Multiplex protein detection using the ECL Plex fluorescent Western blotting system

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Key words: *ECL Plex ● fluorescent Western blotting ● multiplex detection ● dynamic range ● linearity*

The ECL Plex™ system reaches a limit of detection of 1.2 pg, with a dynamic range over 3.6 orders of magnitude. In the multiplex application, two proteins can be detected in the same blot with minimal cross-reactivity between antibodies or dyes.

Introduction

Enhanced chemiluminescence is the most common technology for protein detection in Western blot applications (1, 2). Detection of the proteins is usually performed with X-ray film or with a CCD camera. Although enhanced chemiluminescence combined with detection on film is very sensitive, it offers a limited dynamic range and only qualitative or semiquantitative analysis. A range of chemiluminescent systems is available from GE Healthcare—ECL™, ECL Plus™, and ECL Advance™.

The aim of this study was to evaluate a new Western blotting detection kit based on fluorescence, the ECL Plex system. By introducing fluorescent dye-labeled, instead of peroxidase-labeled, antibodies into the Western blotting application, highly quantitative data with broad dynamic range and high sensitivity can be obtained. The sensitivity of the CyDye™ fluorescent dyes, along with low-fluorescent membranes and the dynamic range of the Typhoon™ scanner, are part of a complete solution for fluorescent Western blotting.

More importantly, because the trend in biological research is towards detection of more than one protein in the same blot, we evaluated a multiplex approach by combining two ECL Plex fluorescent conjugated antibodies in the same reaction. The combination of two antibodies at the same time, with secondary antibody dye conjugates carefully selected and optimized for minimal cross-reactivity, enables quantitative differential analysis. This eliminates the need to strip and reprobe blots when using two antibodies, a source of protein loss and less quantitative data.

Materials

Products used

ECL Plex goat-α-mouse IgG-Cy™3, 150 µg	PA43009
ECL Plex goat-α-rabbit IgG-Cy5, 150 µg	PA45011
Hybond™ ECL, 20 cm x 3 m	RPN203D
Hybond-LFP, 20 x 20 cm, 10 sheets	RPN2020LFP
ECL Plex Fluorescent Rainbow™ Markers, full range, 500 µl	RPN851
2D Quant Kit	80-6483-56
miniVE Vertical Electrophoresis System	80-6418-77
EPS 301 Power Supply	18-1130-01
TE 22 Mini Tank Transfer Unit	80-6204-26
Typhoon 9410 (includes ImageQuant™ TL software)	9410-PC
Deep Purple™ Total Protein Stain, 5 ml	RPN6305
PlusOne™ Bromophenol Blue	17-1329-01
PlusOne DTT	17-1318-01
PlusOne Glycerol	17-1325-01
PlusOne Glycine	17-1323-01
PlusOne SDS	17-1313-01
PlusOne Tris	17-1321-01

Other materials required

Human apotransferrin (Calbiochem)	616395
Bovine cardiac muscle actin (Sigma-Aldrich)	A3653
Rabbit polyclonal anti–human transferrin (Dako Cytomation)	A0061
Monoclonal Anti-Actin, mouse-α-bovine (Sigma-Aldrich)	A4700
Anti-MAP Kinase (ERK-1, ERK-2), rabbit (Sigma-Aldrich)	M5670
Monoclonal Anti-β-Tubulin, mouse (Sigma-Aldrich)	T4026
Phospho-Akt (Ser473) Antibody, rabbit (Cell Signaling)	9271S
Phospho-p38 MAP Kinase (Thr180/Tyr182) Antibody, rabbit (Cell Signaling)	9211S
Alexa Fluor™ 680 goat-α-mouse IgG (Invitrogen)	A-21058
IRDye™ 800 Conjugated Affinity Purified Anti-Rabbit IgG, Goat (Rockland Immunochemicals)	611-132-003
Odyssey™ Infrared Imaging System (LI-COR Biosciences)	9201
10x PBS (Medicago)	12-9423-5
Novex™ 12% Tris-glycine gel (Invitrogen)	EC60055BOX
Methanol (Merck)	K33730207
Tween™ 20 (Merck)	8.22184.1000

Methods—single protein detection

Gel electrophoresis

Human apotransferrin was loaded onto Novex 12% Tris-glycine gels in a series of two-fold dilutions from 5 ng to 0.6 pg. Gel electrophoresis was performed for 2.5 h at 100 V using the miniVE Vertical Electrophoresis System. Gels were blotted, and then detected with either chemiluminescent or fluorescent systems.

