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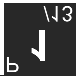

Flexible Method for Imaging Fluorescent Western Blot

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Latest advances in fluorescent dye technology have increased the detection capabilities and raised the sensitivity levels in Western blotting. In contrast to traditional detection methods such as colorimetric and chemiluminescent detection, the newer fluorescent dyes such as DyLights and Cy dyes offer many benefits when conjugated to secondary antibodies. These methods are not suitable for multiplex analysis as they generate a white chemiluminescent emission or are in only one color.

Moreover, in these methods, a series of Western blots need to be produced and compared. This process is not only time-intensive but is also expensive in terms of blots and reagents used. Therefore, users are unable to differentiate between different proteins, especially when they are very close together or have the same molecular weight.

These difficulties can be overcome by using the correct lighting and filter conditions enabling the detection of the different colors emitted by the new fluorescent labels. A blot image is generated by digitally overlaying the images produced, enabling simultaneous detection of several proteins on one blot. With just a one-step image capture of the fluorescent signal, simple and direct imaging of fluorescent-labeled blots is enabled.

Additionally, the extraordinary photostability of the Cy dye and DyLight conjugates allows them to be visualized several times without a drop in signal. The need for film and the excessive time spent analyzing images of different exposures and other drawbacks of performing chemiluminescence are eliminated. Finally, the cost of employing fluorescent reagents is approximately only 10% of the cost of chemiluminescent substrates in Western blotting.

A unique detection system is enabled when these dyes are combined with KPL antibodies to yield exceptional results when analyzed with G:BOX imagers. Using the dyes, the scientists can generate optimal images of each dye and visualize multiplex images of fluorescently-labeled Western blots. This article describes the optimal conditions when several advanced fluorescent conjugates

are used for Western blotting.

G:BOX iChemi – a flexible method for imaging fluorescent Westerns

Syngene has developed the G:BOX iChemi (see Figure 1) for accurate visualization of fluorescently-labeled Western blots. The G:BOX iChemi is an affordable range of CCD-based analyzers that can be used for chemiluminescence and fluorescence imaging.

Learn more about the G:Box Gel Imaging System

It is equipped with a high quality digital camera inside a light tight cabinet and can be fitted with EPI-UV, EPI RGB, or IR lighting modules, and band-pass filters matched to a large range of fluorochromes. Even the faintest fluorescent signals can be visualized by combining high resolution cameras with correct lighting and filters.

Figure 1. Syngene's G:BOX iChemi XR image analyzer.

The GeneSnap software of the G:BOX iChemi allows users to automatically overlay all the different images of each color to produce a single blot image, thus simultaneously showing the different colors.

The GeneTools software of the system enables users to analyze images to determine the molecular weight of proteins and quantify protein amounts. These features make the G:BOX iChemi an economical system for imaging fluorescently-labeled Western blots.

To show the exceptional quality and imaging range achievable by a G:BOX iChemi system, the optimal imaging conditions of common fluorescent dyes were determined using a G:BOX iChemi XR system at KPL, a major reagent manufacturer situated in Gaithersburg, USA.

Method

Fluorescent labeling of dot blots

1 µL of each of the nine dyes conjugated to a Goat anti-Human antibody (KPL, Gaithersburg, MD, USA) were dotted four times onto a nitrocellulose membrane: DyLight™ 405, DyLight™ 448, DyLight™ 549, DyLight™ 594, DyLight™ 633, DyLight™ 649, DyLight™ 680 and DyLight™ 800 (ThermoFisher Scientific, Rockford, IL, USA) and Cy3 (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). The blots were allowed to dry for 10 minutes.

Imaging fluorescent dot blots to determine optimum conditions

The dot blot was loaded into the G:BOX iChemi XR darkroom in order to identify the optimum lighting and filter combination for imaging the fluorescent dyes. One of the below conditions was used to image the blot:

- White light without any filter
- EPI-Red lighting and an FRLP filter
- EPI-Blue lighting and SWSP filter
- Long wave (LW)-UV and a short wave short pass (SWSP) filter

All images were produced by setting the G:BOX iChemi XR's GeneSnap software "series capture" to 30 seconds.

Generating fluorescently labeled Western blots

Four SDS-PAGE gels were run, loaded with seven dilutions of Human IgG

(Lampire, Pipersville, USA) (500 ng, 250 ng, 125 ng, 62.5 ng, 31.25 ng, 15.6 ng, and 7.8 ng). The gels were also loaded with a set of pre-stained protein markers (Blue Protein Molecular Weight Marker, ThermoFisher, Waltham, MA).

