

# Key steps for performing sensitive quantitative chemiluminescent western blot analysis

## Introduction

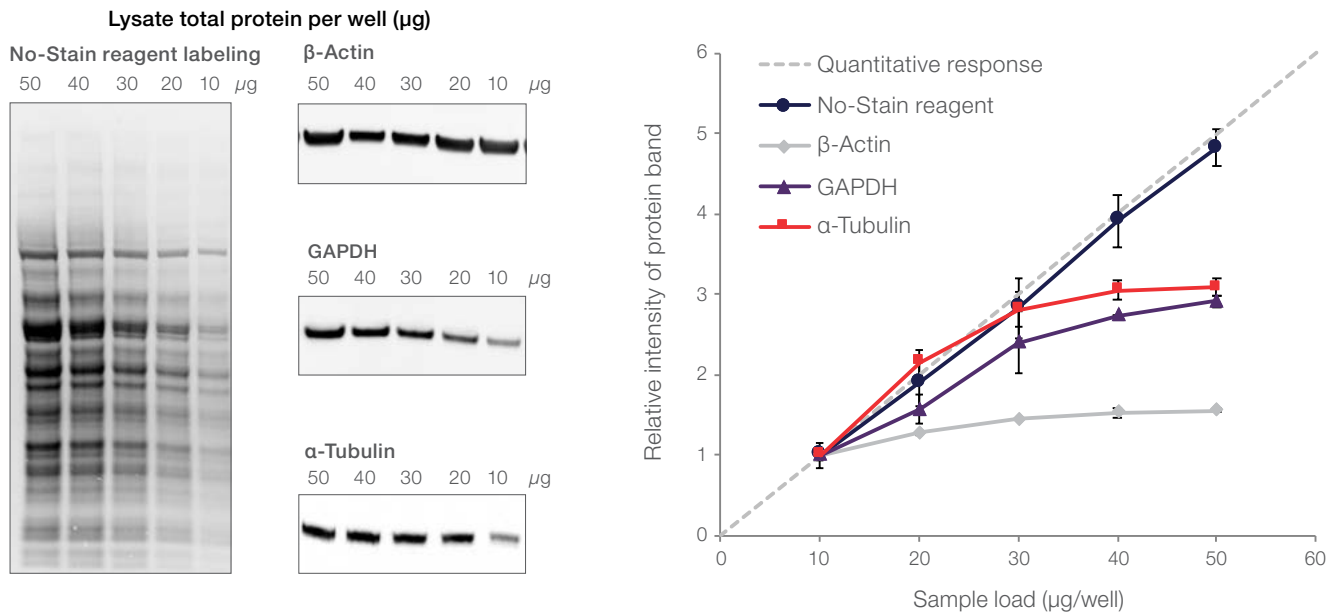
Traditional western blotting is useful for detecting the presence or absence of a specific protein in a lysate or complex mixture. Furthermore, it can provide a qualitative assessment of changes in protein expression levels in response to stimuli or interventions. Western blotting is no longer limited to generating qualitative data and can be used to assess the magnitude of these expression differences. However, simply using current densitometric techniques to quantify expression differences can be misleading due to signal saturation and suboptimal normalization of variation between samples. Publishers agree—several journals have adopted more stringent standards in recent years for quantitative reporting methods [1,2]. Here we discuss key parameters needed for effective quantitative chemiluminescent western blot analysis to generate more accurate data and reduce the number of blots lost to optimization.