Chemiluminescent detection

After electrophoretic separation the gels were blotted onto a Hybond ECL membrane for 2.5 h at 25 V using a TE 22 Mini Tank Transfer Unit followed by incubation with the recommended blocking solution for each detection system (ECL, ECL Plus, or ECL Advance).

Incubations were performed using the following antibody dilutions:

	Primary (anti-transferrin)	Secondary (HRP-conjugated anti-rabbit IgG)
ECL	1:1500	1:10000
ECL Plus	1:10000	1:100000
ECL Advance	1:100000	1:400000

The corresponding chemiluminescent detection reagent was then used to visualize the protein bands according to the manufacturer's instructions (GE Healthcare).

Fluorescent detection

After electrophoretic separation the gels were blotted onto either Hybond ECL (low-fluorescent nitrocellulose) or Hybond-LFP (low-fluorescent PVDF) membranes for 2.5 h at 25 V using a TE 22 Mini Tank Transfer Unit followed by incubation in PBS + 0.1% Tween-20 (PBST) blocking solution overnight at 4 °C.

The blots were then incubated with the rabbit anti–human transferrin primary antibody (1:750 dilution in PBST) for 1.5 h at room temperature. They were washed twice quickly, then twice for 5 min each in PBST, and then incubated for 1 h, protected from light, with the secondary antibody, ECL Plex goat-α-rabbit IgG-Cy5 (1:2500 dilution in PBST).

The membranes were then washed three times quickly, then four times for 5 min each in PBST followed by two brief washes in PBS before scanning on the Typhoon scanner. Imaging was performed using the 633-nm (red) laser with a 670BP30 band pass filter. The images were then analyzed using ImageQuant software. Limit of detection, linearity, and reproducibility between three separate blots were evaluated.

Total protein staining

An evaluation of total protein staining of blots was also performed using Deep Purple Total Protein Stain. A four-fold dilution series of apotransferrin from 5 ng to 0.6 pg was loaded onto a gel, separated, and then blotted onto Hybond ECL and Hybond-LFP membranes (see Fluorescent detection section above). The protein blots were stained with Deep Purple Total Protein Stain according to the manufacturer's protocol (GE Healthcare), and then scanned with the Typhoon scanner. Sensitivity was then evaluated using ImageQuant software.

Methods—multiplex protein detection

Model system

A protein mixture of human apotransferrin and bovine cardiac muscle actin was loaded onto Novex 12% Tris-glycine gels in four-fold dilutions from 5 ng to 1.2 pg (transferrin) and in two-fold dilutions from 150 to 2.34 ng (actin).

The electrophoresis, protein transfer, blocking, and washing steps were identical to the single protein detection protocol. Both the Hybond ECL and Hybond-LFP membranes were used.

After the blocking step, the blots were incubated in a mixture of rabbit anti–human transferrin and mouse anti–bovine actin primary antibodies (diluted 1:750 in PBST) for 1.5 h at room temperature. Following washing in PBST, the blots were incubated with the secondary antibodies, ECL Plex goat-α-rabbit IgG-Cy5 and ECL Plex goat-α-mouse IgG-Cy3 (both diluted 1:2500 in PBST), for 1 h at room temperature and protected from light.

The membranes were scanned for both Cy3 (532-nm green laser) and Cy5 (633-nm red laser) in a single scan. The images were then analyzed using ImageQuant software. An alternative two-color Western blotting system was also evaluated. The secondary antibodies Alexa Fluor 680 goat-α-mouse IgG and IRDye 800 goat-α-rabbit IgG were also run in parallel on Hybond ECL, followed by imaging on the Odyssey scanner. Limit of detection and cross-reactivity between the two antibodies were analyzed for all alternatives.

Applications

TGF-β is a potent growth factor stimulating a number of cellular responses including growth inhibition, cell differentiation, and apoptosis. In this application, human T293 epithelial kidney cells were activated with TGF-β and harvested at different time points. Lysates of these cells were prepared, run on gels, transferred to Hybond ECL membranes, and then blotted for selected proteins according to the protocol above. Three different protein kinases were studied: unphosphorylated ERK1/2, a highly abundant protein, and phosphorylated Akt (pAkt) and p38 (pp38) as examples of low-abundant proteins.

