Accurate Western Blot Normalization with AzureRed Fluorescent Protein Stain

Introduction

An important component of quantitative Western blotting is normalization of the band intensity obtained from the target protein against a reference whose intensity should only vary proportionally to the quantity of material in the sample. Traditionally, "housekeeping proteins" – proteins thought to be consistently expressed across different sample types – are used as such a reference. However, in some cases, housekeeping proteins may not be expressed consistently between samples. Studies have emerged showing that experimental conditions may alter the expression of some commonly used housekeeping proteins.[1] In other situations, housekeeping proteins may not be present in the sample at a level that is similar enough to the protein of interest to fall within the linear range of detection.[2-3]

An increasingly popular alternative normalization method that avoids many of the drawbacks of relying on housekeeping proteins is "total protein normalization" or TPN. In this method, the volume of the protein of interest is compared to the total amount of protein loaded in the lane. TPN presents advantages over using a single protein as a standard including not being susceptible to unexpected changes in the amount of the standard protein between samples. Instead of looking at the intensity of one protein, the total density for each lane is measured. Because TPN technology eliminates many of the issues related to signal normalization, publications are increasingly requiring the use of total protein signal for normalization.[4]

AzureRed Fluorescent Protein Stain is a quantitative total-protein stain for gels and blots that is compatible with downstream protein analysis, including Western blotting. AzureRed is a perfect choice for staining applications, including post-transfer staining to confirm uniform protein transfer from gel to membrane, and staining quantitative Western blots as part of a TPN protocol.

In this application note, we demonstrate that AzureRed Fluorescent Protein Stain is ideally suited for TPN because of its wide, linear dynamic range and sensitivity. Additionally, it is fully compatible with standard Western blot workflows, which we demonstrate by normalizing a tricolor fluorescent Western blot (three different proteins detected) to total protein using AzureRed stain.

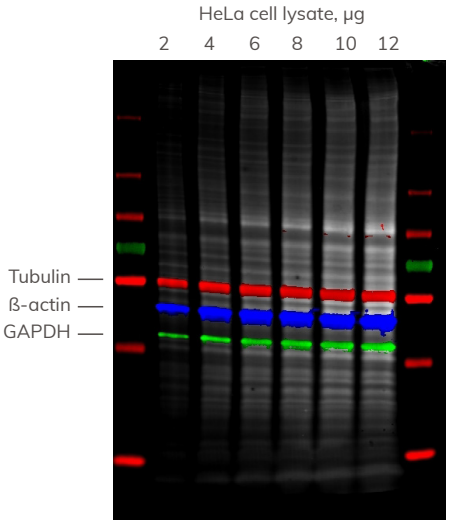

Figure 1. AzureRed is imaged simultaneously with three proteins of interest. The gel was loaded with dilutions of HeLa cell lysate. After transfer, the blot was stained with AzureRed and then probed for tubulin, ß-actin, and GAPDH without a destaining step. The blot was scanned with each of the four lasers of the Sapphire Biomolecular Imager. In this overlay of the four channels, total protein (AzureRed stain) is shown in gray; tubulin in red, ß-actin in blue, and GAPDH in green.

Methods

Samples of HeLa cell lysate were separated by polyacrylamide gel electrophoresis and proteins transferred from the gel to a low-fluorescence PVDF membrane (Azure Biosystems product AC2127; AC2105). After protein transfer, the blot was stained with AzureRed Fluorescent Protein Stain (Azure Biosystems product AC2124), according to the PVDF staining protocol (available at www.azurebiosystems.com).

Following transfer, the blot was washed briefly in water then stained in AzureRed stain solution according to the protocol. After the 30-minute staining procedure, the blot was blocked using Azure Fluorescent Blot Blocking Buffer (Azure Biosystems product AC2190) and incubated with primary antibodies for Western blot detection (Table 1).

The blot was imaged using the Sapphire Biomolecular Imager by scanning the blot with all four laser simultaneously. The three target proteins were detected with the lasers as shown in Table 1, while AzureRed staining was detected using the 520nm laser.

Images were analyzed using AzureSpot software.