A standard electroblotting method was used to transfer the proteins from acrylamide gels onto nitrocellulose membranes. Each membrane was incubated overnight in 1X Detector Block solution (KPL, Cat. No. 71-83-00) with 1% Detector Block Powder. The blots were incubated in 1X Detector Block at 25 ºC for one hour with secondary antibodies labeled with the following dyes:

- DyLight™ 549 and DyLight™ 649 which were applied to blots one and two
- Cy3 and Cy5 (GE Healthcare Life Sciences) which were applied to the third and fourth blots, respectively

Imaging fluorescent Western blots to determine sensitivity

To determine the sensitivity of different lighting and filter combinations, the Western blot membranes were loaded into the G:BOX iChemi XR darkroom for imaging. One of the following conditions was used to image the blots:

- EPI-RED lighting and the FRLP filter for DyLight 649 and Cy5
- EPI-Blue lighting and the EtBr filter for DyLight 549 and Cy3

Each image was captured by setting the G:BOX iChemi's GeneSnap software "capture series" to 30 seconds.

Generating a multiplex labeled Western blot

An SDS-PAGE gel was run, loaded with seven dilutions of Mouse IgG protein (Lampire) (500 ng, 250 ng, 125 ng, 62.5 ng, 31.25 ng, 15.6 ng, and 7.8 ng) and Goat IgG Fab fragment (500 ng, 250 ng, 125 ng, 62.5 ng, 31.25 ng, 15.6 ng, and 7.8 ng (KPL).

A standard electroblotting method was used to transfer the proteins from acrylamide gels onto nitrocellulose filters. The membrane was incubated overnight in 1X Detector Block solution (KPL, Cat. No. 71-83-00) containing 1% Detector Block Powder.

The blot was incubated at 25 ºC for one hour in 1X Detector Block consisting of

a DyLight 680 labeled rabbit anti-goat antibody and DyLight 800 labeled goat anti-mouse antibody from KPL in accordance with the manufacturer's instructions.

Imaging multiplex fluorescent blots to determine dye applications

The membrane was imaged using one of the following conditions after placing it inside the G:BOX iChemi XR darkroom:

- EPI-Red lighting and FRLP filter
- EPI-IR lighting and an IR 780 filter

Each image was generated by setting the G:BOX iChemi's GeneSnap software "capture series" to 30 seconds. After selecting the best image of each color, a composite image was created by overlaying the different colored images.

Results and discussion

Determining optimum imaging conditions

Figure 2 displays the image generated by the G:BOX iChemi of the dot blots, revealing that the DyLight 549, Cy3, and DyLight 594 can be seen in the white light but are difficult to see on white background. However, it is not recommended to use white light for imaging any of these dyes.

The FRLP filter and the EPI-Red lighting combination provides good images of the DyLight 594, 633, 649 and 680 dyes owing to the generation of a very distinct, sharp signal by the light emitted that is free from any bleed effects or halos of light around the protein dots.

The SWSP filter and the LW-UV light combination produces good images of DyLight 405, 488, Cy3, and DyLight 549. However, the signal is not discrete with the DyLight 594 and a slight halo of light around the dot is visible.

Therefore, the FRLP filter and EPI-Red light combination is preferred for imaging DyLight 594. The combination of the EPI-Blue lighting and SWSP filter provides stronger signal with DyLight 549 and Cy3. However, the signal is not discrete with DyLight 488 and major light bleed effects can be seen around each dot. Thus, the FRLP and the EPI-Red light combination is preferred option

for imaging this dye.

