

The Tactical Guide on Normalization

Strategies for Success



Get the full review article:

Western Blot Normalization: Challenges and
Considerations for Quantitative Analysis

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This guide explains how common normalization strategies work – and how they can affect the accuracy and reproducibility of your Western blot data.

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Part 1:
What Is
Normalization?

What Is Normalization?

In general terms, **normalization** compares target protein signal intensity to an **internal loading control**, to generate accurate and reproducible quantitative Western blot data.

Internal loading controls are endogenous sample protein(s) that are stably expressed and unaffected by experimental treatments.

Normalization uses these internal loading controls to mathematically correct for unavoidable sample-to-sample and lane-to-lane variation.

Normalization is a Strategy – Not Just a Step in the Protocol

Chess Grandmasters strategize to ensure the best possible outcome. Why not do the same with your experiments?

Successful normalization reduces the variability of your Western blot data. Your research will be more reproducible, meaning others will be able to generate the same data in a different lab at a different time, with the same conclusions and trends. It will also be more precise, for the most credible, publication-quality data.

Effective normalization gets you precise, reproducible data.

Why Is Normalization so Important for Quantitative Westerns?

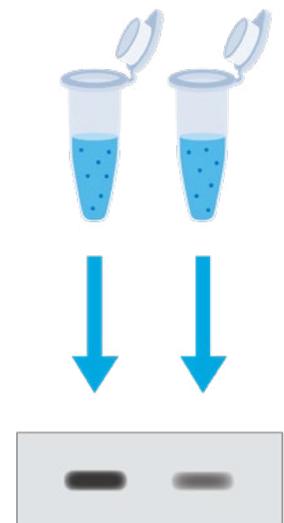
Normalization gives you confidence that the differences you see on your Western blot reflect actual differences in your experiment. It corrects for variations between samples, so you can reliably analyze your target protein levels.

Without proper normalization, you can't know if your data reflect real biological change or unexpected experimental error. Accurate quantitative analysis depends on effective normalization.



“[W]ith numbers comes great responsibility. There are straightforward ways to diagnose immunoblot accuracy and precision.”

— Dr. Kevin Janes¹



What Does Normalization Correct For?

Immunoblotting steps introduce unavoidable error. Normalization can help correct for variability introduced during:



Sample preparation:

Cell lysis procedures
Varying protein concentrations



Gel loading:

Pipetting technique
Overloading lanes



Transfer from gel to membrane:

Transfer method (wet, dry, semi-dry, or fast transfer)
Protein transfer efficiency

How Can You Maximize the Accuracy of Your Data?

Understand

Understand how common normalization methods work and how they affect your data. Different normalization strategies have different effects on normalized data.

Design

Design your experiment to minimize the need for normalization. Reduce error and variation whenever possible, so only small data corrections are needed.

Choose

Choose a normalization strategy that fits the biological conditions and goals of your experiment. You may need to validate that experimental variability doesn't affect your chosen strategy.

What Are the Requirements of an Effective Internal Loading Control?

Linear, proportional response

Signal intensity of the internal control accurately reflects protein concentration across a wide range.

Low biological variability

Your experimental treatments may affect the biological expression of commonly-used internal loading control proteins. For example, the expression of a “housekeeping” protein may vary in response to experimental conditions.

Corrects for variation at all stages of immunoblotting

Variation occurs throughout the Western blot process, including gel loading and transfer.

Compatible with immunodetection

The strategy you choose shouldn't interfere with effective downstream detection of your target proteins.

A solution that works for your biological context

Pick a method that you can adapt to your experimental demands.

It's important to choose a normalization strategy that fits the context and biology of your experiment. Regardless of your experimental conditions, Odyssey® imaging systems can provide accurate results based on any normalization strategy.



Part 2:
Normalization
Strategies

Types of Internal Loading Controls

Internal Reference Protein

Measure signal intensity from a reference point found within the sample.

Compare target intensity to this reference to correct for sample-to-sample variation.

Examples of Housekeeping Proteins	Examples of Phospho-Analysis
Actin	Phospho-ERK and pan-ERK
Tubulin	Phospho-Akt and pan-Akt

Total Protein Controls

Combine signal intensities from all proteins found within the sample.

Compare target intensity to this total measurement to correct for sample-to-sample variation.