Target	Primary antibody	Secondary antibody	Detected using laser
Tubulin	Rat anti-tubulin	Goat anti-rat AzureSpectra 700	658nm
ß-actin	Rabbit anti–ß-actin	Goat anti-rabbit AzureSpectra 490	488nm
GAPDH	Chicken anti-GAPDH	Goat anti-chicken AzureSpectra 800	784nm

Figure 1. Overview of a Western blotting protocol using indirect antigen detection.

Results

Scanning the tricolor Western blot stained with AzureRed Fluorescent Protein Stain on the Sapphire Biomolecular Imager results in a four-channel composite image (Figure 1). All four channels are imaged and aligned as part of image capture, removing the need to align the images at a later point, and not adding any additional imaging steps in the workflow. The composite image with an overlay of the four resulting images allows easy comparison of the three proteins with each other and with total protein load.

Viewing the single channel images allows visual inspection of the dynamic range of AzureRed and the three proteins (Figure 1A-D). The signal intensity for total protein in each lane (for AzureRed stain) or band (for each of the three proteins detected) was quantified using AzureSpot software. Figure 2E shows the relationship between each signal and the amount of protein loaded on the gel. The quantitative nature of AzureRed stain is observed in the excellent correlation of signal to amount of protein loaded (R^2 = 0.994). This correlation was superior to that of any of the three individual proteins (Figure 2E), each of which is frequently used as a housekeeping protein.

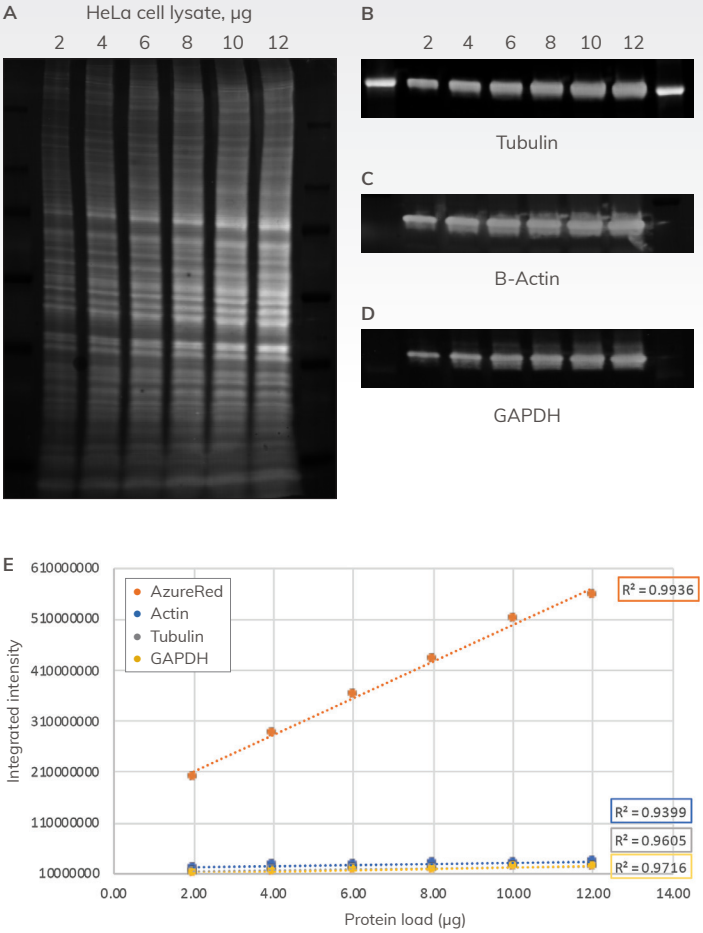

Figure 2. AzureRed has a wider linear dynamic range compared to common housekeeping proteins. Single color channel images for A) AureRed staining for total protein B) Tubulin C) B-actin and D) GAPDH. The superior dynamic range of the AzureRed signal to the actual amount of protein loaded is displayed in image (A). The signals from the total protein and the housekeeping proteins were quantified using the AzureSpot software, and the resulting values are plotted against the amount of protein loaded for each sample (E). AzureRed showed superior correlation and a much broader dynamic range than the common housekeeping proteins.

