Cell Biosciences™

SpectraPlex™ Western Blot Kit and the FluroChem® Q Provide a Complete Solution for Multicolor Fluorescent Western Blotting

Application Note 123

Abstract

SpectraPlex is a kit for fluorescent Western blotting, developed for the FluorChem Q imaging system. This application note demonstrates the superior sensitivity and fast imaging times obtained with SpectraPlex and the FluorChem Q, which together provide a complete solution for fluorescent Western blotting. SpectraPlex is shown to be more sensitive than competitor fluorescent antibodies, both those that fluoresce in the visible as well as those that fluoresce in the near IR spectrum. SpectraPlex allows detection of low picogram levels of protein with both a traditional scanner and the FluorChem Q, but imaging times on the FluorChem Q are 25-times faster. Together, SpectraPlex and the FluorChem Q provide quick, sensitive, and quantitative imaging of multicolor Western blots.

Introduction

Western blotting is commonly used by biologists to confirm the presence of a protein of interest in a sample, and to detect changes in the level of a protein of interest between samples. The most widespread method of imaging Western blots has been the use of secondary antibodies labeled with the enzyme horseradish peroxidase (HRP), with the chemiluminescent signal detected using x-ray film. Chemiluminescence is sensitive, but only allows the detection of a single protein per Western blot experiment unless the proteins of interest are spatially well resolved. In many cases, the blot must be stripped and re-probed for each protein, which is time-consuming and could introduce artifacts if the stripping is uneven. Therefore, the use of fluorescence detection is becoming more prevalent in Western blotting.

With fluorescent Western blotting, it is possible to detect two or more proteins on a single Western blot. Multiplex blots are conducted by choosing secondary antibodies labeled with fluorophores having non-overlapping excitation and emission spectra (Figure 1). A loading control can be assayed simultaneously with a protein of interest, increasing the quantitative nature of the data that can be obtained. Additionally, proteins that migrate closely together, such as phosphorylated isoforms, can be assayed simultaneously. With fluorescence, more information can be obtained from a single experiment.

SpectraPlex is a kit for fluorescent Western blotting, developed for the FluorChem Q imaging system. The SpectraPlex antibodies are conjugated to fluorescent proteins, the phycobiliproteins, which are isolated from algae and cyanobacteria. Phycobiliproteins are among the brightest fluorophores known, having very high quantum efficiencies. In addition to being brighter than synthetic dyes, the phycobiliproteins are protected from quenching, are water soluble, and are stable over a high pH range, making them perfect reporters for fluorescent Western blotting. The phycobiliproteins used in SpectraPlex have excitation and emission spectra compatible with common fluorescence instrumentation (Figure 2).

In this application note, the superior sensitivity available through the SpectraPlex kit is demonstrated, as well as the faster imaging that is

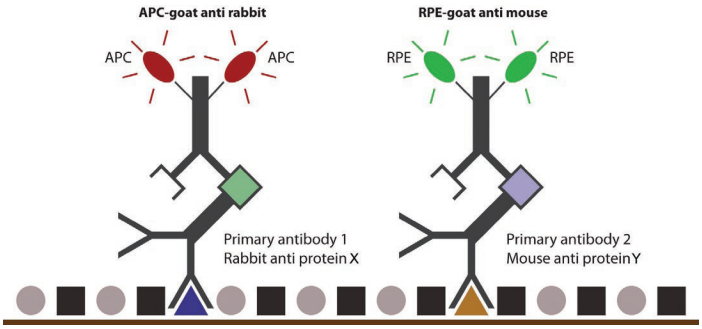


Figure 1: The principle of two-color fluorescent Western blotting. Two secondary antibodies having non-overlapping excitation and emission spectra are used to detect two different primary antibodies. The blot can be incubated with both secondary antibodies at once, and then each can be imaged in its own fluorescent channel.