The combinations of primary and secondary antibodies used were:

- Anti-MAP Kinase (ERK-1, ERK-2) and Monoclonal Anti-β-Tubulin targeted with ECL Plex goat-α-rabbit IgG-Cy5 and ECL Plex goat-α-mouse IgG-Cy3, respectively
- Phospho-Akt (Ser473) Antibody and Monoclonal Anti-β-Tubulin targeted with ECL Plex goat-α-rabbit IgG-Cy5 and ECL Plex goat-α-mouse IgG-Cy3, respectively
- Monoclonal Anti-Actin and Phospho-p38 MAP Kinase (Thr180/Tyr182) Antibody targeted with ECL Plex goat-α-mouse IgG-Cy3 and ECL Plex goat-α-rabbit IgG-Cy5, respectively

Optimization of ECL Plex conjugated antibodies

To achieve the highest specificity and sensitivity in the ECL Plex system, the CyDye-conjugated antibodies have been carefully selected and the protocols thoroughly optimized. A number of conjugate variants were tested to find the best candidates, minimizing the dye interaction effect often seen in immunohistochemical applications with fluorescence (data not shown here). To prevent cross-reaction, highly cross-absorbed secondary antibodies made in the same host species (goat) were chosen. A comparison with a multiplex system from an alternative supplier was also made.

Results and discussion

Sensitivity

Three chemiluminescent detection systems from GE Healthcare (ECL, ECL Plus, and ECL Advance) were compared with ECL Plex Cy5-conjugated fluorescent detection (Fig 1). The displayed blots are representative of an experiment performed in triplicate. The detection limits are shown in Table 1.

Our results demonstrate that ECL Plex has similar sensitivity to ECL Advance. Unlike previous reports where the chemi-luminescent approach reached a higher sensitivity than CyDye-labeled fluorescent antibodies (3), the optimized ECL Plex Cy5 conjugate is as sensitive as the ECL Advance kit, and superior to ECL and ECL Plus.

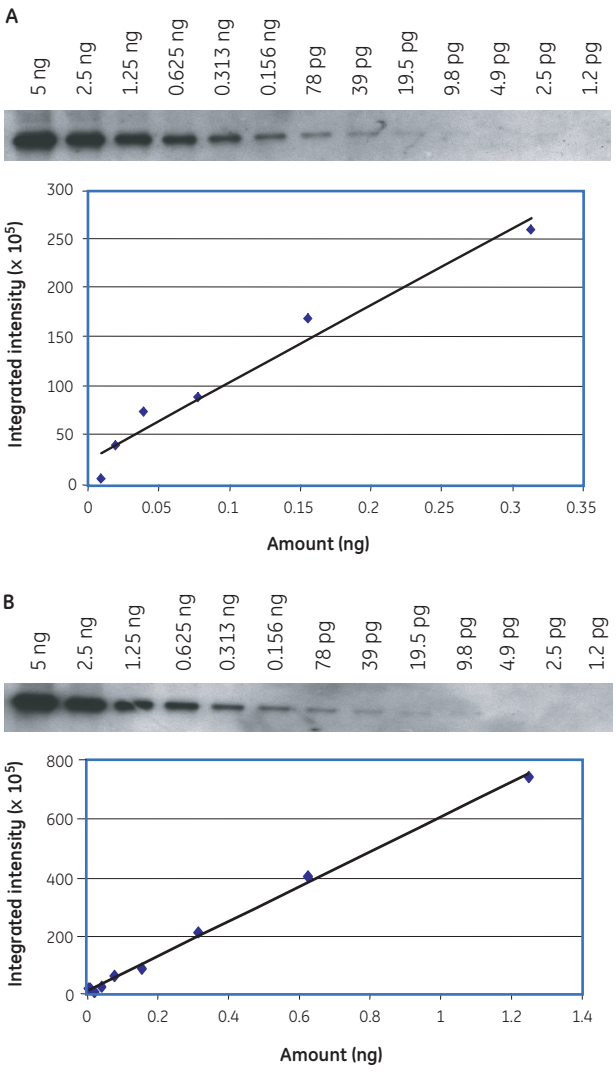
Linearity

To determine the linearity of the data the square of the correlation coefficient (R^2) was used. Pixel intensities from the scanned membranes or films were plotted against the amount of protein loaded onto the gel, and a linear curve fit was performed. The graphs are displayed below the corresponding blot images in Figure 1.

