ECL Plex Western Blotting Detection System: new fluorescent Cy2 and Cy3 conjugates for multiplex protein detection

Introduction

With the ECL Plex™ Western Blotting Detection System, GE Healthcare introduced a range of new products under the well-established Amersham™ ECL™ brand (ECL, ECL Plus, and ECL Advance™). ECL Plex uses direct fluorescent light detection—in contrast to the earlier products, which are based on detection of chemiluminescent or chemifluorescent signals. The ECL Plex system reaches a limit of detection of 1.2 pg in a model system, with a dynamic range over 3.6 orders of magnitude (1). In the multiplex application, two proteins can be detected in the same blot with minimal cross-reactivity between antibodies or dyes (2, 3).

The ECL Plex system is now complemented with an improved ECL Plex goat-anti-mouse IgG-Cy™3 antibody and a new ECL Plex goat-anti-rabbit IgG-Cy3 antibody. The new and improved Cy3 conjugates have significantly lower background than the original Cy3 conjugate, therefore giving sensitivity at the same levels as the Cy5 conjugates. Furthermore, two new ECL Plex goat-anti-rabbit IgG-Cy2 and goat-anti-mouse IgG-Cy2 antibodies allow for multiplex applications using the Storm™ imager.

Benefits

ECL Plex offers:

- Compatibility with Typhoon™ scanner, Ettan™ DIGE Imager, and Storm imager in addition to other multipurpose imagers.* No requirement for additional capital equipment.
- Multiplex analysis with high sensitivity. Proven CyDye™ technology enables multiwavelength detection. No need to strip and reprobe blots: avoids protein loss and saves time.
- Quantitative analysis with broadest dynamic range and highest linearity: detects significant differences in protein levels with high accuracy. Rescanning of membranes is possible after months.
- Optimized system giving the highest specificity: increased accuracy of results.
- Simple protocol for fast analysis with nontoxic products.

Western blotting is an important tool in protein analysis. Current techniques based on enhanced chemiluminescence are very sensitive but offer limited dynamic range and accuracy of quantitation. Fluorescent Western blotting, on the other hand, can have problems with high background from membranes and cross-talk (spectral overlap) between dyes. These issues have been addressed by the ECL Plex system.

The ECL Plex system consists of products selected and optimized for best performance regarding sensitivity, dynamic range, linearity, and signal-to-noise ratio. Together with a high-performance multipurpose imager, such as the Typhoon scanner, this provides high-quality data for single or multiplex analyses (Fig 1).

It is also possible to perform direct in-gel detection with ECL Plex, without blotting onto a membrane. However, this is only recommended for highly expressed proteins and is not quantitative.

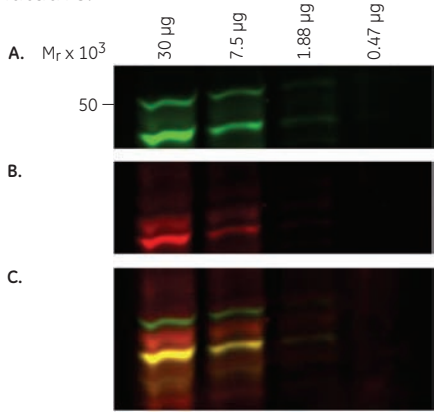

Fig 1. Color images of multiplex Western blot scans. Using a mixture of ECL Plex goat-α-mouse IgG-Cy3 and ECL Plex goat-α-rabbit IgG-Cy5 fluorescent antibodies, GSK3β and phospho-GSK3β (Ser 9) were simultaneously detected in one blot. Four-fold dilutions of human prostate carcinoma (PC-3U) cells from 30 to 0.47 µg of total protein were applied to 1-D gels and transferred to Hybond-LFP membranes. A: GSK3β was detected with rabbit anti-GSK3β primary antibody and ECL Plex Cy3-conjugated secondary antibody; B: phospho-GSK3β was detected with mouse anti–phospho-GSK3β (Ser 9) primary antibody and ECL Plex Cy5-conjugated secondary antibody; C: overlay of A and B. The yellow color of the 48-kDa band shows that the signals from the two primary antibodies overlap: the 48-kDa band contains phosphorylated GSK3β.

* Tested on Typhoon 9410, Ettan DIGE Imager, Storm 860, and a range of other imagers capable of detecting Cy2, Cy3, and Cy5 fluorescence (data available on request).

