Stain-Free™ Gels as Part of the Western Blotting Workflow: Imaging and Total Protein Normalization

azure biosystems

Introduction

Stain-Free gel technology enables detection of protein bands in gels and on transfer membranes without using colorimetric or fluorescent stains. Stain-Free gels (Bio-Rad) contain a trihalocompound within the gel matrix that produces a fluorescent product when covalently crosslinked to protein tryptophan residues. After electrophoretic separation of proteins, crosslinking is carried out by exposing the gel to UV light. The resulting crosslinked products fluoresce under UV light and protein bands can be detected using an imaging system equipped with a UV illumination source such as the Azure cSeries Imaging Systems.

With Stain-Free technology, gels can be imaged almost immediately after the completion of electrophoresis, without the need to stain the gel by soaking the gel in stain solution. Additionally, the crosslinked product can be detected on the membrane after protein transfer from the gel, without any additional crosslinking steps. This allows the efficiency of transfer to be monitored by imaging the gel before and the membrane after transfer.

The Stain-Free chemistry is compatible with downstream Western blotting and can be used to measure the total protein content of a sample for use in total protein normalization (TPN) of Western blots. To carry out TPN using stain-free technology, total protein on the blot is detected by imaging the membrane under UV light before antibody incubation. The total amount of protein in each lane is calculated by quantifying the fluorescent signal off all the bands in the lane. After antibody detection is complete, the signal for the protein of interest in each lane is normalized to the total protein amount in the same lane.

TPN is an alternative to the common practice of normalizing to a housekeeping protein. In some cases, housekeeping proteins may not be expressed consistently between samples, or may not be present in the sample at a level that is similar enough to the protein of interest that both proteins are within the linear range of detection. Also, normalizing to a housekeeping protein requires optimizing a second antibody to detect the housekeeping protein. TPN avoids all of these concerns. However, if transfer artifacts are present on part of the membrane, or if the total protein composition of different samples varies substantially, normalizing to a housekeeping protein may be preferable.

Here we describe how to image Stain-Free gels using the Azure imaging systems, and how to carry out TPN of chemiluminescent Western blots with AzureSpot software.

Materials and Methods

Gel Electrophoresis

Protein samples consisting of different amounts of the same HeLa cell lysate were separated by SDS-PAGE on a 4-15% Mini-PROTEAN® TGX Stain-Free™ Precast Gel (Bio-Rad). Immediately after completion of electrophoretic separation, the gel was placed directly on the UV transilluminator of an Azure c600 for activation and imaging.

Activation and Imaging: To activate the stain-free gel, the gel was exposed to UV light (302 nm) for 5 minutes. An image of the crosslinked gel was then captured. In the cSeries Capture software, **GEL** was selected in the top menu bar, **302 nm** was selected as the light source, and the **UV OVERRIDE** button was clicked. **LIGHT ON 5 MINUTES** was selected, and the box to **Automatically acquire an image when done** was clicked (Figure 1). The resulting gel image is shown in Figure 2.

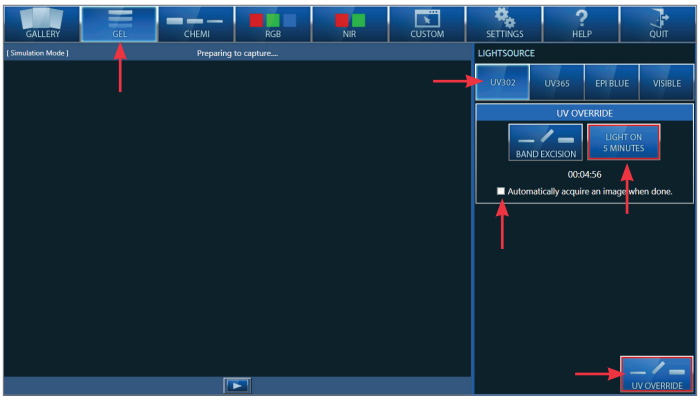

Figure 1. Settings to automatically acquire gel image in cSeries Capture.

