Cell Biosciences™

The FluorChem Q® and ECL Plex™: Sensitive and Quantitative Imaging of Multicolor Fluorescent Westerns

Application Note 117

Abstract

Multiplex fluorescent detection allows the detection and analysis of multiple proteins 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 which can be achieved in using the FluorChem Q with ECL Plex labeled Westerns, and the compatibility of the FluorChem Q with multicolor Western blots.

Introduction

Multiplex analysis, or multiple fluorescent channel imaging, allows for the simultaneous analysis of multiple RNA samples on a microarray, or multiple antigen locations in a cell via fluorescence microscopy. Recently, this technology has become available for Western blotting, a powerful tool for the protein chemist.

The most commonly used detection method for Western blotting, chemiluminescence, is a well-characterized technique for sensitive detection of proteins (1). However, this method can present limitations in applications where multiple proteins are of interest. If two proteins of interest are similar in size and migrate closely together on a gel, as is the case for phosphorylated isoforms, only one protein can be assayed at a time. The blot must be stripped and reprobed independently for each protein, which is time-consuming and may result in protein loss. Alternatively, duplicate blots may be run, which consumes precious sample.

When quantifying a protein across multiple samples, a housekeeping or reference protein is used to control for differences in gel loading. Frequently this also requires stripping and reprobing of the chemiluminescent Western blot, unless the reference protein is well differentiated in size from the protein of interest. If the housekeeping protein and the protein of interest are probed simultaneously on a chemiluminescent blot, and the proteins have very different abundances, a large difference in signal strength between the two proteins may result. Under such conditions, multiple exposures of the Western blot to film may be required to identify conditions under which one signal is not saturated. Direct fluorescent labeling of the secondary antibodies, combined with fluorescent light detection, overcomes these limitations. Multiple proteins can be assayed on one blot, regardless as to whether they overlap or are in different relative abundance.

The ECL Plex Western Blotting Detection System (GE Healthcare) uses CyDye-conjugated secondary antibodies. The direct fluorescent detection of the antibodies allows for excellent sensitivity, linearity, and dynamic range (2). 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 (Figure 1). 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.

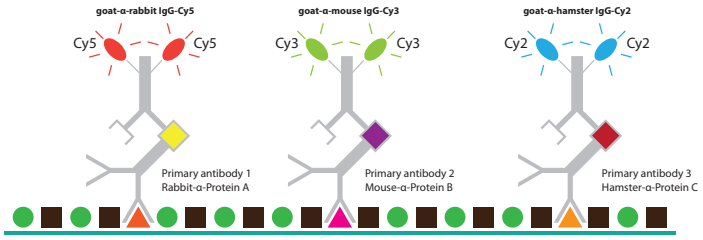

Figure 1. The concept of fluorescent western detection. Three primary antibodies are depicted, binding to three independent antigens (triangles) on a membrane. Each primary antibody is bound by a secondary antibody that has been directly labeled with a CyDye, Cy5 (red), Cy3 (green) or Cy2 (blue).

The FluorChem Q imaging system is designed to acquire high quality images from fluorescently labeled, multicolor westerns. Three excitation 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 Cy2, Cy3 and Cy5 dyes used in the ECL Plex system 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 fixed lens provides fast image acquisition, shortening analysis time and helping prevent fluorescence bleaching.

In this article, we demonstrate the performance of the FluorChem Q in detecting and quantifying multicolor Western blots labeled with the ECL Plex fluorescent detection system. The linearity, dynamic range, and detection limit of Transferrin detected with a Cy5 labeled antibody are presented, and the ability to image three proteins on a single blot using three different fluorescent channels is demonstrated.

Methods

Electrophoresis and transfer. Proteins were separated on 10-20% polyacrylamide Tris-HCl gels (Bio-Rad 161-1124), using a standard Tris-Glycine-SDS buffer system in the Mini-PROTEAN Tetra Cell from Bio-Rad. Transfers were conducted in Tris-Glycine buffer containing 12% methanol.

Experiment 1: Two-fold serial dilutions of Transferrin were prepared, spanning the range from 0.6 pg to 9.3 ng, and loaded on the gel in a

final volume of 10 µL. Electrophoresis was carried out at 90 V for two hours. After separation, proteins were transferred to PVDF membrane (Immobilon-FL, Millipore IPVH00010) overnight at 15V.

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 1:10 dilution of BSA blocker (Pierce 18587) as per manufacturers instructions.

The blot was incubated with the primary antibody, Rabbit-anti-Transferrin (Abcam ab1223-1) at 5 µg/mL for two hours at room temperature.

The blot was washed five times with Pierce wash buffer (1860498) diluted 1:30 as per manufacturers instructions.

