Protocol

Housekeeping Protein Validation Protocol

Published March 2017. Revised July 2020. The most recent version of this document is posted at licor.com/bio/support.

Visit us on protocols.io! Explore an interactive version of this protocol at bit.ly/HKP-Validation.

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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 13.

Using a Housekeeping Protein for Normalization

Housekeeping proteins (HKPs) are routinely used for Western blot normalization. For common HKPs (such as actin, tubulin, or GAPDH), stable protein expression is generally assumed. However, expression of several HKPs is now known to vary in response to certain experimental conditions, including cell confluence, disease state, drug treatment, and cell or tissue type. Because HKP normalization uses a single indicator of sample loading, changes in HKP expression will introduce error and may alter data analysis and interpretation.

Before using a housekeeping protein for Western blot normalization, it is critical to validate that its expression is constant across all samples and unaffected by the specific experimental context and conditions.

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.

> **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.

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.

III. Required Reagents

1. **Treated and untreated samples**
   Protein concentration must be determined for all samples.
2. **Revert™ 700 Total Protein Stain Kit** (licor.com/revertkit)
   Revert 700 Total Protein Stain is used to assess sample protein loading in each lane as an internal loading control. After transfer and prior to immunodetection, the membrane is treated with this near-infrared fluorescent protein stain and imaged. Membrane staining can verify that sample protein was uniformly loaded across the gel, and assess the quality and consistency of protein transfer.
3. **Odyssey Loading Indicator, 800 nm (LI-COR, P/N 926-20002)**
   Odyssey Loading Indicator (OLI) is an external loading control that is added to your samples just before electrophoresis and is used to verify that a similar sample volume was loaded in each lane. Because it is an exogenous protein, it does not provide information about the amount of sample protein loaded or transferred.
4. **Electrophoresis reagents**
5. **Transfer reagents**
6. **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 samples representing the specific range of treatments or conditions you will use in your experiment (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. Determine the Final Volume per well for all samples to be loaded based on the well capacity of your gel (see Table 1).
4. Determine the Sample Volume required to add the proper amount of Odyssey Loading Indicator. Sample Volume should be equal to 75% of the Final Volume per well.

For example, if Final Volume per well is 10 µL, Sample Volume should be 7.5 µL

5. Dilute samples to equal concentration with appropriate sample buffer to equal the Sample Volume determined in step 3.

> **Note:** All samples should have the same final protein concentration and volume.

Table 1 Volumes of Odyssey Loading Indicator per Sample Amount

Sample Volume Buffer (µL)	Odyssey Loading Indicator (with β-ME, µL)	Final Volume per Well (µL)
7.5	2.5	10
9.0	3.0	12
10.5	3.5	14
12.0	4.0	16
13.5	4.5	18
15	5.0	20

6. Prepare samples for gel loading according to the instructions for Odyssey Loading Indicator (800 nm).
7. Denature sample by heating at 95 °C for 3 min (or 70 °C for 10 min).
8. Load an equal volume of sample, and uniform amount of sample protein, in each lane.
9. Separate sample proteins by SDS-PAGE.
10. Transfer proteins to blotting membrane.
11. After transfer is complete, stain membrane with Revert™ 700 Total Protein Stain using the Single-Color Western Blot protocol:

> **More Info:** For more information about the Revert protocol, see licor.com/revert.

a. Add methanol to the stain reagents as indicated on each bottle.

b. Rinse membrane in water, and incubate in 5 mL of Revert 700 Total Protein Stain solution for 5 minutes, with gentle shaking.

c. Decant Total Protein Stain solution thoroughly. Rinse membrane two times for 30 seconds with approximately 5 mL of Wash Solution. Briefly rinse membrane with water.
Revert 700 Wash Solution (P/N 926-11012): 6.7% (v/v) Glacial Acetic Acid, 30% (v/v) Methanol, in water.

12. Image the membrane in the 700 nm channel with an Odyssey® imaging system (Classic, CLx, or Fc).
Adjust settings so that no saturation appears in the bands to be quantified.

13. Rinse membrane briefly with water, and proceed immediately to blocking and immunodetection steps.

14. Perform immunoblotting using IRDye® 800CW conjugated secondary antibody to detect the HKP.

15. Image the membrane in the 800 nm channel with an Odyssey imaging system.
Adjust settings so that no saturation appears in the bands to be quantified.

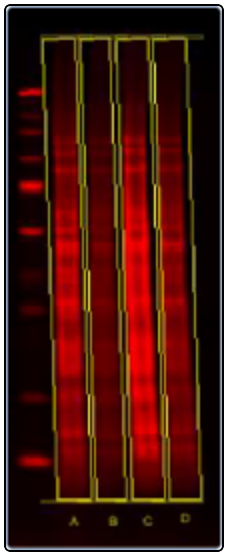
V. Total Protein and HKP Quantification

Use Image Studio™ Software (licor.com/islite) to quantify the fluorescent signals from the total protein stain (700 nm), HKP (800 nm), and loading indicator (800 nm).

> **More Info:** Empiria Studio® Software provides a dedicated workflow for this process. Go to licor.com/empiria to learn more.