NOTE: Do not soak gel in buffer or water after electrophoresis and before activation: the stain-free moiety can diffuse out of the gel.

NOTE: Do not place the gel on plastic for activation or imaging; place it directly on the transilluminator.

Protein Transfer

After imaging the gel, the proteins from the gel were transferred to a PVDF membrane. The membrane was activated by incubation with methanol for 15 seconds, water for 5 minutes, and then 1X Azure Transfer Buffer for 5 minutes.

Transfer was performed using Azure Transfer Buffer at 19 V for 35 minutes with an Idea Scientific GENIE electrophoretic transfer device.

Immediately after transfer was complete, the wet blot was placed directly on the UV transilluminator for imaging without rinsing.

Imaging: The blot was imaged on the UV transilluminator using UV light (302 nm) for 1 min. The image is shown in Figure 3.

NOTE: Once activation is carried out within the gel, the same protein bands will be visible on the membrane after transfer. No additional activation step is required.

Western Blotting

After imaging, the membrane was blocked for 30 minutes at room temperature. The membrane was then incubated for 1 hour with 2 µg mouse anti-GAPDH antibody (EMD Millipore) in blocking buffer. The membrane was rinsed two times quickly with 1X PBST then washed three times, for 5 minutes each wash, with 1X PBST. The membrane was then incubated for 30 minutes with an HRP-conjugated goat anti-mouse antibody (Bio-Rad) in blocking buffer and the membrane was washed as before.

Radiance ECL chemiluminescent substrate (Azure Cat #AC2101) was applied to the blot for 5 minutes. Excess substrate was drained from the membrane and the blot was placed in a black tray for imaging.

Imaging: In the cSeries Capture software, the **CHEMI** tab was selected in the top menu, and **Normal** sensitivity (Figure 3). The blot was imaged using **AUTO EXPOSURE** (Figure 4).

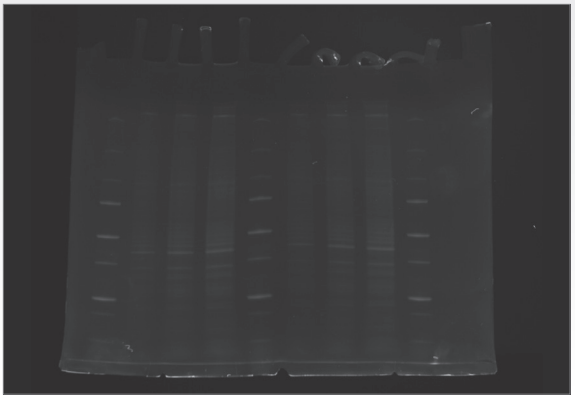

Figure 2. Image of total protein on Stain-Free gel after crosslinking.

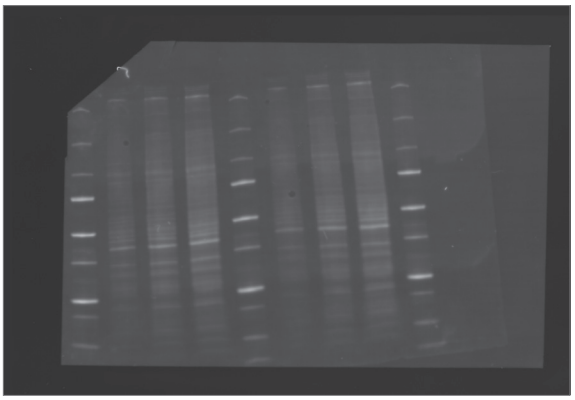

Figure 3. Image of total protein on membrane after transfer of proteins from gel shown in Figure 2.