The blot was incubated one hour at room temperature with the secondary antibody, Cy5-labeled Goat-anti-Rabbit IgG (GE Healthcare PA45012V) diluted 1:2500 in wash buffer.

The blot was then washed six times in Pierce wash buffer for five minutes each. After a final rinse in PBS, the blot was imaged while still damp, approximately one hour after the PBS rinse. The blot was placed on a low-fluorescent plastic sheet on the sample holder for imaging.

Experiment 2: 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 α1-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,

After separation, proteins were transferred to a PVDF membrane (Immobilon-FL, Millipore IPVH00010). Transfer was conducted at 100 V for one hour with an ice pack inserted in the tank.

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 (GE Healthcare CPK1075) dissolved in PBST.

After blocking, the membrane was washed with PBST for 5 minutes and incubated with a mixture of primary antibodies, containing Rabbit-anti-Transferrin (Abcam ab1223-1) and Mouse-anti-AFP (Meridian Life Sciences H45301M) 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 a mixture of fluorescently labeled secondary antibodies for one hour at room temperature. The secondary antibodies used were Cy3-Goat-anti-Mouse IgG at 1:2500 dilution, and Cy5-Goat-anti-Rabbit IgG at 1:2500 dilution (both from GE Healthcare). A Cy2-Goat-anti-Human IgG antibody was also added at 1:2500 dilution, to detect Human IgG directly. The Cy2-Goat-anti-Human IgG antibody was prepared from non-labeled antibody (Vector Labs AI-3000) using the Cy2 labeling kit from GE (GE Healthcare PA32000).

After hybridization, 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.

The dry membrane was imaged with the FluorChem Q imaging system at 1.7x magnification. The membrane was positioned on the sample holder.

Single channel images of each of the three fluorescent dyes were acquired on the FluorChem Q. The spectral characteristics of the filter sets used are shown in Table 1. The exposure times were adjusted manually for each of the three channels.

Channel	Excitation	Emission Filter	Compatible Dyes
Blue	475/42 nm	537/35 nm	Cy2, Alexa 488, FITC
Green	534/30 nm	614/62 nm	Cy3, Alexa 546
Red	632/22 nm	699/62 nm	Cy5, Alexa 680

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)
- BSA blocker (Pierce 1858753)

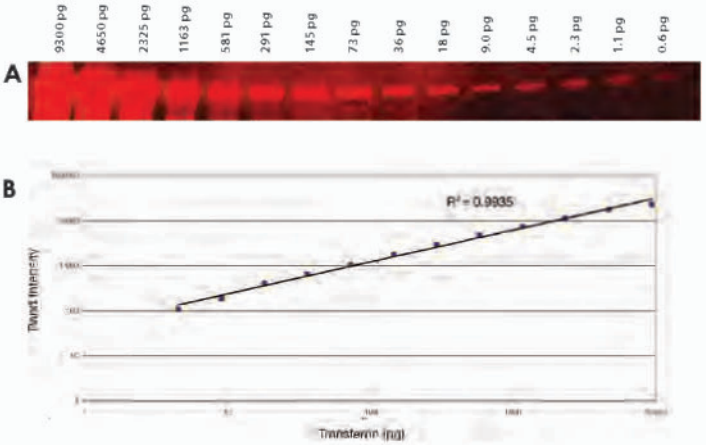

Figure 2. Serial dilutions of Transferrin detected by Western blot and imaged on the FluorChem Q. (a) Image acquired with a 16 second exposure. A lower limit of 1.1 pg Transferrin was detected, and the fluorescence intensity was linear from 4.5 pg to 9300 pg (b), providing a dynamic range over 3.3 orders of magnitude.

- Pierce wash buffer (Pierce 1860498)
- Rabbit-anti-Transferrin antibody (Abcam ab1223-1)
- Mouse-anti-AFP (Meridian Life Sciences H45301M)
- ECL Plex goat-α -mouse IgG-Cy3 (GE Healthcare PA43009)
- ECL Plex goat- α -rabbit IgG-Cy5 (GE Healthcare PA45011)
- Goat-anti-Human IgG (Vector Labs AI-3000)
- Cy2 Ab Labeling Kit (GE Healthcare PA32000)
- 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
- PBST buffer

Results and Discussion

Experiment 1: The performance of the FluorChem Q was tested by preparing a Western blot of a dilution series of Transferrin, a protein normally present in Human serum. An image of the Western blot, acquired on the FluorChem Q with a 16 second exposure is shown in Figure 2. A measurable linear dynamic range of over 2000-fold was achieved (Figure 2). Exposing a duplicate blot for 256 seconds gave a lower limit of detection of 0.6 pg (Figure 3)

Experiment 2: The compatibility of the FluorChem Q and ECL Plex fluorescent western blotting system when imaging multicolor Western blots was tested by preparing a Western blot containing serial dilutions of two proteins, Transferrin and AFP, as well as a constant level of a third protein, Human IgG. The three proteins were imaged in one imaging protocol, each in a different fluorescent channel. Though each of the proteins can be imaged 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 4a. The power of multiplexing is demonstrated by this image, in which Transferrin (73-76 kDa) and AFP (70kDa) are easily distinguished.

