Pan/Phospho Analysis For Western Blot Normalization

LI-COR Biosciences

Abstract

QWB is often used for relative analysis of protein phosphorylation and other post- translational modifications (PTMs). This method combines two primary antibodies raised in different hosts: a phospho-specific or other PTM-specific antibody, and a pan-specific antibody that recognizes the target protein regardless of its modification state. Fluorescently-labeled secondary antibodies are used to simultaneously detect and discriminate the two signals. Phospho-specific signals are then normalized against the total level of the target protein, using that protein as its own internal loading control for maximum accuracy.

This multiplex fluorescent method is used to analyze changes in target protein phosphorylation, glycosylation, acetylation, ubiquitination, and other PTMs.

Note: This protocol is intended for use with near-infrared fluorescent Western blots.

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Guidelines

I. Introduction

In quantitative Western blotting (QWB), normalization mathematically corrects for unavoidable sample-to-sample and lane-to-lane variation by comparing the target protein to an internal loading control. The internal loading control is used as an indicator of sample protein loading, to correct for loading variation and confirm that observed changes represent actual differences between samples.

More Info: For more normalization related resources, see 'Further Reading' in section IX.

Pan-Specific Antibodies as an Internal Loading Control

QWB is often used for relative analysis of protein phosphorylation and other post- translational modifications (PTMs). This method combines two primary antibodies raised in different hosts: a phospho-specific or other PTM-specific antibody, and a pan-specific antibody that recognizes the target protein regardless of its modification state. Fluorescently-labeled secondary antibodies are used to simultaneously detect and discriminate the two signals. Phospho-specific signals are then normalized against the total level of the target protein, using that protein as its own internal loading control for maximum accuracy.

This multiplex fluorescent method is used to analyze changes in target protein phosphorylation, glycosylation, acetylation, ubiquitination, and other PTMs.

Note: This protocol is intended for use with near-infrared fluorescent Western blots.

II. Keys for Success

Saturation and linear range. Saturated bands and sample overloading frequently compromise the accuracy of QWB. Use a dilution series to verify that you are working within the linear range of detection, and signal intensity is proportional to sample loading. See the protocol: Determining the Linear Range for Quantitative Western Blot Detection for more information.

Replication. Replicate samples provide information about the inherent variability of your methods, to determine if the changes you see are meaningful and significant. A minimum of three technical replicates is recommended for each sample. Replicates are discussed further in section VII.

Uniform sample loading. Uniform loading of total sample protein across the gel is critical for accurate QWB analysis. A protein concentration assay (BCA, Bradford, or similar assay) must be used to adjust sample concentration and load all samples as consistently as possible.

Antibody validation. Verify specificity of the phospho-antibody to ensure that it does not cross-react with the unmodified target protein, and to identify possible interference from background bands. Important guidelines are provided in Section IV.

More Info: The Antibody Publication Database can help you find antibody pairs that work for your experiment (licor.com/antibodyrequest).

Phosphorylation stoichiometry. This protocol is intended for relative comparison of pan- protein and phospho-protein signals, and results do not indicate the stoichiometry of phosphorylation (1).

III. Required Reagents

1. Treated and untreated samples

Protein concentration must be determined for all samples

2. Electrophoresis reagents

3. Transfer reagents

4. Pan-specific and modificiation-specific antibodies against target protein

Antibody selection and validation are described in Section IV.

5. Western blot detection reagents (near-infrared fluorescence)

Perform near-infrared Western blot detection according to the Near-Infrared Western Blot Detection Protocol

LI-COR NIRWesternProtocol with PVDF Membrane

LI-COR NIRWestern Protocol with Nitrocellulose Membrane

IV. Selection of pan- and phospho-specific antibodies

Antibody selection and validation are critical for pan/phospho analysis and pan/PTM analysis of other modifications. Follow these important guidelines:

- Pan- and modification-specific primary antibodies must be raised in different host species, so they can be discriminated by the secondary antibodies. For example, a rabbit polyclonal pan-antibody can be used with a mouse monoclonal phospho-antibody.
- Use highly cross-adsorbed secondary antibodies labeled with spectrally distinct NIR fluorescent dyes.
- The primary antibodies must recognize different epitopes on the protein, to avoid binding interference. Polyclonal pan-specific antibodies less likely to demonstrate interference.
- To validate the primary antibody pair, three Western blots are recommended. Perform a separate Western blot with each individual primary antibody, and a third blot with the two primary antibodies combined. These controls will characterize the banding patterns and verify that the primary antibodies can be multiplexed.