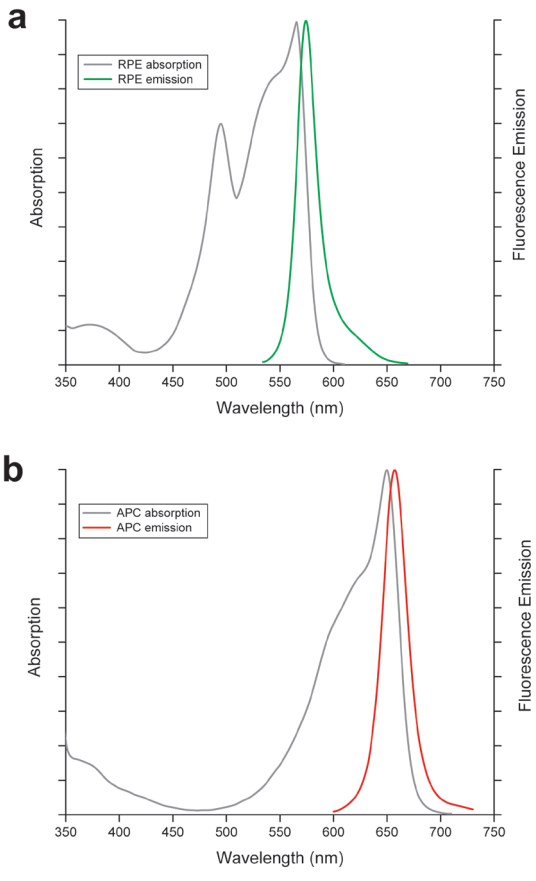


Figure 2: Excitation and emission spectra of the SpectraPlex fluorophores. The excitation (grey) and emission (green or red) spectra of RPE (a) and APC (b) are shown. The excitation and emission maxima are similar to those for the commonly used dyes Cy3 and Cy5, and are compatible with common fluorescence detection systems.

possible with the FluorChem Q as compared to traditional laser scanners.

Methods

Limit of Detection, SpectraPlex. A serial dilution of transferrin was made and separated on a 10% SDS-PAGE gel. A carrier protein with no known reactivity with the antibodies used was included in the transferrin dilutions to ensure no protein loss due to adsorption. Electrophoresis was carried out at 30 V for 30 minutes, and then at 120 V for 1 more hour. After separation, the protein was transferred to a low-autofluorescence membrane (Immobilon-FL) using a standard tank protocol.

Subsequent antibody incubations and washes were carried out according to the SpectraPlex protocol. Briefly, the membrane was incubated for one hour in primary antibody (rabbit anti-transferrin, Abcam) diluted 1:5000 in SpectraPlex Blocking Solution. The membrane was washed in SpectraPlex Washing Solution, and then incubated for one hour with APC-goat anti-rabbit secondary antibody diluted 1:2000 in SpectraPlex Blocking Solution. The blot was imaged after a final wash with SpectraPlex Washing Solution.

Comparison of sensitivity of the FluorChem Q and Typhoon imaging systems. The gel used in the limit of detection assay was dried and re-imaged 10 days later with both the FluorChem Q and the Typhoon Trio™ (GE Healthcare). The stability of the SpectraPlex fluorescent signal can be obtained when the SpectraPlex protocol is followed, and is dependant on using the SpectraPlex blocking solution.

Limit of Detection, IRDye™-680. A serial dilution of transferrin was made and separated on a 8-16% SDS-PAGE gel (Bio-Rad). Electrophoresis was carried out using a standard Tris-Glycine-SDS system at 90 V for 3 hours. After separation, the protein was transferred to a low-autofluorescence membrane (Immobilon-FL) using a standard tank transfer procedure.

The membrane was rinsed two times with PBS containing 0.05% Tween-20 (PBST) for 5 minutes, then blocked with a 2% solution of ECL Advance blocking agent (GE Healthcare) for one hour at room temperature. The membrane was washed with PBST for 5 minutes, and then incubated for one hour with primary antibody (rabbit-anti-transferrin, Abcam) diluted 1:5000. The membrane was washed two times with PBST, once more for 15 minutes, and four times for 5 minutes each. The membrane was then incubated with the secondary antibody (goat anti-rabbit IRDye-680, LI-COR) for one hour. The antibody was reconstituted according to the manufacturer's instructions and used at a dilution of 1:2500.

The membrane was washed three times for 5 minutes each with PBST, one time with PBS for 5 minutes, and then briefly rinsed with water and dried at room temperature before imaging. The blot was then imaged on the LI-COR Odyssey with an 8-minute scan.

Comparison of SpectraPlex to CyDye™ Labeled Secondary Antibodies. Samples of AFP and CEA were separated on 12% polyacrylamide gels, and transferred to PVDF membrane. The protein amounts in each lane were:

Lane	1	2	3	4	5	6	7
CEA (ng)	–	–	0.4	1.1	3.3	10	30
AFP (ng)	–	8.3	50	17	5.5	1.9	0.6
MW marker	+	–	–	–	–	–	–

Two identical blots were probed with the same mixture of mouse-anti-AFP and rabbit-anti-CEA antibodies, each diluted 1:5000. Then, one blot was stained with a mixture of Cy3-anti-mouse and Cy5-anti-rabbit secondary antibodies (diluted 1:2500), while the other was stained with RPE-anti-mouse and AP-anti-rabbit secondary antibodies (diluted to have the same molar concentration as the CyDye-labeled antibodies). Both blots were imaged on the FluorChem Q one hour after the final wash. The exposure time for the red channel was 8 seconds, and for the green channel was 2 seconds for both blots.

