Western blotting

Easy and accurate normalization of western blot data using No-Stain Protein Labeling Reagent and iBright 1500 Series Imaging Systems

Introduction

As the amount of protein loaded on an electrophoresis gel can vary greatly from sample to sample, leading journals have developed protein normalizing guidelines for submitting quantitative western blot data.

One common method of protein normalization relies on measuring the abundance of a stably expressed housekeeping protein that is endogenous to all the samples in an experiment. Proteins such as α-tubulin, β-actin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are commonly used as loading controls for protein normalization. But there are potential drawbacks, as the expression of housekeeping proteins can vary with experimental conditions, and they often have saturated western blotting signals. These drawbacks can be minimized by optimization, but this is time-consuming. One must first confirm that the signal obtained for the housekeeping protein is linear over a wide concentration range, such that it can be used as a reliable reference for normalization, and that it is unaffected by the conditions of the experiment. The use of antibodies to detect housekeeping proteins is also time-consuming, requires the use of costly antibodies, and may require the extra steps of stripping and reprobing the blot to detect the experimental targets.

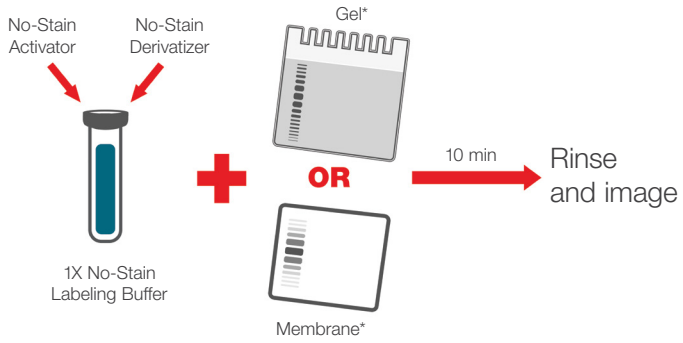
Another protein normalization method is total target protein normalization for posttranslationally modified proteins. Targets with posttranslational modifications (e.g., phosphorylation, glycosylation, acetylation, and ubiquitination) are normalized to their respective total protein using a pan antibody that recognizes the target regardless of modification state. There are multiple disadvantages to using this method to normalize for posttranslationally modified target proteins. The pan or total target protein antibody must recognize all posttranslationally modified versions of the target as well as the unmodified target. In addition, the expression of the total protein target used for normalization must be constant across all experimental conditions. Further, the linear dynamic range for this method is often narrow.

Total protein normalization (TPN) is a better alternative to using housekeeping proteins or total target protein normalization for posttranslationally modified proteins. It avoids the variability and inaccuracy of using housekeeping proteins, the time-consuming effort to optimize conditions, and the cost of the immunodetection reagents to detect the normalization targets.

For TPN, the total protein on a blot is labeled or stained to enable the relative amount of protein loaded in each lane to be compared and normalized to a reference lane. Ponceau S and several reversible fluorescent dyes are available to stain the total protein on a blot for TPN. A shortcoming of these dyes is that they must be removed before the immunoblotting steps. The membrane, in this case, must be imaged twice, once immediately after staining and again after western blotting detection. The added steps make the process cumbersome and inefficient. Additionally, those stains can cause increased background during the immunoassay detection step, impacting the downstream imaging. For example, Ponceau S is not compatible with fluorescence imaging.

Specialized gels, formulated with the addition of a photoactivatable molecule, offer an alternative way to perform total protein normalization. However, the limited gel chemistry availability (Tris-glycine only) and the need for a fluorescence imager with UV excitation to capture the image make them a suboptimal solution. Furthermore, imaging the membrane during the detection step often results in high background, resulting in a low signal-to-noise ratio.

Invitrogen™ No-Stain™ Protein Labeling Reagent offers the benefits of TPN and also allows you to use your gel of choice. Labeling the proteins in a gel before transfer or on a membrane after transfer is easy (Figure 1). Prepare a working solution of No-Stain Protein Labeling Reagent by adding No-Stain Activator and No-Stain Derivatizer to 1X No-Stain Labeling Buffer, and incubate with the gel or the membrane for 10 minutes.



* For Tris-glycine gels and membranes derived from Tris-glycine gels, washing for 2 minutes with ultrapure water, 4 and 2 times, respectively, is necessary to remove glycine before labeling.

Figure 1. Labeling a protein gel or blot with No-Stain Protein Labeling Reagent.

Since the No-Stain Protein Labeling Reagent forms covalent bonds with a portion of the lysines in the total-protein population, the fluorescent signal is stable and can be imaged at the same time as the fully processed immunoblot. The No-Stain Protein Labeling Reagent also provides a quick and convenient way to assess the quality of the electrophoretic separation before proceeding to the immunoblotting steps. Labeled proteins in the gel can be transferred to a membrane and probed with primary and secondary antibodies, without additional processing. This compatibility between protein gel labeling and protein transfer and immunoblotting is shown in Figure 2. The No-Stain reagent can be used with PVDF or nitrocellulose membranes and is compatible with antibody detection using chemiluminescence or fluorescence methods.

