New and improved ECL Plex Cy3 conjugates for multiplex Western blotting

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

The Amersham™ ECL Plex™ system has been complemented with a new antibody conjugate, ECL Plex goat-anti-rabbit IgG-Cy™3, and an improved ECL Plex goat-anti-mouse IgG-Cy3. These new ECL Plex Cy3 conjugates have a better signal-to-noise ratio than the original Cy3 conjugate, facilitating the detection and quantitation of low-abundant proteins.

Introduction

The ECL Plex Western blotting system—based on sensitive CyDye™ conjugated antibodies, low-fluorescent membranes, and high-performance imagers—allows detection of low-abundant proteins with high linearity and a dynamic range of nearly four orders of magnitude (1). Multiplexing is another important property of the ECL Plex system, where secondary antibody-dye conjugates—carefully selected and optimized for minimal cross-reactivity—enable detection of two proteins simultaneously.

The use of ECL Advance™ blocking agent and Hybond-LFP™ (low-fluorescent PVDF) membranes increases sensitivity and keeps nonspecific detection to a minimum. The ECL Plex Western blotting system is compatible with the Typhoon™ scanner and the new Ettan™ DIGE Imager from GE Healthcare, but many other scanners can also be used. Reliable relative quantitation of two proteins can be performed by using ImageQuant™ TL software.

The ECL Plex system is now complemented with a new ECL Plex goat-anti-rabbit IgG-Cy3 antibody and an improved ECL Plex goat-anti-mouse IgG-Cy3 antibody. These ECL Plex Cy3 conjugates have significantly lower background than the original Cy3 conjugate. This application note describes these new and improved conjugates.

Materials

Products used

ECL Plex goat-α-rabbit IgG-Cy3, 150 µg	28-9011-06
ECL Plex goat-α-mouse IgG-Cy3, 150 µg	PA43009
ECL Plex goat-α-rabbit IgG-Cy5, 150 µg	PA45011
ECL Plex goat-α-mouse IgG-Cy5, 150 µg	PA45009
Hybond-LFP, 20 x 20 cm, 10 sheets	RPN2020LFP
ECL Plex Fluorescent Rainbow™ Markers, full range, 500 µl	RPN851
ECL Advance Blocking Reagent	RPN418
2-D 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
Ettan DIGE Imager, including installation kit	63-0056-42
Ettan DIGE Imager Cassette, with low-fluorescent glass, for naked gels	11-0027-33
Typhoon 9410 and ImageQuant TL	63-0055-80
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
Monoclonal Anti-β-Tubulin, mouse (Sigma-Aldrich)	T4026
Anti-MAP Kinase (ERK-1, ERK-2), rabbit (Sigma-Aldrich)	M5670
Phospho-Akt (Ser 473) Mouse mAb (Cell Signaling)	4051L
Phospho-Akt (Ser473) Antibody, rabbit (Cell Signaling)	9271L
Akt 1 (B-1), mouse monoclonal antibody (Santa Cruz)	5298
Akt 1/2 (H-136), rabbit polyclonal antibody (Santa Cruz)	8312
10 x PBS (Medicago)	12-9423-5
Novex™ 12% Tris-glycine gel (Invitrogen)	EC60055BOX
Methanol (Merck)	1.06009.2511
Tween™ 20 (Merck)	8.22184.1000
Cell Dissociation Buffer, enzyme free, PBS-based (Invitrogen)	13151-014
Chinese hamster ovary (CHO) cells (ECACC)	85050302

Human prostate carcinoma (PC-3U) cells, a subclone of PC-3 [human prostate cancer cells (nr 3); ATCC number CRL-1435] modified for enhanced TGF-β receptor production (2)

Methods

Single-protein detection

Human apotransferrin was loaded onto Novex 12% Tris-glycine gels in a series of two-fold dilutions from 5 ng to 0.6 pg. Bovine cardiac muscle actin was loaded in a two-fold dilution series from 150 ng to 18 pg. Gel electrophoresis was performed for 2.5 h at 100 V using the miniVE Vertical Electrophoresis System.

