Multiplexing LI-COR Infra red 680LT 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.

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 IRDyes® 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-ERK 1:1000 (Cell Signaling) both diluted in Odyssey block buffer and 0.1% Tween.

Secondary antibodies used were Anti-mouse-680LT (1:20,000) and Anti-rabbit-800 (1:5000) both diluted in Odyssey block buffer and 0.1% Tween.

Fluorescence imaging systems

Syngene's G:BOX Chemi IR^6, XT^4, XL$^{1.4}$, XX^8 and XR^5 systems and Odyssey scanner (Li-COR).

Method

HeLa cell lysate was diluted 1:2 (ranging from 29.2 - 0.028µg/well with 10 µl 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. The gel was transferred to a nitrocellulose membrane using the Sure-Lock system (Invitrogen) at 125V for ~1hr 40 mins. The blot was blocked in Odyssey block buffer for 1 hour.

The membrane was then incubated with primary antibodies (Anti-actin and anti-ERK) overnight at 4^{0}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 LI-COR secondary antibodies 680LT 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 IRDye680 and IRDye800 dyes.

	Lighting	Filter
IRDye680	Epi Red (M)	Filt705M
IRDye800	Epi LED IR740 (M)	FiltLY800

Table I - Recommended lighting and filter combinations for visualizing IRDye680 and IRDye800 dyes.

Results

The G:BOX Chemi systems can successfully multiplex between the IRDye680LT and IRDye800 (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 scanner (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 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 systems the scan times are only dependant on the sensitivity.

The Odyssey scanner took a total scan time of 3 minutes 42 seconds whereas the G:BOX Chemi IR^6 for the IRDye680LT dye image was captured in 20 seconds with a 4x4 binning factor and the IRDye800 image was captured in 2 minutes with a 4x4 binning factor.

The exposure time varied depending on which G:BOX Chemi system was used. With image capture for the IRDye680 the exposure time ranged from 20 to 80 seconds and for the IRDye800 the range was from 2 to 10 minutes.