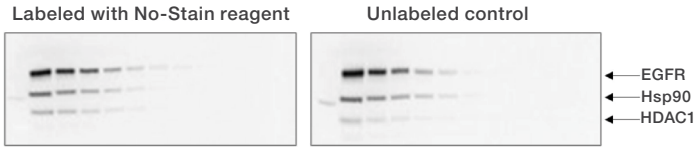


Figure 2. No-Stain Protein Labeling Reagent is compatible with protein transfer and immunoblotting. Invitrogen™ Bolt™ 4–12% Bis-Tris Plus gels were loaded with A431 cell lysate (20 µg to 20 ng serial dilutions) and separated by electrophoresis using MES running buffer. The gels were incubated with No-Stain Protein Labeling Reagent for 10 minutes. Labeled gels were transferred to PVDF membranes using the Invitrogen™ iBlot™ 2 Gel Transfer Device with iBlot 2 Transfer Stacks (P0 protocol for 7 minutes). PVDF membranes were then probed with specific primary antibodies, and proteins were detected using secondary antibodies labeled with Invitrogen™ Alexa Fluor™ 800 dye. Analysis images, without brightness or contrast adjustments, were collected using the Invitrogen™ iBright™ FL1500 Imaging System (exposure time of 1 second).

While a 10-minute incubation is sufficient for efficient protein labeling in most applications, prolonging the incubation time or doubling the concentration of the labeling reagent produces a stronger signal, both in gels and transferred membranes, as shown in Figures 3 and 4. The increased sensitivity is beneficial when analyzing a dilute sample.

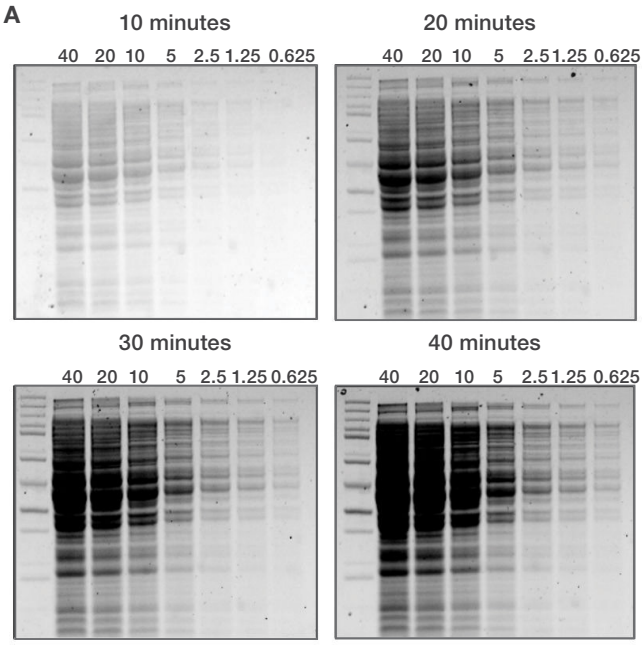


Figure 3. Signal intensity of No-Stain Labeling Reagent increases with incubation time. Transferred PVDF membranes, incubated with No-Stain Protein Labeling Reagent for 10, 20, 30, or 40 minutes, show increased signal intensity over time. Invitrogen™ NuPAGE™ 4–12% Bis-Tris gels were loaded with *E. coli* lysate ranging from 40 to 0.625 µg and separated by electrophoresis using MES SDS running buffer. Proteins from the gels were transferred onto mini PVDF membranes using the iBlot 2 Gel Transfer Device with iBlot 2 Transfer Stacks (P0 protocol for 7 minutes). The PVDF membranes were quickly rinsed with 20 mL of ultrapure water and incubated with 10 mL of a working solution of No-Stain Protein Labeling Reagent on a rotating platform. **(A)** Analysis images, without contrast or brightness adjustment, were collected using the iBright FL1500 Imaging System (manual exposure time of 1 second). **(B)** A linear correlation between the normalized signal intensity plotted against the incubation time can be observed.

