Multiplexing LI-COR® IRDye® 680LT and IRDye® 800CW

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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.

Fluorescent dyes that have wavelengths in the visible spectrum are becoming more popular. However, visible fluorophores do not offer optimal performance for all applications. In particular, cells, tissues and blotting membranes all possess intrinsic autofluorescence that can interfere with detection. 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 dyes from LI-COR which provide enhanced detection sensitivity and quantification of proteins.

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-ERK1/2 (1:1000, Cell Signaling) both diluted in Odyssey® Blocking Buffer with 0.1% Tween.

Secondary antibodies used were anti-mouse-680LT (1:20,000) and anti-rabbit-800 (1:5,000,LI-COR) both diluted in Odyssey blocking buffer with 0.1% Tween.

Fluorescence imaging systems

Syngene's G:BOX Chemi IR[6], XT[4], XL[1.4], XX[8] and XR[5] systems, PXi 4 and 6 and Odyssey® Infrared Imager (LI-COR).

Method

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

2µl of BioRad Precision All Blue marker was loaded on to the gel. The gel was then run at 60V for 30 mins then followed by 125V for 1hr 30 minutes. The gel was transferred to a nitrocellulose membrane using the Sure-Lock system (Invitrogen) at 30V for ~1hr 15 minutes. 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 4^0C on a rocker. After the incubation period the membrane was washed 4 times, 5 minutes each wash with PBS with 0.1% Tween (PBST).

The membrane was incubated with LI-COR secondary antibodies 680LT and 800CW for 45 minutes 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 IRDye 680LT and IRDye 800CW dyes.

	Lighting	Filter
IRDye 680LT	Epi Red (M)	Filt705M
IRDye 800CW	Epi LED IR740 (M)	FiltLY800

Table I - Recommended lighting and filter combinations for visualising IRDye 680LT and IRDye 800CW dyes.

Results

The G:BOX Chemi and PXi systems can successfully multiplex between the IRDye 680LT and IRDye 800CW (LI-COR) using the stated imaging conditions in Table I (**Figure 1b, d and f**). 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 Chemi, PXi 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 Chemi and PXi systems 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 G:BOX Chemi IR[6] for the IRDye 680LT dye image was captured in 20 seconds with a 4x4 binning factor and the IRDye

800CW image was captured in 2 minutes with a 4x4 binning factor.

The exposure time varied depending on which G:BOX Chemi and PXi system was used. With image capture for the IRDye 680LT, the exposure time ranged from 20 to 80 seconds and for the IRDye 800CW the range was from 2 to 10 minutes.