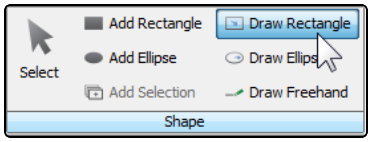
Total Protein Quantification

Use the Draw Rectangle tool in Image Studio™ Software to quantify the Total Protein signal in each lane.



1. Draw a shape around the first lane on the Total Protein Stain image (700 nm channel image).

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

   b. Draw a rectangle around the first lane.

   If lanes are skewed, rotate the shape by clicking Rotate in the Edit group.

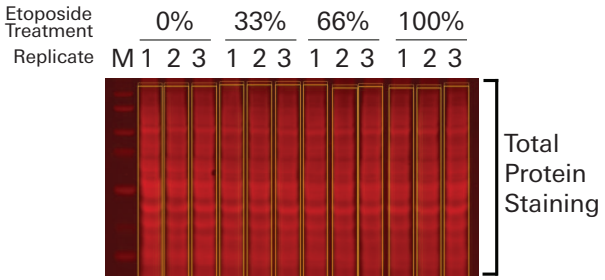
   > **More Info:** For help choosing the right background subtraction method, see licor.com/BgSubtractHelp.

2. Add the shape from the first lane to each remaining lane.

   a. With the first shape selected, click Add Selection.

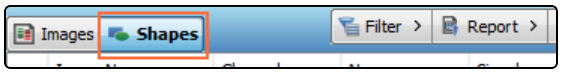
   b. Click the next lane to copy the shape.

   If necessary, adjust the shape borders to ensure that each shape encloses the signal from one, and only one lane.



3. Export total protein quantification data.

   a. Click Shapes to open the Shapes data table.

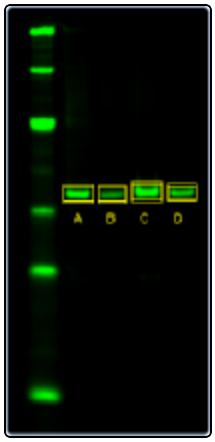
   

   b. Select shape data, then copy and paste data into a spreadsheet.

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

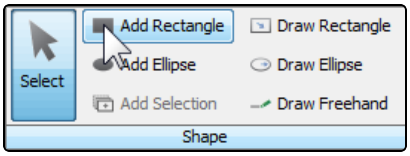
HKP Quantification (800 nm image)

Use the Add Rectangle tool in Image Studio to quantify the HKP bands (800 nm channel). Loading indicator bands will also be analyzed in the 800 nm image.

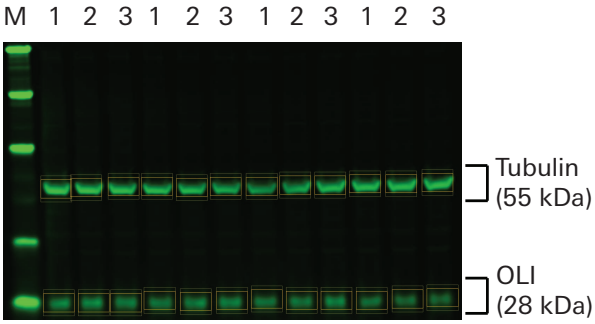


1. Click to select your Western blot 800 channel image, then add shapes to bands.

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

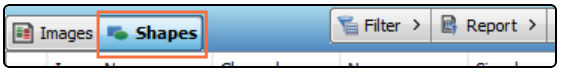
   b. Click each band to be analyzed, and an appropriately sized shape will be added around the band.



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

2. Export quantification data.

   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. Calculations

> **More Info:** Empiria Studio® Software will perform these calculations automatically. Please continue to the Data Interpretation section.

Prepare a spreadsheet that contains all total protein stain, HKP, and OLI quantification values using spreadsheet software.

1. Calculate the average signal intensity for replicate samples.

   "Average" formula in Excel = AVERAGE(rep 1, rep 2, ……)

   ### Average Signal for Total Protein

Treatment	rep 1	rep 2	rep 3	Avg	St Dev	% CV
0%	2,030,000	2,060,000	2,100,000	2,063,333	35,119	2%
33%	2,110,000	2,120,000	2,120,000	2,116,667	5,774	0%
66%	2,080,000	2,100,000	2,120,000	2,100,000	20,000	1%
100%	2,060,000	2,110,000	2,070,000	2,080,000	26,458	1%

   ### Average Signal for HKP (Tubulin)

Treatment	rep 1	rep 2	rep 3	Avg	St Dev	% CV
0%	9,190	9,070	8,780	9,013	211	2%
33%	8,210	7,760	7,340	7,770	435	6%
66%	6,650	7,270	7,780	7,233	566	8%
100%	7,770	7,590	7,370	7,577	200	3%

Average Signal for Odyssey Loading Indicator

Treatment	rep 1	rep 2	rep 3	Avg	St Dev	% CV
0%	5,000	4,980	5,170	5,050	104	2%
33%	5,220	5,320	5,320	5,287	58	1%
66%	5,310	5,220	5,210	5,247	55	1%
100%	5,150	4,880	5,310	5,113	217	4%

2. Calculate the Standard Deviation of the replicate treatment conditions.

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

3. Calculate the Coefficient of Variation (CV) of the replicate treatment conditions.

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

4. Separately plot the relative signal intensities for each data set, HKP, total protein stain, and loading indicator (as in Figure 1).