## Normalization in quantitative western blotting

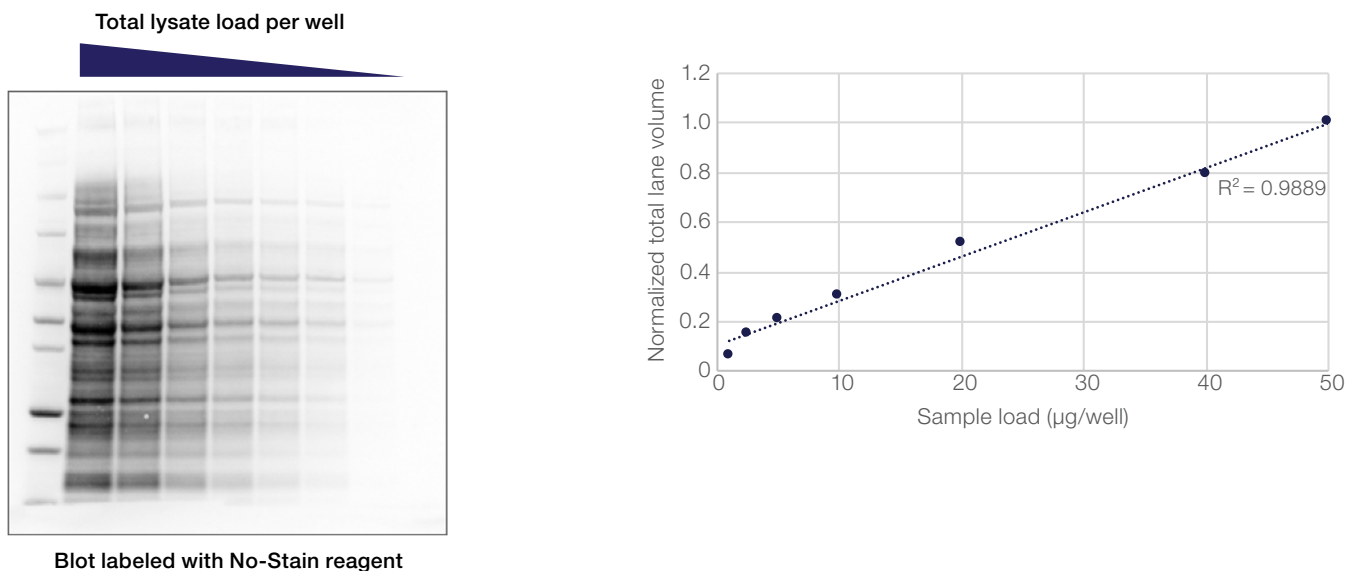
Normalization is required to accurately assess differences in target abundance. Normalization corrects for unavoidable errors that occur during the western blot process, including sample loading or effects from electrophoresis, transfer, or sample concentration. Choosing the correct normalization method for your quantitative western blot is critical for obtaining reliable and reproducible results. When transitioning your western blotting experiments from qualitative to quantitative analysis, the most important attribute to address is signal saturation. When a chemiluminescent signal becomes saturated, the ability to relate signal intensity to protein abundance is lost. Figure 1 shows some common normalization controls known as housekeeping proteins, or HKPs. They include  $\beta$ -actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and  $\alpha$ -tubulin, which have been shown to become saturated at common lysate loading

amounts (e.g., 30–50  $\mu$ g/well). The relative intensity becomes nonlinear in shape and levels off, indicating virtually the same intensity for higher and higher amounts of protein. It is important to avoid signal saturation in the normalization controls as well as the target protein, or accurate quantitation will not be possible. With appropriate calculations, sample signals can be accurately normalized to housekeeping proteins designated as internal loading controls to obtain quantitative western blot data. An accurate loading control should display a linear relationship between sample load and signal intensity in all experimental conditions.

An alternative normalization method that is growing in popularity is total protein normalization (TPN), which normalizes the target signal with the total amount of protein loaded in each lane. One TPN method utilizes Invitrogen™ No-Stain™ Protein Labeling Reagent, which covalently labels the total protein loaded in each lane with a fluorescent label. Once labeled, total protein can then be used to normalize the signal of your target protein. Figure 1 shows the advantage of using No-Stain Protein Labeling Reagent as a substitute for traditional HKPs, which can easily become oversaturated. No-Stain Protein Labeling Reagent provides a linear response curve (Figure 2) with a wide dynamic range, enabling accurate normalization.



**Figure 1. Total protein normalization using No-Stain Protein Labeling Reagent.** No-Stain Protein Labeling Reagent outperforms common housekeeping proteins for signal linearity at higher protein loads. Method: Invitrogen™ Bolt™ 4–12% Bis-Tris Plus gels were loaded with 10–50 µg of HeLa lysate total protein per lane and electrophoresed using MES running buffer. Proteins in the gels were transferred to mini PVDF membranes using the Invitrogen™ iBlot™ 2 Gel Transfer Device with iBlot™ 2 Transfer Stacks (P0 protocol for 7 min). The membranes were washed twice for 2 min each with 20 mL of ultrapure water and then labeled with 10 mL of a working solution of No-Stain Protein Labeling Reagent for 10 min. The membranes were then washed 3 times for 2 min each with 20 mL of ultrapure water, 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™ 680 secondary antibody (Cat. No. A21058). The blot shown was imaged using the Invitrogen™ iBright™ Imaging System, and Invitrogen™ iBright™ Analysis Software was used to quantitate the total protein signal in the lanes. The  $R^2$  of the plotted data for the entire range of total protein signal was determined to be 0.9990, whereas the  $R^2$  for β-actin, GAPDH, and α-tubulin were 0.8851, 0.9438, and 0.8332, respectively.