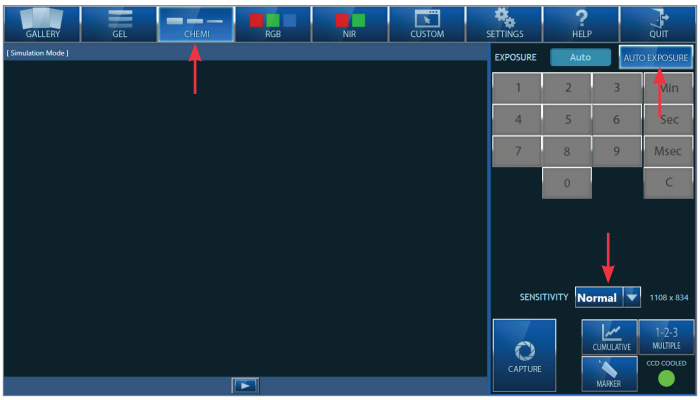

Figure 4. Settings to capture Western blot image in cSeries Capture.

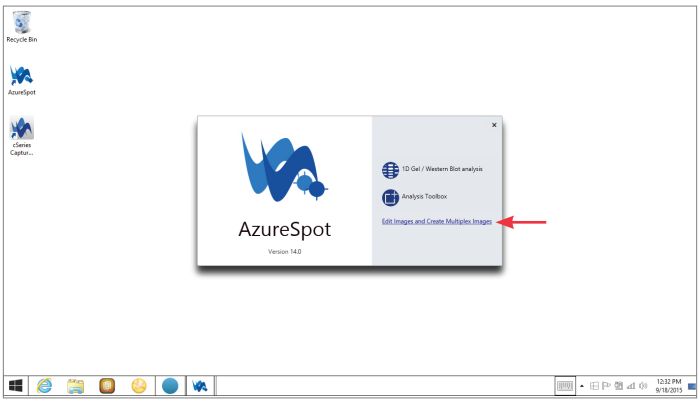

Figure 5. Opening the AzureSpot Image Editor.

Total Protein Normalization

To normalize the amount of GAPDH detected by Western blotting to the total protein in each sample, the signal for the GAPDH band in each lane in the chemiluminescent image was normalized to the total protein amount detected in the stain-free image of the membrane for that lane.

First, a multiplex image was created from the stain-free image of the membrane and the chemiluminescent image of the GAPDH Western blot. In the AzureSpot software, the image editor was opened by selecting **Edit Images and Create Multiplex Images** from the start screen (Figure 5). The two images were selected, and any adjustments for alignment were made using the translate and rotate tools as necessary. Note that to move one image relative to the other, check the box next to the image to be moved under the **Image** heading in the column to the right of the image window (Figure 6). Once the images are aligned, crop both images using the crop tool; be sure to select both images by checking the boxes next to both images in the Image column. The multiplex image was saved and the image editor was closed.

For analysis, **1D Gel / Western Blot analysis** was selected from the AzureSpot start screen (Figure 7). The multiplex image was opened by selecting **Open Multiplex Image**. Lanes were identified using the **Create Lanes** function while viewing the stain-free image (Figure 8). Background correction was conducted using the rolling ball method under the **Background** function (Figure 9). The GAPDH bands were identified using the **Detect Bands** function (Figure 10).

To carry out TPB, the **Normalisation** function was selected from the top menu (Figure 11). **Total Protein Normalisation** was selected, and the channel with the stain-free image of the membrane was selected as the Normalisation Channel. The Reference Lane was set to be lane 1. The software calculates a normalization factor for each lane based on the total protein signal in that lane compared to the reference lane. The normalized results for each lane are reported relative to the intensity of the band in the reference lane. Choosing a different reference lane will have no effect on the relative results, though the absolute numbers reported for the normalized bands will change.

At the bottom of the **Normalisation** screen, **All Lanes** was selected to display the calculated values of the GAPDH band in every lane in the measurements table (Figure 11). The data were then exported by selecting **Export** > **Export to File** > **Measurements Table** from the top dropdown menu (Figure 12).