For the chemiluminescent analysis, ECL (Fig 1A) was qualitative, while ECL Plus (Fig 1B) and ECL Advance (Fig 1C) had semiquantitative dynamic ranges of 255-fold and 260-fold differences in protein abundance, respectively. The ECL Plex system had a linear detection range of > 4000-fold differences, with an R^2 value of 0.998 using the Hybond ECL membrane (Fig 1D) and 0.986 using the Hybond-LFP membrane (Fig 1E). This offers a system where the signal is proportional to the amount of protein over 3.6 orders of magnitude. The ECL Plex system proves to be the method of choice when linear quantitation is important (Table 1).

Table 1. Comparison of sensitivity and linearity data for the ECL, ECL Plus, ECL Advance, and ECL Plex systems.

System	Detection limit (pg)	Linearity (R^2)	Dynamic range	
			Orders of magnitude	Range
ECL	9.8	0.961	1.5	0.313 ng–9.8 pg
ECL Plus	4.9	0.997	2.4	1.25 ng–4.9 pg
ECL Advance	0.6	0.978	2.4	0.156 ng–0.6 pg
ECL Plex				
Hybond ECL	1.2	0.998	3.6	5 ng–1.2 pg
Hybond-LFP	1.2	0.986	3.6	5 ng–1.2 pg