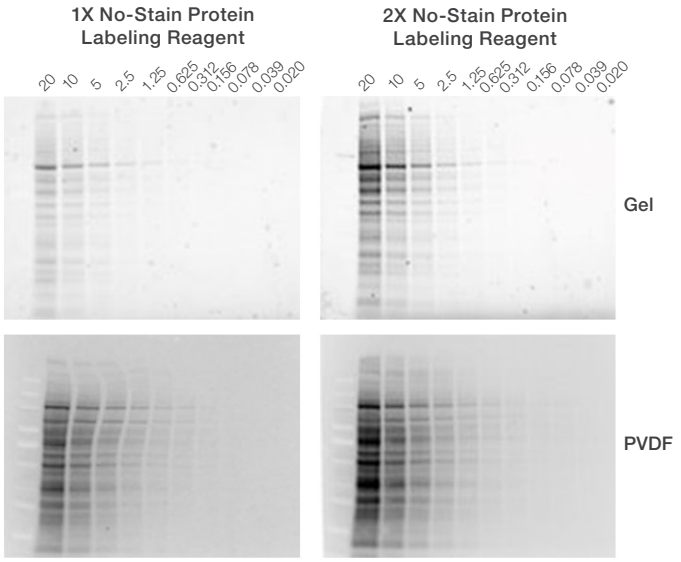


Figure 4. Doubling the concentration of No-Stain Protein Labeling Reagent increases the sensitivity of detection. Bolt 4–12% Bis-Tris Plus gels were loaded with A431 cell lysate (20 µg to 20 ng serial dilutions) and separated by electrophoresis using MES running buffer. Proteins were labeled with 1X or 2X No-Stain Protein Labeling Reagent directly on the gel or on the PVDF membrane after transfer. 1X No-Stain Protein Labeling Reagent was prepared according to the standard protocol. 2X No-Stain Protein Labeling Reagent was prepared by doubling the volume of No-Stain Activator (40 µL) and No-Stain Derivatizer (40 µL). Analysis images, without brightness or contrast adjustment, were collected using the iBright FL1500 Imaging System (exposure time of 1 second).

Using the No-Stain Protein Labeling Reagent enables accurate TPN over a broad linear range for protein detection, allowing the loading of 1–80 µg total protein per gel well. Bands with as little as 20 ng protein can be detected. Labeled blots can be imaged using a wide range of imagers with UV, green LED, or fluorescence (~488 nm) light sources, including the Invitrogen™ iBright™ 1500 Series Imaging Systems. Pairing the No-Stain Protein Labeling Reagent with an iBright 1500 Series Imaging System will elevate your quantitative western blotting accuracy and productivity.

TPN using No-Stain Protein Labeling Reagent is more accurate than using housekeeping proteins

No-Stain Protein Labeling Reagent for TPN avoids the variability and inaccuracy of using housekeeping proteins and reduces the time, effort, and cost to detect the housekeeping protein using immunoblotting, which may involve stripping of the blot and reprobing [1,2].

An accurate loading control should display a linear relationship between signal intensity and sample load under all experimental conditions. The signal intensity obtained from labeling of total proteins on a membrane with No-Stain reagent ensures a linear relationship between signal intensity and sample load in all experimental conditions (Figure 5).

Therefore, the No-Stain reagent enables the use of total protein as an ideal loading control for quantitative western blotting applications [3,4].

The graph in Figure 5 shows the linear signal response versus the amount of protein loaded per well using the No-Stain Protein Labeling Reagent for TPN. Signals from the housekeeping proteins, which were probed at the recommended concentrations, show saturation as lysate load increases, providing inaccurate normalization results.

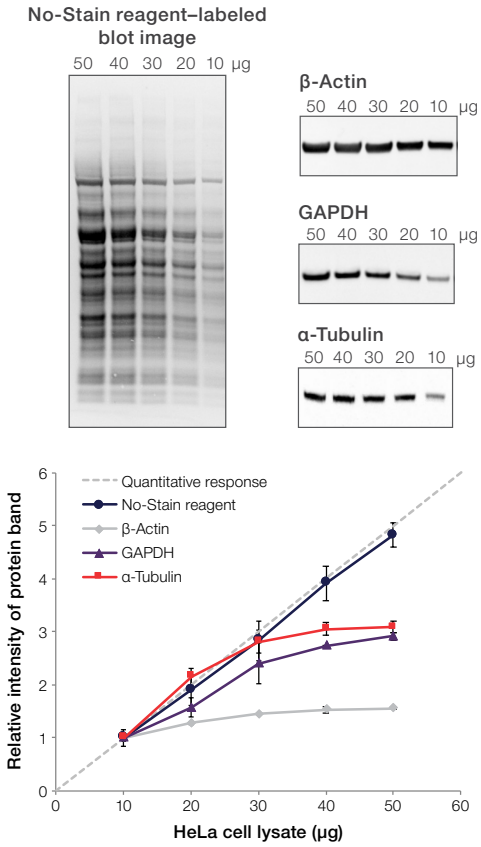


Figure 5. TPN using the No-Stain Protein Labeling Reagent. Bolt 4-12% Bis-Tris Plus gels were loaded with HeLa lysate ranging from 10 to 50 µg and electrophoresed using MES running buffer. Proteins from the gels were transferred onto mini PVDF membranes using the iBlot 2 Gel Transfer Device with iBlot 2 Transfer Stacks (P0 protocol for 7 minutes). The PVDF membranes were washed twice for 2 minutes with 20 mL of ultrapure water on a rotating platform, whereupon they were labeled with 10 mL of No-Stain Protein Labeling Reagent on a rotating platform for 10 minutes. The membranes were then washed 3 times for 2 minutes with 20 mL of ultrapure water on a rotating platform, followed by addition of Invitrogen™ primary antibodies against β-actin (Cat. No. AM4302), GAPDH (Cat. No. 398600), and α-tubulin (Cat. No. 138000), and Invitrogen™ Goat Anti–Mouse IgG Alexa Fluor™ Plus 680 secondary antibody (Cat. No. A32729). The blot was imaged using the iBright FL1500 Imaging System. The iBright software was used to quantitate the total protein signal in the lanes. The R^2 value of the plotted data for the entire range of total protein signal was determined to be 0.9990, whereas the R^2 values for β-actin, GAPDH, and α-tubulin were 0.8851, 0.9438, and 0.8332, respectively.

