Technical Note

Housekeeping Protein Normalization Protocol

LI-COR

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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" on page 12.

Using a Housekeeping Protein (HKP) as an Internal Loading Control

Housekeeping proteins (HKPs) are routinely used as loading controls for Western blot normalization. Accurate normalization requires stable expression of the HKP across all experimental conditions and treatments. Because HKP normalization relies on a single indicator of sample loading, variation in HKP expression leads to inconsistent estimation of sample loading and introduces experimental error that may alter data analysis.

For widely-used HKPs (such as actin, tubulin, and GAPDH), stable expression has generally been assumed. However, expression of common HKPs is now known to vary in response to certain experimental conditions, including cell confluence, disease state, drug treatment, and cell or tissue type.

Important: Before an HKP is used for Western blot normalization, stable expression must be validated for the specific experimental context and treatments. For more information, see the *Housekeeping Protein Validation Protocol* (licor.com/HKP-Validation; LI-COR).

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

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* (licor.com/LinearRange; LI-COR) 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 on page 7.

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. Two-color Western blot detection requires careful selection of primary and secondary antibodies to prevent cross-reactivity. Always perform single-color control blots first to verify antibody specificity, and to identify possible interference from background bands.

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

III. Required Reagents

1. **Treated and untreated samples**
   Protein concentration must be determined for all samples.

2. **Electrophoresis reagents**

3. **Transfer reagents**

4. **Western blot detection reagents (near-infrared fluorescence)**
   Perform near-infrared Western blot detection according to the *Near-Infrared Western Blot Detection Protocol* (licor.com/NIRWesternProtocol; LI-COR).

IV. Protocol

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

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

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

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

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

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

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

7. Separate protein by SDS-PAGE.

8. Transfer proteins to immobilizing membrane.

9. Perform Western blot detection of target protein and HKP, according to the *Near-Infrared Western Blot Detection Protocol* (licor.com/NIRWesternProtocol; LI-COR).

   > **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.

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.

V. Target Protein and HKP Quantification

Use Image Studio™ Software (licor.com/islite) to quantify the fluorescent signals of the HKP (700 nm) and target protein (800 nm).

HKP Quantification (700 nm)

1. Select and view the 700 nm channel image only.

2. Add shapes to bands.

   a. In the Shape group on the Analysis tab, click Add Rectangle.
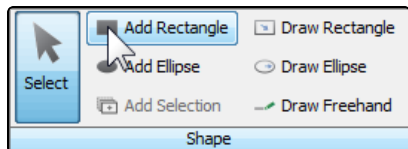
   b. Click each band to be analyzed, and an appropriately sized shape will be added around the band.

   > **More Info:** For help choosing the right background subtraction method, see licor.com/BgSubtractHelp.

3. Export the quantification data for your HKP.

   a. Click Shapes to open the Shapes data table.

   b. Select shape data, then copy and paste data into a spreadsheet.

   > **Note:** All data fields will be exported, but "Signal" is the field of interest for analysis.

Target Protein Quantification (800 nm)

1. Select and view the 800 nm channel image only.

2. Add shapes to bands.

   a. In the Shape group on the Analysis tab, click Add Rectangle.

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

> **More Info:** For help choosing the right background subtraction method, see licor.com/BgSubtractHelp.

3. Export the quantification data for your target.

    a. Click Shapes to open the Shapes data table.
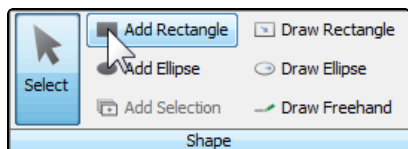

    b. Select shape data, then copy and paste data into a spreadsheet.

> **Note:** All data fields will be exported, but "Signal" is the field of interest for analysis.

VI. 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 ([1], [2]).

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 (HKP, 700 nm)

1. Prepare a spreadsheet that contains the HKP and target protein quantification data.

2. Using the HKP data from the normalization channel, 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, ...)

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

   REVERT Staining (700 nm)

Lane	Sample treatment	Replicate	700 nm signal	Average Signal	St Dev	CV
1	none	1	1,000	900	100	11%
2		2	800			
3		3	900			
4	UV	1	800	833	58	7%
5		2	900			
6		3	800			

   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 the HKP.

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

   $$\text{Lane Normalization Factor} = \frac{\text{HKP Signal for Each Lane}}{\text{HKP Signal from Lane with Highest HKP Signal}}$$

HKP (700 nm)

Lane	Sample treatment	Replicate	700 nm signal	Highest Signal	Lane Normalization factor
1	none	1	1,000	1,000	1
2		2	800	1,000	0.8
3		3	900	1,000	0.9
4	UV	1	800	1,000	0.8
5		2	900	1,000	0.9
6		3	800	1,000	0.8

Important: Normalization factors must be calculated for each blot. Normalization factors and standard curves cannot be reused between blots.

Calculate the Normalized Target Protein Signals (800 nm)

1. Using the Target Protein data from the 800 nm channel, calculate the Average, Standard Deviation, and % Coefficient of Variation of the replicate samples.

Target protein (800 nm), not normalized

Lane	Sample treatment	Replicate	Target (800 nm)	Average Signal	St Dev	CV
1	none	1	650	600	50	8%
2		2	550			
3		3	600			
4	UV	1	450	477	25	5%
5		2	500			
6		3	480			

Example values shown for illustration only.

2. Calculate the Normalized Target Signal for each target band by applying the LNF for that lane.

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

   $$\text{Normalized Signal} = \frac{\text{Target Band Signal}}{\text{Lane Normalization Factor}}$$

   b. Calculate the Average, Standard Deviation, and % Coefficient of Variation of the replicates.

Target protein (not normalized)				Normalization		Normalized to total protein			
Lane	Sample treatment	Replicate	Target (800 nm)	LNF (HKP)	Norm. of Target	Norm. Target	Average signal	St Dev	CV
1	none	1	650	1	650 / 1	650	668	19	3%
2		2	550	0.8	550 / 0.8	688			
3		3	600	0.9	600 / 0.9	667			
4	UV	1	450	0.8	450 / 0.8	563	573	44	4%
5		2	500	0.9	500 / 0.9	556			
6		3	480	0.8	480 / 0.8	600			

VII. Data Interpretation

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

   In the example above, Target Protein level is 14% lower in UV-treated samples than in untreated samples.

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 measurement precision.

      ○ 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), a CV of 10% or less 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.

VIII. 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.

Pan/Phospho Analysis For Western Blot Normalization

licor.com/PanProteinNormalization

This protocol describes how to use pan-specific antibodies as an internal loading control for normalization.

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. 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

2. Naegle K, Gough NR, and Yaffe MB. *Sci Signal.* 8:fs7 (2015). https://www.ncbi.nlm.nih.gov/pubmed/25852186