High sensitivity and linearity with wide dynamic range

Optimization of conjugated antibodies, membranes with low fluorescence characteristics, and blocking buffer provides high-quality results. Now, six different ECL Plex secondary antibody conjugates have been developed for best performance. The following membranes have been selected: Hybond-LFP™, a low-fluorescent PVDF membrane recommended when stripping is required and for detecting low-abundant proteins; and Hybond™ ECL, nitrocellulose membrane for high-abundant proteins. Many blocking solutions are compatible (Table 1), but we recommend 2% ECL Advance Blocking Reagent in PBST (PBS + 0.1% Tween™ 20) or TBST (TBS + 0.1% Tween-20) used with Hybond-LFP membranes to reduce nonspecific detection. The blocking solution should be optimized for each new primary antibody used with ECL Plex because different antibodies will perform differently under different blocking conditions.

Table 1. Compatibility of blocking solutions with the ECL Plex system

Membrane	2% ECL Advance Blocking Reagent in PBST/TBST	5% BSA in PBS/TBS	5% ECL Blocking Agent in PBST/TBST	10% gelatin
Hybond-LFP	++++	+++	++	–
Hybond ECL	+++	+++	++	–

++++ = high performance, +++ = good performance, ++ = acceptable performance, + = poor performance, – = not compatible

Ratings are based on overall performance, including level of autofluorescence/background, nonspecific detection, and signal intensity.

Figures 2 and 3 show the performance of the improved and new ECL Plex secondary antibodies on Hybond-LFP membranes, scanned on the Typhoon 9410.

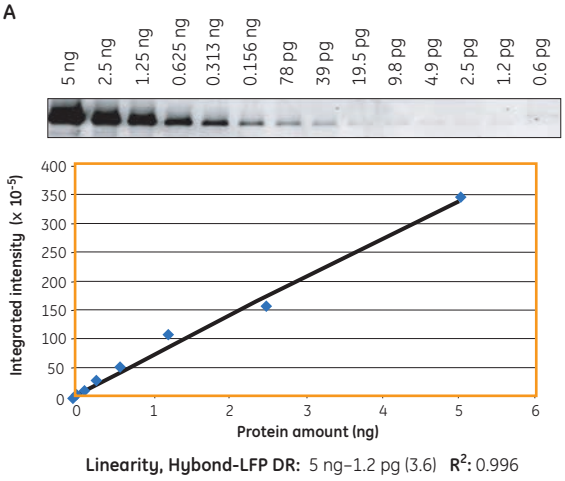

Fig 2. ECL Plex single protein detection on Hybond-LFP. A: human apotransferrin, 5 ng–0.6 pg (two-fold dilutions). Primary antibody: rabbit polyclonal anti-human transferrin; secondary antibody: new ECL Plex goat-α-rabbit IgG-Cy3. B: bovine cardiac muscle actin 150 ng–18 pg (two-fold dilutions). Primary antibody: mouse α-actin; secondary antibody: improved ECL Plex goat-α-mouse IgG-Cy3. Dynamic range = DR, linearity = R^2.