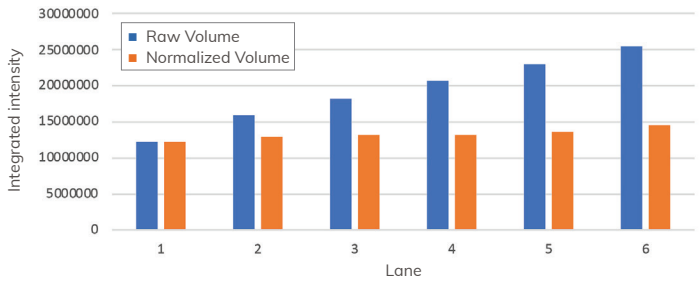

Figure 3. Tubulin signal normalized to total protein as assessed using AzureRed Fluorescent Protein Stain. The tubulin band was quantified using AzureSpot software. Raw tubulin band volume (blue bars) and band volume normalized to total protein (orange bars) are shown. Normalizing the signal volume using AzureRed corrects for any errors in sample loading or differences in transfer across the membrane.

The use of the AzureRed signal for total protein as a standard to normalize protein signal is shown in Figure 3. Normalizing the tubulin signal for each lane, by the total protein signal for the same lane, results in excellent agreement across samples, with a coefficient of variation of normalized values of only 4%.

Conclusion

AzureRed Fluorescent Protein Stain is an ideal choice for quantitative fluorescent Western blotting. The wide, linear dynamic range ensures that AzureRed acts as an effective standard to account for variations in protein load. A key feature of AzureRed is the simplicity of fitting it into your existing workflow (Figure 4).

AzureRed dye can be detected simultaneously alongside commonly used fluorescent probes without interference, allowing multiple proteins to be assessed on a single blot. Using the quantitative AzureRed signal for total protein normalization of multicolor blots makes it possible to quantify multiple proteins on a single blot without stripping or destaining. The protocol is quick, and imaging occurs simultaneously with the proteins of interest.

Learn more about AzureRed Fluorescent Protein Stain at www.azurebiosystems.com.

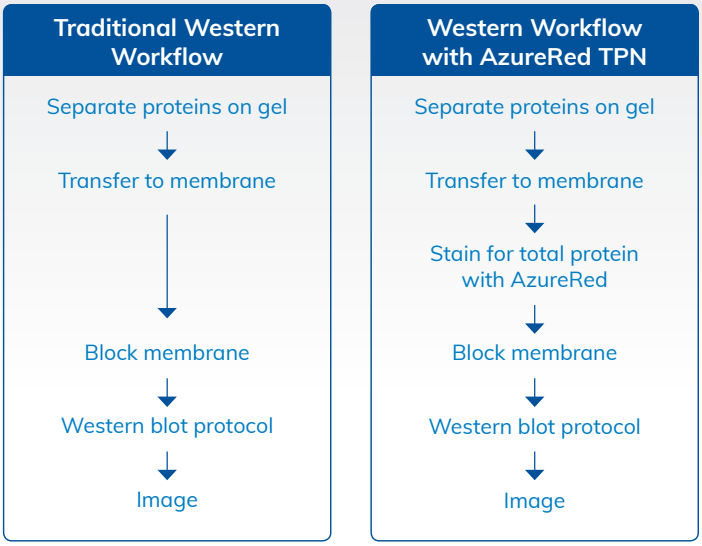

Figure 4. Total protein normalization with AzureRed fits easily into any Western blotting workflow.

References

1. R. E. Ferguson, H. P. Carroll, A. Harris, E. R. Maher, P. J. Selby, R. E. Banks, *Proteomics* 2005, 5, 566.
2. J. Petrak, R. Ivanek, O. Toman, R. Cmejla, J. Cmejlova, D. Vyoral, J. Zivny, C. D. Vulpe, *Proteomics* 2008, 8, 1744.
3. M. Wilhelm, J. Schlegl, H. Hahne, A. M. Gholami, M. Lieberenz, M. M. Savitski, E. Ziegler, L. Butzmann, S. Gessulat, H. Marx, T. Mathieson, S. Lemeer, K. Schnatbaum, U. Reimer, H. Wenschuh, M. Mollenhauer, J. Slotta-Huspenina, J.-H. Boese, M. Bantscheff, A. Gerstmair, F. Faerber, B. Kuster, *Nature* 2014, 509, 582.
4. A.J. Fosang, R.J. Colbran, *Journal of Biological Chemistry*, 2015, 290, 50.

azure biosystems

www.azurebiosystems.com • info@azurebiosystems.com