Protocol

Revert™ 700 Total Protein Stain Normalization Protocol

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

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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 14.

Total Protein Staining as an Internal Loading Control

Total protein detection is becoming the "gold standard" for normalization of protein loading. After transfer, but prior to immunodetection, the membrane is treated with a total protein stain to assess actual sample loading across the blot. Because this internal loading control uses the combined signal from many different sample proteins in each lane, error and variability are minimized. This antibody-independent method corrects for variation in both sample protein loading and transfer efficiency, and monitors protein transfer across the blot at all molecular weights.

Revert™ 700 Total Protein Stain (licor.com/revert) is a near-infrared fluorescent membrane stain used for total protein detection and normalization. Revert staining is imaged at 700 nm, and fluorescent signals are proportional to sample loading.

This protocol describes how to use Revert 700 Total Protein Stain 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 9.

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)
3. **Electrophoresis reagents**
4. **Transfer reagents**
5. **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

This protocol highlights the main steps to use Revert™ 700 Total Protein Stain for single-color detection.

Step 1. Prepare and Transfer Proteins

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 minutes (or 70 °C for 10 minutes).
6. Load a uniform amount of sample protein in each lane.
7. Separate sample proteins by SDS-PAGE.
8. Transfer proteins to blotting membrane.

Step 2. Stain with Revert™ 700 Total Protein Stain

1. Add methanol to the stain reagents as indicated on each bottle.
2. After transfer is complete, fully dry the membrane. Place the membrane on top of a piece of clean filter paper and allow it to dry:
   - 40 to 60 minutes at room temperature.
   - 10 minutes in an oven at 37 °C.
   - Overnight at room temperature as a stopping point.
3. Rehydrate the membrane after fully drying.
   - For nitrocellulose membranes, incubate the membrane in TBS or PBS (no detergent) for 5 minutes at room temperature with gentle shaking.
   - For PVDF membranes, first rehydrate using 100% methanol for 30 seconds. Then rinse in TBS or PBS (no detergent) for 5 minutes at room temperature with gentle shaking.
4. Rinse the membrane with ultrapure water.

> **Note:** Before moving to the next step, ensure the membrane container provides a minimum clearance of 1/8th of an inch on all sides. Revert 700 Total Protein Stain will cause the membrane to swell. Without clearance, staining may be uneven.

5. Stain membrane with Revert 700 Total Protein Stain. Incubate the membrane in 5 mL of Revert 700 Total Protein Stain solution for 5 minutes at room temperature with gentle shaking.

> **Note:** Do not allow the membrane to dry from this point on.

6. Decant total protein stain solution thoroughly. Using approximately 5 mL of Revert 700 Wash Solution (P/N 926-11012), rinse the membrane two times for 30 seconds at room temperature with gentle shaking.
7. Decant wash solution thoroughly, then briefly rinse the membrane with ultrapure water.

Step 3. Image Membrane

> **Note:** Do not allow the membrane to dry during imaging. To prevent drying, you may add ultrapure water on top of the membrane.

1. Immediately image the membrane in the 700 nm channel using an Odyssey® Imaging System. If saturation occurs, reduce the scan intensity or acquisition time, or use AutoScan if your instrument includes this.
2. Proceed immediately to blocking and follow your normal Western blot protocol using IRDye® 800CW Secondary Antibody to detect your target in the 800 nm channel.
3. Image the membrane in the 800 nm channel with an Odyssey® Imaging System. If saturation occurs, reduce the scan intensity or acquisition time, or use AutoScan if your instrument includes this.

> **Note:** Visible color from stain will wash off during processing and residual total protein signal may be detected in the 700 nm channel.