V. Protocol

See 'STEPS'

VI. Pan Protein and Phospho-Protein Quantification

Use Image Studio™ Software (free a free version of the analysis software, download Image Studio Lite) to quantify the fluorescent signals for the pan protein (700 nm) and phosphorylated target protein (800 nm).

See 'STEPS'

VII. Normalization Calculations and Analysis of Replicates

Replication is an important part of QWB analysis, and is used to confirm the validity of observed changes in protein levels. Biological and technical replicates are both important, but meet different needs (2, 3).

Biological replicates: Parallel measurements of biologically distinct samples, used to control for biological variation and examine the generalizability of an experimental observation.

Technical replicates: Repeated measurements used to establish the variability of a protocol or assay, and determine if an experimental effect is large enough to be reliably distinguished from the assay noise.

Technical replication can be performed by testing the sample multiple times on the same gel or membrane (intra-assay variation) or by testing the sample multiple times in several Western blot experiments. This procedure describes the normalization and analysis of technical replicates that were tested multiple times on the same membrane.

Calculate the Lane Normalization Factor for Each Lane (pan protein, 700 nm)

1. Prepare a spreadsheet containing the pan protein and phospho-protein quantification data.

2. Using the pan-antibody data from the normalization channel (700 nm), calculate the Average (arithmetic mean), Standard Deviation, and % Coefficient of Variation (% CV) of the replicate samples.

“Average” formula in Excel = AVERAGE(rep 1, rep 2, ...)

Standard Deviation formula in Excel = STDEV(rep 1 value, rep 2 value, ...)

$$\%\ \text{Coefficient of Variation} = \frac{\text{Standard Deviation of replicates}}{\text{Average Signal of replicates}} \times 100$$

Pan Protein (700 nm)

Treatment	Replicate	Primary Antibody	Signal	Average	St Dev	% CV
-	1	pan	990	950	36	4%
-	2	pan	940			
-	3	pan	920			
+	1	pan	930	943	51	5%

+	2	pan	900
+	3	pan	1000

Example values shown for illustration only

3. Calculate the Lane Normalization Factor (LNF) for each lane.

a. Identify the lane with the highest signal for total protein staining.

b. Use this value to calculate the Lane Normalization Factor for each lane.

$$\text{Lane Normalization Factor} = \frac{\text{Pan Protein Signal for Each Lane}}{\text{Highest Pan Protein Signal in Any Lane}}$$

Treatment	Replicate	Primary Antibody	Signal	Highest Signal	LNF
-	1	pan	990	1,000	0.99
-	2	pan	940	1,000	0.94
-	3	pan	920	1,000	0.92
+	1	pan	930	1,000	0.93
+	2	pan	900	1,000	0.90
+	3	pan	1,000	1,000	1.00

Calculate the Normalized Phospho-Target Signals (phospho antibody, 800 nm)

1. Using the phospho-antibody data from the 800 nm channel, calculate the Average (arithmetic mean), Standard Deviation, and % Coefficient of Variation (% CV) of the replicate samples.

Phospho-target (800 nm)

Treatment	Replicate	Primary Antibody	Signal	Average	St Dev	% CV
-	1	phospho	160	182	26	14%

-	2	phospho	175			
-	3	phospho	210			
+	1	phospho	19,500	18,833	1,607	9%
+	2	phospho	17,000			
+	3	phospho	20,000			

Example values shown for illustration only.

2. Calculate the Normalized Phospho-Target Signal for each target band by applying the Lane Normalization Factor for that lane.

a. Divide the Target Signal for each lane by the corresponding LNF.

$$\text{Normalized Signal for Each Lane} = \frac{\text{Modified Protein Signal for Each Lane}}{\text{Lane Normalization Factor}}$$

Phospho-target (800 nm) - Normalization

Treatment	Replicate	Primary Antibody	Signal	LNF (pan - Ab)	St Dev	Norm. Signal
-	1	phospho	160	0.99	160/0.99	162
-	2	phospho	175	0.94	175/0.94	186
-	3	phospho	210	0.92	210/0.92	228
+	1	phospho	19,500	0.93	19,500/0.93	20,968
+	2	phospho	17,000	0.9	17,000/0.9	18,889
+	3	phospho	20,000	1	20,000/1	20,000

b. Use this value to calculate the Lane Normalization Factor for each lane

Treatment	Replicate	Primary Antibody	Signal	Norm Signal	Average	St Dev	% CV
-	1	phospho	160	162	192	34	18%
-	2	phospho	175	186			
-	3	phospho	210	228			
+	1	phospho	19,500	20,968	19,952	1,040	5%

+	2	phospho	17,000	18,889
+	3	phospho	20,000	20,000

VIII. Data Interpretation

1. Use the normalized Target Protein values for relative comparison of samples.

In the example above, treatment induced a 100-fold increase in phosphorylation of the target protein.