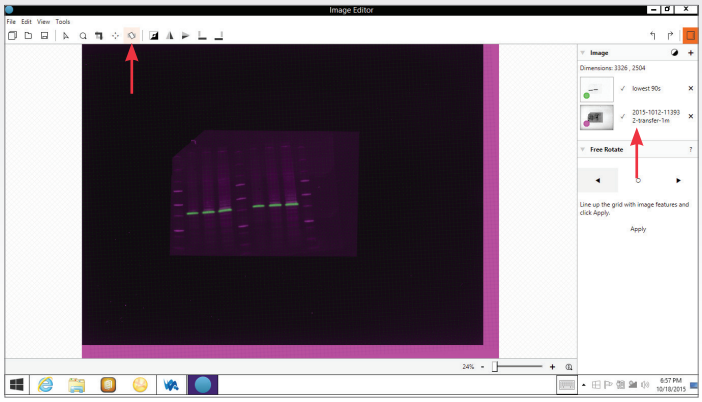

Figure 6. Aligning two images to create a multiplex image in AzureSpot Image Editor.

Figure 7. Opening the AzureSpot Western blot analysis tool.

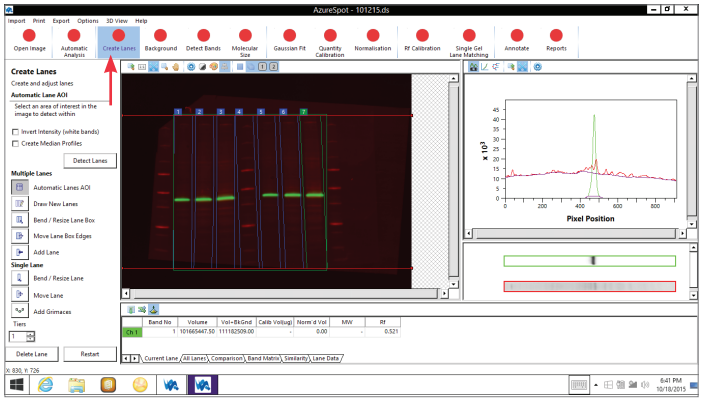

Figure 8. Automatic lane detection.

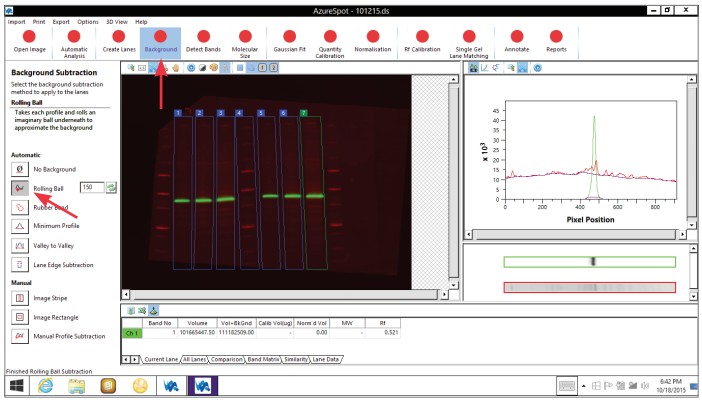

Figure 9. Background subtraction.

The resulting unnormalized and normalized values for the GAPDH band were plotted (Figure 13). Though the amounts of sample loaded varied between lanes, the normalized values for GAPDH levels using TPN are identical across samples.