**Figure 2. Signal response from proteins labeled with No-Stain reagent on membranes is linear from 1 to 50 µg/well of lysate total protein.** A Bolt 4–12% Bis-Tris Plus mini gel was loaded with 1–50 µg of HeLa lysate total protein per lane, and Thermo Scientific™ PageRuler™ Unstained Protein Ladder (Cat. No. 26614) was loaded in lane 1. After electrophoresis using MES running buffer, the proteins were transferred to a PVDF membrane using the Invitrogen™ Power Blotter System and Power Blotter Select Transfer Stacks (10 min). The membrane was washed twice for 2 min each with 20 mL of ultrapure water, and then labeled with 10 mL of a working solution of No-Stain Protein Labeling Reagent for 10 min. The membrane was then washed 3 times for 2 min each with 20 mL of ultrapure water. The image was captured using the iBright Imaging System with the “No-Stain Labeled Membrane” settings (455–485 nm excitation and 565–615 nm emission).

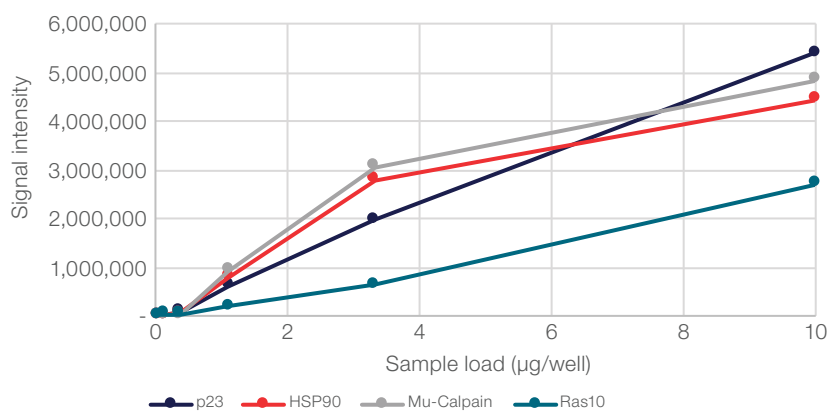
## Avoiding common pitfalls in quantitative western blotting

Once proper normalization techniques are in place, the traditional western blot protocol will need some additional optimization before it can provide truly quantitative results. We have identified three parameters that have the greatest impact on quantitative western blotting and have detailed strategies to optimize them appropriately.

### 1. Optimizing protein loading

The most common problem contributing to signal saturation is adding too much protein sample to a gel well. It is important to optimize the amount of protein loaded based on target protein abundance to avoid signal saturation. High-abundance proteins such as HSP90 will tend to saturate with lysate loads greater than 3  $\mu\text{g}$ .

Low- and medium-abundance proteins such as p23 can show linear detection with as much as a 20  $\mu\text{g}$  lysate load. Detection of low-abundance targets such as Ras10 may be linear with up to a 40  $\mu\text{g}$  load of lysate (Figure 3). To obtain linear signals with the majority of western blots, we recommend loading smaller amounts of protein sample between 1 and 10  $\mu\text{g}$  per well. To avoid under- or overloading samples, determine the protein concentration of each sample prior to electrophoresis with a compatible protein assay. For quick and accurate protein quantitation, we recommend the Invitrogen™ Qubit™ Protein BR Assay or the Thermo Scientific™ Pierce™ Rapid Gold BCA Protein Assay.