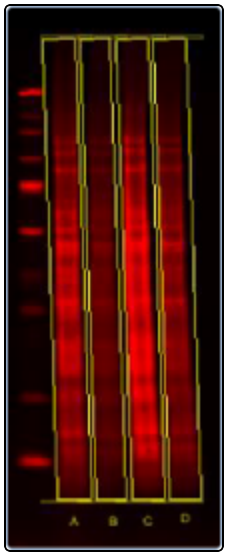
V. Total Protein and Target Quantification

Use Image Studio™ Software (licor.com/islite) to quantify the fluorescent signals from Revert staining (700 nm) and your target protein (800 nm).

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

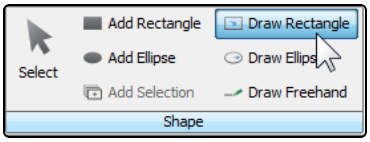
Total Protein Quantification Using Image Studio™ Software

Before you begin, under the **Analysis** tab change the type to **Manual.** 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 of 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.

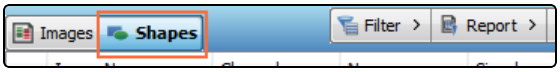
   a. With the first shape selected, click **Add Selection**.

   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.

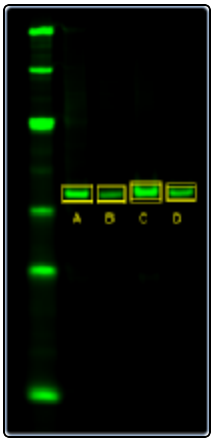
   

   b. Select shape data, then copy and paste data into a spreadsheet, or click **Report** to export the data into an external spreadsheet.

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

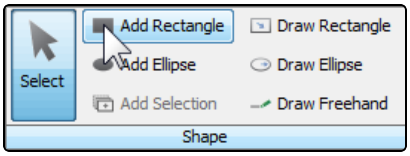
Target Protein Quantification Using Image Studio™ Software

Before you begin, under the **Analysis** tab change the type to **Manual.** Use the **Add Rectangle** tool in Image Studio Software to quantify the target bands.



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

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

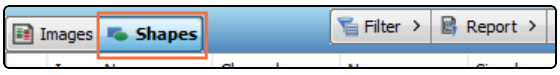
   

   b. Click each band to be analyzed, and an appropriately sized shape will be added around the band.

> **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, or click **Report** to export the data into an external 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.

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

Calculate the Lane Normalization Factor for Each Lane (Total Protein Stain, 700 nm)

1. Prepare a spreadsheet that contains the total protein staining and target protein quantification data.
2. Using the total protein stain 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	10,000	9,000	1000	11%
2		2	8,000			
3		3	9,000			
4	UV	1	8,000	8,333	577	7%
5		2	9,000			
6		3	8,000			

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.

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

Revert Staining (700 nm)

Lane	Sample treatment	Replicate	700 nm signal	Highest Signal	Lane Normalization factor
1	none	1	10,000	10,000	1
2		2	8,000	10,000	0.8
3		3	9,000	10,000	0.9
4	UV	1	8,000	10,000	0.8
5		2	9,000	10,000	0.9
6		3	8,000	10,000	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 (%CV) 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 lane normalization factor for that lane.

   a. Divide the target signal for each lane by the corresponding LNF.

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

Target protein (not normalized)				Normalization		Normalized to total protein
Lane	Sample treatment	Replicate	Target (800 nm)	LNF (Revert)	Apply LNF	Norm. Target
1	none	1	650	1	650 / 1	650
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
5		2	500	0.9	500 / 0.9	556
6		3	480	0.8	480 / 0.8	600

   b. Calculate the average, standard deviation, and % coefficient of variation of the replicates.

Target protein (not normalized)				Target protein (normalized to total protein)			
Lane	Sample treatment	Replicate	Target (800 nm)	Normalized Target	Average signal	St Dev	% CV
1	none	1	650	650	668	19	3%
2		2	550	688			
3		3	600	667			
4	UV	1	450	563	573	24	4%
5		2	500	556			
6		3	480	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. 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

IX. 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.

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