Examples of Irreversible Covalent Labeling	Examples of Total Protein Staining
Stain-Free™	Revert™ 700 Total Protein Stain
CyDye™	

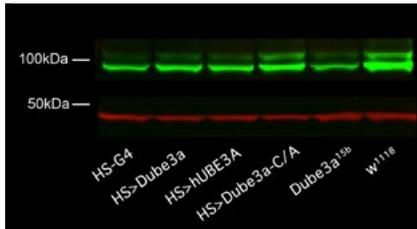
Which method is best?

There are many ways to normalize, and no single normalization strategy is always the best choice. Choose a strategy that fits best for the context, biology, and goal of your experiment. Understanding the limits of each strategy will help you select the best method for accurate analysis.

Housekeeping and Pan Proteins

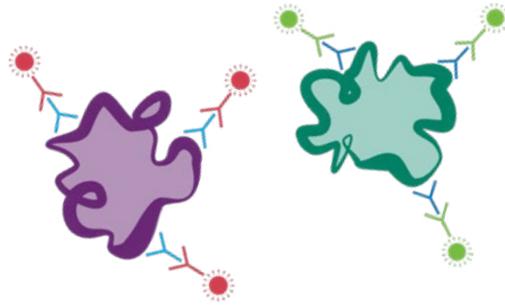
Types of Internal Reference Proteins:

Housekeeping proteins: a second, unrelated protein found in all samples



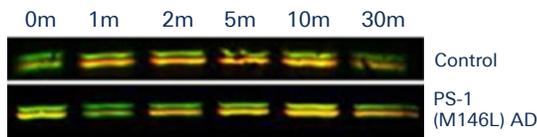
Housekeeping protein

Jensen et al. (2013) *PLoS ONE* 8(4): e61952.



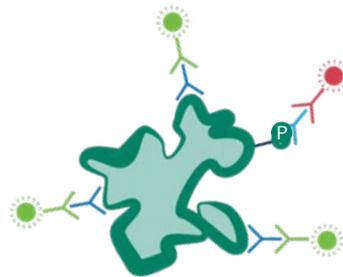
- Housekeeping protein
- Target protein
- Fluorescent detection of internal loading control
- Fluorescent detection of target protein

Pan proteins: multiplex phospho-analysis where the target protein is used as its own internal control



Multiplex phosphorylation analysis

Mendonso et al. (2009) *PLoS ONE* 4(2):e46655.



- Target protein
- Fluorescent detection of phospho-protein
- Fluorescent detection of pan-protein
- P** Phospho-epitope

How it works: Detect a reference protein in the same lane as your target protein. Then use the reference as a surrogate marker of sample concentration to correct for experimental variation.

Advantages of this Technique

Corrects for variation at all stages of immunoblotting. If your internal reference proteins are detected on the same blot, and in the same lane as your target proteins, any membrane transfer issues should affect them in the same way. However, be aware that highly expressed proteins will transfer differently than less abundantly expressed proteins.

Compatible with immunodetection. Internal reference proteins don't interfere with downstream detection of your target proteins, unless they migrate too closely together.

Challenges of this Technique

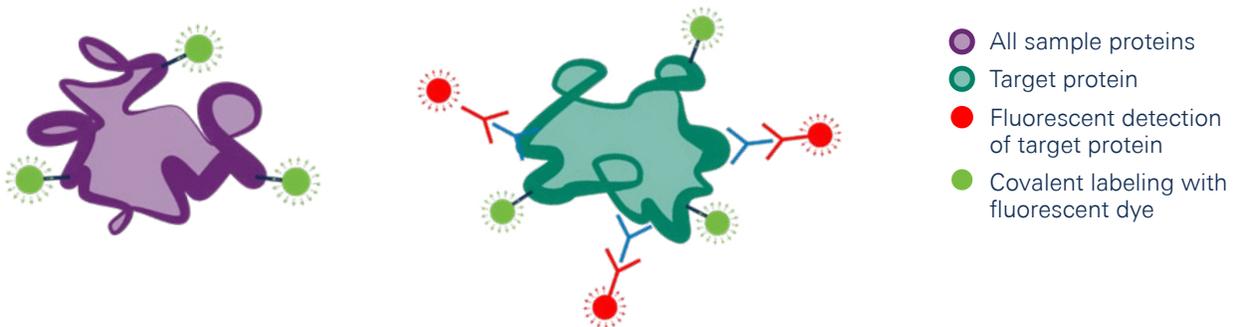
Beware saturated signals. Saturated bands hide real protein level differences and make normalization less accurate. Since reference proteins can be highly abundant, they may have a different linear range of detection than your target protein. Adjust sample loading amounts to avoid saturation (see page 14).