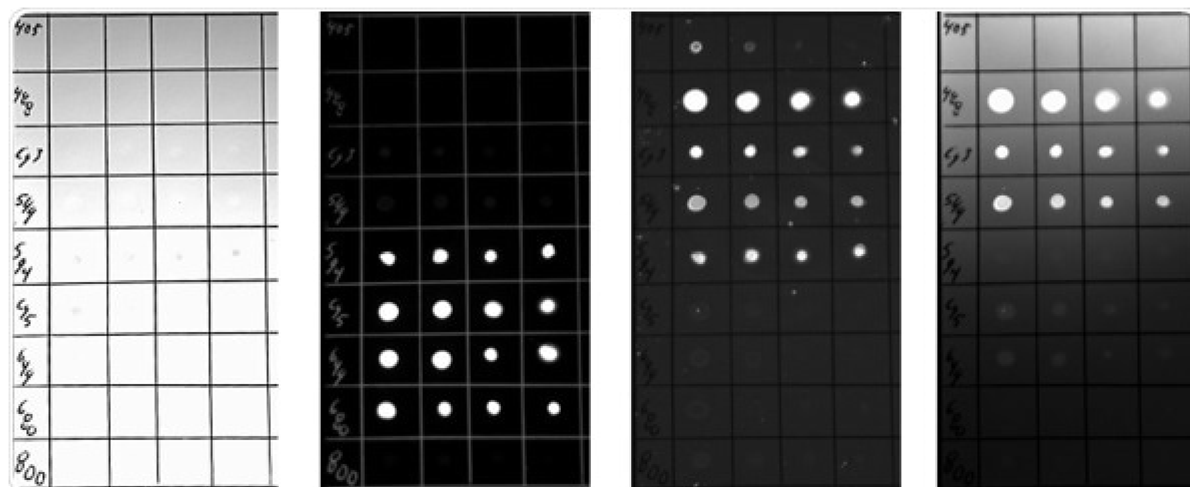

Figure 2. Dot blot images generated by G:BOX iChemi XR showing from left to right the following lighting and filter combinations: white light without any filter; EPI-Red lighting and an FRLP filter; Long wave UV and a SWSP filter; and EPI-Blue and SWSP filter from top to bottom the following fluorescent dye conjugates: DyLight 405, DyLight 488, Cy3, DyLight 549, DyLight 594, DyLight 615, DyLight 649, DyLight 680 and DyLight 800. (Figure kindly provided by KPL)

Table 1 lists the full results of this blot and analysis.

Table 1. Analysis of filter and lighting conditions for imaging fluorescent dyes using a G:BOX iChemi XR

Lighting and filter	DyLight 405	DyLight 488	DyLight 549 Cy3	DyLight 594	DyLight 633	DyLight 649	DyLight 680	DyLight 800
White lighting without filter	x	x	√	√	x	x	x	x
EPI-Red and FRLP	x	x	x	√	√	√	√	x
LW-UV and SWSP	√	√	√	√	x	x	x	x

EPI-Blue and SWSP	x	√	√	√	x	x	x	x

Determining sensitivity

Figure 3 displays the image of the four fluorescent Western blots produced by the G:BOX iChemi XR, where seven bands can be detected with the Cy3 and DyLight 549 dyes using an EtBr filter and EPI-Blue lighting combination.

The same seven bands are also visible with DyLight 649 and Cy5 using the combination of the FRLP filter and the EPI-Red lighting. Therefore, when these dyes and filter/lighting combination were used, it is possible to achieve a sensitivity of <10 ng using direct fluorescent detection.

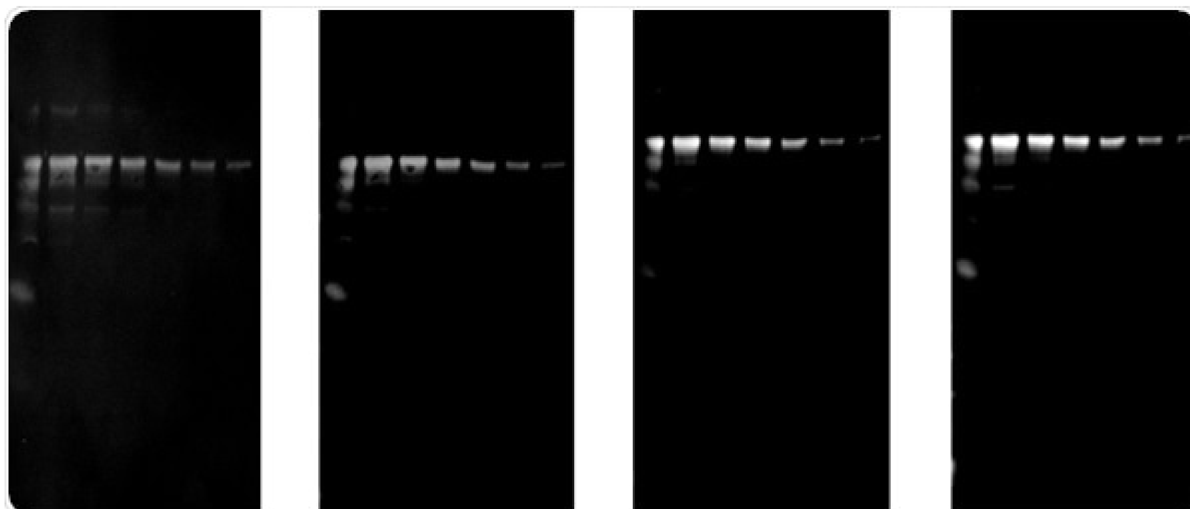

Figure 3. From left to right DyLight 549, DyLight 649, Cy3 and Cy5 labeled Western blot image captured by the G:BOX iChemi XR showing from left to right on each blot a protein marker, 500 ng, 250 ng, 125 ng, 62.5 ng, 31.25 ng, 15.6 ng and 7.8 ng of human IgG. (Figure kindly provided by KPL)

Determining dye applications for multiplexing

Figure 4 shows the image of the multiplex Western blot produced by the G:BOX iChemi XR, where all seven bands of the Mouse IgG (red) are visible. Here, 5ng of protein can be detected using the DyLight 800 as it is a spectrally well-separated fluorophore.

