Cell Biosciences™

The FluorChem® Q and Pierce DyLight™-labeled Antibodies: Multiplex Imaging Fluorescent Western Blots

Application Note 119

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 demonstrates the sensitivity and linear range that can be achieved in using the FluorChem Q with DyLight 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.

The DyLight Multiplex Fluorescent Western Blotting Kits (Pierce) provide DyLight dye-labeled secondary antibodies. The direct fluorescent detection of the antibodies allows for excellent sensitivity, linearity, and dynamic range. Additionally, direct fluorescent detection 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 fluorescent 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 DyLight 549, DyLight 649, and DyLight 488 dyes is shown in Table I.

Important to the collection of fluorescence 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 and helping prevent fluorescence quenching.

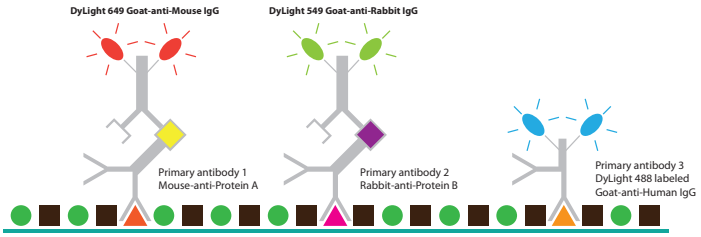

Figure 1. The concept of fluorescent western detection. Three primary antibodies are depicted, binding to three independent antigens (triangles) on a membrane. Two primary antibodies are bound by secondary antibodies that are labeled with DyLight fluorescent dyes, either DyLight 649 (red), or DyLight 549 (green). The third primary antibody has been labeled with DyLight 488 (blue).

In this article, we provide protocols for developing Western blots with the DyLight kit with the FluorChem Q. We also demonstrate the performance of the FluorChem Q in detecting and quantifying multicolor Western blots labeled with DyLight fluorescent antibodies.

Methods

Electrophoresis and transfer. Proteins were separated on 10-20% polyacrylamide Tris-HCl gels.

All samples were loaded in equal volumes (10 µL). Each lane contained 30 ng of Human IgG, corresponding to approximately 20 ng of heavy chain. Two-fold serial dilutions of Transferrin, and of α_1-fetoprotein (AFP), were loaded on the gel as follows:

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

Separation was carried out at 150 V for one hour, using a standard Tris-Glycine-SDS buffer system in the Mini-PROTEAN Tetra Cell from Bio-Rad.

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.

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 a mixture of primary antibodies for one hour at room temperature.

The primary antibodies were Rabbit-anti-Transferrin and Mouse-anti-AFP antibodies, each diluted 1:5000 in PBST.

The membrane was subjected to 2 quick rinses, a 15 minute rinse and four 5 minute washes all in PBST.

The membrane was then incubated with a mixture of fluorescently labeled secondary antibodies for one hour at room temperature.

The secondary antibodies were DyLight 649-Goat-anti Mouse IgG, and DyLight 549-Goat-anti-Rabbit IgG diluted 1:2500. DyLight 488-Goat-anti-Human IgG was also added, diluted 1:2500. This antibody was prepared from non labeled antibody using DyLight 488 NHS Ester according to the manufacturer's instructions.

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. The exposure time in the red channel was 16 seconds, in the green channel was 32 seconds, and in the blue channel was 8 seconds.

Channel	Excitation	Emission Filter	Compatible Dyes
Blue	475/42 nm	537/35 nm	Cy2, DyLight488, FITC
Green	534/30 nm	606/68 nm	Cy3, DyLight 549
Red	632/22 nm	699/62 nm	Cy5, DyLight 649

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

Cell Biosciences products used:

- FluorChem Q

Other materials required:

- 10-20% linear gradient Ready Gel (Bio-Rad 161-1124)
- Immobilon-FL PVDF membrane (Millipore IPVH00010)
- ECL Advance blocking agent (GE Healthcare CPK1075)
- Rabbit-anti-Transferrin antibody (Abcam ab1223-1)
- Mouse-anti-AFP (Meridian Life Sciences H45301M)
- DyLight649-goat-anti-mouse IgG (Pierce 22854)
- DyLight549-goat-anti-rabbit IgG (Pierce 22854)
- Goat-anti-Human IgG (Vector Labs AI-3000)
- DyLight488 NHS Ester (Pierce 46403)
- ECL Advance blocking agent (GE Healthcare CPK1075)
- 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 and DyLight-labeled antibodies when imaging multicolor Western blots was tested by preparing a Western blot containing serial dilutions of two proteins, Transferrin and α_1-Fetoprotein (AFP), as well as a constant level of a third protein, Human IgG. The three proteins were imaged independently, each in a different fluorescent channel. The three color image was captured with a total exposure time of 56 seconds.

Though each of the proteins can be viewed 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 merged 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. Single channel images are also shown for AFP (Figure 2b), Transferrin (Figure 2c) and Human IgG (Figure 2d).

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.

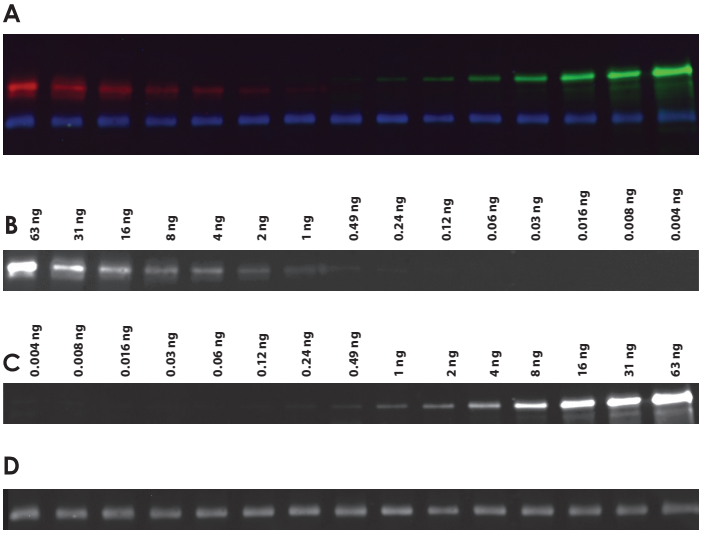

Figure 2. FluorChem Q generated image of DyLight labeled Western blot. (a) The composite image formed from the images acquired in the three fluorescence channels is shown. (b) Red channel: α1-Fetoprotein. (c) Green channel: Transferrin. (d) Blue channel: Human IgG.

Conclusion

The ability to detect multiple proteins on a single Western blot provides tools for accurate protein quantification, with a minimization of time and sample usage.

The FluorChem Q provides a powerful means to image and quantify multicolor fluorescent Western blots using DyLight labeled antibodies. The protocols used in these experiments proved to be compatible for developing and imaging Westerns blots with the DyLight dyes and the FluorChem Q. The FluorChem Q also performed in multiplex analysis, acquiring images of three proteins, detected with three unique fluorophores on a single blot.

Acknowledgement

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