Cell Biosciences™

The FluorChem® Q and Alexa Fluor® Labeled Antibodies: Multiplex Imaging of Fluorescent Western Blots

Application Note 120

Abstract

Multiplex fluorescent detection allows the analysis of multiple proteins, including proteins having similar molecular weights, on a single Western blot. This approach facilitates quantification of proteins via the use of an internal reference protein, removing the need to strip and reprobe a blot. The FluorChem Q provides a sensitive means to acquire high-quality images of multicolor fluorescent Westerns. This report describes a protocol for using the FluorChem Q with Alexa dye labeled Westerns, and the compatibility of the FluorChem Q with multicolor Western blots.

Introduction

Recently, multiplex fluorescent technology has become available for Western blotting, a powerful tool for a protein chemist. Direct fluorescent labeling of antibodies used in Western blotting, combined with fluorescent light detection, allows multiple proteins to be assayed on one blot, regardless of whether they overlap in size or are in different relative abundance. This is especially useful in quantitative analysis using Western blotting, since the reference protein used for normalization can be imaged on the same blot as the protein of interest.

Alexa Fluor conjugated antibodies (Invitrogen) are available in multiple excitation and emission combinations, and provide strong fluorescent signals and excellent photostability. Direct fluorescent detection of proteins on Western blots allows for multiplex applications in which multiple proteins can be detected simultaneously on the same blot by the use of secondary antibodies labeled with different Alexa Fluor dyes. Each dye is chosen to have unique spectral properties so that, with proper selection of excitation and emission wavelengths, they can be independently excited and imaged (Figure 1).

The FluorChem Q imaging system is designed to acquire high quality images from fluorescently labeled, multicolor Westerns. Three excitation light sources and three emission filters on the FluorChem Q provide three unique spectral detection channels, optimized for the most commonly used fluorescent reagents. The compatibility of the FluorChem Q filters with the excitation and emission spectra of the Alexa Fluor 488, Alexa Fluor 546, and Alexa Fluor 647 dyes is shown in Table I.

Important to the collection of fluorescent images useful for quantitative analysis is a large dynamic range, accomplished through a combination of high sensitivity and low background noise. The FluorChem Q has a high power illumination field with consistent intensity across its large field of view, allowing for uniform imaging across the entire blot. An integrated 4.2 megapixel, peltier-cooled camera collects high-resolution images while minimizing noise, and a f/0.95 manual fixed lens provides fast image acquisition, shortening analysis time.

In this article, we demonstrate the performance of the FluorChem Q in imaging multicolor Western blots labeled with Alexa Fluor fluorescent antibodies. The ability to image three proteins on a single blot using three different fluorescent channels is demonstrated.

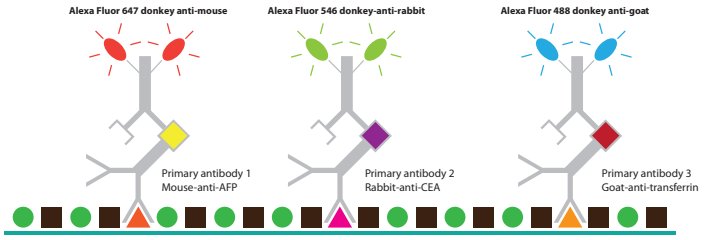

Figure 1. Multiplex fluorescent Western detection. Three primary antibodies are depicted, binding to three independent antigens (triangles) on a membrane. The primary antibodies are bound by secondary antibodies that are labeled with Alexa Fluor fluorescent dyes, either Alexa Fluor 647 (red), Alexa Fluor 546 (green) or Alexa Fluor 488 (blue).

Methods

Electrophoresis and transfer. Proteins were separated on 8-16% polyacrylamide Tris-HCl gels (Bio-Rad). Separation was carried out at 150 V for two hours, using a standard Tris-Glycine-SDS buffer system in the Mini-PROTEAN Tetra Cell from Bio-Rad.

