

G:BOX iChemi DyLight Conjugates for Imaging Fluorescent Western Blots Optimum lighting and filter combinations for maximum sensitivity

Introduction

Recent advances in fluorescent dye technology have enabled greater capabilities in detection and a new level of sensitivity in Western blotting. The newer fluorescent dyes such as Cy dyes and DyLights, when conjugated to secondary antibodies, offer a range of benefits over traditional detection methods such as colorimetric and chemiluminescent detection. These methods are not well suited for multiplex analysis because they produce only one colour or a white chemiluminescent emission, and involve producing and comparing a series of Western blots, which is both time-consuming and expensive in terms of reagents and blots used. Thus, users cannot distinguish between different proteins, especially when these proteins have the same molecular weight or are very close together.

With the new fluorescent labels these difficulties may be overcome. The new fluorescent labels emit different colours that can be detected using the correct lighting and filter conditions, and the images digitally overlaid to generate a blot image that allows simultaneous detection of multiple proteins on one blot. In other words they enable direct and simple imaging of fluorescent-labelled blots with just a one step image capture of the fluorescent signal. In addition, due to their exceptional photostability, DyLight and Cy dye conjugates can be visualised multiple times without a decrease in signal. The drawbacks of performing chemiluminescence, such as the need for film and the excessive time spent analysing images of different exposures, are eliminated. Last but not least, the cost of using fluorescent reagents is approximately one tenth the cost of chemiluminescent substrates in Western blotting.

Combining these dyes with KPL antibodies allows for a unique detection system that yields exceptional results when analysed with G:BOX imagers. They enable scientists to generate optimal images of each dye and to visualise multiplex images of fluorescently labelled Western blots. This application note describes

the optimal conditions when using a range of advanced fluorescent conjugates for Western blotting.

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

For accurate visualization of fluorescently labelled Western blots, Syngene has developed the G:BOX iChemi (Figure 1), an affordable range of CCD-based analysers that can be used for both fluorescence and chemiluminescence imaging. The G:BOX iChemi comes with a high quality digital camera inside a light tight cabinet and can be fitted with EPI RGB, EPI-UV or IR lighting modules, as well as band-pass filters matched to a large range of fluorochromes. The correct lighting and filters, combined with high resolution cameras ensures visualisation of even the faintest fluorescent signals.

The G:BOX iChemi features GeneSnap software which allows users to automatically overlay all the different images of each colour to produce a single blot image showing the different colours simultaneously. The system also includes GeneTools software so users can analyse images to determine the molecular weight of proteins and quantify protein amounts. The combination of these features makes the G:BOX iChemi a cost-effective system for imaging fluorescently labelled Western blots.

To demonstrate the exceptional quality and imaging range a G:BOX iChemi system can achieve, the G:BOX iChemi XR system was utilised at a major reagent manufacturer, KPL, in Gaithersburg, USA to determine the optimal imaging conditions of commonly used fluorescent dyes.



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

Method

Fluorescent labeling of dot blots

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

Imaging fluorescent dot blots to determine optimum conditions

To determine the optimum filter and lighting combination for imaging these fluorescent dyes, the dot blot was placed inside the G:BOX iChemi XR darkroom. The blot was imaged using one of the following conditions: white light without any filter; EPI-Red lighting and an FRLP filter; long wave (LW)-UV and a short wave short pass (SWSP) filter; and EPI-Blue lighting and SWSP filter. Each image was produced by using the G:BOX iChemi XR's GeneSnap software "series capture" set at 30 seconds.

Generating fluorescently labeled Western blots

Four SDS-PAGE gels were run, loaded with 7 dilutions of Human IgG (Lampire, Pipersville, USA) (500ng, 250ng, 125ng, 62.5ng, 31.25ng, 15.6ng and 7.8ng). Each gel was also loaded with a set of pre-stained protein markers (Blue Protein

Molecular Weight Marker, ThermoFisher, Waltham, MA). The proteins were transferred from acrylamide gels onto nitrocellulose membranes using a standard electroblotting method. All the membranes were incubated overnight in 1X Detector Block solution (KPL, Cat. No. 71-83-00) with 1% Detector Block Powder. The blots were incubated (1 hour, 25°C) in 1X Detector Block with secondary antibodies labelled with the following dyes: DyLight™ 549 and DyLight™ 649 were applied to blots one and two and Cy3 and Cy5 (GE Healthcare Life Sciences) were applied to the third and fourth blot respectively all according to each manufacturer's instructions.