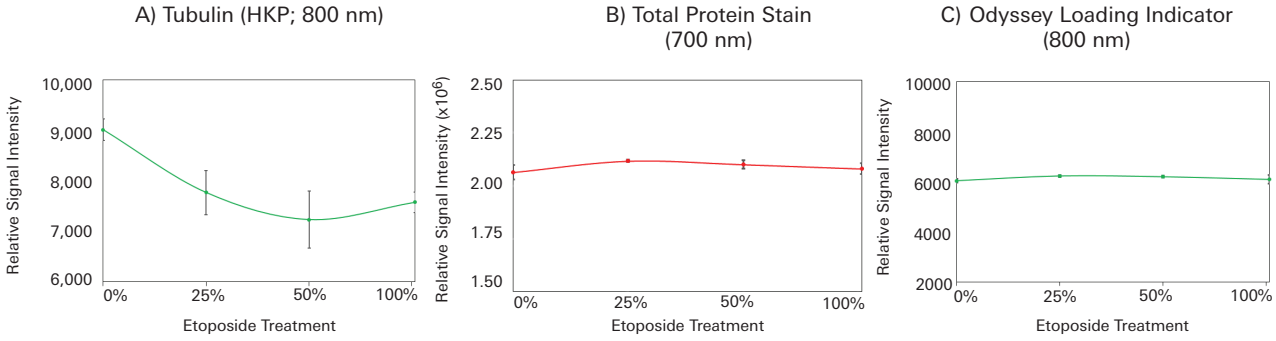


Figure 1. HKP expression may be affected by experimental treatments. Jurkat cells were treated with etoposide to induce apoptosis. Cell lysate samples were loaded in triplicate. Relative signal intensities are shown. Intensity of tubulin bands (A) decreased by 16% across the blot, suggesting that etoposide treatment may lower the expression of this HKP. Total protein staining (B) and Odyssey Loading Indicator (C) showed consistent signals in all samples, confirming that sample protein loading and volume were uniform across the gel. This indicates that the observed changes in HKP band intensity reflect actual change in tubulin levels in the etoposide-treated cells, not variation in sample loading.

VII. Data Interpretation

1. Evaluate results to determine if expression of this HKP is stable across samples.

    a. Were samples uniformly loaded across the gel?

    - Consistent signals for Revert total protein stain (as in Fig. 1B, with an approximately horizontal line) confirm that the amount of sample protein loaded was consistent and uniform.
    - Consistent signals from the Odyssey Loading Indicator (Fig. 1C) indicate that a uniform sample volume was loaded.
    - Inconsistent signals indicate a problem with sample loading, and HKP expression cannot be accurately evaluated. Repeat the Western blot experiment with uniformly loaded samples.

    b. If samples were uniformly loaded, the intensity of HKP bands should reflect actual HKP expression levels in your samples.

    - Stable HKP bands indicate that actual HKP protein levels were stable in these samples. This HKP may be suitable for Western blot normalization.

    > **Important:** The HKP must be revalidated if there are changes in experimental treatments, conditions, cell lines, etc.

    - Variation in HKP band intensity indicates that actual HKP expression was altered by your experimental conditions (Fig. 1A). This HKP should not be used for normalization.

    c. If samples were not uniformly loaded, HKP bands may not accurately reflect actual HKP expression in these samples.

    HKP validation results cannot be accurately interpreted, and the Western blot experiment should be repeated with careful attention to uniform sample loading.

2. If results indicate that HKP expression is affected by experimental conditions, this HKP should not be used for normalization. Next steps should be:

    a. Repeat this process to evaluate additional HKPs, or

    b. Consider total protein staining of the membrane for Western blot normalization. Revert 700 Total Protein Stain is a robust alternative to HKPs.

    - Total protein staining is not affected by changes in the expression of a single internal reference protein.

- It provides an accurate readout of sample loading across a wide range, from 1 - 60 µg of total sample protein.
- Re-validation is not required for different experimental treatments, cell lines, or other experimental parameters.

VIII. Further Reading

Please see the following for more information about QWB analysis.

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™ 700 Total Protein Stain Normalization Protocol

licor.com/RevertNormalization

This protocol describes how to use Revert 700 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.

LI-COR Biosciences

4647 Superior Street
Lincoln, NE 68504
Phone: +1-402-467-0700
Toll free: 800-645-4267
biosales@licor.com
licor.com/bio

Regional Offices

LI-COR Biosciences GmbH

Siemensstraße 25A
61352 Bad Homburg
Germany
Phone: +49 (0) 6172 17 17 771
bio-eu@licor.com

LI-COR Biosciences UK Ltd.

St. John's Innovation Centre
Cowley Road • Cambridge
CB4 0WS • United Kingdom
Phone: +44 (0) 1223 422104
bio-eu@licor.com

Doc # 988-18497
07/20