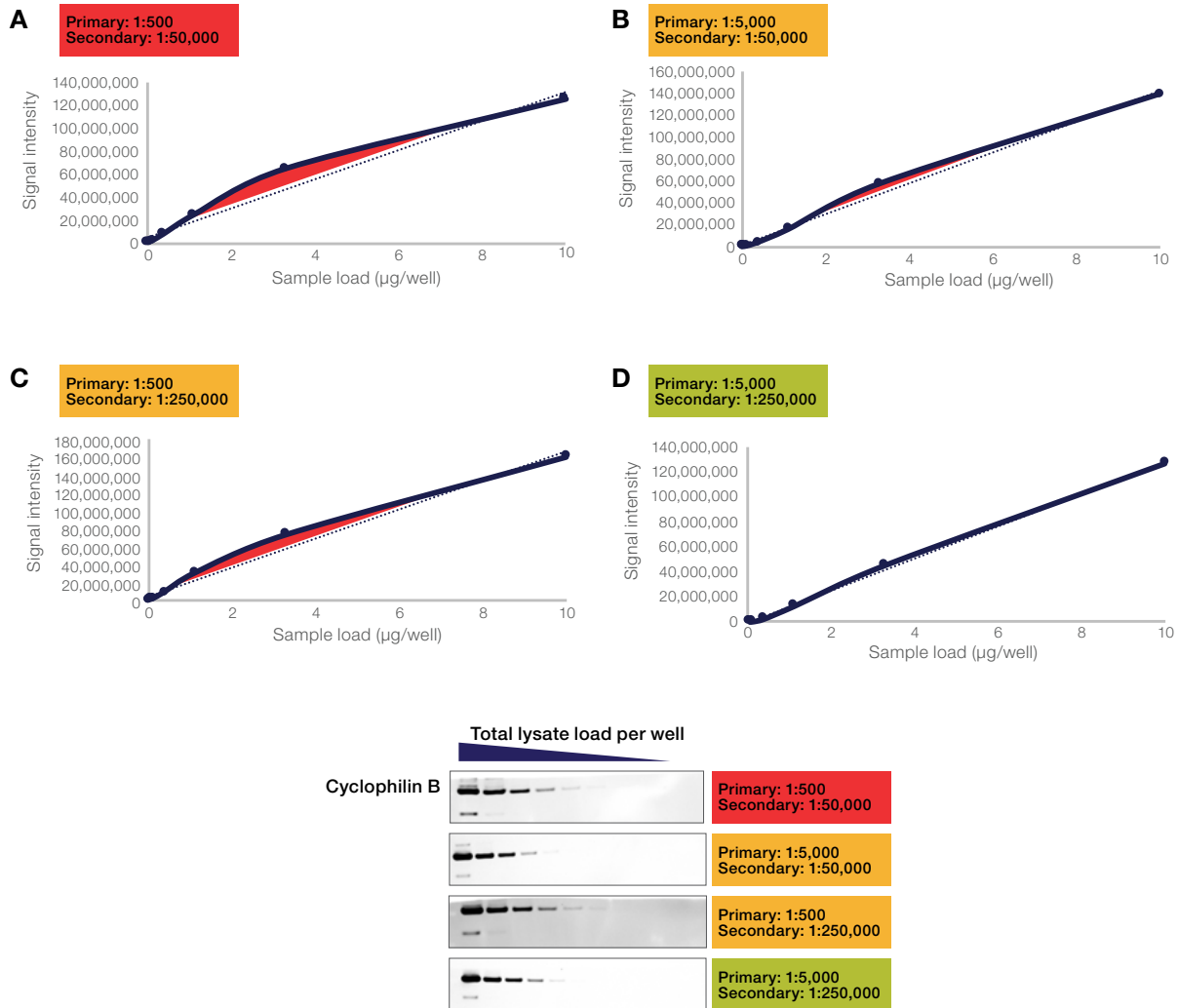


**Figure 3. Linear relationships between sample load and band intensity are based on protein target abundance.** High-abundance proteins such as HSP90 and mu-calpain may exhibit a linear signal only in lysate loads between 1 and 3  $\mu\text{g}$ , while medium- and low-abundance proteins show linear signals with up to 10  $\mu\text{g}$  of lysate loads. A431 or A549 cell lysates serially diluted 3-fold starting at 10  $\mu\text{g}/\text{well}$  were loaded onto Bolt 4–12% Bis-Tris Plus gels and electrophoresed at 200 V for 20 min. Gels were blotted to nitrocellulose membranes using the iBlot 2 Gel Transfer Device (P0, 7 min). Immunoblot processing was performed using the Invitrogen™ Bandmate™ Automated Western Blot Processor (Cat. No. BW1000). Membranes were blocked in Thermo Scientific™ Pierce™ 1X Clear Milk Blocking Buffer (Cat. No. 37587). Each membrane was probed with a single Invitrogen™ primary antibody diluted in 1X Clear Milk Blocking Buffer: mouse anti-p23 (1:20,000, Cat. No. MA3-414), rabbit anti-HSP90 (1:25,000, Cat. No. PA3-013), mouse anti-pan Ras10 (1:5,000, Cat. No. MA1-012), or mouse anti-mu-calpain (1:5,000, Cat. No. MA3-940). Secondary antibody incubation was performed using Invitrogen™ HRP-conjugated goat anti-rabbit IgG (1:50,000, Cat. No. 32460) or goat anti-mouse IgG (1:50,000, Cat. No. 32430). Chemiluminescence detection was performed using Thermo Scientific™ SuperSignal™ West Dura Extended Duration Substrate (Cat. No. 34076). Membranes were imaged on the iBright Imaging System.