All samples were loaded in equal volumes (10 µL). Each lane contained 4 ng of Transferrin, and two-fold serial dilutions of α1-fetoprotein (AFP) and CEA as follows:

After separation, proteins were transferred to a PVDF membrane using a standard tank transfer procedure in a Tris-Glycine buffer containing 12% methanol. Transfer was conducted at 100 V for one hour with an ice pack inserted in the tank.

Lane	CEA (ng)	α1-Fetoprotein (ng)	Transferrin (ng)
1	63	0.004	4
2	31	0.008	4
3	16	0.015	4
4	8	0.03	4
5	4	0.06	4
6	2	0.12	4
7	1	0.24	4
8	0.49	0.49	4
9	0.24	1	4
10	0.12	2	4
11	0.06	4	4
12	0.03	8	4
13	0.015	16	4
14	0.008	31	4
15	0.004	63	4

Western blot assay. The membrane was washed with PBS containing 0.05% Tween-20 (PBST) for 5 minutes. The membrane was then blocked for one hour at room temperature in a 2% solution of ECL Advance blocking agent dissolved in PBST. After blocking, the membrane was washed with PBST for 5 minutes and incubated with the primary antibody mixture for one hour at room temperature. The mixture of primary antibodies contained Goat-anti-Transferrin, Mouse-anti-AFP, and Rabbit-anti-CEA antibodies, each diluted at 1:5000 in PBST. The membrane was then subjected to two brief, one 15 minute, and four five-minute washes in PBST.

The membrane was then incubated with fluorescently labeled secondary antibodies for one hour at room temperature. A mixture of secondary antibodies was used, containing Alexa-Fluor-488-donkey-anti-goat IgG, Alexa-Fluor-546-donkey-anti-rabbit IgG, and Alexa-Fluor-647-donkey-anti-mouse IgG, all diluted 1:2500.

After the incubation, the membrane was washed three times with PBST for five minutes each, once with PBS for five minutes, then briefly rinsed with water and dried at room temperature.

Imaging and analysis. The dry membrane was imaged with the FluorChem Q imaging system at 1.7x magnification. The optical filters used are described in Table 1. The membrane was positioned on the sample holder. The exposure times for image acquisition in each channel were adjusted independently. Imaging times were 32 sec blue channel, 64 sec green channel, and 64 sec red channel.

Channel	Excitation	Emission Filter	Compatible Dyes
Blue	475/42 nm	537/35 nm	Cy2, Alexa 488, FITC
Green	534/30 nm	606/68 nm	Cy3, Alexa 546
Red	632/22 nm	699/62 nm	Cy5, Alexa 680, Alexa 647

Table 1. Spectral characteristics of FluorChem Q filter sets, and compatible fluorescent dyes.

Cell Biosciences products used:

- FluorChem Q

Other materials required:

- 8-16% linear gradient Ready Gel (Bio-Rad 161-1123)
- Immobilon-FL PVDF membrane (Millipore IPVH00010)
- ECL Advance blocking agent (GE Healthcare CPK1075)
- Goat-anti-Transferrin antibody (Abcam ab19771-1)
- Mouse-anti-AFP (Meridian Life Sciences H45301M)
- Rabbit-anti-CEA (Abcam ab15987-250)
- Alexa-Fluor-488-donkey-anti-goat IgG (Invitrogen A11055)
- Alexa-Fluor-546-donkey-anti-rabbit IgG (Invitrogen A10040)
- Alexa-Fluor-647-donkey-anti-mouse IgG (Invitrogen A31571)
- Mini PROTEAN Tetra cell (Bio-Rad 165-8004)
- Tris-Glycine running buffer
- Tris-Glycine transfer buffer
- PBS buffer
- Tween-20
- PBST buffer

Results and Discussion

The compatibility of the FluorChem Q with multicolor Western blots labeled with Alexa Fluor-conjugated fluorescent secondary antibodies was tested. A Western blot was prepared containing serial dilutions of two proteins, CEA and AFP, as well as a constant level of a third protein, Transferrin. The three proteins were imaged independently, each in a different fluorescent channel. The resulting images captured in the green channel (CEA, Figure 2b), and red channel (AFP, Figure 2c), demonstrate the large dynamic range and low limit of detection achieved for each protein.