2. % CV can be used to evaluate the robustness of QWB results, and determine if the magnitude of observed changes in Target Protein levels is large enough to be reliably distinguished from assay variability.

a. The percent coefficient of variation (% CV) describes the spread or variability of measured signals by expressing the standard deviation (SD) as a percent of the average value (arithmetic mean). Because % CV is independent of the mean and has no unit of measure, it can be used to compare the variability of data sets and indicate the precision and repeatability of an assay.

- A low % CV value indicates low signal variability and high precision of measurement.
- A larger % CV indicates greater variation in signal and reduced precision

b. On a Western blot, a change in band intensity is meaningful only if its magnitude substantially exceeds the % CV.

- Generally speaking, the magnitude of the reported change should be at least 2X greater than the % CV.
- **Example:** To report a 20% difference between samples (0.8-fold or 1.2-fold change in band intensity), % CV 10% would be recommended for replicate samples.
- For a specific measurement, this threshold for the magnitude of change would correspond to the mean + 2 SD.
- Faint bands or subtle changes in band intensity are more difficult to detect reliably. In these situations, QWB analysis requires more extensive replication and low % CV.
- This is a general guideline only. Replication needs and data interpretation are specific to your experiment, and you may wish to consult a statistician.

3. Compare the % CV of Target Protein replicates before and after normalization.

a. Normalization should not greatly increase the % CV of replicate samples.

b. The purpose of normalization is to reduce the variability between replicate samples by correcting for lane-to-lane variation. A large increase in % CV after normalization of replicates is a warning sign that the normalization method is not sufficiently robust, and may be a source of experimental error.

IX. Further Reading

Please see the following for more information about normalization.

Western Blot Normalization Handbook

licor.com/handbook

The Normalization Handbook describes how to choose and validate an appropriate internal loading control for normalization.

Good Normalization Gone Bad

licor.com/GNGB

Good Normalization Gone Bad presents examples of normalization that have been adversely affected by common pitfalls and offers potential solutions.

Western Blot Normalization White Paper

licor.com/normalizationreview

This white paper comprehensively reviews the literature of Western blot normalization.

Determining the Linear Range for Quantitative Western Blot Detection

licor.com/LinearRange

This protocol explains how to choose an appropriate amount of sample to load for QWB analysis.

REVERT™ Total Protein Stain Normalization Protocol

licor.com/RevertNormalization

This protocol describes how to use REVERT™ Total Protein Stain for Western blot normalization and analysis.

Housekeeping Protein Validation Protocol

licor.com/HKP-Validation

This protocol explains how to validate an HKP for use as an internal loading control, by demonstrating that HKP expression is stable in the relevant experimental samples.

Housekeeping Protein Normalization Protocol

licor.com/HKP-Normalization

This protocol describes how to use a housekeeping protein for Western blot normalization and quantitative analysis.

References

1. Janes KA (2015) An analysis of critical factors for quantitative immunoblotting. Sci Signal. 8(371): rs2.

2. Robasky, K, Lewis NE, and Church GM. Nat. Rev. Genet. 15: 56–62 (2014).

http://www.nature.com/nmeth/journal/v11/n9/pdf/nmeth.3091.pdf

3. Naegle K, Gough NR, and Yaffe MB. Sci Signal. 8:fs7 (2015).

https://www.ncbi.nlm.nih.gov/pubmed/25852186

Materials

β-Actin Rabbit Monoclonal Antibody, 100 µL 926-42210 by Licor

β-Tubulin Rabbit Polyclonal Antibody, 100 µL 926-42211 by Licor

β-Actin Mouse Monoclonal Antibody, 100 µL 926-42212 by Licor

α-Tubulin Mouse Monoclonal Antibody, 100 µL 926-42213 by Licor

COX IV Rabbit Monoclonal Antibody, 100 µL 926-42214 by Licor

REVERT™ Total Protein Stain Kit 926-11010 by Licor

Odyssey 28 KD Loading Indicator – 800 nm, 2 x 1 mL 926-20002 by Licor

Odyssey 28 KD Loading Indicator – 700 nm, 2 x 1 mL 926-20004 by Licor

Protocol

Step 1.