Beware biological variability. Normalization with a housekeeping protein will not account for biological variability. For example, if you're studying cell cycle changes, a structural protein (like actin or tubulin) may be affected by experimental treatments. Validation of stable expression is critical with housekeeping proteins.^{2,3,4}

Must be validated for each biological context. Internal reference proteins must be repeatedly validated for each experiment, to prevent biological variability from affecting data analysis.

Irreversible Covalent Labeling

How it works: Irreversible covalent labeling of your target proteins with fluorescent molecules occurs before or during electrophoresis. The covalently-modified proteins are then detected with UV or visible fluorescence imaging.



Advantages of this Technique

Biological variability no longer affects the loading control. The internal loading control measures the total amount of sample protein in each lane.

Challenges of this Technique

Poor detection sensitivity on the membrane. High membrane autofluorescence at UV and visible wavelengths limits the detection sensitivity and linear range compared to gel detection.

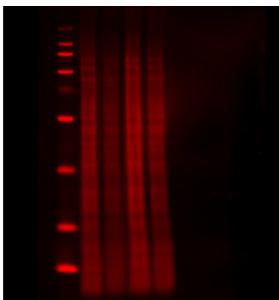
Increased variation. Variability may actually increase, because it's difficult to keep time-sensitive factors like crosslinking and dye-labeling consistent between experiments. This unavoidable variability is difficult to measure or correct for.

Target detection issues. Irreversible chemical modification of your target proteins may block antibody binding and prevent accurate immunodetection. It may also interfere with transfer of sample proteins to membranes.

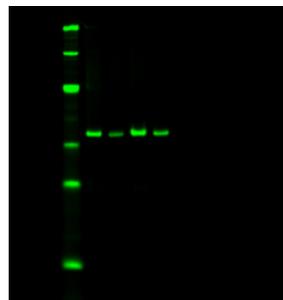
Less flexibility. Methods may require vendor-specific gels, reagents, transfer equipment, and imagers throughout electrophoresis and detection.

Total Protein Staining

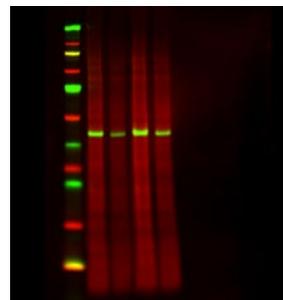
How it works: Typically, you detect total protein on your blotted membrane by applying a total protein stain. Then normalize the measured values of each of your targets to the total protein stain measurement. Your protocol may vary, since a variety of protein stains are available commercially.



Total Protein Stain



Immunostain



Overlay

Revert™ 700 membrane staining corrects for variation at all stages of immunoblotting. After transfer, this blot was stained with Revert 700 Total Protein Stain. The stain was removed with Reversal Solution, followed by Western blot detection of ERK.

Advantages of this Technique

Biological variability no longer affects the loading control. The internal loading control is a direct measure of the total amount of sample protein in each lane.

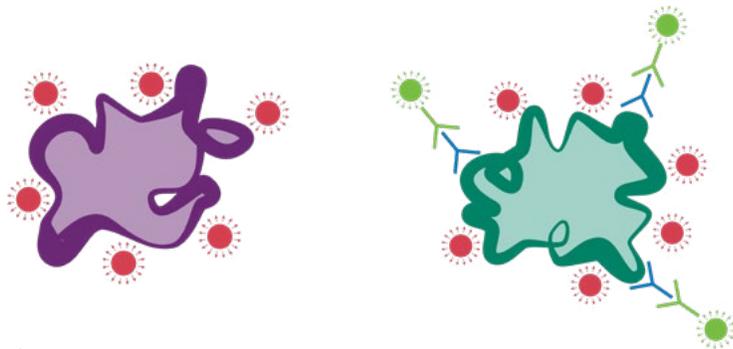
Corrects for variation at all stages of immunoblotting. Total protein stains should stain all proteins on the membrane equally.

Compatible with immunodetection. A membrane based (post-transfer) stain doesn't interfere with immunodetection.