Imaging fluorescent Western blots to determine sensitivity

To establish the sensitivity of different filter and lighting combinations, the Western blot membranes were placed inside the G:BOX iChemi XR darkroom for imaging. The blots were imaged using one of the following conditions: EPI-Blue lighting and the EtBr filter for DyLight 549 and Cy3; and EPI-RED lighting and the FRLP filter for DyLight 649 and Cy5. Each image was produced by using the G:BOX iChemi's GeneSnap software "capture series" set at 30 seconds.

Generating a multiplex labeled Western blot

An SDS-PAGE gel was run, loaded with seven dilutions of Mouse IgG protein (Lampire) (500ng, 250ng, 125ng, 62.5ng, 31.25ng, 15.6ng and 7.8ng) and Goat IgG Fab fragment (500ng, 250ng, 125ng, 62.5ng, 31.25ng, 15.6ng and 7.8ng (KPL). The proteins were then transferred from acrylamide gels onto nitrocellulose filters using a standard electroblotting method. The membrane was incubated overnight in 1X Detector Block solution (KPL, Cat. No. 71-83-00) with 1% Detector Block Powder. The blot was incubated (1 hour, 25°C) in 1X Detector Block containing DyLight 800 labelled goat anti-mouse antibody and a DyLight 680 labelled rabbit anti-goat antibody from KPL according to the manufacturer's instructions.

Imaging multiplex fluorescent blots to determine dye applications

The membrane was placed inside the G:BOX iChemi XR darkroom and was imaged using one of the following conditions: EPI-IR lighting and an IR 780 filter or EPI-Red lighting and FRLP filter. Each image was produced by using the G:BOX iChemi's GeneSnap software "capture series" set at 30 seconds. The best image of each colour was chosen and the different coloured images were overlaid to create a composite image.

Results and discussion

Determining optimum imaging conditions

The image produced by the G:BOX iChemi of the dot blots (Figure 2) shows that the DyLight 549, Cy3 and DyLight 594 are visible with white light but are very difficult to see on white background (white light is not recommended for imaging any of these dyes). The EPI-Red lighting and the FRLP filter produces good images of the DyLight 594, 633, 649 and 680 dyes because the light emitted produces a very sharp, distinct signal without any halos or bleed effects of light around the protein dots. The combination of the LW-UV light and the SWSP filter generates good images of DyLight 405, 488, Cy3, and DyLight 549. However, with the DyLight 594 the signal is not discrete and there is a slight halo of light around the dot. Thus, the combination of EPI-Red light and the FRLP is the preferred option for imaging DyLight 594. The EPI-Blue lighting and SWSP filter combination produces stronger signal with Cy3, and DyLight 549. However, with DyLight 488 the signal is not discrete and includes major light bleed effects around each dot. Thus, again, the combination of EPI-Red light and the FRLP is the preferred option for imaging this dye.

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

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

Determining sensitivity

The image of the four fluorescent Western blots (Figure 3) generated by the G:BOX iChemi XR shows seven bands are visible with DyLight 549 and Cy3 dyes using EPI-Blue lighting and an EtBr filter. The same seven bands are also detected with DyLight 649 and Cy5 using an EPI-Red lighting and FRLP filter combination. And so, when using these dyes and filter/lighting combination, a sensitivity of less than 10ng can be achieved using direct fluorescent detection.

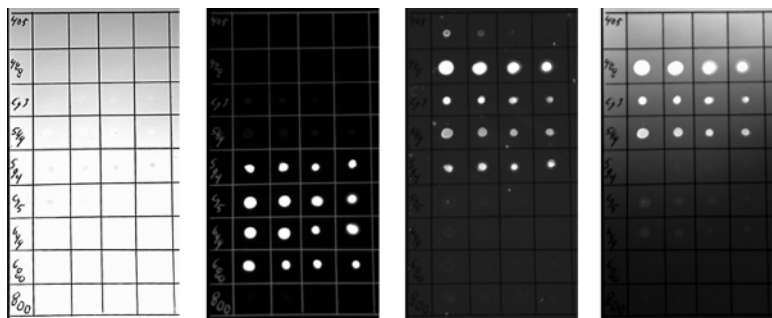


Figure 2: Dot blot images generated by G:BOX iChemi XR showing from left to right the following lighting and filter combinations: white light without any filter; EPI-Red lighting and an FRLP filter; Long wave UV and a SWSP filter; and EPI-Blue and SWSP filter from top to bottom the following fluorescent 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)