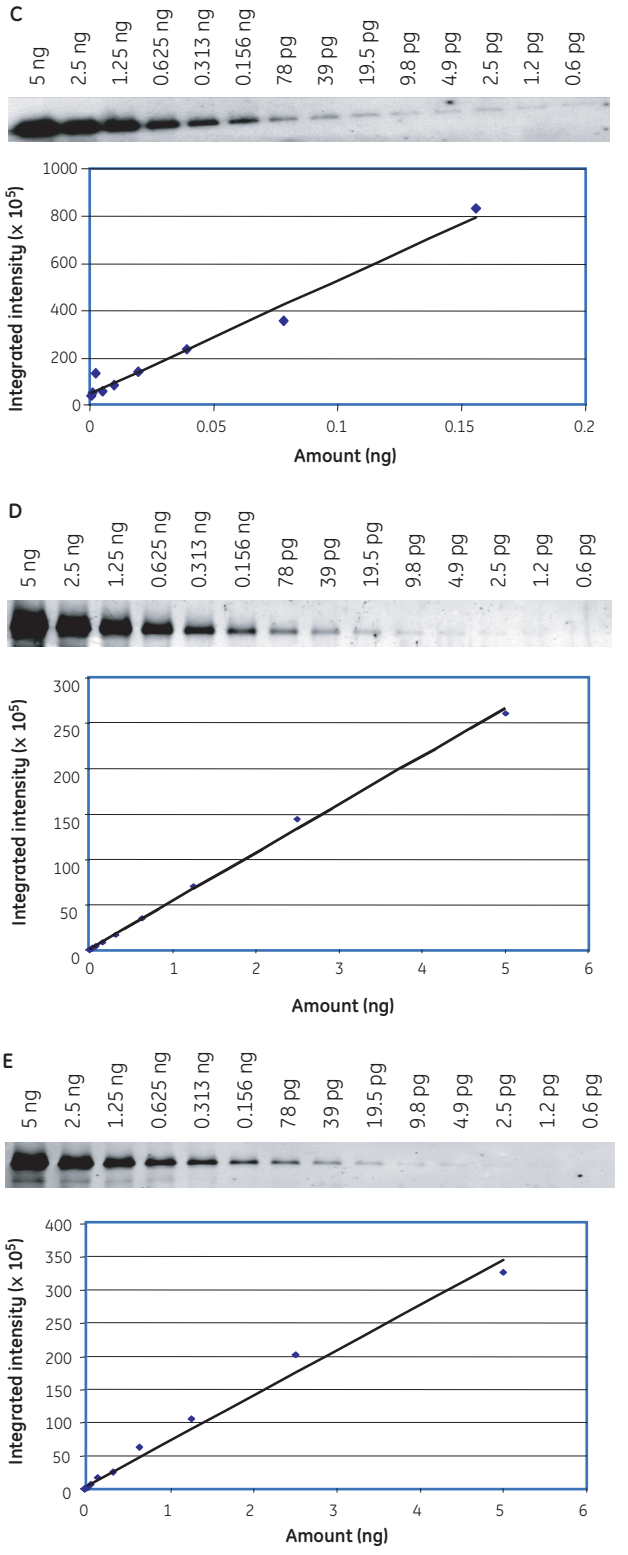


Fig 1. Human apotransferrin, two-fold dilutions starting at 5 ng. Blotted on Hybond ECL and detected with ECL (A), ECL Plus (B), or ECL Advance (C); blotted on Hybond ECL or Hybond-LFP and detected with ECL Plex goat-α-rabbit IgG-Cy5 (D, Hybond ECL; E, Hybond-LFP). Scanned blots (top); resulting linearity plots based on data from ImageQuant software (below). R^2 values are shown in Table 1.

Reproducibility

In Figures 2A and 2B, the reproducibility of the ECL Plex system is shown. Reproducibility between triplicate gels was evaluated by statistical analysis (SAS JMP v.5.1). All three data points from the scanned blots were plotted in the same diagram, and the variation from the mean in a log-log scale was calculated. Evaluations were performed with both the Hybond ECL and Hybond-LFP membranes (Table 2).

For Hybond ECL (Fig 2A), the relative standard deviation (RSD) of the volume readings was less than 1% in the range from 5 ng to 78 pg of protein and between 1% and 5% in the range from 39 to 1.2 pg of protein. A higher relative variation is expected as the amount of protein decreases. The corresponding values for Hybond-LFP (Fig 2B) were similar or slightly higher. The standard deviation of the linear fit was 0.27 for Hybond-LFP and 0.11 for Hybond ECL, indicating a better reproducibility with the latter. On the other hand, the slope of the line, which is an indication of the sensitivity of the assay, was 2.34 for Hybond-LFP and 0.88 for Hybond ECL. High reproducibility is achieved by a low standard deviation and a steep slope. Thus, both membrane types have their advantages regarding reproducibility.

The overall levels of variation were low, showing that the ECL Plex system delivers reliable data in a reproducible way. Note also that the signal from blots with the ECL Plex antibodies is stable for up to three months.

Table 2. Reproducibility of the ECL Plex system, indicated by RSD of log intensity values.

Protein amount (ng)	RSD, Hybond ECL (%)	RSD, Hybond-LFP (%)
0.0012	5.4	3.2
0.0025	1.9	2.3
0.0049	2.5	4.8
0.0098	0.7	6.0
0.0195	2.1	2.4
0.039	1.2	0.6
0.078	0.9	0.5
0.156	0.6	2.0
0.313	0.6	1.0
0.625	0.7	0.8
1.25	0.3	1.4
2.5	0.4	1.2
5	0.3	1.0

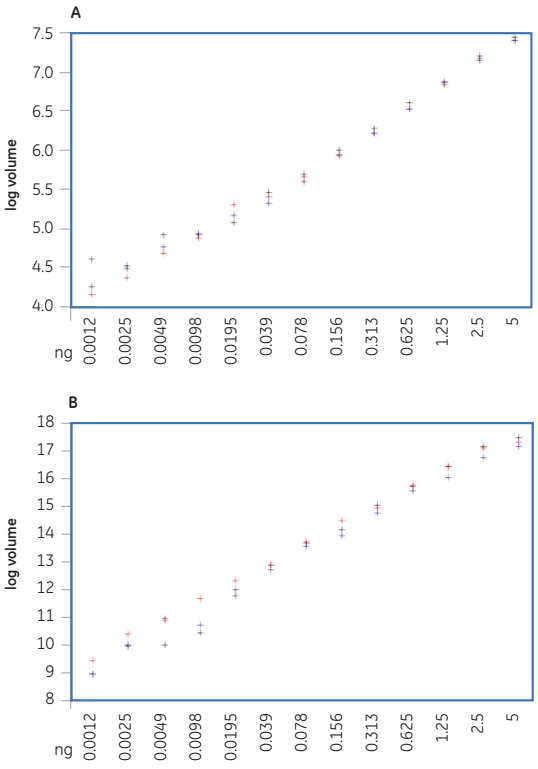


Fig 2. Reproducibility plots for ECL Plex on Hybond ECL (A) and Hybond-LFP (B). Shown are the variability charts from SAS JMP v.5.1 software for log volume in a triplicate experiment. The resulting standard deviations for each protein amount are shown in Table 2.