Cell Biosciences materials used:

- SpectraPlex Western Blot Kit (Cell Biosciences 60-14242-00)
- FluorChem Q (Cell Biosciences 92-14095-00)

Other materials used:

- 10%, 12%, and 8-16% polyacrylamide minigels (Bio-Rad 161-1119, 161-1120, 161-1223)
- Immobilon-FL PVDF membrane (Millipore IPFL00010)
- Rabbit-anti-transferrin antibody (Abcam ab11223-1)
- Goat anti-rabbit IRDye-680 (LI-COR 926-32221)
- Rabbit-anti-CEA (Abcam ab15987-250)
- Mouse-anti-AFP (Meridian Life Sciences H45301M)
- Cy3-goat-anti-mouse IgG (GE Healthcare PA43009)
- Cy5-goat-anti-rabbit IgG (GE Healthcare PA45011)
- ECL Advance blocking agent (GE Healthcare CPK1075)
- Odyssey, LI-COR
- Typhoon Trio, GE Healthcare

Results

The SpectraPlex Western blot kit provides high sensitivity detection. Figure 3 shows a Western blot containing a serial dilution of transferrin, detected with rabbit-anti-transferrin primary antibody and SpectraPlex APC-goat-anti-rabbit secondary antibody. As little as 1.1 pg of transferrin could be detected, with imaging times as short as 3 seconds.

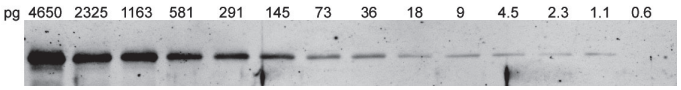


Figure 3: Sensitivity of the SpectraPlex system imaged with the FluorChem Q. A serial dilution of transferrin was blotted and detected using the APC-goat-anti-rabbit antibody from the SpectraPlex kit. As little as 1.1 pg of transferrin was detected.

In contrast, only 15 pg of transferrin was detected using an IRDye-680 conjugated antibody, imaged with an 8-minute scan on the Odyssey imaging system from LI-COR (Figure 4).

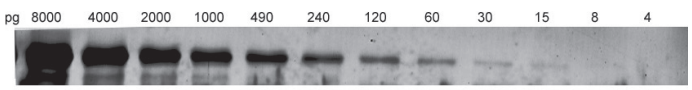


Figure 4: Sensitivity of near IR detection imaged with a laser scanning system. A serial dilution of transferrin was blotted and detected using the IRDye-680-goat-anti-rabbit antibody from LI-COR and detected with an 8 minute imaging scan on the LI-COR Odyssey instrument. 15 pg of transferrin was detected.

The blot depicted in Figure 3 was dried completely, and imaged 10 days later on the FluorChem Q (a CCD imaging system) and the Typhoon

(GE Healthcare, a laser scanner system). The 1.1 pg band was visible with a 5-minute scan on the Typhoon system (Figure 5b). A similar image, with the same detection limit, could be obtained using the FluorChem Q in only 12 seconds (Figure 5a), 25 times faster.

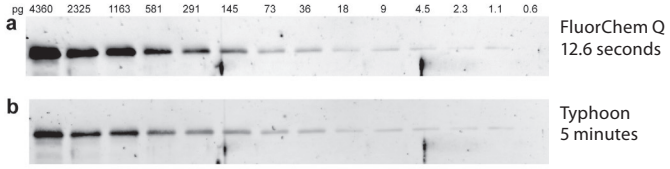
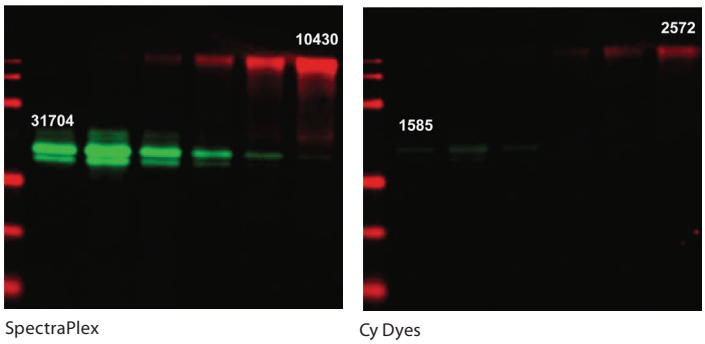


Figure 5: The FluorChem Q outperforms laser scanners. The same Western blot containing a serial dilution of transferrin was imaged on either the FluorChem Q for 12 seconds (a) or the Typhoon laser scanner for 5 minutes (b). The same limit of detection, 1.1 pg, was obtained by the two systems, but the FluorChem Q image was obtained 25-times faster.