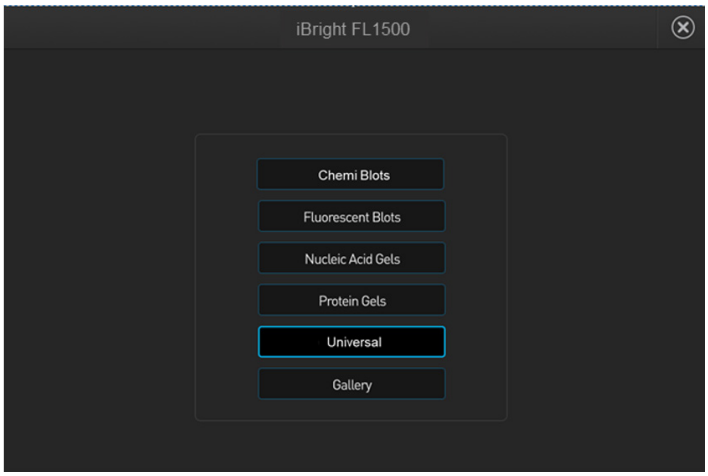
Easy workflow using No-Stain Protein Labeling Reagent with iBright 1500 Series Imaging Systems

After the No-Stain reagent–labeled membrane is prepared, whether by labeling the gel or membrane directly as previously described, the western blot is processed as usual.

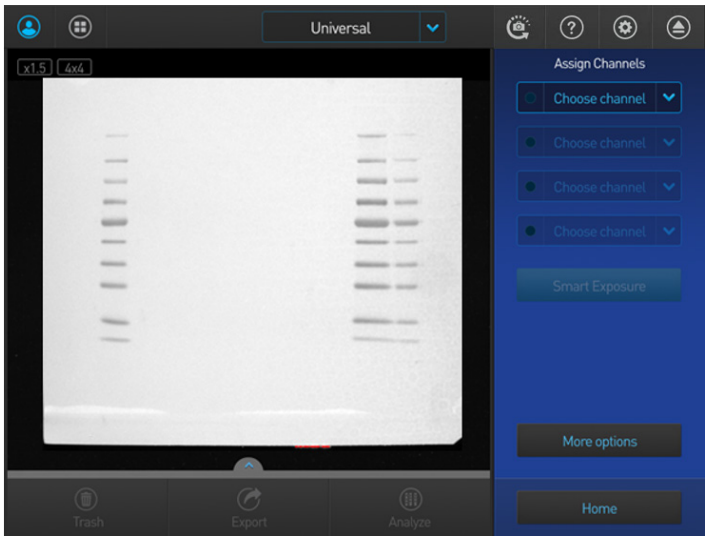
After processing, the western blot can be imaged and normalized on an iBright 1500 Series Imaging System. The on-instrument analysis software allows for normalization to be performed directly on the instrument, saving busy scientists extra time and effort. There are 9 simple on-instrument steps to perform TPN analysis with the iBright 1500 Series Imaging Systems and No-Stain Protein Labeling Reagent, which take less than 5 minutes to complete.

Note: The normalization workflow can also be completed on the cloud-based or desktop-based version of Invitrogen™ iBright™ Analysis Software. For more details on how to perform normalization in this off-instrument software, visit **thermofisher.com/ibright** to download the instruction manual.

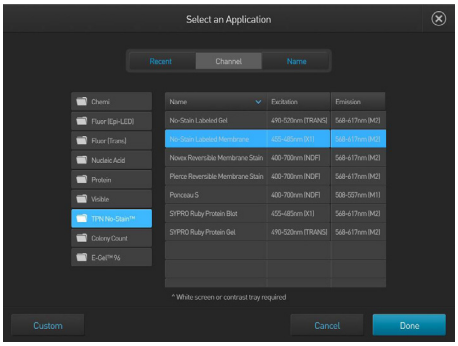
1. On the main Invitrogen™ iBright™ FL1500 or CL1500 screen, select **Universal** mode.

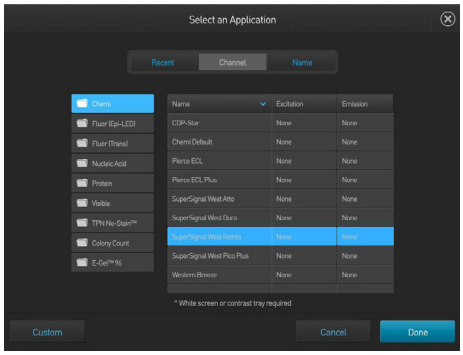


2. Next, assign channels based on your western blot detection strategy.



– For a No-Stain reagent–normalized chemiluminescent western blot, touch **Channel**. Under the **Channel** chevron, touch **TPN No-Stain**; and under the list of application names, touch **No-Stain Labeled Membrane**. For the next channel, select **Chemi** and then the corresponding substrate in the right-hand menu—in this particular example, SuperSignal West Femto (default false color assignment is white).

