

## Multiplexing DyLight® Infra red 680 and 800 dyes

### Introduction

Life sciences research has recently seen a trend towards the application of fluorescence-based detection of proteins on Western blots. Using fluorescence based fluorophores increases sample throughput and can make multiplexing several fluorophores less time consuming.

Near-infrared (NIR) (spectral region 650-900nm) has been shown to significantly reduce background autofluorescence and this has therefore generated a trend towards NIR fluorophores such as, IRDye® 680LT and IRDye® 800CW from LI-COR® and DyLight® dyes from ThermoFisher which provide enhanced detection sensitivity and quantification of proteins.

The DyLight dyes offer intense signal whilst maintaining excellent photo-stability and water solubility across a broad range of pH values. Another advantage of using DyLight dyes is that they are competitively priced and permit the use of lower conjugate concentrations to be used.

### Materials and methods

#### Materials

Pre-cast Invitrogen gels (4-12% Bis-Tris, 12 well, 1mm), running buffer used was MOPS. HeLa cell lysate (2.92mg/ml sample) was loaded on to the pre-cast gel. The marker used was BioRad Precision All Blue.

For transfer semi-wet Invitrogen Sure-Lock system was used. Nitrocellulose membrane (Invitrogen) and transfer buffer (50ml 20X NuPAGE transfer buffer, 100ml methanol and 850ml MilliQ water) were used.

Primary antibodies used were Anti-actin monoclonal (1:5000) (Abcam, UK), Anti-ERK 1:1000 (Cell Signaling) both diluted in Odyssey® Blocking bBuffer and 0.1% Tween.

Secondary antibodies used where Anti-mouse-680 (1:15,000) and Anti-rabbit -800 (1:15,000) both diluted in Odyssey blocking buffer and 0.1% Tween.

#### Fluorescent imaging system

Syngene G:BOX IR<sup>6</sup> and Odyssey Infrared Imager scanner (LI-COR).

#### Method

HeLa cell lysate was diluted 1:2 (ranging from 29.2 - 0.028µg/well when 10µl of each concentration is loaded on to the gel).

2µl of BioRad Precision All Blue marker was loaded on to gel. The gel was then run at 60V for 30 mins then 120V for 1hr. The gel was transferred to a nitrocellulose membrane using the Sure-Lock system (Invitrogen) at

125V for ~1hr. The blot was blocked in Odyssey blocking buffer for 1 hour.

The membrane was then incubated with primary antibodies (Anti-actin and anti-ERK) overnight at 4°C on a rocker. After the incubation period the membrane was washed 4 times, 5 minutes each wash with PBS and 0.1% Tween (PBST).

The membrane was incubated with DyLight secondary antibodies 680 and 800 for 1 hour at room temperature in the dark. The membrane was then washed 3 times, 5 minutes each wash PBST and 1 wash, 5 minutes with PBS.

### Visualization

The following table shows the recommended lighting and filter combinations that should be used when multiplexing between the DyLight 680 and DyLight 800 dyes.

	Lighting	Filter
DyLight 680	Epi Red (M)	Filt705M
DyLight 800	Epi LED IR740(M)	FiltLY800