Generate a set of experimental samples (drug treatment, time course, dose-response, etc).

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Note: A minimum of three replicates should be performed for each sample.

Step 2.

Determine the protein concentration of each sample using a BCA, Bradford, or similar protein assay.

Step 3.

Dilute the samples to equal concentrations to enable consistent, uniform loading of total sample protein across the gel.

Step 4.
Prepare samples to be loaded on the gel with sample loading buffer.

Step 5.
Denature sample by heating at 95 °C for 3 min (or 70 °C for 10 min).

Step 6.
Load a uniform amount of sample protein in each lane.

Step 7.
Separate protein by SDS-PAGE.

Step 8.
Transfer proteins to immobilizing membrane.

Step 9.

Perform Western blot detection of target protein and HKP, according to the Near-Infrared Western Blot Detection Protocol.

LI-COR NIRWesternProtocol with PVDF Membrane

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Note: Use the 800 nm channel to detect the lowest-abundance target (typically, the modified protein). This channel provides the lowest membrane background and will maximize the sensitivity of detection.

Step 10.

Image membrane with an Odyssey imaging system (Classic, CLx or Fc) in the 700 and 800 nm channels.

Adjust settings so that no saturation appears in the bands to be quantified.

Quantification of Pan Protein (700 nm image)

Step 11.

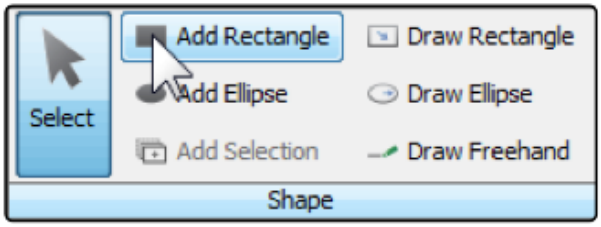
Use Image Studio™ Software (free a free version of the analysis software, download Image Studio Lite) to quantify the fluorescent signals for the pan protein (700 nm) and phosphorylated target protein (800 nm).

Select and view the 700 nm channel image only.

Quantification of Pan Protein (700 nm image)

Step 12.

Add shapes to bands. In the Shape group on the Analysis tab, click Add Rectangle.



Quantification of Pan Protein (700 nm image)

Step 13.

Click each band to be analyzed, and an appropriately sized shape will be added around the band.

Quantification of Pan Protein (700 nm image)

Step 14.

Export the pan-protein quantification data. Click Shapes to open the Shapes data table.

Quantification of Pan Protein (700 nm image)

Step 15.

Select shape data, the copy and paste data into a spreadsheet.

For information on how to perform the normalization calculations and interpret the data, go to

Sections VII - VIII in the Guidelines

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Note: All data fields will be exported, but “Signal” is the field of interest for analysis.

Target Protein Quantification (800 nm image)

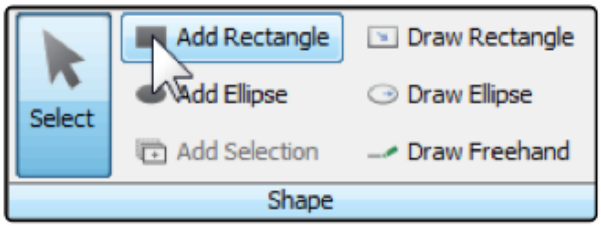
Step 16.

Select and view the 800 nm channel image only.

Target Protein Quantification (800 nm image)

Step 17.

Add shapes to bands. In the Shape group on the Analysis tab, click Add Rectangle.



Target Protein Quantification (800 nm image)

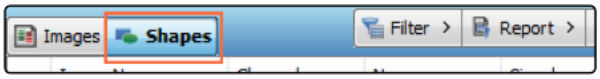
Step 18.

Click each band to be analyzed, and an appropriately sized shape will be added around the band.

Target Protein Quantification (800 nm image)

Step 19.

Export the phospho-specific quantification data. Click Shapes to open the Shapes data table.



Target Protein Quantification (800 nm image)

Step 20.

Select shape data, the copy and paste data into a spreadsheet.

For information on how to perform the normalization calculations and interpret the data, go to Sections VII - VIII in the Guidelines.

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Note: All data fields will be exported, but “Signal” is the field of interest for analysis.

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