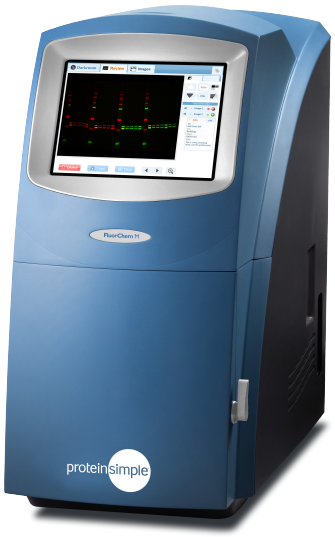
Total Protein Normalization with FluorChem Imagers

Introduction



Total protein normalization is the preferred method when it comes to normalizing Western blot protein signals, as it simplifies the quantitation workflow and eliminates the need for a housekeeping protein to normalize against. This method also avoids situations where the housekeeping protein expression changes between samples or is expressed in levels outside the dynamic linear range of your protein of interest. You also eliminate the need to strip and re-probe the membrane if your housekeeping gene migrates too close to your protein of interest.

Stain-Free™ gel technology simplifies the process further by removing the need for detecting total protein amounts after protein separation with a colorimetric or fluorescent stain. Stain-Free gels (Bio-Rad) contain a trihalo compound that crosslinks with tryptophan residues in proteins, producing a fluorescent signal under UV light. The signal can be detected right after separation in the gel and after transfer to a membrane.

In this application note, we demonstrate how to combine the ease-of-use of FluorChem™ imaging systems with Stain-Free gels to normalize the chemiluminescent signal from Western blots using AlphaView® software.

Stain-Free gel preparation and imaging

All FluorChem imagers have UV transilluminators and can detect chemiluminescent signals. So while we used a FluorChem M in this application note, any FluorChem imager can easily be used for your total protein normalization experiments.

Brain lysate at 0.6 mg/mL was prepared in 2X Laemmli Buffer (Bio-Rad, PN 161-0737) and denatured at 95 °C for 5 minutes. 10 µL was loaded into four wells on a 4-20% TGX Stain-Free gel (Bio-Rad, PN 567-8095) along with SDS-PAGE Broad Range Molecular Weight Standard (Bio-Rad, PN 161-0317) and separated at 200 V for approximately 1 hour.

The gel was removed from the cassette and placed directly on the FluorChem UV transilluminator without a screen and without washing. A custom protocol was then created by selecting Trans UV light for excitation and the orange filter (593/40 nm) in Channel 1 for emission. The trihalo compound linking was activated using this protocol to expose the gel to UV light for 5 minutes. The same

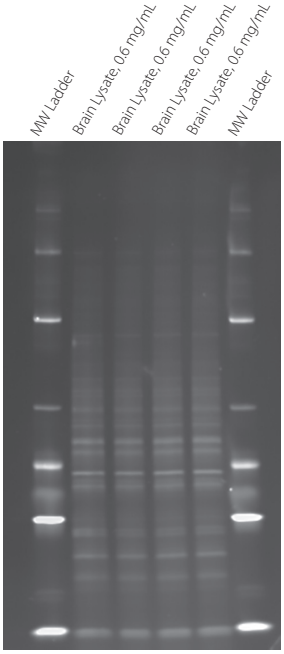


FIGURE 1. Total protein image of a 0.6 mg/mL brain lysate on a Stain-Free gel. Imaged on the FluorChem M system.

protocol was then used to image the gel with a 1 minute exposure (**Figure 1**).

Western blotting

Proteins were transferred from the gel to a nitrocellulose membrane at 100 V for 1 hour using a Criterion Blotter transfer chamber. The membrane was placed onto the FluorChem UV transilluminator without rinsing. The total protein was imaged for 1 minute using the same custom protocol created previously, exciting with Trans UV light and the orange filter for detection (**Figure 2**). You can also image the membrane after immunostaining if you're concerned about losing proteins during wash steps.

The membrane was then blocked with blocking buffer (5% milk in TBST) for 1 hour before incubating overnight with anti-alpha-synuclein antibody (Novus, PN NBP1-05194) diluted 1:1000 in blocking buffer. After primary incubation, the membrane was washed 5X with TBST for 5 minutes before a 1 hour incubation with goat anti-mouse-HRP secondary antibody (Cell Signaling, PN 7076S) diluted 1:1000 in blocking buffer. The membrane was then washed 5X with TBST for 5 minutes, placed on the black chemiluminescent screen, then placed on the UV transilluminator. Luminol/Peroxide XDR (ProteinSimple, PN 040-652 and 043-379) mixed at a 1:1 ratio was dropped onto the membrane and the chemiluminescent signal was detected using either the Chemi with Markers protocol or the Chemiluminescence protocol (**Figure 3**).