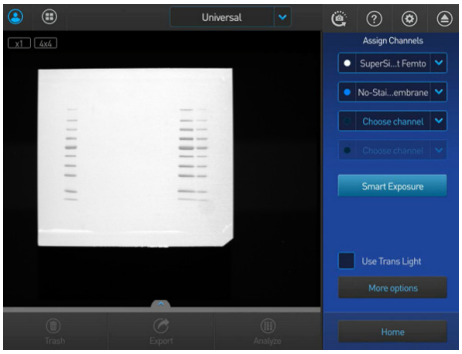


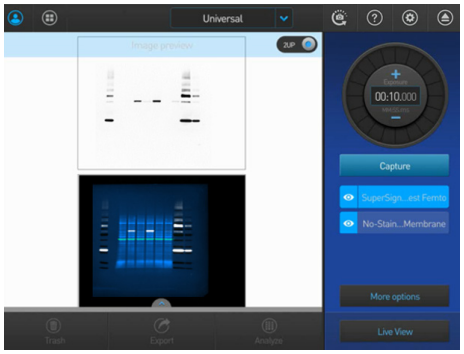


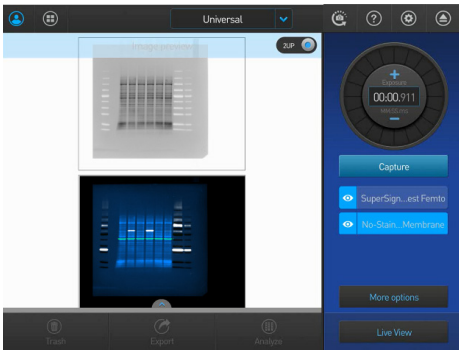
– For a No-Stain reagent–normalized fluorescent western blot, select **TPN No-Stain** and then **No-Stain Labeled Membrane** for the first channel. Next, assign the relevant fluorescence channels based on the fluorophores used.

Note: Avoid fluorophores that have an excitation maximum at <575 nm and an emission maximum at <645 nm, as fluorescent spectral overlap will occur with the No-Stain Protein Labeling Reagent.

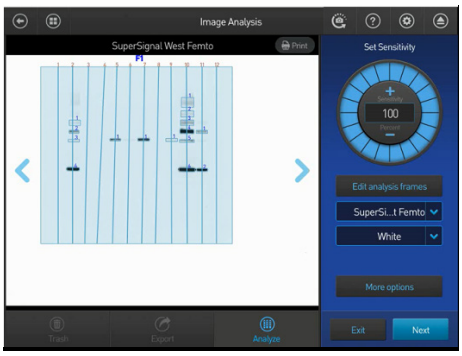
3. Touch **Smart Exposure** to determine the optimal exposure time. An image preview will be displayed to show how each channel image will appear after capture, based on the recommended exposure time for each channel. If the preview is acceptable, touch **Capture**. If exposure time adjustment is desired, use the interactive exposure dial or numeric pad to increase or decrease the exposure time. As exposure time changes, the resulting image preview adjusts in real time.



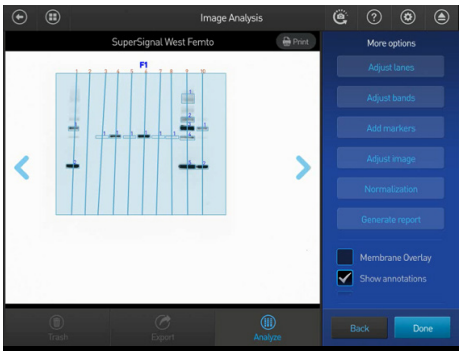


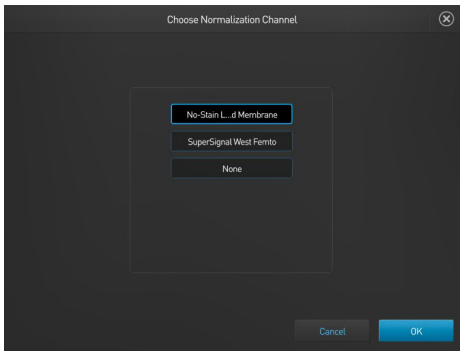


4. Next, touch the **Analyze** icon directly beneath the image viewport. The software will automatically identify analysis frame(s), lanes, and bands. The band sensitivity dial raises and lowers the minimum volume intensity threshold for band identification (default sensitivity is 100, can be adjusted between 0 and 100). The analysis frame should border the data-containing portions of the blot, leaving an adequate border around the outer boundaries of data.

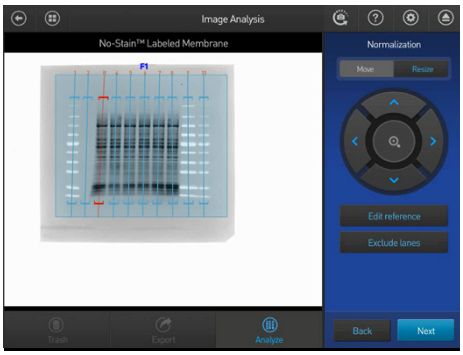


5. After any necessary adjustments to analysis frames, lanes, or bands, touch the **More options** button, followed by the **Normalization** button. Then, designate the normalization channel (the channel that will be used to generate normalization factors)—in this example, the **No-Stain Labeled Membrane** channel.