Works in any biological context. Total protein staining is easy to incorporate into your existing protocols.

Challenges of this Technique

Different stains have different linear ranges. The linear range of detection for total protein stains is dependent on which stain you choose.



- All sample proteins
- Target protein
- Reversible total protein stain
- Fluorescent detection of target

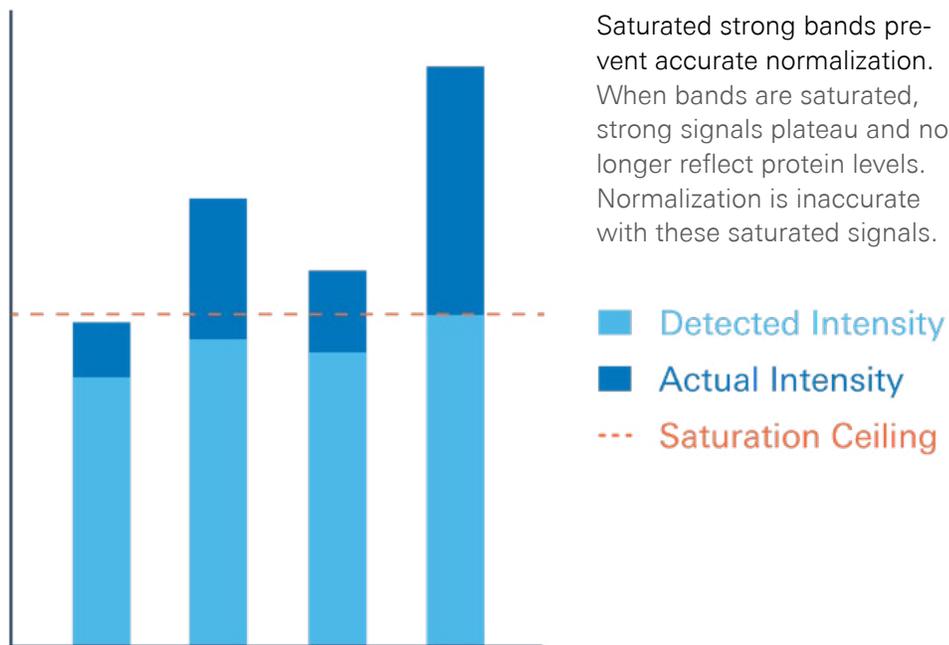


Part 3: Normalization Considerations

Beware Saturated Signals

Signal saturation is when the signal intensity of a protein is outside of its linear response range. This commonly happens with very abundant proteins like housekeeping proteins.

Saturated bands mask real differences in protein levels, and prevent accurate normalization. Whenever possible, the amount of sample protein you load should produce a signal intensity that lies in the middle of its linear range of detection. To use any normalization strategy correctly, it is necessary to detect both target and reference proteins in their appropriate linear ranges. Determine the optimal amount to load by running a dilution series of your sample.

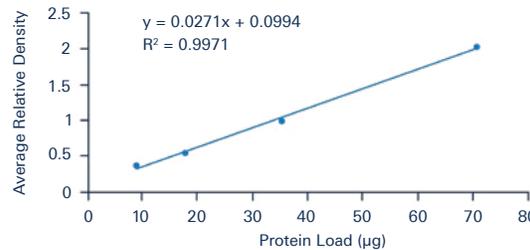
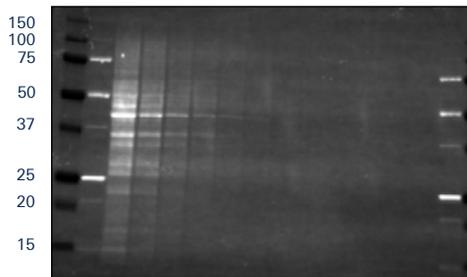
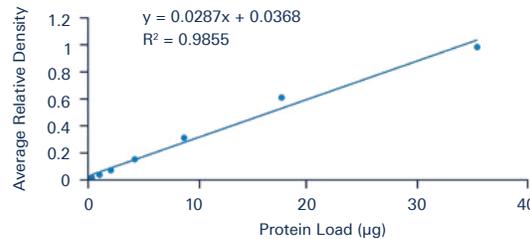
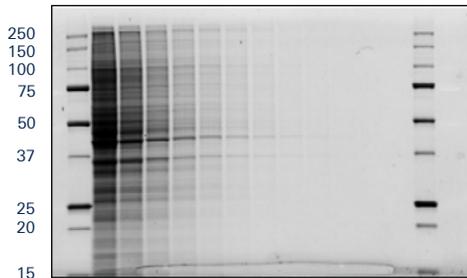


Did you know?