Total protein normalization with AlphaView software

The chemiluminescent alpha-synuclein signal was then normalized to the total protein imaged on the nitrocellulose membrane. First, we used AlphaView software's image and enhancement tools to make sure the signal to noise was optimized.

To determine the densitometry of the total protein signal on the membrane, we used the Multiplex Band Analysis in the Analysis Tool tab. The Single Region Tool box in the Region tab was used to identify the bands in the first lane and then the Multi Region Copy tool was used to draw similarly sized boxes around the remaining lanes (**Figure 4**). Background was then subtracted using the

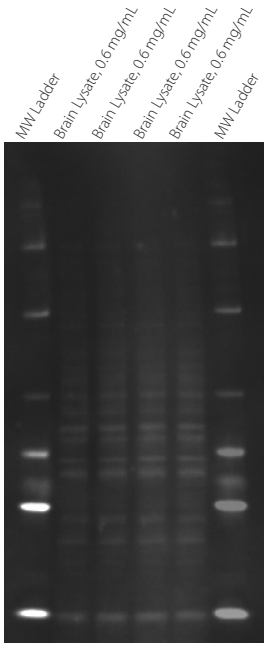


FIGURE 2. Total protein image of a brain lysate on a nitrocellulose membrane after transfer. Imaged on a FluorChem M system.

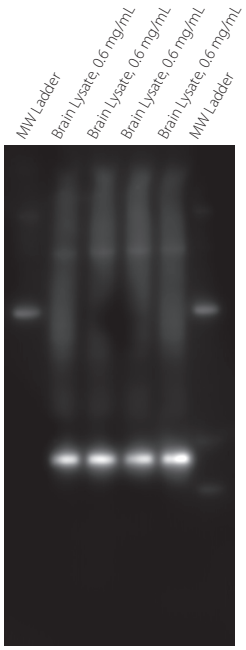


FIGURE 3. Chemiluminescent signal for alpha-synuclein imaged on the FluorChem M system.

tools in the Bkgrnd tab and the data was then exported into an Excel® spreadsheet.

To determine the signal from the alpha-synuclein we again used Multiplex Band Analysis to keep analysis

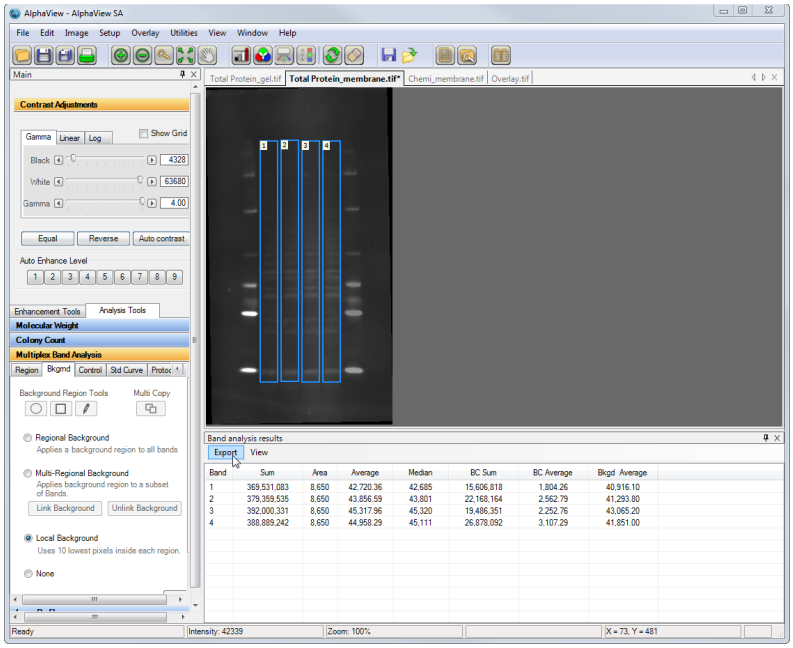


FIGURE 4. Quantitation of the total protein signal using Multiplex Band Analysis in AlphaView software.

methods between the total protein densitometry and the chemiluminescent densitometry consistent. The Single Region Tool box was used to draw a box around just the alpha-synuclein signal and the Multi-Region Copy tool was used to draw similarly sized boxes around the signal in the remaining lanes (**Figure 5**). The data was then copied into the Excel spreadsheet, where the sum of the alpha-synuclein signal was normalized to the sum of the total protein signal.