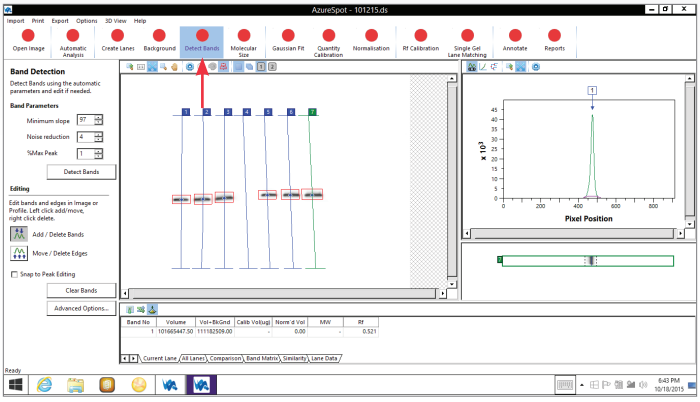

Figure 10. Automatic band detection.

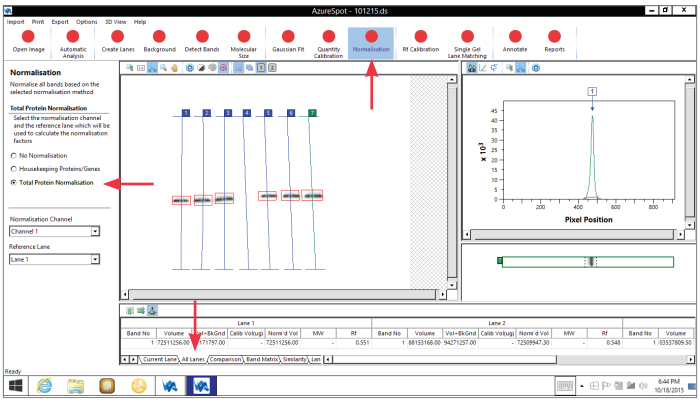

Figure 11. Total protein normalization.

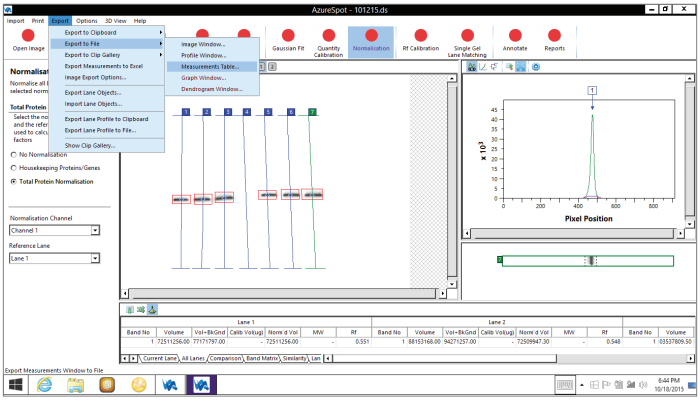

Figure 12. Exporting analysis results.

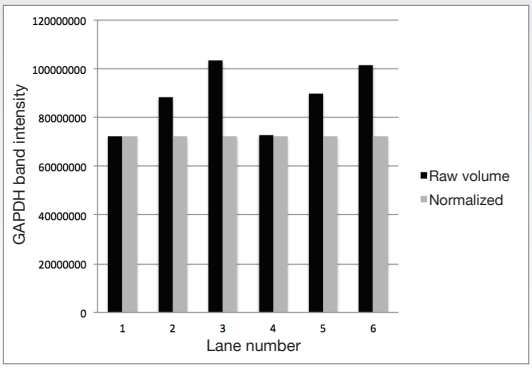

Figure 13. GAPDH band intensity before and after TPN.

Conclusion

Azure Biosystems' imaging systems and software make total protein normalization of Western blots easy and efficient. TPN provides a simple way to normalize quantitative Western blots and has several advantages over the use of housekeeping proteins as normalization standards. Using Stain-Free gel technology, total protein on the membrane can be measured by Azure's cSeries instruments immediately after protein transfer without any extra staining or destaining steps. The c200 through c600 imaging systems are compatible with this approach. AzureSpot software includes TPN as a built-in normalization method, streamlining the analysis workflow. Scientists need only capture two images, the total protein image and the Western image, and the software guides the rest.

azure biosystems

www.azurebiosystems.com • info@azurebiosystems.com