After electrophoresis, proteins were transferred onto 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) solution overnight with shaking at 4°C.

The membranes were then incubated with the primary antibodies, rabbit anti–human transferrin or mouse anti-actin (1:750 dilutions in PBST) for 1.5 h with shaking at room temperature. After two quick washes followed by two times 5 min each in PBST, membranes were incubated for 1 h, protected from light, with the secondary antibody: ECL Plex goat-anti-rabbit IgG-Cy3 or ECL Plex goat-anti-mouse IgG-Cy3 (1:2500 dilution in PBST).

The membranes were washed three times quickly, then four times for 5 min each in PBST, followed by three quick washes in PBS. Membranes were dried for 1 h at 40°C to obtain a low and even background before being scanned on the Ettan DIGE Imager or the Typhoon scanner. Imaging on the Typhoon scanner was performed using the 633-nm (red) laser with a 670BP30 emission filter for Cy5 conjugates and the 532-nm (green) laser with a 580BP30 emission filter (TAMRA) for Cy3 conjugates. On the Ettan DIGE Imager, scanning was performed using the 635BP30 excitation filter with the 680BP30 emission filter for Cy5 conjugates and the 540BP30 excitation filter with the 595BP25 emission filter for Cy3. The PMT values (Typhoon) or exposure levels (Ettan DIGE Imager) were adjusted until the most intense band nearly reached saturation.

Note: An emission filter setting of 555BP20 for Cy3 on the Typhoon scanner (default Cy3 setting on Typhoon 8600) will result in suboptimal sensitivity (weak signals and high background) and should therefore be avoided.

Images were analyzed using ImageQuant TL software to determine the limit of detection, linearity, and the linear dynamic range.

Reproducibility

Reproducibility data was collected by performing single-protein detection using a two-fold dilution series of transferrin or actin as described above. The new ECL Plex goat-anti-rabbit IgG-Cy3 and improved ECL Plex goat-anti-mouse IgG-Cy3 were tested in duplicate on three separate days. For each day, different experimenters prepared the dilution series and buffers, and performed the experiments, thereby introducing as much experimental variation as possible. In addition, different batches of membranes and gels were used. Only the batches of primary and secondary antibodies were the same.

Reproducibility between duplicate blots of the ECL Plex system for the three experiments was evaluated by statistical analysis (SAS JMP v 5.1). Data points were excluded from the data set if they deviated more than 50% from the predicted value derived from the regression line.

All data points (signal intensity) for each ECL Plex conjugate were plotted against protein amount in the same diagram on a log-log scale, and the variation from the mean was calculated.

Multiplex protein detection

Chinese hamster ovary (CHO) cells and human prostate cancer (PC-3U) (2) cells were grown using standard cell culture methods in recommended culture media. The cell culture media were removed and the cells were washed with PBS, detached by treatment with enzyme-free cell-dissociation media, washed twice with ice cold PBS, and then lysed with ice-cold lysis buffer (150 mM NaCl, 50 mM Tris, pH 8, 0.5% deoxycholate, 1% NP-40, 10% glycerol, 1 mM aprotinin, 1 mM Pefabloc™, and 1 mM phenylmethylsulfonyl fluoride). After centrifugation, the supernatants were collected and protein concentrations were determined using the 2-D Quant Kit.

The electrophoresis, protein transfer, and washing steps were identical to the single-protein detection protocol.

CHO cell lysates were applied to gels in four-fold dilution series from 5 mg to 78 ng (total protein), and then transferred to Hybond-LFP membranes. After transfer, Hybond-LFP membranes were blocked in 2% ECL Advance blocking agent (in PBST) overnight, shaking at 4°C. After washing, the membrane was incubated with a mixture of rabbit anti-MAP kinase ERK 1/2 (diluted 1:5000 in PBST) and mouse anti-β-tubulin (diluted 1:1000 in PBST) primary antibodies, followed by a mixture of anti-rabbit and anti-mouse IgG CyDye-conjugated ECL Plex secondary antibodies (both diluted 1:2500 in PBST) in all combinations. The membranes were washed in PBST and PBS, dried, and scanned. Limit of detection for the endogenous protein of interest, signal-to-noise ratio, and level of nonspecific detection were determined for all combinations of ECL Plex Cy3 and Cy5 conjugates using Image Quant TL software.