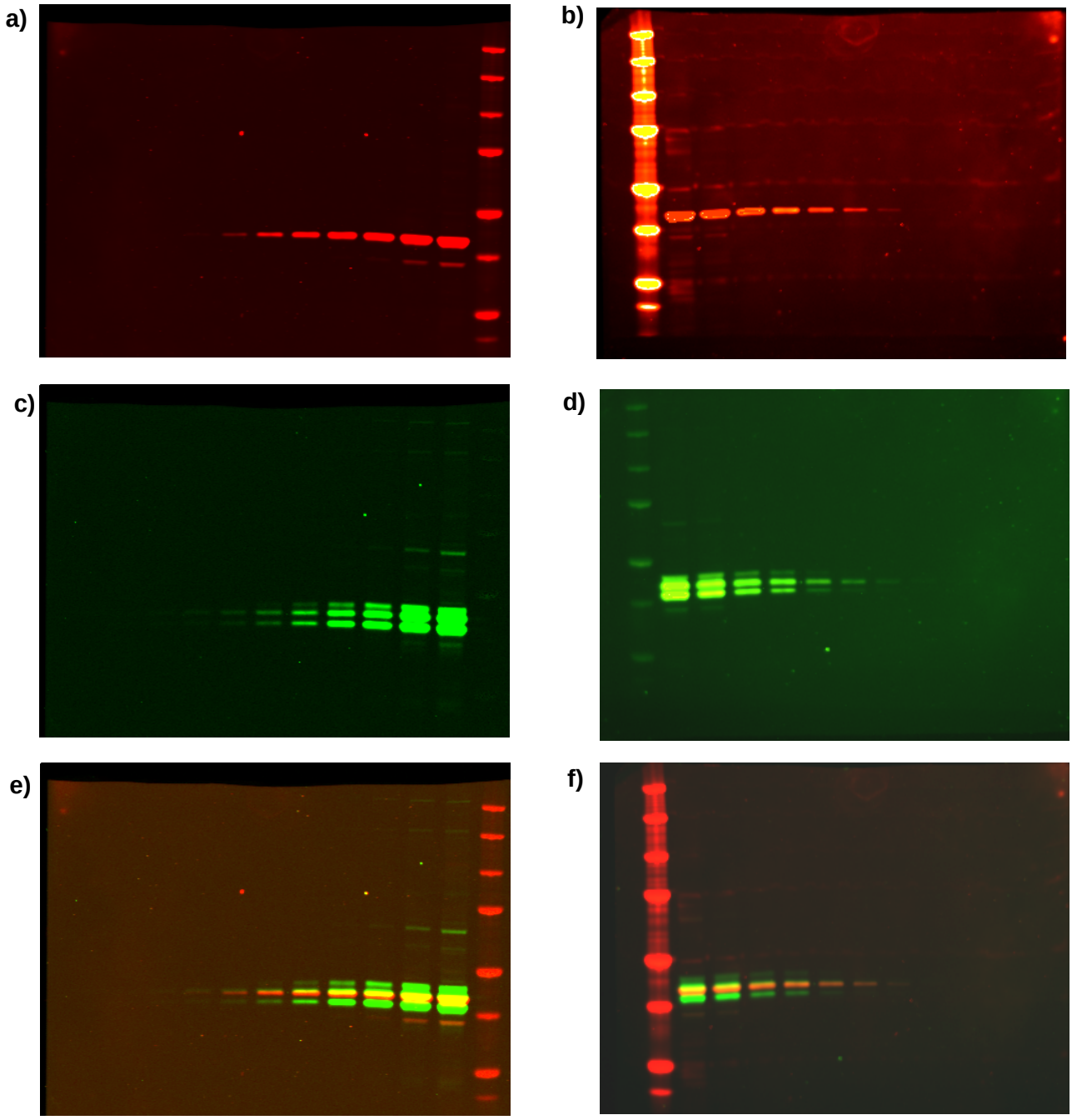


Figure 1- Comparison of scanned images taken with the Li-COR Odyssey system and images captured with the G:BOX Chemi IR[6] (Syngene)
a) Actin (anti-actin 1:5000, Abcam; anti-mouse-680LT 1:1000) scanned with the Odyssey system at an intensity set to 4.5 and **b)** captured with the G:BOX Chemi IR[6] system **c)** ERK (anti-ERK 1:1000, Cell signalling; anti-rabbit- 800 1:5000) scanned with the Odyssey system 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 each of the IRDyes the amount of HeLa protein loaded on to the gel was plotted against the integrated intensity (Kcounts mm^2) for the Odyssey system (**Figure 2a and 3a**) and against raw volume (Signal Intensity) for GeneTools software (**Figure 2b and Figure 3b,c,d,e and f**).

With the Odyssey and G:BOX Chemi IR^6 systems the IRDye680LT was not linear over the series dilution used in this experiment (**Figure 2a and 2b**). This was also observed with all G:BOX Chemi systems tested (data not shown).

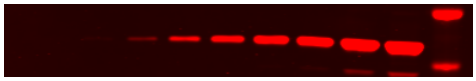
The IRDye800 was linear with Odyssey and G:BOX Chemi systems over the dilution series used in this experiment. To compare the linearity of the data and to determine LOD ERK p44 (IRdye800) was used. The linearity of ERK p42 was calculated but the data is not shown.

The Odyssey system 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**).

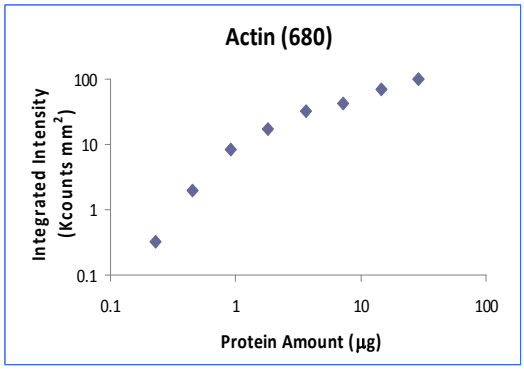
The G:BOX Chemi systems produced a linear range for both ERK p42 and p44 with R^2 values of 0.98. The LOD with the G:BOX Chemi IR^6 system is 0.11µg (**Figure 3 Table II**).

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**).

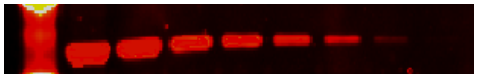
a)



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 (µg)	Actin
29.2	5420889
14.6	4388313.5
7.3	2730295
3.65	2029880.13
1.825	984526.25
0.9125	453259
0.45625	88478.55
0.228125	4386.52