## 2. Optimizing antibody dilution

The concentrations of both primary and secondary antibodies can contribute to nonlinear saturated signals in chemiluminescent western blotting. Applying too much enzyme conjugate or secondary antibody to a western blot is one of the greatest causes of signal variability, high background, short signal duration, and low sensitivity. Although antibody dilutions are not quite as important as ensuring the proper lysate load, an optimal antibody

concentration can elevate your blot from semi-quantitative to fully quantitative (Figure 4). If you are experiencing signal saturation, we recommend diluting both the primary and secondary antibodies. In most cases this will have some benefit, particularly with high-abundance targets. However, excessive dilution can result in a minor reduction in the detection limit at the lowest target concentrations. It is important to try several different combinations of dilutions to understand what is best for your experiment.

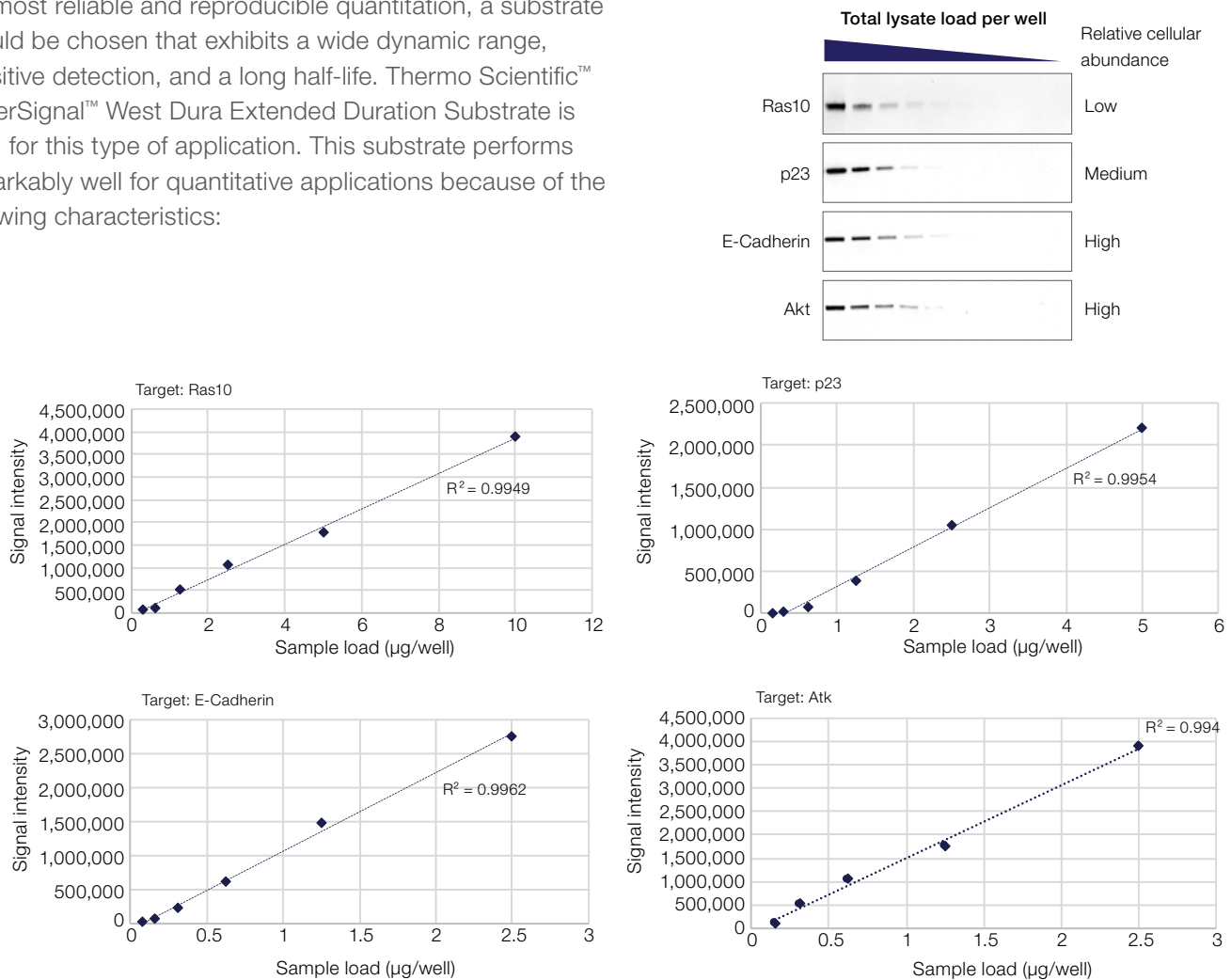


**Figure 4. Proper dilution of primary and secondary antibodies can increase linearity of signal.** Signals become more linear as primary antibody is diluted from 1:500 to 1:5,000 (A to B) and as secondary antibody is diluted from 1:50,000 to 1:250,000 (A to C). Maximum linearity is achieved when both are diluted (D). Method: A 3-fold dilution series of A549 cell lysate starting at 10  $\mu\text{g}/\text{well}$  was loaded onto a Bolt 4–12% Bis-Tris Plus gel and electrophoresed at 200 V for ~20 min. Gels were blotted to nitrocellulose membranes using the iBlot 2 Gel Transfer Device (P0 protocol for 7 min). Immunoblot processing was performed using the Bandmate