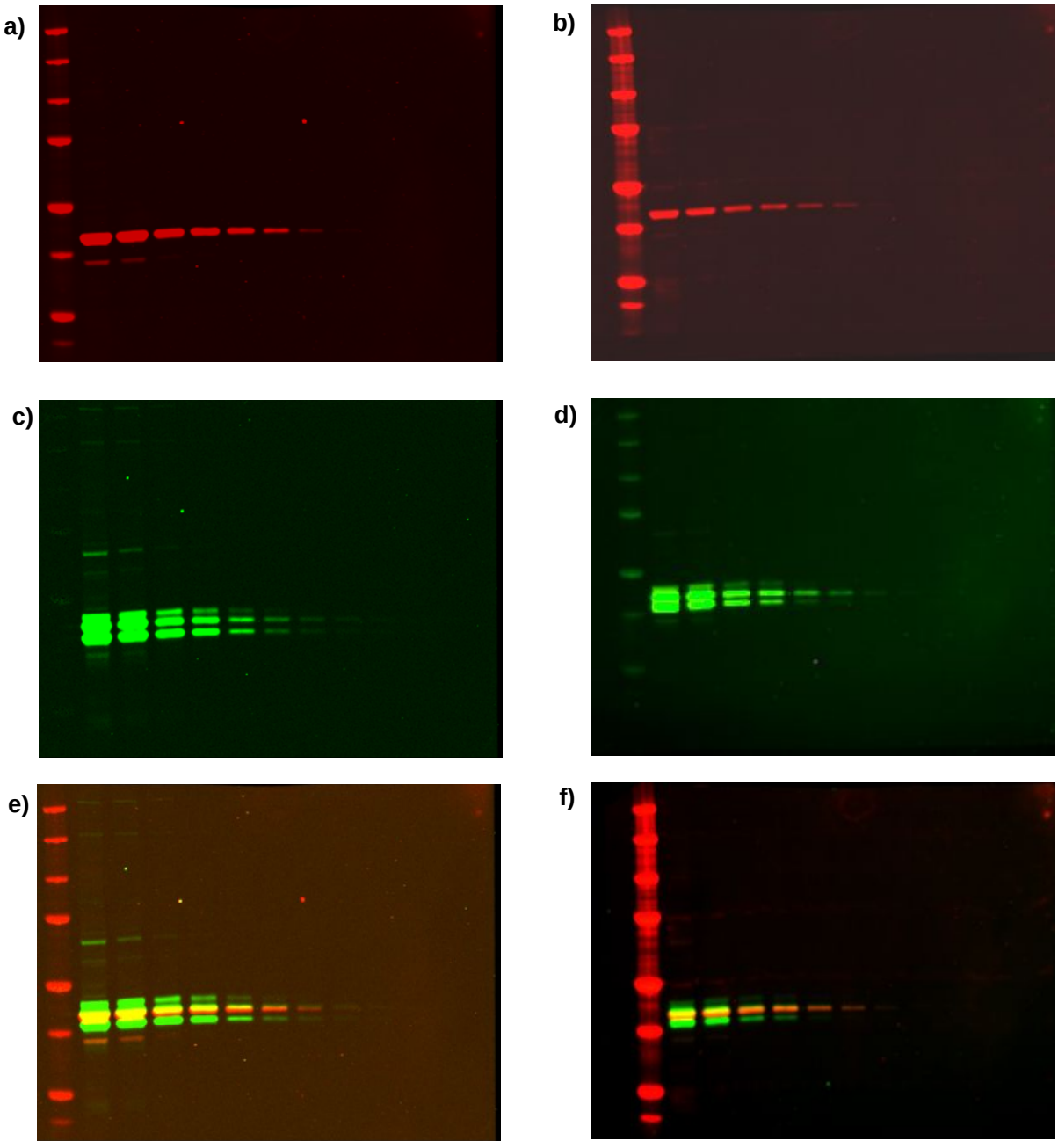


Figure 1- Comparison of scanned images taken with the LI-COR Odyssey Infrared Imager and images captured with the G:BOX Chemi IR[6] (Syngene)
a) Actin (anti-actin 1:5000, Abcam; anti-mouse-680LT 1:20,000) scanned with the Odyssey Infrared Imager at an intensity set to 5 and **b)** captured with the G:BOX Chemi IR[6] system **c)** ERK (anti-ERK 1:1000, Cell signalling; anti-rabbit- 800 1:5000) scanned with the Odyssey Infrared Imager at an intensity set to 5 and **d)** captured with the G:BOX Chemi IR[6]. **e)** Overlay image of **a)** and **c)**. **f)** Overlay image of **b)** and **d)**.

Determining linear range and LOD

To determine the linear range and limit of detection (LOD) of IRDye 680LT and IRDye 800CW dyes, the amount of HeLa protein loaded on to the gel was plotted against the integrated intensity (Kcounts mm^2) for the Odyssey Infrared Imager (**Figure 2a and 3a**) and against raw volume (Signal Intensity) for GeneTools software (**Figure 2b and Figure 3b,c,d,e,f, g and h**).

With the Odyssey and G:BOX Chemi IR^6 systems the IRDye 680LT was not linear for the detection of actin over the series dilution used in this experiment (**Figure 2a and 2b**). This was also observed with all G:BOX Chemi and PXi systems tested (data not shown).

The IRDye 800CW detecting ERKp42/p44 was linear with Odyssey and G:BOX Chemi and PXi systems over the dilution series used in this experiment. To compare the linearity of the data and to determine LOD ERK p44 (IRDye 800CW) was used. The linearity of ERK p42 was calculated but the data is not shown.

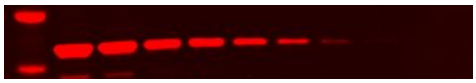
The Odyssey Infrared Imager produced linear range for both ERK p42 and p44 with R^2 values of 0.98 and 0.99 respectively and has a LOD of 0.11µg (**Figure 3 Table II**).

The G:BOX Chemi systems produced a linear range for both ERK p42 and p44 with R^2 values of 0.98. The LOD with the G:BOX Chemi IR^6 system is 0.11µg (**Figure 3 Table II**).