Transforming growth factor β (TGF-β) is a potent growth factor stimulating a number of cellular responses including growth inhibition, cell differentiation, and apoptosis. In one application, human prostate cancer cells (PC-3U) cells, transfected with a plasmid containing a gene X or an empty plasmid, were starved in culture media containing 1% fetal calf serum for 24 h and then stimulated with 10 ng/ml TGF-β for different lengths of time. In a second application, PC-3U cells were first starved for 12 h in culture media containing 1% fetal calf serum, and then pretreated for 1 h with or without 10 µM phosphatidylinositol-3 kinase inhibitor (LY) followed by stimulation with 10 ng/ml TGF-β for different lengths of time. Cells were harvested at different time points of TGF-β stimulation, lysates prepared as described above, and volumes corresponding to 35 mg of protein were applied to 1-D SDS-PAGE gels and transferred to Hybond-LFP membranes. After transfer, the Hybond-LFP membranes were blocked in 2% ECL Advance blocking agent (in TBST) overnight with shaking at 4°C.

The targeted proteins were the phosphorylated form of Akt (pAkt) and total Akt protein, which are low-abundant proteins involved in the TGF-β signaling pathway.

In the first application, after washing the membrane was incubated with rabbit anti-pAkt (diluted 1:1000 in TBST) and mouse anti–total Akt (diluted 1:1000 in TBST) primary antibodies, followed by incubation with ECL Plex goat-anti-mouse IgG-Cy5 and new ECL Plex goat-anti-rabbit IgG-Cy3 secondary antibodies (both diluted 1:2500 in TBST), as described above. In the second application, after washing the membrane was incubated with mouse anti-pAkt (diluted 1:1000 in TBST) and rabbit anti–total Akt (diluted 1:1000 in TBST) primary antibodies, followed by incubation with ECL Plex goat-anti-rabbit IgG-Cy5 and improved ECL Plex goat-anti-mouse IgG-Cy3 secondary antibodies (both diluted 1:2500 in TBST), as described above.

The membranes were washed in TBST and TBS, dried, and then scanned. Quantitation of the pAkt signals in response to TGF-β stimulation was performed using Image Quant TL software and related to the amount of total Akt by dividing the pAkt signal by the total Akt signal. Note that TBST and TBS were used throughout the protocol, which is recommended for detection of phosphorylated proteins.

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. To prevent cross-reaction, highly cross-absorbed secondary antibodies made in the same host species (goat) were chosen.

Results and discussion

Sensitivity, linearity, and dynamic range

The improved ECL Plex goat-anti-mouse IgG-Cy3 has a reduced background (increased signal-to-noise ratio) compared to the original ECL Plex goat-anti-mouse IgG-Cy3 (Figs 1A and 1B, Table 1). Also, the new ECL Plex goat-anti-rabbit IgG-Cy3 conjugate showed a higher signal-to-noise ratio compared to a candidate goat-anti-rabbit IgG-Cy3 conjugate during development (Figs 1C and 1D, Table 1).