A significant advantage of fluorescent Western blotting is the ability to assay more than one protein per blot. Figure 6a shows a two-color blot detected using SpectraPlex. Imaging a replicate blot using fluorescent antibodies labeled with Cy Dyes (Figure 6b) results in an image four-times less bright in the red channel, and 20-times less bright in the green channel, than the SpectraPlex blot when imaging times are identical.



SpectraPlex Cy Dyes

Figure 6: SpectraPlex outshines the competition. Replicate Western blots were incubated with the same primary antibodies, and then detected using either SpectraPlex (a) or Cy Dye-labeled secondary antibodies (b). Representative band densities are shown. SpectraPlex was four times brighter in the red channel, and 20 times brighter in the green channel, under identical imaging times (8 seconds red channel, 2 secconds green chanel) and conditions.

The SpectraPlex kit provides all the reagents needed to conduct two-color fluorescent Western blots, and has been designed to work with the FluorChem Q imaging system. Superior results are obtained with respect to both sensitivity and speed relative to other systems for imaging fluorescent Westerns.

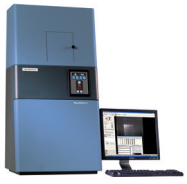
SpectraPlex provides very high sensitivity; as little as 1.1 pg of protein can be detected (Figure 3) using SpectraPlex secondary antibodies. High sensitivity is possible because the SpectraPlex secondary antibodies are conjugated to extremely bright phycobiliprotein fluorophores. Due to high fluorescence intensity and low background, SpectraPlex imaged with the FluorChem Q outperforms near-IR fluorescing secondary antibodies imaged with a laser scanner (Figure 4), with which only 15 pg of transferrin could be detected. Near IR fluorophores are often thought to provide a cleaner signal, due to less light scatter and less autofluorescence in the near IR part relative to the visible part of the spectrum. However, with the optimized protocol developed for SpectraPlex, there is no advantage to using near IR fluorescence with a laser scanner to develop Western blots. SpectraPlex also outperforms competitor secondary antibodies that fluoresce in the same region of the visible spectrum (Figure 6), with signals 4 to 20-times brighter than those obtained with Cy5 or Cy3.

SpectraPlex is compatible with common instrumentation for fluorescence detection, including laser scanners (Figure 5). However, SpectraPlex has been optimized for CDD imagers, giving the same limit of detection but with much faster imaging times. An image comparable to the image captured by a 5-minute scan on a laser scanner system (Figure 5b) could be captured 25 times faster, in only 12 seconds, on the FluorChem Q (Figure 5a).

Two-color Western blots substantially increase the power of the Western blotting technique. With two-color fluorescent blots, it is possible to measure a loading control at the same time as a protein of interest, or to measure different phosphorylated isoforms of a protein on the same blot. This increases confidence in the quantitative analysis of Western blot data, and saves time and money by removing the need to sequentially strip and re-probe a blot for each protein of interest, as is necessary with chemiluminescent Western blotting. SpectraPlex provides clean, two-color fluorescent blots (Figure 6a) with no cross-talk between the fluorescent channels.

SpectraPlex, the FluorChem Q and AlphaView® Q image acquisition and analysis software combine to provide a complete solution from Cell Biosciences for multicolor fluorescent Western blotting, designed to work together with streamlined blotting and analysis protocols to ensure success. With SpectraPlex and the FluorChem Q, fluorescent Western blotting is faster and more sensitive than with traditional laser based systems.

Cell Biosciences gratefully acknowledges the researchers at Advansta Corporation in Menlo Park, California, for the information contained in this Application Note.



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

SpectraPlex is a complete system for multicolor fluorescent Western blots, developed for the FluorChem Q. The kit includes all the reagents needed to conduct two-color fluorescent blots using mouse and/or rabbit primary antibodies. A carefully optimized protocol ensures success.

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