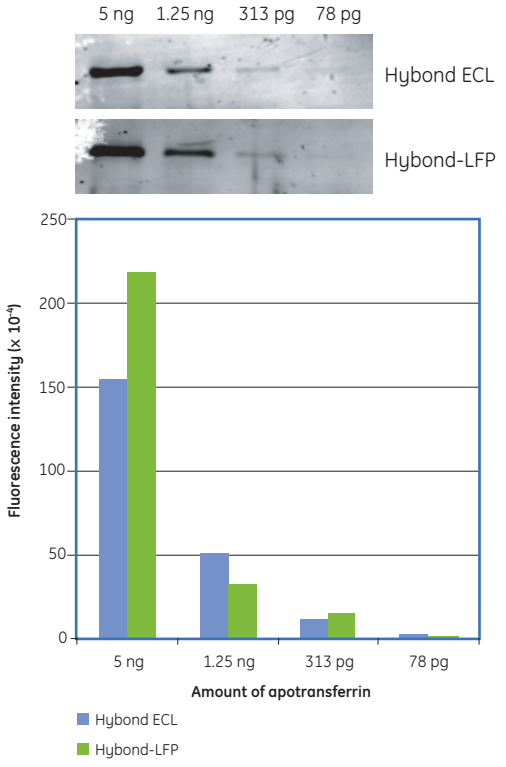


Fig 3. Blot staining with Deep Purple Total Protein Stain. Resulting blots (top) and the plotted fluorescence intensity vs protein loading (bottom). The binding capacity was measured by detecting the resulting protein bands on the membrane, correcting for background intensity, and integrating the volumes. Both the Hybond ECL and Hybond-LFP membranes were evaluated as indicated.

Total protein blot stain

The sensitivity of Deep Purple Total Protein Stain was evaluated in ImageQuant software. The resulting blots and graph is shown in Figure 3. The detection limit was 78 pg of protein in the membrane, for both the Hybond ECL and Hybond-LFP membranes, confirming Deep Purple Total Protein Stain as a sensitive fluorescent stain.

Membranes can be destained before antibody labeling.

Multiplex model system

Using a mixture of ECL Plex goat-α-mouse IgG-Cy3 (green) and ECL Plex goat-α-rabbit IgG-Cy5 (red) fluorescent antibodies, actin and transferrin were detected in one reaction simultaneously (Fig 4A). Both the overlaid color image and the separate images from the respective Cy3 and Cy5 channels are displayed.

The indicated cross-reactivity in this application originates from a dye interaction effect between Cy3 and Cy5. It is defined as percent signal in the Cy3 channel coming from the protein labeled with Cy5, divided by signal from the true Cy3-labeled protein (largest amount), and vice versa.

The ECL Plex products and protocols are optimized to produce minimal levels of cross-reactivity. In an application from another supplier (LI-COR Biosciences), the level of cross-reactivity is higher when running according to the manufacturer's recommendations (Fig 4B) with the same model system.

Multiplex applications

Three different multiplex applications are shown. Figure 5 shows an ECL Plex application on a TGF-β activated cell line, targeted with antibodies against β-tubulin and ERK 1/2. The images clearly show that specificity is very high, resulting in no detectable cross-reactivity. In another experiment (Fig 6), TGF-β–mediated phosphorylation was evaluated by detection of phospho-Akt (pAkt) and phosphorylated p38 (pp38). Quantitation of activation was also determined, where increasing time of TGF-β stimulation results in a proportional increase of pp38. In the pAkt experiment, the same linear increase of phosphorylated protein was not observed. One reason for this might be the cell-line dependence of TGF-β activation that is often seen. These results clearly indicate the quantitation power of the ECL Plex System, and that the results are trustworthy, even when not as expected.

Optimization of ECL Plex

The importance of selecting dye conjugates with minimal cross-reactivity is shown in Figure 7, where an example of a suboptimal combination of conjugated antibodies was used. The conjugates included in the ECL Plex system are shown as a reference.