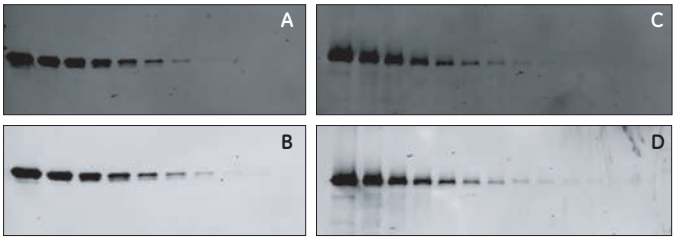

Fig 1. A two-fold dilution series of actin starting at 150 ng (A and B) or transferrin starting at 5 ng (C and D) was applied to 1-D gels and transferred to Hybond-LFP membranes. Actin was detected with mouse anti-actin primary antibody and either the original ECL Plex goat-anti-mouse IgG-Cy3 (A), or the improved ECL Plex goat-anti-mouse IgG-Cy3 (B). Transferrin was detected with rabbit anti-transferrin primary antibody and either a candidate goat-anti-rabbit IgG-Cy3 (C), or the new ECL Plex goat-anti-rabbit IgG-Cy3 (D). The membranes were dried and scanned side by side simultaneously with the same PMT setting using the Typhoon 9410.

Table 1. Sensitivity, dynamic range, linearity, and signal-to-noise ratio (S/N) for ECL Plex Cy3 conjugates.

Secondary antibody	Detection limit	Dynamic range (orders of magnitude)	Linearity (R^2)	S/N
Original Cy3 anti-mouse	0.6 ng	150 ng–0.6 ng (2.4)	0.988	493
Improved Cy3 anti-mouse	0.6 ng	150 ng–0.6 ng (2.4)	0.992	994
Candidate Cy3 anti-rabbit	9.8 pg	5 ng–9.8 pg (3.3)	0.982	230
New Cy3 anti-rabbit	1.2 pg	5 ng–1.2 pg (3.6)	0.996	629

The improved ECL Plex goat-anti-mouse IgG-Cy3 detected 0.6 ng of the model protein actin with a linear dynamic range of 2.4 orders of magnitude (Figs 2A and 2B, Table 2). The new ECL Plex goat-anti-rabbit IgG-Cy3 detected as low as 1.2 pg of the model protein transferrin with a linear dynamic range of 3.6 orders of magnitude (Figs 2C and 2D, Table 2). The new ECL Plex Cy3 conjugates both performed similarly to the corresponding ECL Plex Cy5 conjugates (1). Actin was not detected at as low amounts as transferrin, most likely due to limitations of the primary antibody directed towards actin. The Ettan DIGE Imager and Typhoon detected proteins probed with ECL Plex Cy3 or Cy5 conjugates equally well and gave the same sensitivity, linearity, and dynamic range using the ECL Plex Western blotting system (Fig 2, Table 2).