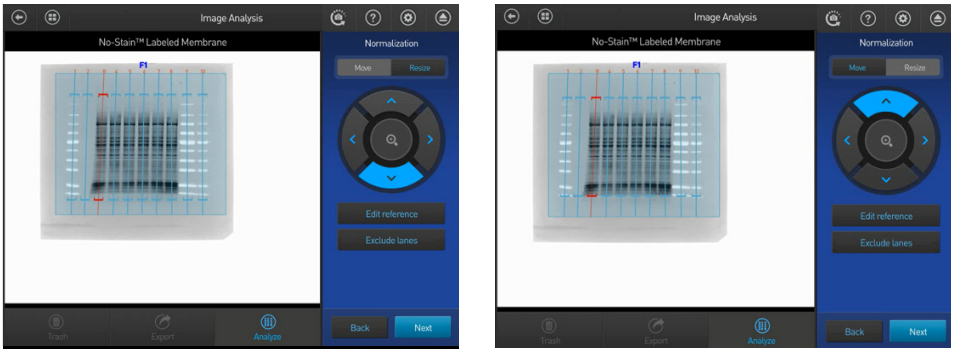




6. If necessary, simultaneously move and/or resize the lane normalization region for all lanes. The normalization region for each lane lies between the top and bottom brackets. The normalization regions identify the portion of each lane that will be used to quantify total protein by densitometry and to calculate normalization factors.

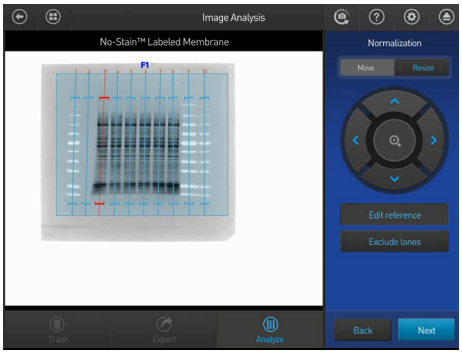


The position of the normalization region for all lanes can be moved up and down inside of the analysis frame by touching the **Move** button followed by using the up (Λ) or down (V) arrow to incrementally move the normalization region. The normalization region height for all lanes can be adjusted up and down by touching **Resize** followed by the up (Λ) or down (V) arrow to incrementally increase or decrease the height. The height of the normalization region should fully encompass the protein bands in the lane. Adjustments can be made for gel separation or transfer artifacts should they occur. Touch **Next**.



Note: For the Total Protein Normalization workflow, the software defines the normalization region for each lane with a bracket at the top and bottom of the lane with a line running through the middle. Lane width is automatically set by an algorithm and is not adjustable. The area of the normalization region is identical for all lanes.

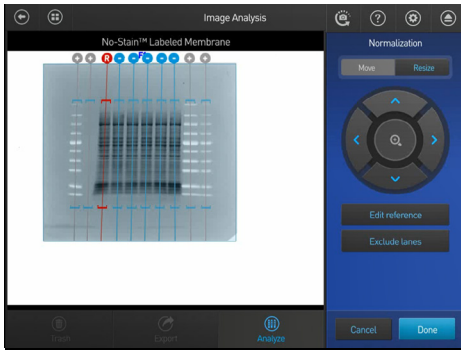
7. Assign one reference lane. The reference lane is the lane that all other lanes are normalized to and is set to a value of 1.000. The reference lane will be highlighted in red. By default, the software assigns as the reference lane the lane with the highest volume intensity.



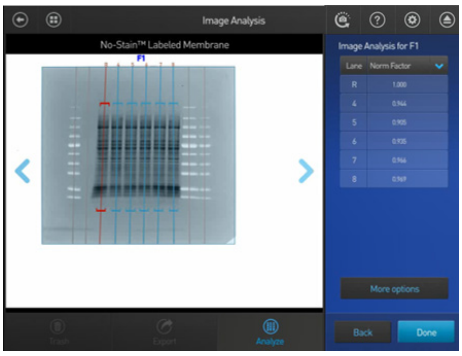
Note: Sometimes the protein ladder will be the default assigned reference lane, but it should not be used as the normalization reference lane; rather, a sample lane should be chosen.

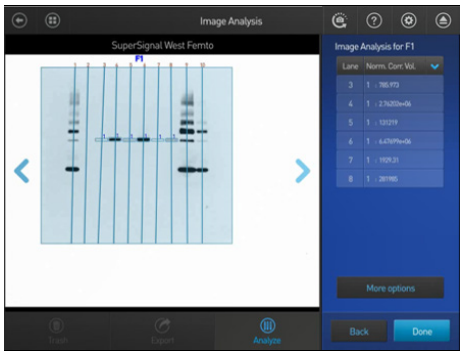
If desired, change the reference lane by touching the **Edit reference** button, followed by selecting the lane of choice. After selecting the lane of choice, touch **Done** and then **Next**.

