of interest in each lane by multiplying the protein signal by the normalization factor for that lane
- Now you have the normalized signal for your protein of interest, and the relative expression of the proteins can be accurately compared between lanes

The Stain-Free Western Blotting Workflow Meets New Publication Guidelines

Several major academic publishers have recently revised their editorial guidelines for data publication, emphasizing reproducibility and quantitation criteria. For example, the Journal of Biological Chemistry (JBC) provides guidelines for collecting and presenting data that specifically address quantitative western blot publication, guidance on normalization methods and supporting data, and concerns regarding HKP in protein normalization (see Table 1).

Table 1. Journal of Biological Chemistry (JBC) guidelines for publication and their compatibility with Bio-Rad western blotting workflow solutions.

JBC Guidelines: Quantitative Blots	How to Meet Requirements	Bio-Rad Western Blotting Workflow Solutions
"Housekeeping proteins should not be used for normalization without evidence that experimental manipulations do not affect their expression"	Use TPN instead of HKPs	The Stain-Free Western Workflow suite simplifies Stain-Free TPN. This technology is proven with over 100 publications, allows for improved accuracy, and will make your data more reliable
"Methods including detection of enhanced chemiluminescence using X-ray film have a very limited dynamic range"	Use an imaging system with at least 4 logs of dynamic range	ChemiDoc MP Imaging Systems are Stain-Free imagers with the sensitivity of film. Image Lab Software enables simple image generation and quantitation
"A description of the data supporting the specificity of all antibodies is required"	Use fully validated antibodies	PrecisionAb™ Antibodies offer documented specificity and validation protocols

Further Reading

Nature Portfolio. Image integrity and standards. https://www.nature.com/nature-portfolio/editorial-policies/image-integrity, accessed April 21, 2023.

Fosang AJ and Colbran RJ (2015). Transparency is the key to quality. J Biol Chem 290, 29,692–29,694.

McNutt M (2014). Journals unite for reproducibility. Science 346, 679.

Yadav G and Oh K (2018). Defining the new normal in quantitative western blot data. https://www.bioradiations.com/defining-the-new-normal-in-quantitative-western-blot-data, accessed April 21, 2023.

Considerations When Using Stain-Free Technology

Because Stain-Free technology requires the modification of tryptophan residues, one potential concern is the ability to apply this technology to all proteins. Therefore, before deploying Stain-Free technology, determine whether your protein or proteins of interest contain tryptophan residues. Proteins that lack tryptophan residues, such as the drug aprotinin, are not detected using this technology (Figure 11). Theoretically, even one tryptophan residue is sufficient for signal activation, and proteins with as few as two tryptophan residues are readily detected and quantified using Stain-Free technology.

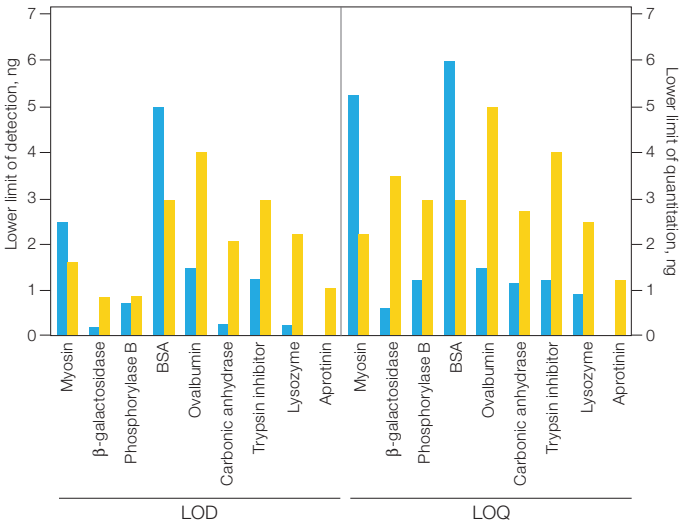


Fig. 11. Limits of detection (LOD) and limits of quantitation (LOQ) of proteins on Criterion TGX Stain-Free Precast Gels (■) and Bio-Safe Coomassie (G-250)–stained gels (■). Individual protein bands from broad range unstained standards from four replicate gels were used to determine visual LOD and LOQ. Averaged numbers were used to generate the graph. BSA, bovine serum albumin.

For most applications, the requirement of tryptophan residues for visualization is not a concern. Data available from UniProt show that only about 10% of proteins from all organisms lack tryptophan, and most of those proteins are less than 10 kD in size (https://www.ebi.ac.uk/uniprot/index). In most organisms, approximately 90% of proteins are greater than 10 kD in size (in the 10–260 kD range) and possess tryptophan residues (Table 2).

Table 2. Tryptophan content of the predicted proteomes of several model organisms.*

Species	Total Number of Proteins	Number of Proteins Lacking Tryptophan	Percentage of Total Proteins Lacking Tryptophan	Number of Proteins >10 kD	Number of Proteins >10 kD Lacking Tryptophan	Percentage of Proteins >10 kD Lacking Tryptophan
Homo sapiens	40,827	4,209	10.31	37,548	2,754	7.33
Escherichia coli O1:K1/APEC	4,865	458	9.41	4,754	408	8.58
Escherichia coli (strain K12)	4,181	456	10.91	3,879	325	8.38
Escherichia coli O6:K15:H31	4,604	562	12.21	4,147	365	8.80
Rattus norvegicus	12,022	1,081	8.99	11,421	745	6.52
Mus musculus	35,344	3,435	9.72	33,262	2,480	7.46
Saccharomyces cerevisiae	5,815	648	11.14	5,563	491	8.83

* Data obtained from UniProt in 2014.

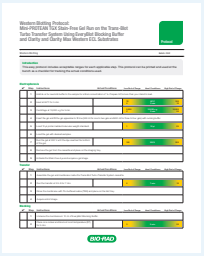
Conclusion and Resources

You have now finished The Complete Guide to Stain-Free Western Blotting. We have discussed how Stain-Free technology fits in a traditional western blotting workflow and offers some key advantages. Stain-Free technology allows you to visualize gels without staining and verify transfer efficiency. It also provides more sensitivity and dynamic range than Coomassie stains and is compatible with downstream applications. Not only does Stain-Free technology save time and labor, it also allows you to perform total protein normalization, which is more accurate and preferred over normalization with HKP. See the resources below to learn more about specific Stain-Free products and our general western blotting resources. Thank you for your interest, and we hope you consider switching to Stain-Free technology.

Additional Resources

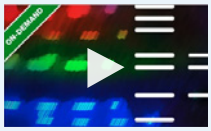
New to western blotting? Visit our Western Blot Learning Center.

Interested in an immersive experience? Enroll in our Western Blot University courses.

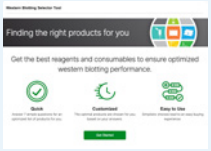


Visit our Western Blotting Protocols Library for detailed custom Stain-Free western blotting protocols.

Check out the Stain-Free Western Blotting Workflow Quick Order Guide.



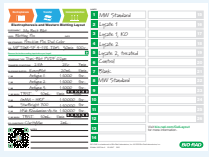
Check out our Western Blotting webinar series.



Find the right products for you with the Western Blotting Selector Tool.

Watch our video — Quick Tips: How to Evaluate Western Transfer Using Stain-Free Gels and a Bio-Rad Imaging System

Request free magnetic guides for interpreting protein gel migration and protein standards.



Request a free western blotting layout sticky note pad.

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Visit bio-rad.com/Stain-Free for more information.

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