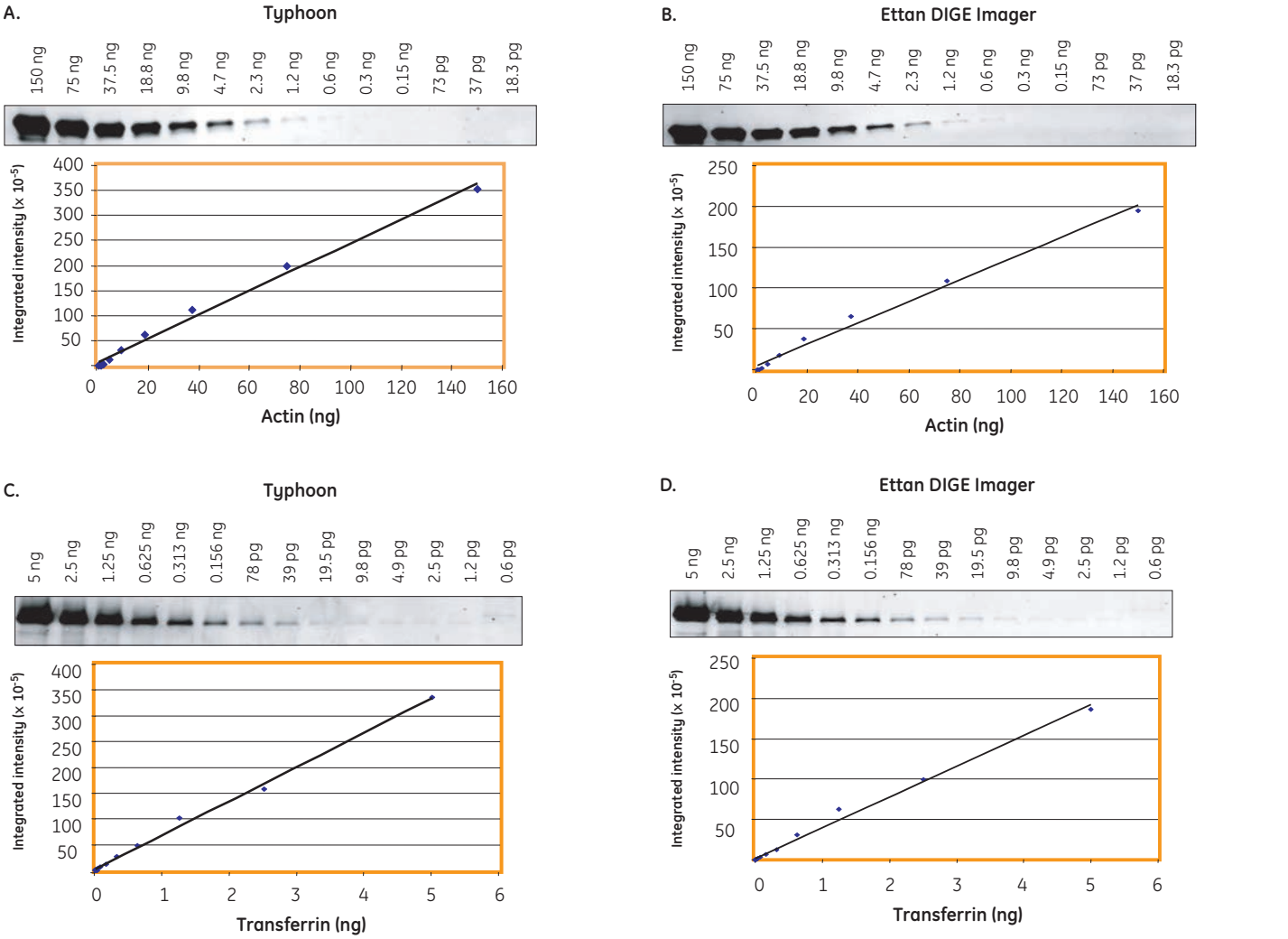

Fig 2. A two-fold dilution series of actin starting at 150 ng was detected with a mouse anti-actin primary antibody and the improved ECL Plex goat-anti-mouse IgG-Cy3 (A and B). A two-fold dilution series of transferrin starting at 5 ng was detected with a rabbit anti-transferrin primary antibody and the new ECL Plex goat-anti-rabbit IgG-Cy3 (C and D). The Typhoon 9410 (A and C) or Ettan DIGE Imager (B and D) was used.

Table 2. Sensitivity, dynamic range, and linearity for the new and improved ECL Plex Cy3 conjugates on the Typhoon scanner and Ettan DIGE Imager.

Secondary antibody	Instrument	Detection limit	Dynamic range (orders of magnitude)	Linearity (R^2)
Improved Cy3 anti-mouse	Typhoon	0.6 ng	150 ng–0.6 ng (2.4)	0.992
	Ettan DIGE Imager	0.6 ng	150 ng–0.6 ng (2.4)	0.989
New Cy3 anti-rabbit	Typhoon	1.2 pg	5 ng–1.2 pg (3.6)	0.996
	Ettan DIGE Imager	1.2 pg	5 ng–1.2 pg (3.6)	0.993

Reproducibility

In Figures 3A and 3B, the reproducibility of the ECL Plex system is demonstrated. The relative standard deviation (RSD) was calculated by dividing the standard deviation by the mean of the data points for each protein concentration. The RSD for the new ECL Plex goat-anti-rabbit IgG-Cy3 varied between 1.2 and 2.4%, and the RSD values for the improved ECL Plex goat-anti-mouse IgG-Cy3 varied between 2.4 and 6.6%. A higher relative variation was seen and is expected as the amount of protein decreases. Despite all of the variables introduced (see Methods) and the natural variation expected for all the steps involved in Western blotting, the levels of variation are low, showing that the ECL Plex system delivers reliable data in a reproducible way.