Signal saturation can happen with as little as 10 μg lysate per lane.⁵

Limits of Irreversible Covalent Labeling

In addition to irreversible chemical modification of your target proteins, some covalent labeling techniques may have limited detection sensitivity. Using the “Stain-Free” method limits your sensitivity, due to high background from autofluorescence of the PVDF membrane.



Stain-Free™ imaging of transferred membranes is less sensitive than gel imaging. UV detection of the gel has a 35-fold linear dynamic range (1-35 μg) compared to a linear range of only 7-fold with membrane detection (10-70 μg).

Reprinted from Taylor SC et al. (2013) *Mol Biotechnol.* 55:217-26.

Strategize to Outmatch Error

Effective normalization always begins with experimental design.

For the most precise and reproducible data, choose a normalization strategy that fits the context and biology of your experiment.

Any normalization strategy can be accurately implemented with a versatile Odyssey® imaging system.

Normalization is a strategy to improve the accuracy and reproducibility of quantitative Western blotting. Without careful planning, your results might not reflect the actual biology of your samples. Use appropriate internal loading controls in your experiment to correct for sample-to-sample variation – yielding reproducible, precise data. Regardless of your experimental conditions, Odyssey® imagers can provide accurate results with any normalization strategy.

Choose a Normalization Strategy that Fits Your Experiment

Internal Reference Protein	Total Protein Controls	
Housekeeping Protein and Multiplex Phospho-Analysis	Irreversible Covalent Labeling	Total Protein Staining
<ul style="list-style-type: none"> ✗ Beware saturated signals. ✗ Increased variation. ✓ Corrects for variation at all stages of immunoblotting. ✓ Compatible with immunodetection. ✗ Must be validated for each biological context. 	<ul style="list-style-type: none"> ✗ Poor detection sensitivity on the membrane. ✓ Biological variability no longer affects the loading control. ✗ Increased variation. ✗ Target detection issues. ✗ Less flexibility. 	<ul style="list-style-type: none"> ✗ Different stains have different linear ranges. ✓ Biological variability no longer affects the loading control. ✓ Corrects for variation at all stages of immunoblotting. ✓ Compatible with immunodetection. ✓ Works in any biological context.

Go In Depth with the Full Review Article

Types of experimental factors that affect your analysis. Learn which factors influence your choice of a normalization strategy. (page 7)

Advantages of multiplexing. Learn how multi-color detection can give you a strategic advantage. (page 9)

How to defeat saturation. Learn about unexpected sources of saturation in your Western blot experiments, and how they affect your data. (page 10)



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References

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3. Greer S, Honeywell R, Geletu M, Arulanandam R, and Raptis L. (2010) Housekeeping genes: expression levels may change with density of cultured cells. *J Immunol Meth.* 355(1-2): 76-9.
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5. Taylor SC and Posch A. (2014) The design of a quantitative Western blot experiment. *BioMed Res Intl.* doi: 10.1155/2014/361590.



Global Headquarters

Serving the United States and Canada

4647 Superior Street
Lincoln, NE 68504

Phone: +1-402-467-0700
Toll free: 800-645-4267
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LI-COR Biosciences GmbH
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Phone: +49 (0) 6172 17 17 771
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LI-COR Ltd., United Kingdom

Serving Denmark, Finland, Iceland, Ireland, Norway, Sweden, and UK.

LI-COR Biosciences UK Ltd.
St. John's Innovation Centre
Cowley Road
Cambridge
CB4 0WS
United Kingdom

Phone: +44 (0) 1223 422104
Fax: +44 (0) 1223 422105

bio-eu@licor.com

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976-18492 09/19

The LI-COR board of directors would like to take this opportunity to return thanks to God for His merciful providence in allowing LI-COR to develop and commercialize products, through the collective effort of dedicated employees, that enable the examination of the wonders of His works.

"Trust in the LORD with all your heart and do not lean on your own understanding. In all your ways acknowledge Him, and He will make your paths straight."

— Proverbs 3:5,6