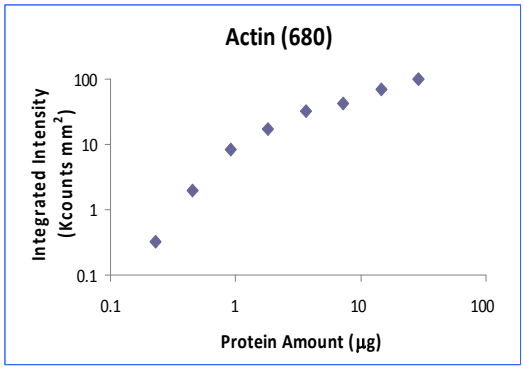
The G:BOX Chemi XT^4, XL$^{1.4}$, XX^8 and XR^5 systems all produced a linear range for both ERK p42 and p44 around 0.98 with a LOD of 0.22µg except the G:BOX Chemi XR^5 system which had a LOD of 0.45µg (**Figure 3 Table II**).

The PXi systems also produced a linear range for both ERK p42 and p44 with a R^2 value around 0.98 with LOD of 0.11mg (**Figure 3 Table II**).

a)



Protein Amount (µg)	Actin
29.2	99.59
14.6	69.61
7.3	41.94
3.65	32.44
1.825	17.47
0.9125	8.34
0.45625	2.01
0.228125	0.33



b)

Protein Amount (ug)	Actin
29.2	54.20889
14.6	43.883135
7.3	27.30295
3.65	20.2988013
1.825	9.8452625
0.9125	4.53259
0.45625	0.8847855
0.228125	0.0438652

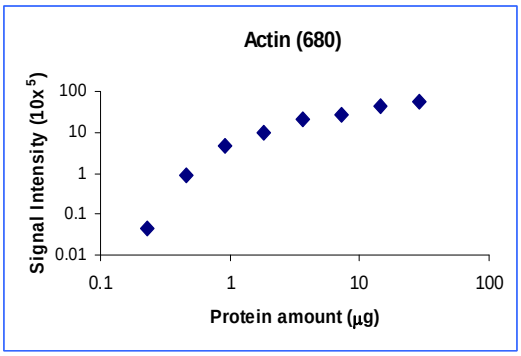
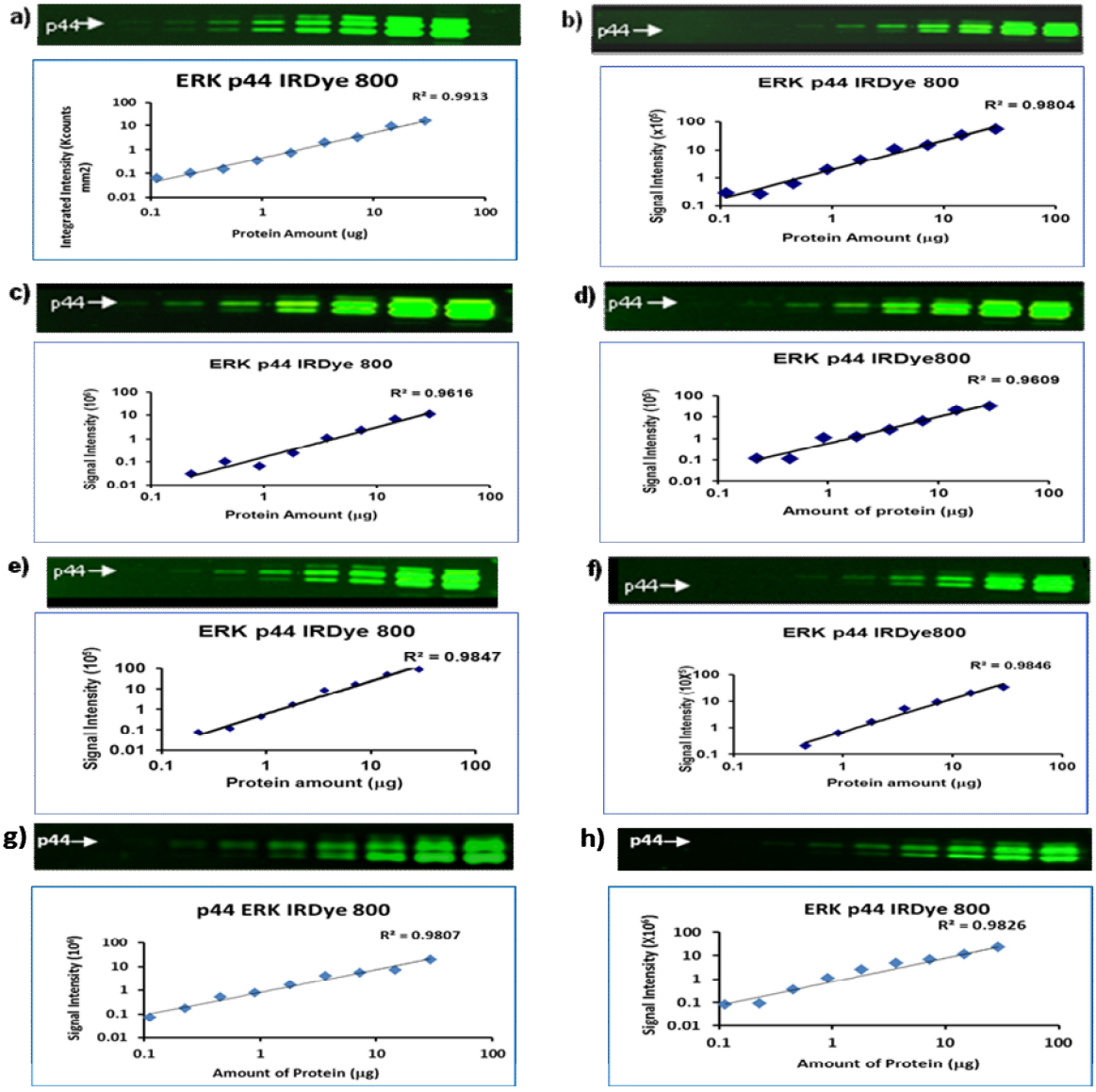