The data improved significantly after normalization, with CVs between the four lanes decreasing from 17.5% before

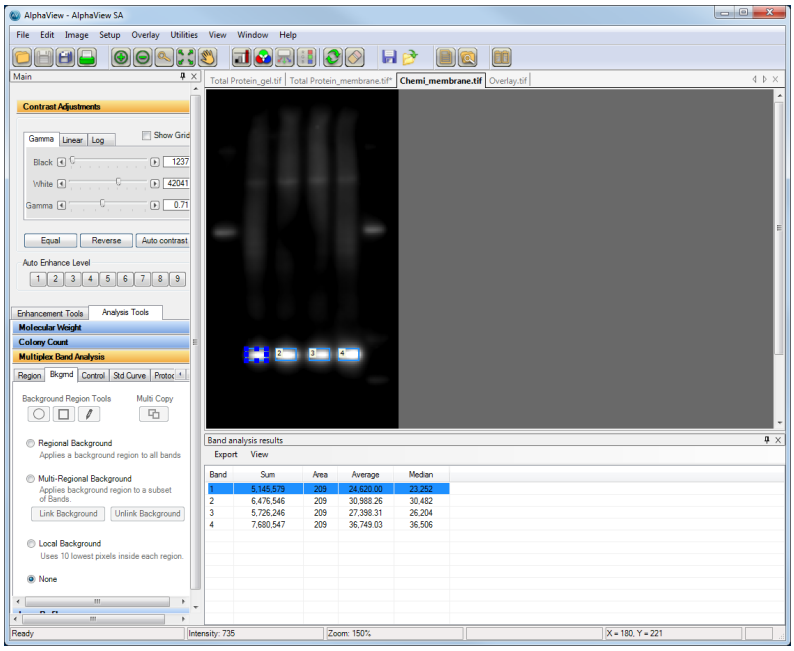


FIGURE 5. Quantitation of the alpha-synuclein signal in AlphaView software.

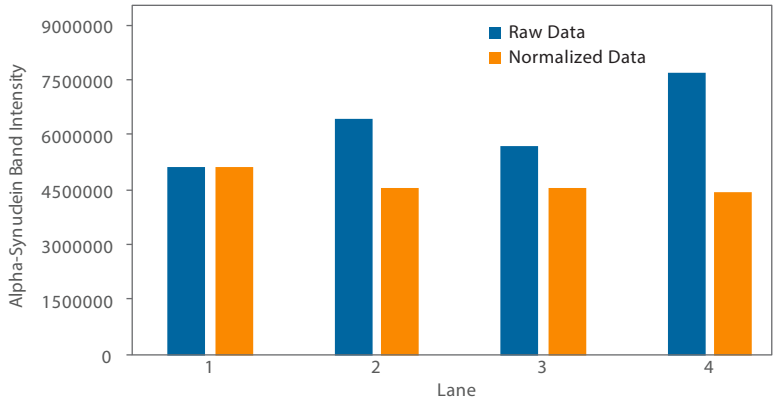


FIGURE 6. Alpha-synuclein signal before and after total protein normalization. CVs for the replicates (n=4) decreased from 17.5% to 6.6% after normalization.

normalization to 6.6% after normalization (**Figure 6**). This confirmed the differences in loading between gels were factored out with the normalization against the total protein signal.

Conclusion

Using total proteins to normalize gel loading has become more common when performing a traditional Western blot as it saves time by eliminating the multiple steps that come with using housekeeping proteins and the data is more accurate. You can easily use FluorChem imagers to perform this normalization using custom application protocols with Stain-Free gels. Simply place your gel or membrane in the instrument, load your protocol, and select **Expose**.

AlphaView software's Multiband Analysis tools then let you quantitate the total protein and chemiluminescent signal and export the data to Excel for normalization. Our results showed a CV decrease from 17.7% to 6.6% for alpha-synuclein after normalization, demonstrating the improved data quality you'll get with total protein normalization methods. The ease-of-use and flexibility in detection that comes with FluorChem imagers makes them an ideal choice for total protein normalization assays.

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