**Table 1 - Recommended lighting and filter combinations for visualizing DyLight 680 and DyLight 800 dyes.**

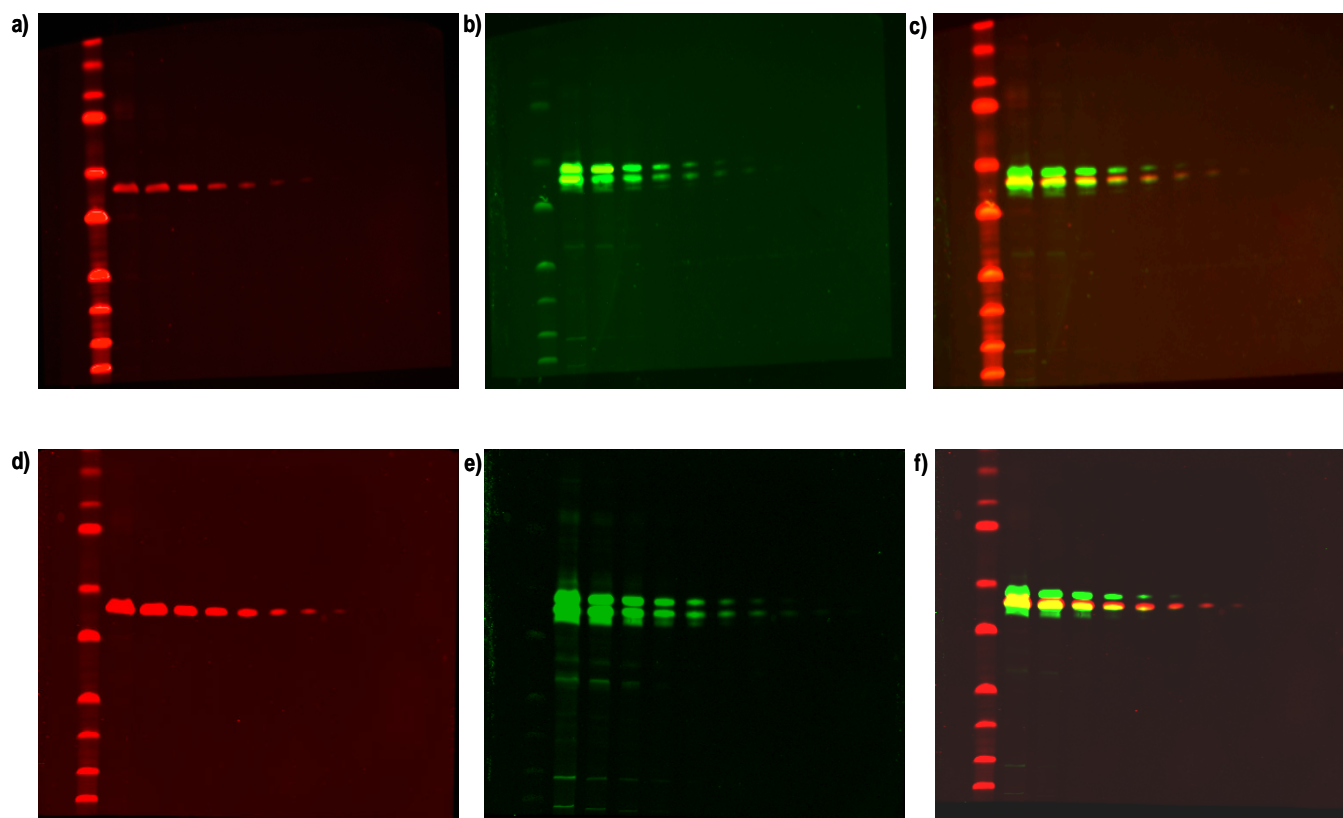
### Results

The G:BOX IR<sup>6</sup> system can successfully multiplex between the DyLight 680 and DyLight 800 (ThermoFisher) using the stated imaging conditions in Table 1 (**Figure 1d, e and f**) and across the whole G:BOX Chemi range (images not shown). The results showed comparable sensitivity to those obtained by the Odyssey Infrared Imager (LI-COR) (**Figure 1 a, b and c**).

#### Imaging exposure times

From the user's perspective the most significant difference between using the G:BOX IR<sup>6</sup> and Odyssey systems is in the imaging times. The scan times on Odyssey are solely determined by the scanning resolution and intensity setting. On the G:BOX IR<sup>6</sup> the scan times are only dependant on the sensitivity.

The Odyssey Infrared Imager took a total scan time of 3 minutes 42 seconds whereas the IR<sup>6</sup> for the DyLight 680 dye image was captured in 20 seconds with a 4x4 binning factor and the DyLight 800 image was captured in 2 minutes with a 4x4 binning factor.



**Figure 1- Comparison of scanned images taken with the LI-COR Odyssey Infrared Imager and images captured with the G:BOX Chemi IR<sup>6</sup> (Syngene)**

**a)** Actin (anti-actin 1:5000, Abcam; anti-mouse – DyLight 680 1:15000) captured with G:BOX Chemi IR<sup>6</sup> system and **d)** scanned with the Odyssey Infrared Imager at an intensity set to 4.5 **b)** ERK (anti ERK 1:1000, Cell signalling; anti-rabbit DyLight 800 1:15000) captured with the G:BOX Chemi IR<sup>6</sup> and **e)** scanned with the Odyssey Infrared Imager at an intensity set to 5. **c)** Overlay image of **a)** and **b)**. **f)** Overlay image of **d)** and **e)**.