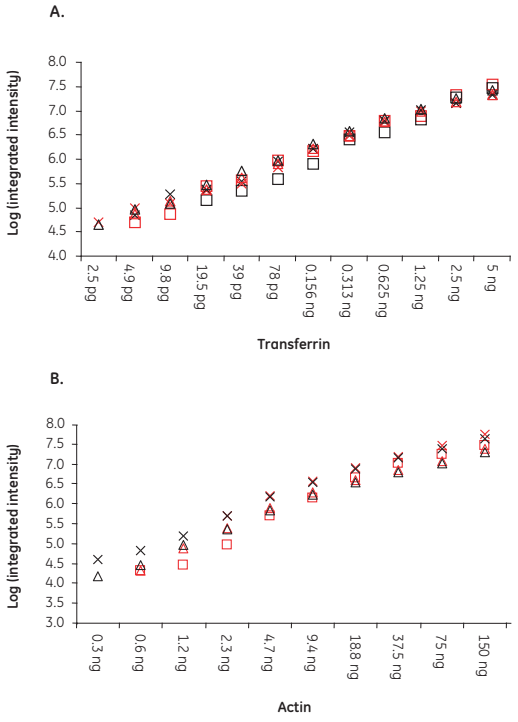

Fig 3. Variability of ECL Plex direct fluorescence detection of two-fold dilution series of the model proteins transferrin and actin performed in duplicate on three separate days (see Methods). Reproducibility plots for the new ECL Plex anti-rabbit Cy3 (A) and the improved ECL Plex anti-mouse Cy3 (B) are shown for Hybond-LFP membranes scanned dry using the Typhoon 9410. For each protein concentration, the different symbols (multiplication sign, triangle, and square) represent the different days of the experiment and the different colors (red or black) discriminate between the replicate sample series.

Multiplex applications

The improved ECL Plex goat-anti-mouse IgG-Cy3 has a reduced background and approximately two-fold higher signal-to-noise ratio compared to the original ECL Plex goat-anti-mouse IgG-Cy3 (Fig 4A vs 4B). Also, the new ECL Plex goat-anti-rabbit IgG-Cy3 was superior in signal-to-noise ratio compared to another candidate Cy3 conjugate tested (Fig 4C vs 4D). The candidate anti-rabbit IgG-Cy3 conjugate showed higher background and had nonspecific detection (Fig 4D). A reduced background facilitates the detection and quantitation of low-abundant proteins.

The ECL Plex secondary antibodies were tested for detection of tubulin and MAP kinase ERK 1/2 in cell lysates. These endogenous proteins were detected down to 78 ng of total CHO cell lysate protein. Only a small fraction of the sample was the protein of interest, indicating that the ECL Plex system can detect endogenous proteins at picogram levels. Both the improved ECL Plex goat-anti-mouse IgG-Cy3 and the new ECL Plex goat-anti-rabbit IgG-Cy3 detected MAP kinase ERK 1/2 and β-tubulin, respectively, in 313 ng of CHO cell lysate (Figs 4A and 4C). The level of nonspecific detection was very low using the CHO cell lysate. The extra band seen below tubulin is a degradation product and not nonspecific detection.

The same experiments were performed using several other cell lysates such as 3T3 fibroblasts, PC-3U cells, and mouse embryonic fibroblasts showing the same sensitive and specific detection (data not shown).