Figure 2- Determination of linear range and limit of detection (LOD) of IRDye 680LT
a) Odyssey Infrared Imager Actin (680/700 channel) the data is not linear for the detection of actin over the dilution series used. Signal intensity was calculated in Kcounts mm^2 using Odyssey software.
b) G:BOX Chemi IR^6 Actin (680/700 wavelengths) the data is not linear over the dilution series used. Signal intensity was calculated by raw volume using GeneTools software.



System	Detection Limit (LOD) (µg)	Linearity (R^2)
Odyssey (LI-COR)	0.11	0.9913
G:BOX Chemi IR[6]	0.11	0.9804
G:BOX Chemi XT[4]	0.22	0.9616
G:BOX Chemi XL[1.4]	0.22	0.9609
G:BOX Chemi XX[8]	0.22	0.9847
G:BOX Chemi XR[5]	0.45	0.9846
PXi 6	0.11	0.9807
PXi 4	0.11	0.9826

Table II

Figure 3- Determination of linear range and limit of detection (LOD) of IRDye 800CW
HeLa cell lysate diluted by two-fold dilution starting at 29.2µg. Blot was incubated with IRDye 800CW and imaged using **a)** Odyssey system, **b)** G:BOX Chemi IR[6] **c)** G:BOX Chemi XT[4], **d)** G:BOX Chemi XL[1.4], **e)** G:BOX Chemi XX[8], **f)** G:BOX Chemi XR[5], **g)** PXi 6 and **h)** PXi 4
For the Odyssey system signal intensity was calculated in Kcounts mm^2 using Odyssey software. For G:BOX Chemi and PXi systems signal intensity was calculated by raw volume using GeneTools software. R^2 values are shown in table II.

Conclusions

Using the recommended lighting and filter combinations stated in Table I users can successfully multiplex between LI-COR dyes IRDye 680LT and IRDye 800CW using Syngene's G:BOX Chemi and PXi imaging systems.

The G:BOX Chemi IR^6 and PXi 4 and 6 systems have comparable sensitivity and linear range to that of the Odyssey Infrared Imager.

The complete G:BOX Chemi and PXi range can be used to successfully image LI-COR IRDye 680LT and IRDye 800CW dyes so there is a system to suit every budget. One of the main advantages of using a G:BOX Chemi and PXi system is the faster imaging exposure times compared to that of the Odyssey Infrared Imager and also that the G:BOX Chemi and PXi systems offer great versatility enabling the imaging system to be used for a variety of different applications.

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