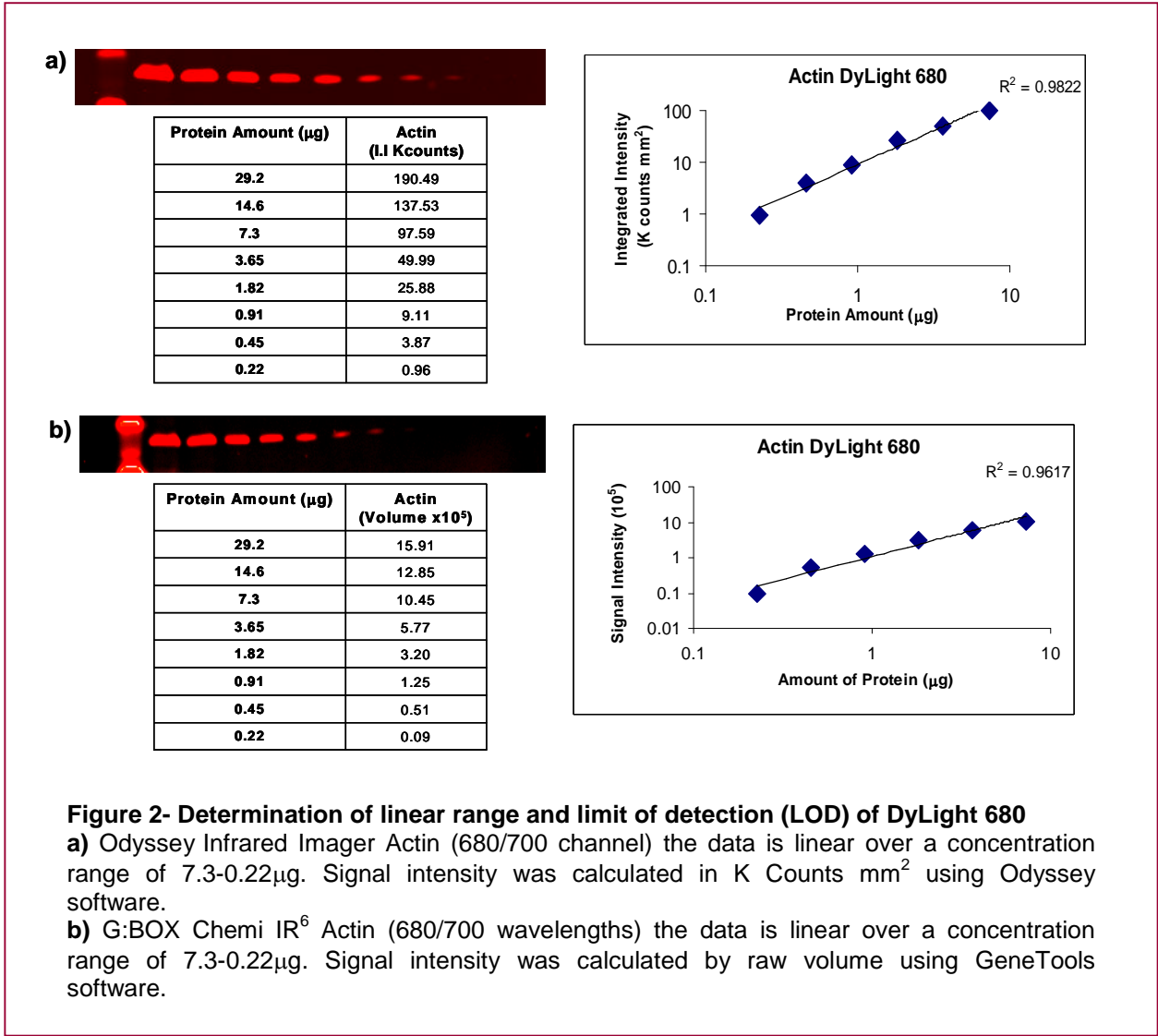
### Determining linear range and LOD

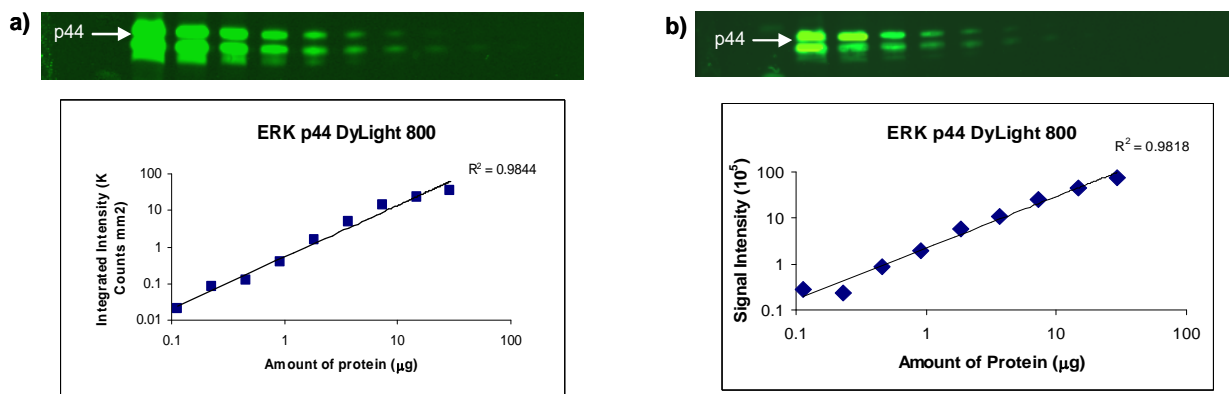
To determine the linear range and limit of detection (LOD) of each of the DyLight dyes the amount of HeLa protein loaded on to the gel was plotted against the integrated Intensity (Kcounts mm<sup>2</sup>) for the Odyssey Infrared Imager (**Figure 2a and 3a**) and against raw volume (Signal Intensity) for GeneTools software (**Figure 2b and Figure 3b**).

With the Odyssey and G:BOX IR<sup>6</sup> systems the DyLight 680 was linear over the following range (7.3-0.22µg) series dilution used in this experiment (**Figure 2a and 2b**).

The DyLight 800 was linear with Odyssey and G:BOX IR<sup>6</sup> systems over the dilution series used in this experiment. To compare the linearity of the data and to determine LOD ERK p44 (DyLight 800) was used. The linearity of ERK p42 was calculated but the data is not shown.

The Odyssey Infrared Imager produced linear range for both ERK p42 and p44 with R<sup>2</sup> values of 0.99 and 0.98 respectively and has a LOD of 0.11µg (**Figure 3 Table II**). The G:BOX IR<sup>6</sup> system produced a linear range for both ERK p42 and p44 with R<sup>2</sup> values of 0.98. The LOD with the G:BOX IR<sup>6</sup> system is 0.11µg (**Figure 3 Table II**).





System	Detection Limit (LOD) (µg)	Linearity (R <sup>2</sup> )
<b>Odyssey (LI-COR)</b>	0.11	0.9844
<b>G:BOX Chemi IR<sup>6</sup></b>	0.11	0.9818

Table II