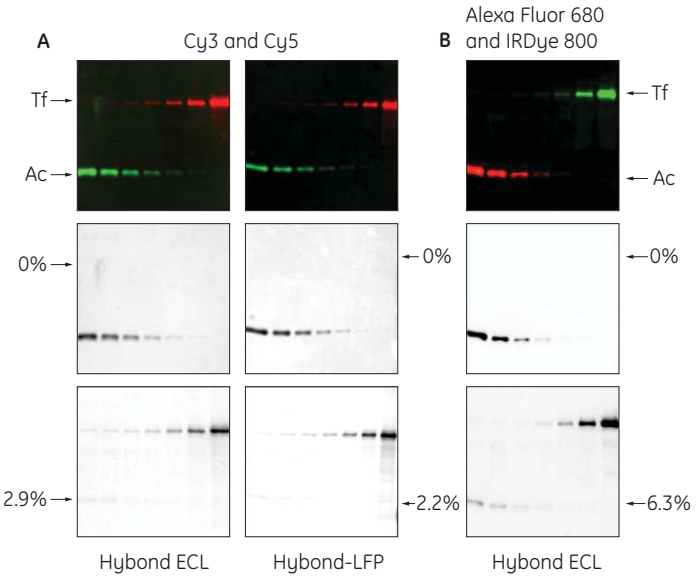


Fig 4. A: ECL Plex multiplex model system with transferrin (Tf) and actin (Ac) on Hybond ECL (left) and Hybond-LFP (right), imaged on the Typhoon 9410. Overlaid color image (top), Cy3 channel (middle), and Cy5 channel (bottom). B: Two-color Western blot with infrared fluorescence with Alexa Fluor 680 and IRDye 800 dyes, imaged on the Odyssey Infrared Imaging System. Level of cross-reactivity is indicated on each image.

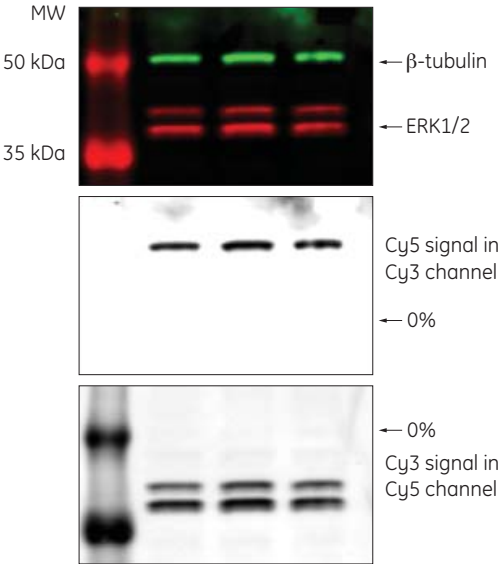


Fig 5. T293 cells stimulated 0, 5, or 15 min with TGF-β. β-Tubulin and ERK1/ERK2 detected with ECL Plex antibodies. False signal in respective channel due to dye interaction is shown (%).