Automated Western Blot Processor. Membranes were blocked in 1X Clear Milk Blocking Buffer. Four membranes were probed with Invitrogen™ rabbit anti-cyclophilin B (Cat. No. PA1-027A) diluted in 1X Clear Milk Blocking Buffer at either 1:500 or 1:5,000 dilution, followed by incubation with HRP-conjugated goat anti-rabbit IgG (Cat. No. 32460) at either 1:50,000 or 1:250,000 dilution. Chemiluminescence detection was performed using SuperSignal West Dura Extended Duration Substrate. Membranes were imaged on the iBright Imaging System.

### 3. Optimizing chemiluminescent signal

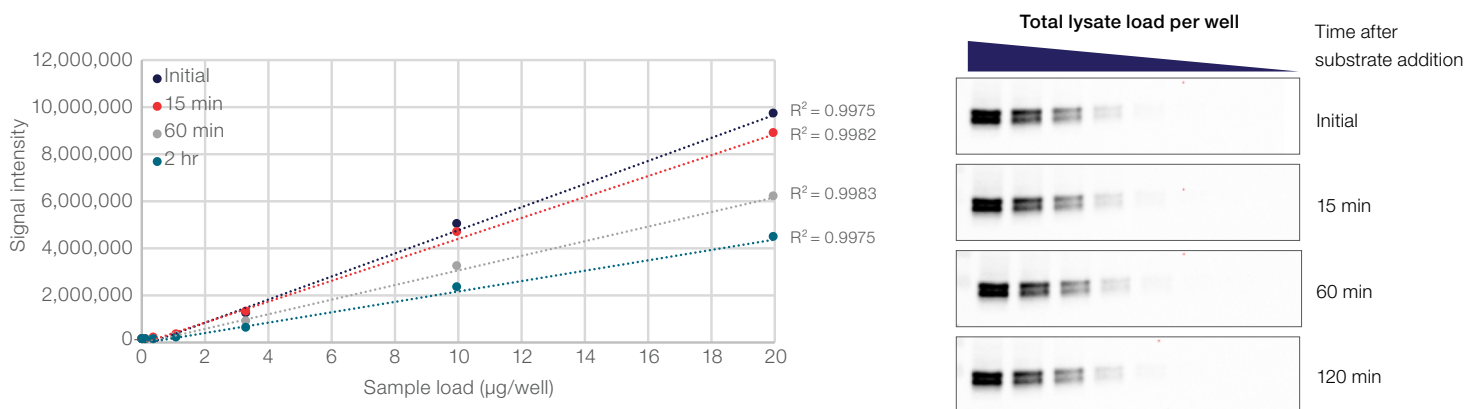
The final common pitfall to avoid is choosing an incompatible or low-performing chemiluminescent HRP substrate. Ultrasensitive chemiluminescent substrates are pushing the boundaries of sensitivity, which can be very important for detecting low-abundance proteins. However, for high- or medium-abundance targets, ultrasensitive substrates may contribute to signal saturation, making quantitation impossible. On the other end of the spectrum, standard ECL substrates may not be sensitive enough for the detection of medium- to low-abundance protein targets. Quantitative chemiluminescent western blotting requires an optimal substrate that provides sensitive, linear signals over a broad range of protein concentrations. For the most reliable and reproducible quantitation, a substrate should be chosen that exhibits a wide dynamic range, sensitive detection, and a long half-life. Thermo Scientific™ SuperSignal™ West Dura Extended Duration Substrate is ideal for this type of application. This substrate performs remarkably well for quantitative applications because of the following characteristics:

- **Greater linearity**—SuperSignal West Dura substrate exhibits linearity with both high- and low-abundance proteins. It is less likely to oversaturate with high-abundance protein targets than a high-sensitivity substrate, such as Thermo Scientific™ SuperSignal™ West Atto Ultimate Sensitivity Substrate, which would require careful optimization. However, it is still sensitive enough to detect low-abundance proteins down to the mid-femtogram level. SuperSignal West Dura substrate provides linear signals across proteins of high (Akt, E-cadherin), medium (p23), and low (Ras10) abundance (Figure 5).



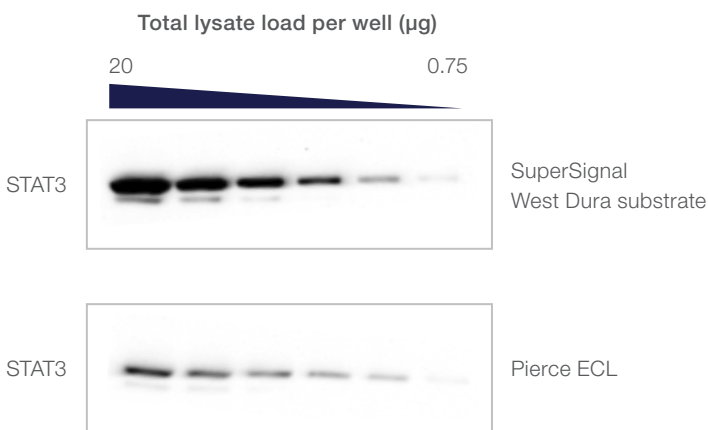
**Figure 5. SuperSignal West Dura substrate provides linear signals with  $R^2 > 0.99$  for high-, medium-, and low-abundance targets.** Dilution series of A549 cell lysate starting at 10 µg/well or 2.5 µg/well were loaded onto Bolt 4–12% Bis-Tris Plus gels and electrophoresed at 200 V for ~20 min. Gels were blotted to nitrocellulose membranes using the iBlot 2 Gel Transfer Device (P0 protocol for 7 min). Immunoblot processing was performed using the Bandmate Automated Western Blot Processor. Membranes were blocked in 1X Clear Milk Blocking Buffer. The membranes were probed with a single primary antibody diluted in 1X Clear Milk Blocking Buffer, either with mouse anti-pan Ras10 (1:5,000, Cat. No. MA1-012), mouse anti-p23 (1:20,000, Cat. No. MA3-414), rabbit anti-E-cadherin (1:25,000, Cat. No. MA5-29711), or rabbit anti-Akt (1:2,000, Cat. No. 44-609G). This was followed by incubation with HRP-conjugated goat anti-mouse IgG (1:25,000 or 1:250,000, Cat. No. 32430) or goat anti-rabbit IgG (1:25,000, Cat. No. 32460). Chemiluminescence detection was performed using SuperSignal West Dura Extended Duration Substrate. The membranes were imaged on the iBright Imaging System.

- **Consistent linear signal**—SuperSignal West Dura substrate is well known for its ability to provide a consistent signal over an extended period of time. Letting blots sit in this substrate for extended times does not cause different light production rates between high- or low-abundance bands, and the data remain linear (Figure 6).



