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Quantitative Analysis of 3-Color Fluorescent Western Blots with the FluorChem® Q

Application Note 118

Abstract

Multiplex detection methodologies are rapidly becoming popular for Western blot imaging due to their ability to provide rapid quantitation across multiple protein samples. In this article, we discuss the power of multicolor fluorescent Westerns in multiplex detection. The FluorChem Q, a CCD – based detection platform able to perform traditional chemiluminescent detection and multicolor fluorescent detection, makes fluorescent Western imaging rapid and easy. Data analysis of multicolor Westerns is streamlined with AlphaView™ Q software, a software package that provides the ability to normalize to lane loading controls and to easily calculate fold change between samples. Together, the FluorChem Q and AlphaView Q simplify the process from image acquisition to image analysis, providing a powerful tool for imaging and analyzing multicolor Westerns.

Introduction

Western blot analysis detects the expression level of a single protein within the complex mixture of proteins present in a cell or tissue sample. This specificity is achieved by probing for the protein of interest using an antibody (the primary antibody) specific to the target protein. The primary antibody itself is then detected using a reporter molecule coupled to a secondary antibody that recognizes the primary antibody. Currently, the most commonly used detection method for Western blotting is chemiluminescence due to its high sensitivity and the ease of access to commercial antibodies labeled with HRP or AP (1).

Frequently, Western blots are used to compare levels of a protein of interest between samples. While chemiluminescence can be ideal for detection of a single target, it can present limitations for applications in which multiple proteins are to be detected. This is especially true if the proteins of interest are of similar size, as is the case for phosphorylated isoforms, or are present in dramatically different amounts within the sample.

To analyze multiple overlapping targets or closely resolved targets on a blot using chemiluminescence, the blot is typically stripped and re-probed sequentially for each additional target of interest. This method takes additional time, and if uneven stripping of the blot occurs, the ability to gain quantitative information from the resulting blot is lost.

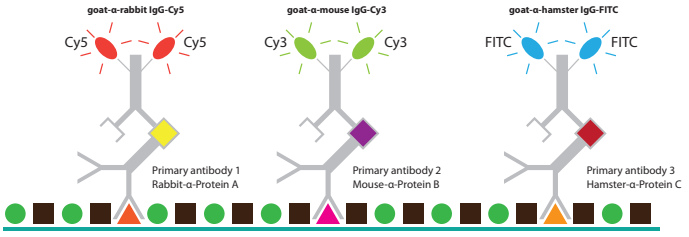

Figure 1. The concept of fluorescent western detection. Three primary antibodies are depicted, binding to three independent antigens (triangles) on a membrane. Each primary antibody is bound by a secondary antibody that has been directly labeled with a dye such as Cy5 (red), Cy3 (green) or FITC (blue).

In situations where multiple proteins are to be detected on a blot, the power of Western blot analysis can be improved by using multicolor fluorescence. In multicolor fluorescence, each antibody probe is conjugated with a different fluorophore with emission spectra at wavelengths distinguishable from the other probes (Figure 1). Because each fluorophore emits in its own channel, proteins can be resolved that migrate in overlapping bands without the need for stripping and re-probing the blot.

Multicolor fluorescent Westerns also increase the accuracy of quantitation when one fluorescent probe is used to detect a protein of interest, and a second fluorescent probe is used to detect a sample loading control used for normalization. With powerful software such as the AlphaView Q, it can quickly be determined whether there were any inconsistencies in loading, and if so, the intensities of the bands for the protein of interest can be normalized to the loading control.

Here we present studies demonstrating independent three-color detection of proteins on Western blots using the FluorChem Q, an imaging system designed for both the detection and analysis of multicolor western blots, as well as for chemiluminescent imaging. We show the rapid acquisition of a three color image, and the workflow for analysis of the data.

Methods

Western blot preparation. A multicolor fluorescence Western blot of human serum samples, probed with antibodies to Transferrin, AFP and IgG, was prepared to provide a multichannel image with features useful for illustrating the analysis process. Samples of human serum were prepared with variable amounts of purified α1-Fetoprotein (AFP) added to represent an experimental protein of interest. Endogenous Transferrin with variable amounts of added purified Transferrin represented a second experimental protein of interest, and the heavy chain of IgG was used as the loading control.