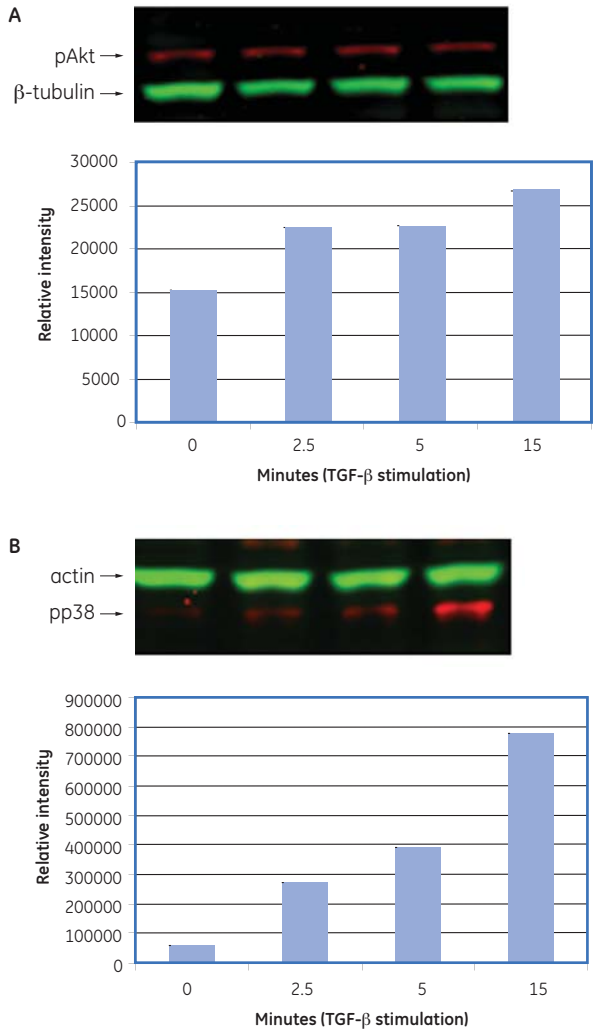


Fig 6. T293 cells stimulated 0, 2.5, 5, or 15 min with TGF-β. Phosphorylated and total proteins detected with polyclonal anti-phospho-Akt (Ser 473) and monoclonal anti-β-tubulin (A) on Hybond ECL membrane, and monoclonal anti–bovine actin and phospho-p38 MAP kinase antibody (B) on Hybond LFP membrane, targeted with ECL Plex secondary antibodies. The graphs below the figures display the scored intensity of pAkt and pp38 relative to the amount of anti-β-tubulin and anti-actin, respectively.

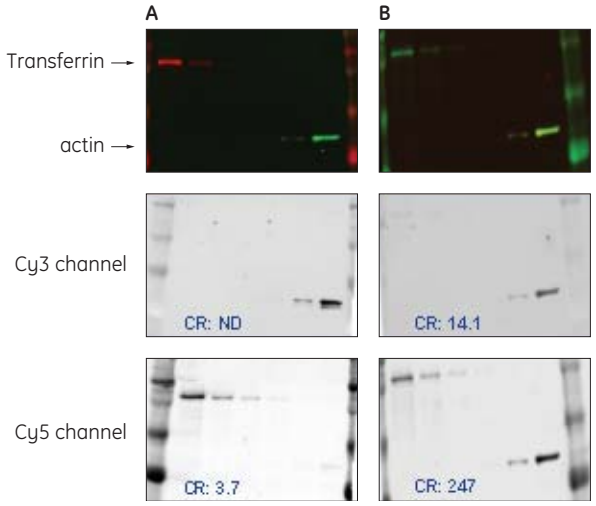


Fig 7. Multiplex protein detection on Typhoon scanner: calculation of cross-reactivity with different sets of dye conjugates. Transferrin and actin loaded on gel in two-fold and four-fold dilutions, respectively, as described. ECL Plex secondary antibodies (A) vs other CyDye conjugates tested during development (B). Cross-reactivity (CR) % noted in each image. (ND = Not detectable)

Conclusions

Detecting two proteins in the same blot has many benefits. One advantage is the possibility of normalizing one protein against another without the need for stripping and reprobing of the blot, enabling reliable differential analysis. Stripping can cause protein loss, which in turn affects the protein quantitation, making the results less reliable.

ECL Plex demonstrated the same sensitivity as the most sensitive chemiluminescent kit, ECL Advance, with a wider dynamic range. In addition, fluorescence has a more straightforward process for getting results than chemiluminescence, long-lasting signal stability, and no need for a darkroom.

Previous reports using CyDye-conjugated antibodies have not shown the same sensitivity as ECL Plex (4). Among the reasons why the fluorescent Western blotting system here demonstrates such high sensitivity are the selection of dye conjugates, the use of low-fluorescent membranes, and the use of an imager with high sensitivity and broad dynamic range.

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Asia Pacific	Tel: +852 2811 8693	Fax: +852 2811 5251
Australasia	Tel: + 61 2 9899 0999	Fax: +61 2 9899 7511
Austria	Tel: 01/57606-1619	Fax: 01/57606-1627
Belgium	Tel: 0800 73 888	Fax: 03 272 1637
Canada	Tel: 1 800 463 5800	Fax: 1 800 567 1008
Central, East, & South East Europe	Tel: +43 1 982 3826	Fax: +43 1 985 8327
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GE Healthcare
Amersham Place
Little Chalfont
Buckinghamshire
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