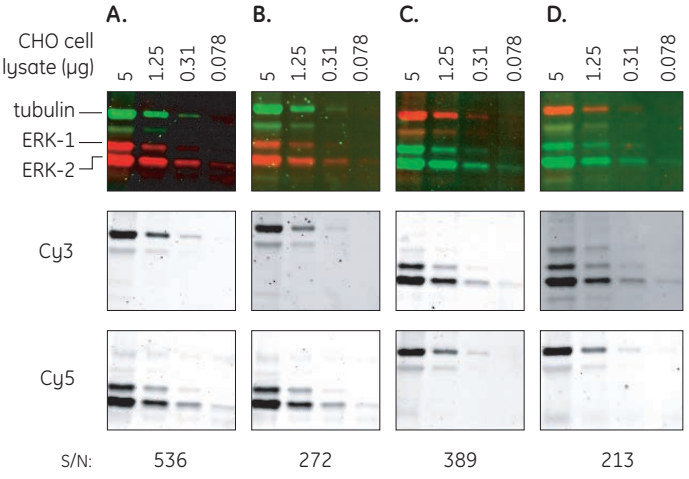

Fig 4. Multiplex detection of endogenous proteins. Four-fold dilution series of CHO cell lysate, starting at 5 µg total protein, were subjected to multiplex ECL Plex Western blotting using mouse anti-tubulin and rabbit anti-MAP kinase ERK 1/2 primary antibodies. Secondary antibody pairs were: ECL Plex anti-rabbit Cy5 and improved anti-mouse Cy3 (A), anti-rabbit Cy5 and the original ECL Plex anti-mouse Cy3 (B), the new ECL Plex anti-rabbit Cy3 and anti-mouse Cy5 (C), and a candidate anti-rabbit Cy3 and anti-mouse Cy5 (D). The Hybond-LFP membranes were dried before scanning on the Typhoon 9410.

Multiplexing is very useful for relative quantitation of a protein of interest against a housekeeping protein, such as tubulin or GAPDH. ECL Plex in combination with Image Quant TL software is a powerful tool for accurate and reliable quantitation in applications were variations in protein amount in response to, for example, stimulation time or dose is investigated.

Two different multiplex applications using PC-3U cells and studying TGF-β signaling are shown. Figure 5 shows an experiment using the improved ECL Plex anti-mouse Cy3, in which increased expression of gene X following transfection causes an increased phosphorylation of Akt in response to TGF-β stimulation compared to control. The same experiment using the original ECL Plex anti-mouse Cy3 gave a lower signal-to-noise and weaker pAkt signals (data not shown).

Figure 6 shows another experiment using the new ECL Plex anti-rabbit Cy3. The expected phosphorylation pattern of Akt in response to TGF-β stimulation was completely absent when the cells were treated with a phosphatidylinositol-3 kinase inhibitor (LY). Again, this shows an example of ECL Plex as a powerful tool for detecting variation in levels of low-abundant phosphoproteins using both the new and improved ECL Plex CyDye conjugates.