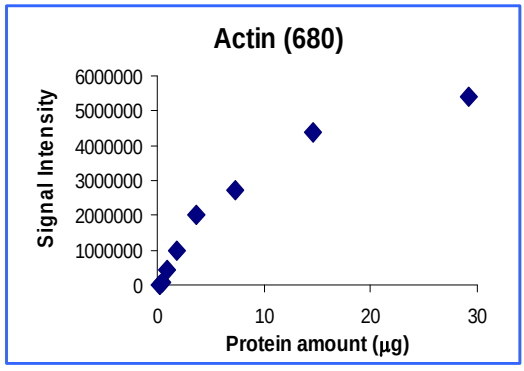
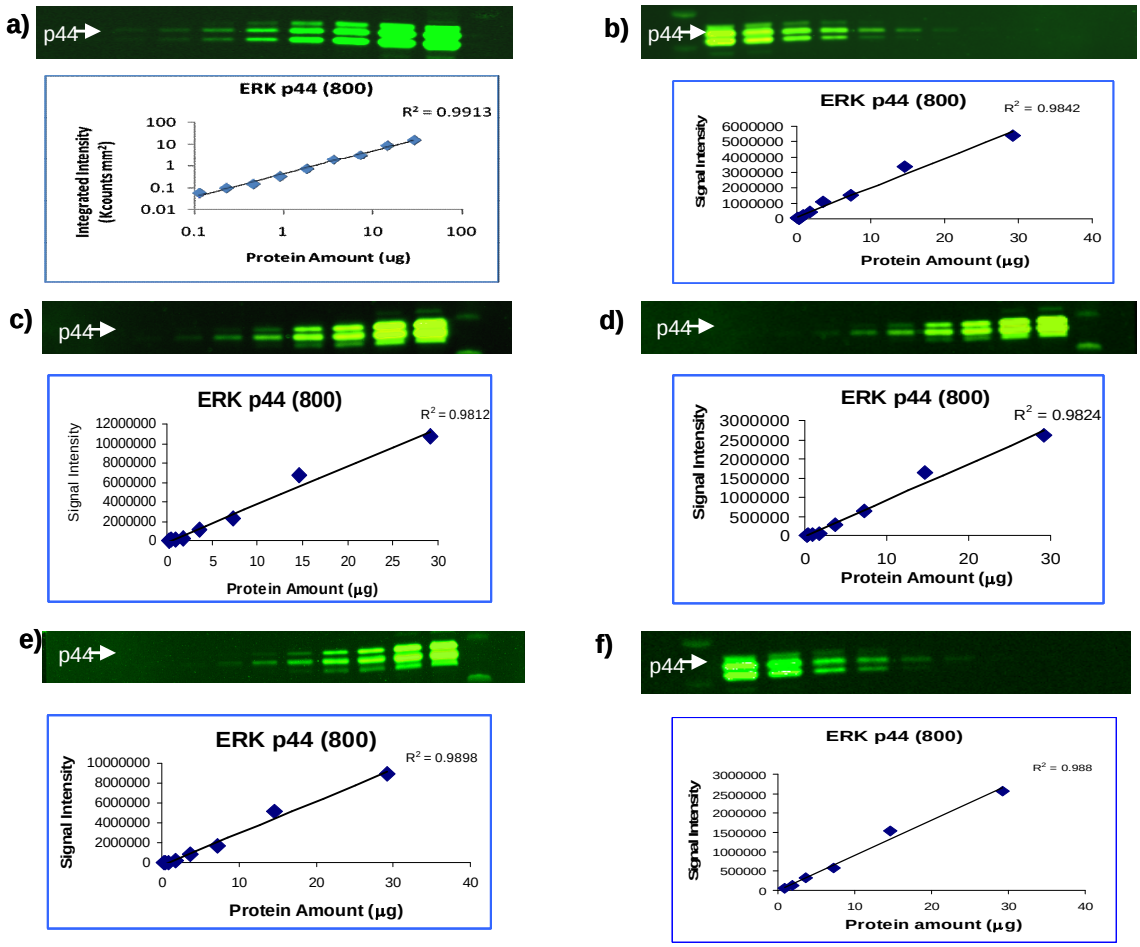


Figure 2- Determination of linear range and limit of detection (LOD) of IRDye680
a) Odyssey scanner Actin (680/700 channel) the data is not linear over the dilution series used. Signal intensity was calculated in Kcounts mm^2 using Odyssey software.
b) G:BOX Chemi IR^6 Actin (680/700 wavelengths) the 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	0.982
G:BOX Chemi XT[4]	0.22	0.9812
G:BOX Chemi XL[1.4]	0.22	0.9824
G:BOX Chemi XX[8]	0.22	0.9898
G:BOX Chemi XR[5]	0.45	0.9893

Table II

Figure 3- Determination of linear range and limit of detection (LOD) of IRDye800
HeLa cell lysate diluted by two-fold dilution starting at 29.2µg. Blot was incubated with IRDye800 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] and **f)** G:BOX Chemi XR[5].
For the Odyssey system signal intensity was calculated in Kcounts mm^2 using Odyssey software. For G:BOX Chemi 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 IRDye680LT and IRDye800 using a Syngene G:BOX Chemi imaging system.

The G:BOX Chemi IR[6] system has comparable sensitivity and linear range to that of the Odyssey system.

The complete G:BOX Chemi range can be used to successfully image Li-COR IRDyes so there is a system to suit every budget. One of the main advantages of using a G:BOX Chemi system is the faster imaging exposure times compared to that of the Odyssey scanner and also that the G:BOX Chemi systems offer great versatility enabling the imaging system to be used for a variety of different applications.

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