**Figure 6. SuperSignal West Dura Substrate exhibits consistent linear signals over time.** A 3-fold dilution series of A549 cell lysate starting at 20 µg/well was loaded onto a Bolt 4–12% Bis-Tris Plus gel and electrophoresed at 200 V for ~20 min. The gel was blotted to a nitrocellulose membrane using the iBlot 2 Gel Transfer Device (P0 protocol for 7 min). Immunoblot processing was performed using the Bandmate Automated Western Blot Processor. The membrane was blocked in 1X Clear Milk Blocking Buffer, then probed with mouse anti-pan Ras10 (Cat. No. MA1-012) at a 1:5,000 dilution, followed by incubation with HRP-conjugated goat anti-mouse IgG (1:100,000, Cat. No. 32430). Chemiluminescence detection was performed using SuperSignal West Dura substrate. The membrane was placed inside a protective sheet to accommodate multiple image acquisitions without having the blot dry out. The membrane was imaged on the iBright Imaging System at various time points (initial, 15 min, 60 min, 120 min) after substrate addition to follow the signal decay. The same exposure time was used for each image.

- **High sensitivity**—SuperSignal West Dura substrate (Figure 7) is capable of detecting proteins down to the mid-femtogram level. This provides enough sensitivity to detect low-abundance proteins and the ability to use a lower concentration of antibodies when detecting medium- to high-abundance proteins.

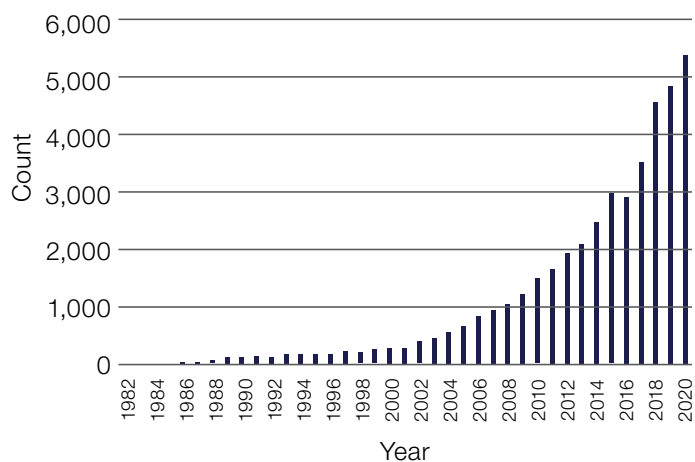


**Figure 7. SuperSignal West Dura substrate provides sensitive, intense signals for western blot detection.** A 1:1 dilution series of HeLa cell lysate starting at 20 µg/well was loaded onto gels and transferred to nitrocellulose membranes using the Invitrogen™ Power Blotter XL System (Cat. No. PB0013). The membranes were probed with Invitrogen™ mouse anti-STAT3 (Cat. No. MA1-13042) and HRP-conjugated goat anti-mouse IgG (Cat. No. 31430). Chemiluminescent detection was performed using SuperSignal West Dura substrate or Thermo Scientific™ Pierce™ ECL Western Blotting Substrate.

## Conclusion

As researchers and publishers acknowledge the shortcomings of current densitometric techniques, there is an ongoing shift from qualitative to quantitative western blotting across protein biology laboratories (Figure 8). To adopt this new and more accurate technique, current western blotting protocols need to be adjusted. In addition to adopting total protein normalization as the normalization method of choice, we also recommend addressing the three parameters discussed in this technical note:

1. Reduce the amount of lysate loaded on a gel to limit signal saturation. We recommend sample loads between 1 and 10  $\mu\text{g}$  of protein per well.
2. Optimize primary and secondary antibody dilutions. In most cases this means reducing the total amount of antibody used (higher dilutions). This will limit the amount of HRP in the system to prevent saturation of signal.
3. Use an optimized chemiluminescent substrate, such as SuperSignal West Dura substrate. The versatility, stability, and wide dynamic range of SuperSignal West Dura substrate make it a great choice for generating linear data.



**Figure 8. PubMed™ searches for “quantitative western blot”.** There is growing usage of quantitative western blot analysis. Search queries for the term have increased exponentially over the past decade. Data obtained from the PubMed search engine. Retrieved 27 April 2021 from <https://pubmed.ncbi.nlm.nih.gov/?term=quantitative+western+blot>.

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