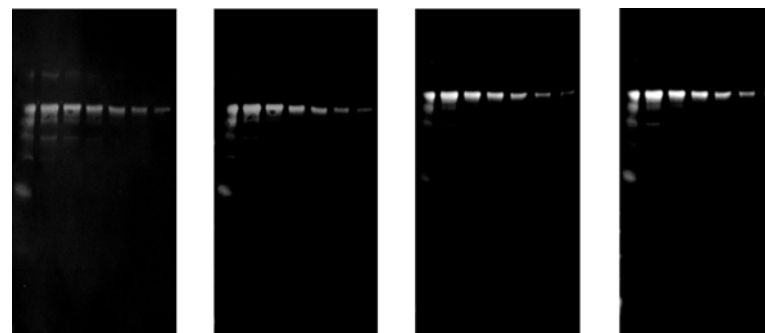


Figure 3: From left to right DyLight 549, DyLight 649, Cy3 and Cy5 labelled Western blot image captured by the G:BOX iChemi XR showing from left to right on each blot a protein marker, 500ng, 250ng, 125ng, 62.5ng, 31.25ng, 15.6ng and 7.8ng of human IgG. (Figure kindly provided by KPL)

The full results of this blot and analysis are listed in Table 1.

Determining dye applications for multiplexing

The image of the multiplex Western blot produced by the G:BOX iChemi XR (Figure 4) shows all seven bands of the Mouse IgG (red). In this application, DyLight 800 is a spectrally well separated fluorophore which enables detection of 5ng of protein, meaning it is useful for low background fluorescence or multiplex assays. Figure 4 also shows five bands of Goat IgG Fab fragment (green) labelled with DyLight 680, enabling detection of 50ng of protein with this dye. This makes DyLight 680 useful for multiplexing with larger amounts of protein. Additionally, the overlaying technique utilised by the G:BOX iChemi GeneSnap software produces good, clear images with the proteins well aligned so users can confidently generate accurate multiplex images quickly and easily using the G:BOX iChemi XR.

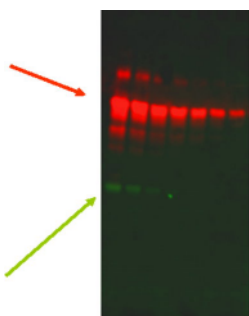


Figure 4: Fluorescently labeled Western blot image generated by G:BOX iChemi XR showing from left to right on each blot 1µg, 500ng, 100ng, 50ng, 10ng and 5ng of Mouse IgG antibody labelled with DyLight 800 (red) and Goat IgG Fab fragment labelled with DyLight 680 (green). (Figure kindly provided by KPL)

A summary of the performance of the different fluorescent dyes with the G:BOX iChemi XR is shown below in Table 2.

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

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

Conclusion

The Syngene G:BOX iChemi XR provides a very flexible method of producing accurate images of fluorescent Western blots because users have the option of a range of tried and tested lighting and filters fitted to this system. The system allows users to try different combinations of filters and lighting to achieve optimum results for each application. 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; EPI-Blue lighting and SWSP filter; and LW-UV light and SWSP filter all generate good results, allowing users to choose the optimum combination depending on whether their application requires discrete bands or higher sensitivity.

Additionally, the lighting and filters available for the G:BOX iChemi XR system make multiplex imaging possible with up to five differently coloured proteins on the same blot. As the system’s GeneSnap software is capable of producing precise overlays of the different coloured images, users can generate accurate blot images in minutes, saving hours by producing and comparing a series of chemiluminescent blots. By choosing the correct filter and lighting combinations, the G:BOX iChemi XR image analysis system offers a cost-effective, yet simple and sensitive alternative to chemiluminescence for detecting small amounts of different proteins on Western blots.

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