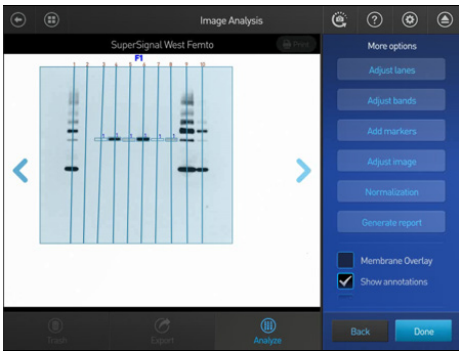
8. To exclude irrelevant lanes from normalization, touch **Exclude lanes** and then touch the (–) symbol above the desired lane(s). The symbol above the selected lane(s) will then turn to a (+) symbol. Touch **Done** and then **Next**.



9. The normalization factors and normalized band data are now available to review directly on the iBright instrument and/or to export as a report or as raw data. Touch **More options** and **Generate report** to create a printable report that contains file information, images, and data. The report can be exported as a PDF file. The raw data will automatically export as a CSV file that can be opened in commercial spreadsheet applications, such as Microsoft™ Excel™ software.







Understanding the report

Normalization factors are calculated from the normalization channel, in this case the No-Stain channel. Normalization factors are calculated by dividing the adjusted total lane volume of each lane by the adjusted total lane volume of the reference lane. The reference lane always has a normalization factor of 1.000, and all other lane normalization factors are relative to the reference lane.

Frame 1				
Channel 2	**Total lane volume**	**Background volume (rolling ball)**	**Adjusted total lane volume**	**Normalization factor**
Lane 3	233,559,000	162,680,000	70,879,200	1.000
Lane 4	231,545,000	164,642,000	66,903,700	0.944
Lane 5	231,837,000	167,711,000	64,126,300	0.905
Lane 6	234,800,000	168,528,000	66,271,800	0.935
Lane 7	236,146,000	167,698,000	68,448,300	0.966
Lane 8	234,745,000	166,097,000	68,648,500	0.969

$$\text{Normalization factor (lane X)} = \frac{\text{Adjusted total lane volume (lane X)}}{\text{Adjusted total lane volume (reference lane)}}$$

$$\text{Normalization factor (reference lane)} = \frac{70{,}879{,}200}{70{,}879{,}200} = 1.000$$

$$\text{Normalization factor (lane 6)} = \frac{66{,}271{,}800}{70{,}879{,}200} = 0.935$$

Normalized corrected volume is calculated in the channel that contains the protein of interest. The signal intensity (local corrected volume) of each band is divided by the normalization factor for the lane it resides in.

Frame 1, channel 4, phospho-AKT						
Name	**Lane**	**Band**	**Volume**	**Local corrected volume**	**Normalization factor**	**Normalized corrected volume**
Untreated	3	1	129,453	786	1.000	786
hIGF	4	1	2,790,000	2,607,000	0.944	2,761,653
hIGF + LY	5	1	318,845	118,717	0.905	131,179
hIGF + Rap	6	1	6,259,000	5,972,000	0.935	6,387,166
hIGF + Rap + LY	7	1	108,734	1,863	0.966	1,929
hIGF + BEZ	8	1	425,214	271,943	0.969	280,643

$$\text{Normalized corrected volume (lane X)} = \frac{\text{Local corrected volume (lane X)}}{\text{Normalization factor (lane X)}}$$

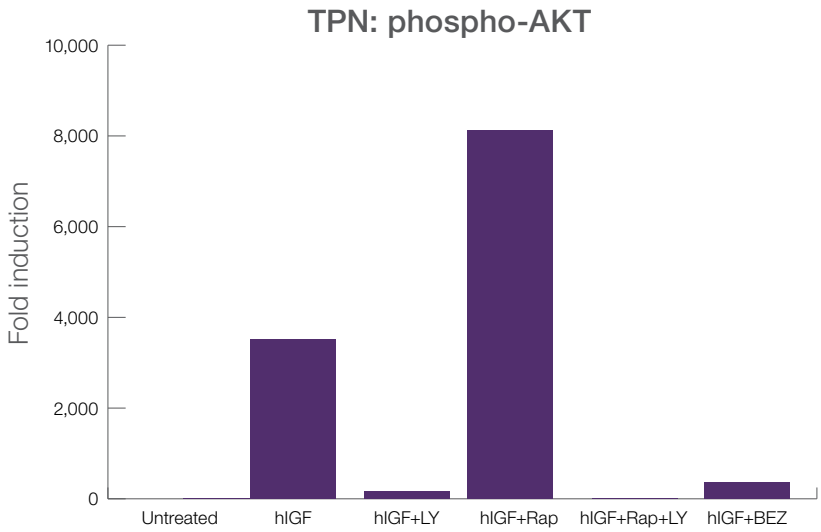
$$\text{Normalized corrected volume (lane 6)} = \frac{5{,}972{,}000}{0.935} = 6{,}387{,}166$$

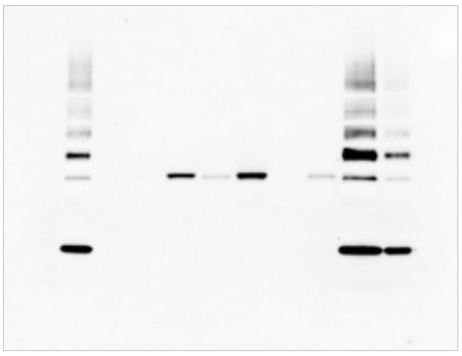
Presenting normalized data

Export data to an Excel spreadsheet or a similar type of application to easily organize and graph the densitometry results.