The serum samples were separated by electrophoresis using a standard Tris-Glycine SDS system at 150 V for one hour. The proteins were transferred onto a PVDF membrane (Immobilon –P, Millipore) using a standard tank transfer procedure at 100 V for one hour in a Tris-Glycine buffer containing 12% methanol.

The membrane blot was blocked for one hour in a 2% solution of ECL Advance blocking agent (GE Healthcare) dissolved in PBST. The blots were washed with PBST and incubated with a solution of the primary antibodies, rabbit anti-human Transferrin and mouse anti-human AFP. The dilution factors for the primary antibodies were determined by preliminary studies. After multiple washings in PBST the blots were incubated with a solution of fluorescently-labeled secondary antibodies containing CY-3- Goat-anti-Mouse IgG (1:5000) (GE Healthcare), and CY5-Goat-anti Rabbit IgG (1:5000) (GE Healthcare). A Fluorescein

labeled anti-Human IgG antibody (1:2500) (Vector Laboratory) was also included to directly detect Human IgG in the serum sample. The blots were washed multiple times in PBS, then a final rinse with water and dried at room temperature.

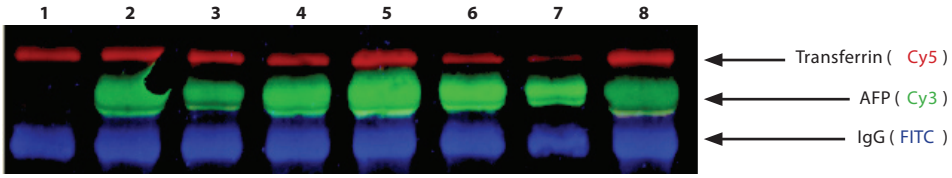

Figure 2. Three-color Western blot of human serum samples hybridized with antibodies to Transferrin, AFP and IgG. Three images were taken sequentially using a multi channel imaging protocol in AlphaView Q, and displayed as a composite three-color image in the software. Total imaging time was 50 seconds.

Image acquisition. To demonstrate multiplex detection of three target proteins in samples containing a complex mixture of proteins, a composite image of the blot was acquired on the FluorChem Q using a three-channel acquisition protocol (Figure 2). The red channel (Transferrin) was acquired with 632 nm excitation and CY5 filter with a 32 sec exposure, the green channel (AFP) was acquired with 534 nm excitation and CY3 filter with a 16 sec exposure and the blue channel (IgG) with 475 nm excitation and Cy2 filter with a 2 sec exposure. The speed /resolution settings were Normal/Ultra for all three channels.

Total imaging time was very rapid (less than 50 seconds). Once the image was acquired, it was immediately displayed by the software as a three color image, and was ready for analysis with AlphaView Q software.

Results

Image analysis. Using a housekeeping gene as a loading control reduces variability in data due to loading errors. In Figure 3, IgG, detected with Fluorescein, serves as the loading control.

Using the Band Analysis module in AlphaView Q, one can quickly extract quantitative data from composite multichannel and single channel images. Once selected within the software, the Band Analysis tool contains clickable tabs taking the scientist step by step through the recommended work flow for data analysis.

Designation of bands and background correction. Beginning with the Region tab, regions of interest are designated, containing images of bands from which quantitative data will be extracted. Next, through the Regional Background Tool tab, background fluorescence signal (which can arise from the fluorescence detection chemistry, the sample matrix or the quality of the sample itself) is removed.

Loading control normalization. Once the regions of interest have been defined and background correction has been performed, loading control normalization takes place by choosing the Control tab. In Figure 3, loading controls were selected by highlighting the IgG bands. AlphaView Q software immediately displays the Loading Control Normalized (LCN) results.

The value of Loading Control Normalization is apparent in comparing the results obtained before and after using this tool. Without Loading Control normalization, it appears that there is less Transferrin in lane 1 than lane 2, when in fact there is more (Figure 3a). Similarly, without loading control normalization, it appears there is less AFP in lane 7 than lane 6, when in fact the levels in each lane are close to being the same (Figure 3b).