The single channel images captured in the red channel (Transferrin, Figure 4b), and green channel (AFP, Figure 4c), demonstrate the large dynamic range and low limit of detection achieved for each protein and dye combination in a true Western blot experiment. The blue channel, IgG, can be used in later analysis as a loading control (Figure 4d).

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 hybridization conditions. The results presented in this report are specific to the materials and conditions described herein.

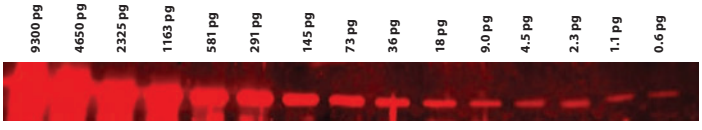

Figure 3. A lower limit of detection of 0.6 pg Transferrin was achieved with a 256 second exposure time.

Conclusion

The FluorChem Q provides a powerful means to image and quantify multicolor fluorescent Western blots labeled using the ECL Plex system. The sensitivity of the system is reflected in a detection limit of 0.6 pg of Transferrin. With an optimized exposure time, the fluorescence intensity of Transferrin staining was linear over three orders of magnitude, covering a range of protein amounts from 4.5 pg to 9.6 ng, which encompasses the range commonly seen in Western blotting.

The FluorChem Q also performed in multiplex analysis, acquiring images of three proteins, detected with three unique fluorophores on a single blot.

The linearity of the response over a large dynamic range, combined with the ability to detect multiple proteins simultaneously on the same blot, demonstrates the potential for accurate protein quantification, with a minimization of time and sample usage.

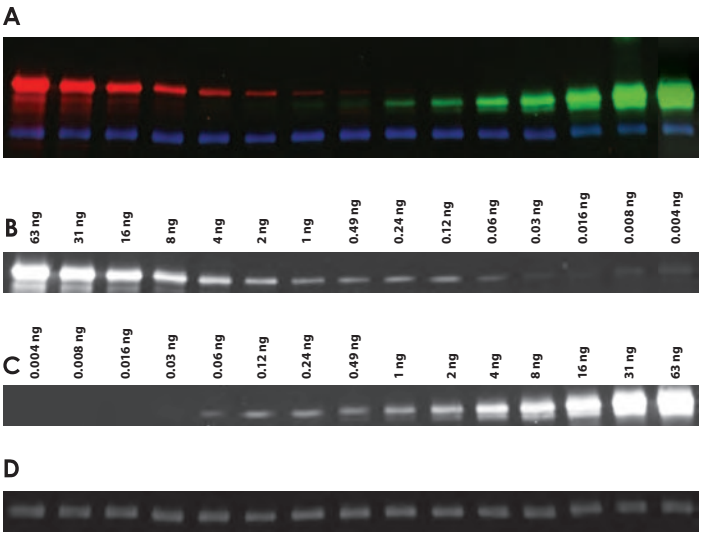

Figure 4. FluorChem Q generated image of ECL Plex labeled western. The Western blot was probed with three different fluorescent secondary antibodies. Panel (a) shows the composite 3-color image, which is 16 bit in all three color channels. Blue: Human IgG. Red: Transferrin. Green: a1-Fetoprotein.Single channel images are shown for (b) the red channel, detecting Transferrin, and (c) the green channel, detecting a1-Fetoprotein and (d) the blue channel detecting IgG.

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.
2. GE Healthcare Application Note 28-4015-40 AB "Multiplex protein detection using the ECL Plex fluorescent Western blotting system"

Acknowledgement

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The FluorChem Q Imaging System provides the sensitivity of chemiluminescence and the quantitative power of fluorescence in one easy-to-use instrument. Designed with fast lens technology, the peltier cooled camera captures high-resolution images with a linear dynamic range that outperforms film, and with speeds 10X faster than a laser scanner. Equipped with three integrated excitation sources for multicolor westerns, the FluorChem Q is compatible with Cy dyes, Alexa dyes, Qdots, and chemiluminescent western blotting kits. Additionally, the FluorChem Q provides the flexibility to image fluorescently stained DNA gels, as well as Coomassie and silver stained protein gels.

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