Though each of the proteins can be imaged and analyzed independently, the images captured in each of the three fluorescent channels can be merged to allow the three proteins to be visualized simultaneously. Such a composite image depicting each of the three fluorescent channels is shown in Figure 2a. The power of multiplexing is demonstrated by this image, in which Transferrin (73-76 kDa) and AFP (70kDa) are easily distinguished.

Note that the linear range and detection limit obtained for a given Western blot experiment will depend on multiple experimental factors including the nature of the protein of interest, the efficiency of transfer of the protein from gel to membrane, the selection of the primary and secondary antibodies, and optimization of the antibody dilution factors. The results presented in this report are specific to the materials and conditions described herein.

Conclusion

The FluorChem Q is designed to image multicolor fluorescent Western blots, and provides a powerful and flexible imaging solution. This note presents a protocol for using the FluorChem Q to image multicolor Western blots hybridized with Alexa Fluor labeled antibodies. The FluorChem Q also has demonstrated compatibility with other fluorescence chemistries, including Cy dyes (Application note 117) and DyLight dyes (Application note 119).

The FluorChem Q was demonstrated to be compatible with Alexa Fluor labeled antibodies, and successfully imaged three different proteins on a single Western blot. Each protein was imaged in a different fluorescent channel, using the three illumination sources and three fluorescence

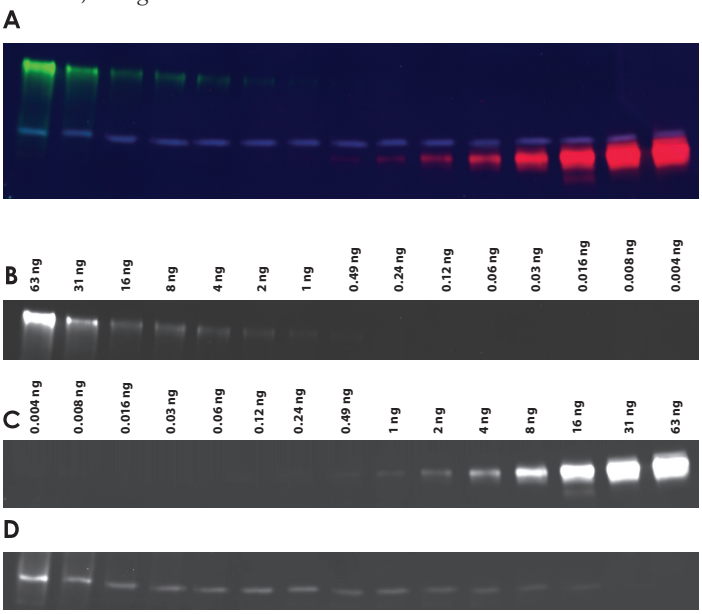

Figure 2. FluorChem Q generated image of Western probed with Alexa Fluor conjugated antibodies. (a) The composite image formed from the images acquired in three fluorescence channels is shown. (b) Image in the green channel, CEA. (c) Image in the red channel, AFP. (d) Image in the blue channel, Transferrin.

excitation and emission filter sets that are standard components of the FluorChem Q. Capturing the three images was quick, requiring less than three minutes total imaging time. The proteins were detected over a range from 120 pg to 63 ng, which encompasses protein amounts commonly found in Western blotting.

The ability to perform multiplex fluorescent analysis facilitates the quantitative analysis of protein levels on Western blots, since a loading control or reference protein can be detected at the same time as the protein of interest. The FluorChem Q provides software tools for automated image alignment and quantitative analysis of Western blot bands.

The ease and speed with which the FluorChem Q can be used to acquire images of multicolor fluorescent Westerns, and its compatibility with multiple fluorescence chemistries, including Alexa Fluor dyes, make the FluorChem Q a powerful choice in Western blot analysis.

Acknowledgement

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