Calculation of fold-change. The ability to rapidly determine fold change between samples is a very important application of multicolor fluorescent Western blotting. In AlphaView Q, it is straightforward to calculate fold change based on a positive control. Once a positive control is selected, the software will immediately display the fold change represented by the band of interest in each lane, relative to the positive control. Figure 4 and Table 1 show the results of computing fold change; lane 1 was chosen as the control, and the greatest increase in Transferrin relative to this lane is in lane 5. Since the samples have been normalized using Loading Control Normalization, the fold-change results are not influenced by variance in sample loading. The values agree with those predicted based on the known amount of serum and spiked pure Transferrin added to each sample (Table 1).

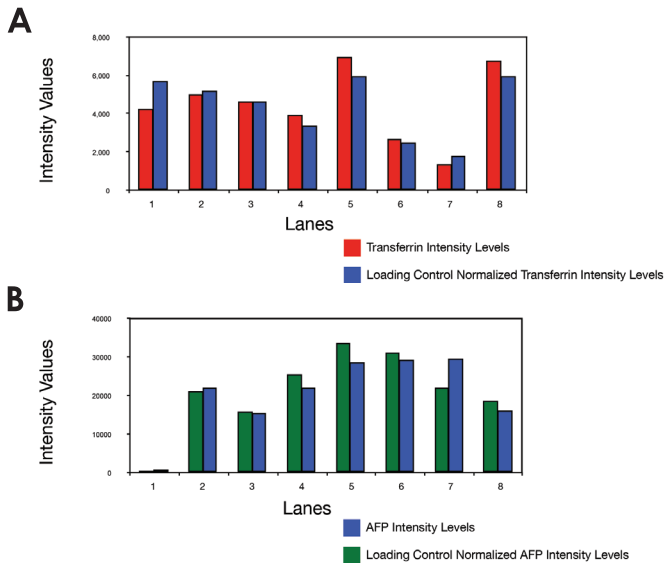

Figure 3. Quantification of Transferrin (a) and AFP (b), before and after Loading Control Normalization, using IgG as the reference band.

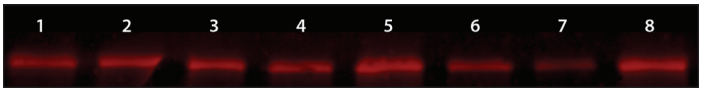

Figure 4. Computation of fold-change for the Transferrin band in each lane relative to the Transferrin band intensity in lane 1. The calculated fold-change values, and the values predicted based on the known amount of Transferrin added to each lane, are shown in Table 2.

Lane	Transferrin (ng)	Predicted
1	1.00	1.00
2	1.17	1.20
3	1.09	1.00
4	-1.08	-1.15
5	1.65	1.60
6	-1.59	-1.67
7	-3.17	-3.33
8	1.58	1.90

Table 1. Fold-change values for Transferrin. The values calculated by the AlphaView Q software are compared to those predicted based on the estimated amount of Transferrin in serum and the known amount of spiked pure Transferrin in each lane.

Conclusions

Multicolor fluorescent imaging increases the power of Western blotting. Multicolor fluorescent Westerns allow the detection of multiple proteins on the same blot, without stripping and reprobing. This facilitates quantitative analysis of Western blots because a gel-loading control or internal standard can be labeled with a different fluorophore, and detected on the same blot but independently from the protein of interest.

The FluorChem Q imager and AlphaView Q software streamline the acquisition and analysis of multicolor fluorescent images. With this powerful combination:

- Images of a blot in three independent fluorescent detection channels can be acquired in under a minute
- The resulting images are easily displayed as either individual or composite three-color images
- Designation of bands of interest involves only a click of the mouse
- Once told which band represents the loading control, normalization is immediate
- Once told which band represents the positive control, calculation of fold-change is immediate

All of these attributes add up to straightforward, accurate and quantitative analysis of multicolor Western blots.

References

1. Patton, Wayne F. (2000) "A thousand points of light: The application of fluorescence detection technologies to two-dimensional gel electrophoresis and proteomics" Electrophoresis. 21: 1123-1144.

Acknowledgement

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