**Figure 3- Determination of linear range and limit of detection (LOD) of DyLight 800**

HeLa whole cell lysate diluted by two-fold dilution starting at 29.2µg. Blot was incubated with DyLight 800 and imaged using a) Odyssey Infrared Imager and b) G:BOX Chemi IR<sup>6</sup>. For the Odyssey system signal intensity was calculated in K counts mm<sup>2</sup> using Odyssey software. For G:BOX Chemi IR<sup>6</sup> system signal intensity was calculated by raw volume using GeneTools software. R<sup>2</sup> values are shown in table II.

**Conclusions**

Using the recommended lighting and filter combinations stated in Table I users can successfully multiplex between DyLight dyes DyLight 680 and DyLight 800 using a Syngene G:BOX Chemi IR<sup>6</sup> imaging system.

The G:BOX IR<sup>6</sup> system has comparable sensitivity and linear range to that of the Odyssey Infrared Imager.

One of the main advantages of using G:BOX Chemi IR<sup>6</sup> system is the faster imaging exposure times compared to that of the Odyssey Infrared Imager and also that the G:BOX Chemi system offers great versatility enabling the imaging system to be used for a variety of different applications.

Another advantage of using a G:BOX Chemi IR<sup>6</sup> system is that when it is used in conjunction with the DyLight IR dyes which perform exceptionally well offering comparable sensitivity and linearity to that of the IRDye 680LT and IRDye 800CW (LI-COR) thus enabling Syngene to offer a complete IR application package which is competitively priced.

Many thanks to Dr Jane Gray of Cancer Research UK, Cambridge for her collaboration with this project.

**Syngene reserves the right to amend or change specifications without prior notice. This Application Note supersedes all earlier versions.**

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