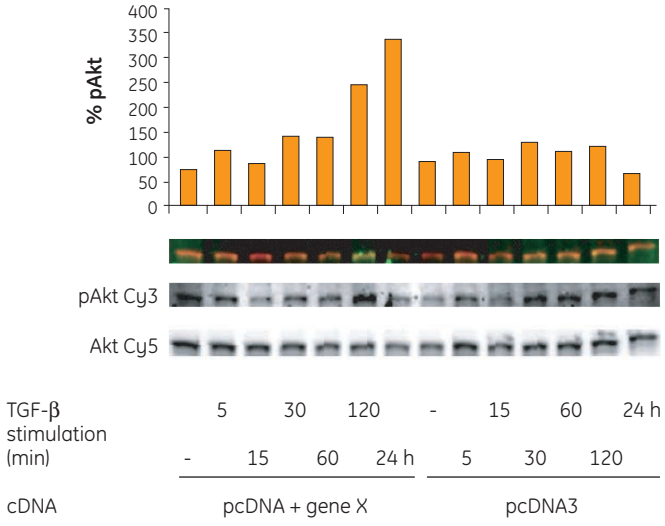

Fig 5. Detection of TGF-β–induced phosphorylation of protein kinase B1 (Akt1) in human prostate cancer (PC-3U) cells. PC-3U cells transfected with plasmid containing gene X (pcDNA3 + gene X) or empty plasmid (pcDNA3) were starved for 24 h, and then stimulated with TGF-β for different lengths of time. Protein extracts were separated on SDS-PAGE gels and then blotted to Hybond-LFP membranes. Primary antibodies: mouse anti-pAkt (Ser 473) and rabbit anti-Akt1. Secondary antibodies: ECL Plex goat-α-rabbit IgG-Cy5 and improved ECL Plex goat-α-mouse IgG-Cy3. The ratio of pAkt/total Akt was calculated and plotted for each sample lane (bar graph). *Data courtesy of Dr. Marene Landström and Anders Marcusson, Ludwig Institute, Uppsala, Sweden.*

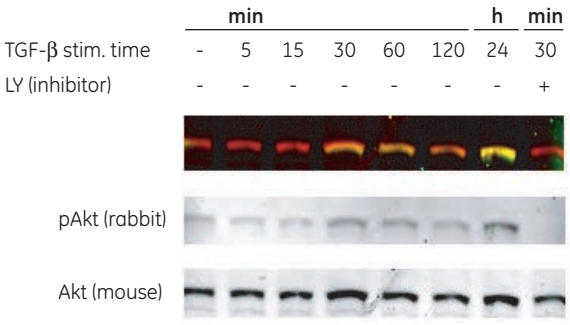

Fig 6. Detection of TGF-β–induced phosphorylation of protein kinase B1 (Akt1) in human prostate cancer (PC-3U) cells. PC-3U cells starved for 12 h, pretreated with or without phosphatidylinositol-3 kinase inhibitor LY for 1 h, and then stimulated with TGF-β for different lengths of time. Protein extracts were separated on SDS-PAGE gels and then blotted to Hybond-LFP membranes. Primary antibodies: rabbit anti-pAkt and mouse anti-Akt1; secondary antibodies: ECL Plex goat-α-mouse IgG-Cy5 and new ECL Plex goat-α-rabbit IgG-Cy3. *Data courtesy of Dr. Marene Landström and Anders Marcusson, Ludwig Institute, Uppsala, Sweden.*

Conclusions

The ECL Plex system has been complemented with an improved goat-anti-mouse IgG-Cy3, as well as a new goat-anti-rabbit IgG-Cy3 with increased signal-to-noise ratio. This allows for more multiplex possibilities. Now, when ECL Plex Cy5 and Cy3 conjugates are available in both anti-rabbit IgG and anti-mouse IgG, the choice of primary antibody is more flexible.

The new and improved ECL Plex Cy3-conjugated antibodies show high sensitivity and specificity, and a wide linear dynamic range. They have a better signal-to-noise ratio than the original Cy3 conjugate. The reduced background for the new and improved ECL Plex Cy3 conjugates facilitates the detection and quantitation of low-abundant proteins. We applied this to the study of low-abundant phosphorylated and nonphosphorylated proteins in various cell lysates.

However, even though the new ECL Plex Cy3 conjugates are shown to be nearly as sensitive as ECL Plex Cy5 conjugates, we still recommend using an ECL Plex Cy5 conjugate for detection of the least-abundant protein of interest in a multiplex experiment. The Cy5 conjugates provide the highest signal-to-noise ratio.

Direct multiplex fluorescent detection by the ECL Plex system gives quantitative, reliable, and reproducible data. ECL Plex CyDye-conjugated antibodies in combination with ECL Advance Blocking Agent and low-fluorescent Hybond-LFP membrane give the highest sensitivity and lowest nonspecific detection and cross-reactivity. The ECL Plex system is easy to use and the fluorescent signals are stable on the membrane for up to three months.

References

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2. Franzén, P. *et al.* Different signals mediate transforming growth factor-α1-induced growth inhibition and extracellular matrix production in prostatic carcinoma cells. *Exp. Cell. Res.* **207**, 1–7 (1993).

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