Therefore, the DyLight 800 can be used for low background fluorescence or

multiplex assays. Five bands of Goat IgG Fab fragment (green) labeled with DyLight 680 are also visible in Figure 4, meaning 50ng of protein can be detected with this dye.

From these results, the DyLight 680 can be used for multiplexing with larger amounts of protein. In addition, utilizing the overlaying technique, the G:BOX iChemi GeneSnap software generates clear, good images with well aligned proteins so that users can quickly and easily generate accurate multiplex images using the G:BOX iChemi XR.

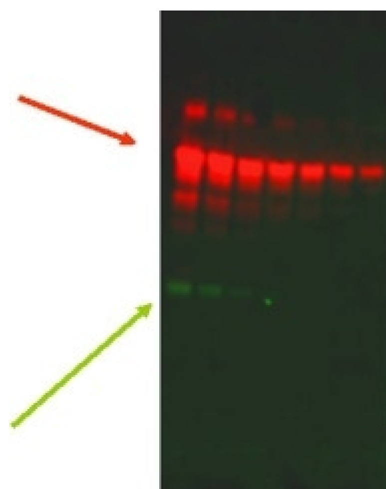

Figure 4. Fluorescently labeled Western blot image generated by G:BOX iChemi XR showing from left to right on each blot 1 µg, 500 ng, 100 ng, 50 ng, 10 ng and 5 ng of Mouse IgG antibody labeled with DyLight 800 (red) and Goat IgG Fab fragment labeled with DyLight 680 (green). (Figure kindly provided by KPL)

Table 2 shows a summary of the performance of the different fluorescent dyes with the G:BOX iChemi XR.

Table 2. Optimum filter and lighting conditions to choose for imaging fluorescent dyes applications with a G:BOX iChemi XR

Fluorescent Dye	**Optimum lighting and filter**
Cy3, and DyLight 549	EPI-Blue lighting and EtBr filter (allows imaging of less than 10ng of Cy3 and DyLight 549)
Cy3, and DyLight 549	EPI-Blue lighting and SWSP filter

DyLight 405, 488, 549 and Cy3	LWUV light and SWSP filter
DyLight 594, 615, 649 and 680, Cy5	EPI-Red light and FRLP filter allows imaging of less than 10ng of Cy5 and DyLight 649
DyLight 800	EPI IR light and IR 780 filter

Conclusion

Using the Syngene G:BOX iChemi XR offers users with a very flexible method of generating accurate images of fluorescent Western blots due to the availability of many tried and tested filters and lighting combinations that can be fitted to this system.

Optimum results for each application can be achieved as the system allows users to try different combinations of lighting and filters. It also enables detection with dyes less than 10 ng size.

For example, with Cy3 and DyLight 549, combinations of EPI-Blue lighting and an EtBr filter; LW-UV light and SWSP filter; and EPI-Blue lighting and SWSP filter all generate good results, allowing users the optimum combination to be chosen depending on whether their application requires higher sensitivity or discrete bands.

In addition, the filters and lighting combinations available for the G:BOX iChemi XR system enable multiplex imaging with up to five differently colored proteins on the same blot.

Tthe system’s GeneSnap software can generate precise overlays of the different colored images, enabling users to generate accurate blot images within a short time, saving hours by generating and comparing a series of chemiluminescent blots.

Using the correct combinations of filters and lighting makes the G:BOX iChemi XR image analysis system an economical, yet simple and sensitive alternative solution to chemiluminescence for detection of trace amounts of different proteins present on Western blots.

About Syngene

Syngene are a division of the Synoptics Group, founded in 1985 by imaging experts from the University of Cambridge. At Syngene we live and breathe image analysis because products specifically for gel documentation and fluorescence/chemiluminescence imaging are all we've ever focused on developing.

Our other divisions in the Synoptics Group, Synbiosis and Syncroscopy, develop imaging solutions for microbial and microscopy applications so we are complete life science imaging specialists. Synoptics Health focuses on imaging techniques within the clinical environment.

We are headquartered in Cambridge, a thriving scientific hub in the UK and have a centrally located US subsidiary in Frederick, USA. Globally, our products are supported by an international network of over 60 highly-trained distributors, all of whom employ imaging specialists.

Our world-leading technology includes a wide range of equipment for instant gel documentation, automated chemiluminescence imaging and TLC plate reading, which comply with current regulations specified by accreditation bodies and regulatory agencies.

Our systems are used globally by more than 75,000 scientists and you'll find them successfully contributing accurate data to important projects in many of the world's top pharmaceutical companies and major research institutes.

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