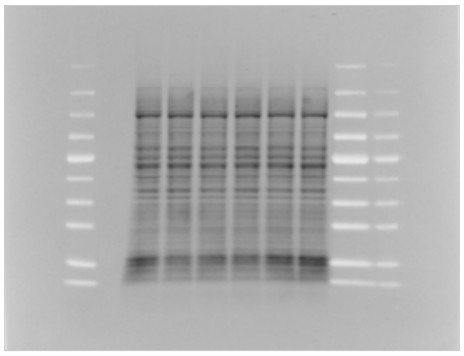
In the example below, the normalized corrected volume of phospho-AKT is presented as fold induction of treated compared to untreated human colon cancer cells.

Frame 1, channel 4, phospho-AKT							
Name	**Lane**	**Band**	**Volume**	**Local corrected volume**	**Normalization factor**	**Normalized corrected volume**	**Fold induction**
Untreated	3	1	129,453	786	1.000	786	1
hIGF	4	1	2,790,000	2,607,000	0.944	2,761,653	3,514
hIGF + LY	5	1	318,845	118,717	0.905	131,179	167
hIGF + Rap	6	1	6,259,000	5,972,000	0.935	6,387,166	8,126
hIGF + Rap + LY	7	1	108,734	1,863	0.966	1,929	2
hIGF + BEZ	8	1	425,214	271,943	0.969	280,643	357

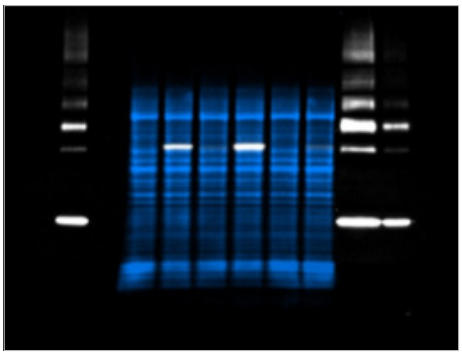




Channel 1: Chemiluminescent detection (SuperSignal West Femto), 10 s exposure



Channel 2: No-Stain reagent–labeled membrane, 911 ms exposure



Composite

Figure 6. HCT116 cells treated with various reagents: human colon cancer cells (HCT116) were serum-starved for 24 hours and pretreated with LY294002 (50 µM, 1 hr), rapamycin (10 nM, 1 hr), and/or BEZ235 (500 nM, 1 hr). Following pretreatment, human insulin-like growth factor-1 (hIGF-1) was added to each sample (12.8 nM, 15 min). Cells were lysed and relative quantities of phospho-AKT (Ser473) were determined using a western blot workflow with No-Stain Protein Labeling Reagent for normalization. hIGF-1 induces the phosphorylation of AKT. Rapamycin inhibits mTOR functionality and induces phosphorylation of AKT through an IGF-1R–dependent mechanism. LY294002 and BEZ235 are inhibitors of the PI3K pathway and block PI3 kinase–dependent AKT phosphorylation.

Results

The normalized western blotting data in Figure 6 show 3,514-fold induction of phospho-AKT by hIGF-1. With the combination of pretreatment with rapamycin before addition of hIGF-1, phospho-AKT is induced 8,126-fold. Pretreatment with LY294002 or BEZ235 before addition of hIGF-1 inhibits the induction of AKT phosphorylation, keeping it to 167-fold and 357-fold, respectively. When the pretreatment was a combination of LY294002 and rapamycin, there was little to no induction of phospho-AKT.

Conclusion

Pairing the No-Stain Protein Labeling Reagent for TPN with an iBright 1500 Series Imaging System will streamline the quantitative western blotting workflow while improving the accuracy of results compared to the use of housekeeping genes. The procedure is fast, easy, and compatible with your current gels of choice along with fluorescent or chemiluminescent detection.

See our video on how to quantitate western blots using the No-Stain Protein Labeling Reagent and the iBright 1500 Series Imaging Systems at thermofisher.com/no-stain.

References

1. Paramanantham A, Asfiya R, Das S et al. (2023) No-stain protein labeling as a potential normalization marker for small extracellular vesicle proteins. *Prep Biochem Biotechnol* 1–11. doi.org/10.1080/10826068.2023.2185897
2. Taylor SC, Rosselli-Murai LK, Crobeddu B et al. (2022) A critical path to producing high quality, reproducible data from quantitative western blot experiments. *Sci Rep* 12(1):17599. doi.org/10.1038/s41598-022-22294-x
3. Lazcano P, Schmidtke MW, Onu CJ et al. (2022) Phosphatidic acid inhibits inositol synthesis by inducing nuclear translocation of kinase IP6K1 and repression of myo-inositol-3-P synthase. *J Biol Chem* 298(9):102363. doi.org/10.1016/j.jbc.2022.102363
4. Matassa DS, Criscuolo D, Avolio R et al. (2022) Regulation of mitochondrial complex III activity and assembly by TRAP1 in cancer cells. *Cancer Cell Int* 22(1):402. doi.org/10.1186/s12935-022-02788-4

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