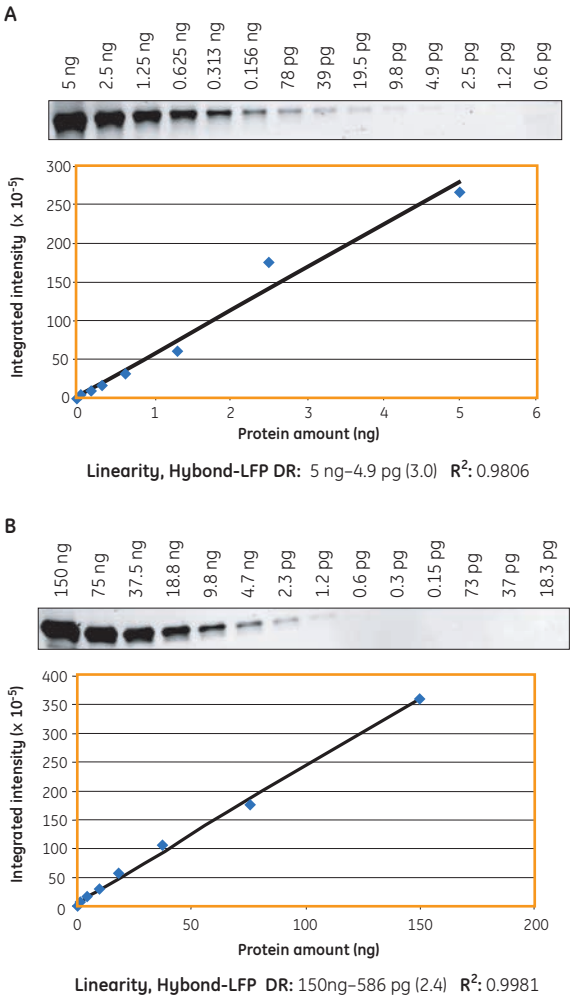

Fig 3. ECL Plex single-protein detection on Hybond-LFP. A: human apotransferrin, 5 ng–0.6 pg (two-fold dilutions). Primary antibody: rabbit polyclonal anti-human transferrin; secondary antibody: new ECL Plex goat-α-rabbit IgG-Cy2. B: bovine cardiac muscle actin 150 ng–18 pg (two-fold dilutions). Primary antibody: mouse α-actin; secondary antibody: new ECL Plex goat-α-mouse IgG-Cy2. Dynamic range = DR, linearity = R^2.

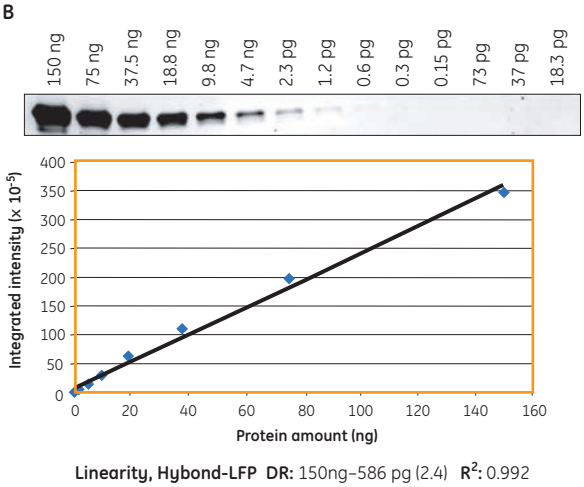

Table 2. Sensitivity and dynamic range of ECL Plex conjugates in a model system

ECL Plex secondary antibody	Detection limit (pg)			Dynamic range* (orders of magnitude)		
	Ettan DIGE Imager	Typhoon	Storm	Ettan DIGE Imager	Typhoon	Storm
Goat-α-mouse IgG-Cy2 (new)	590	590	2300	2.4	2.4	1.8
Goat-α-rabbit IgG-Cy2 (new)	4.9	4.9	78	3.0	3.0	1.8
Goat-α-mouse IgG-Cy3 (improved)	590	590	NA	2.4	2.4	NA
Goat-α-rabbit IgG-Cy3 (new)	1.2	1.2	NA	3.6	3.6	NA
Goat-α-mouse IgG-Cy5	590	590	1170	2.4	2.4	2.1
Goat-α-rabbit IgG-Cy5	1.2	1.2	2.5	3.6	3.6	3.3

*The dynamic range (DR) is defined as DR = log (max/min) with max and min being the upper and lower limit of the detection range, respectively. NA = not applicable.

Table 2 shows the sensitivity and dynamic range of ECL Plex CyDye-conjugated secondary antibodies in a model system using bovine cardiac muscle actin and human transferrin.

Reduced background noise levels in the detection of multiple protein targets

Secondary antibodies labeled with different CyDye labels allow detection of fluorescent signal at different wavelengths. This allows multiple proteins to be analyzed on the same blot without stripping and reprobing the membrane. The optimized ECL Plex secondary antibody conjugates ensure the lowest cross-reactivity and highest confidence for quantitation. Low background noise levels are particularly important when low-abundance proteins are being analyzed.

Figure 4 shows dual-protein detection on Chinese hamster ovary (CHO) and human prostate carcinoma (PC-3U) cell protein lysate using the new optimized antibody pairs. The new ECL Plex goat-α-rabbit IgG-Cy3 is shown in comparison with a candidate antibody (Fig 4A and 4B, respectively). The improved ECL Plex goat-α-mouse IgG-Cy3 gives significantly lower background when compared to the old ECL Plex goat-α-mouse IgG-Cy3 (Fig 4C and 4D, respectively). Also included in Figure 4 are new ECL Plex goat-α- rabbit IgG-Cy2 and goat-α-IgG-Cy2 (Fig 4E and 4F, respectively).

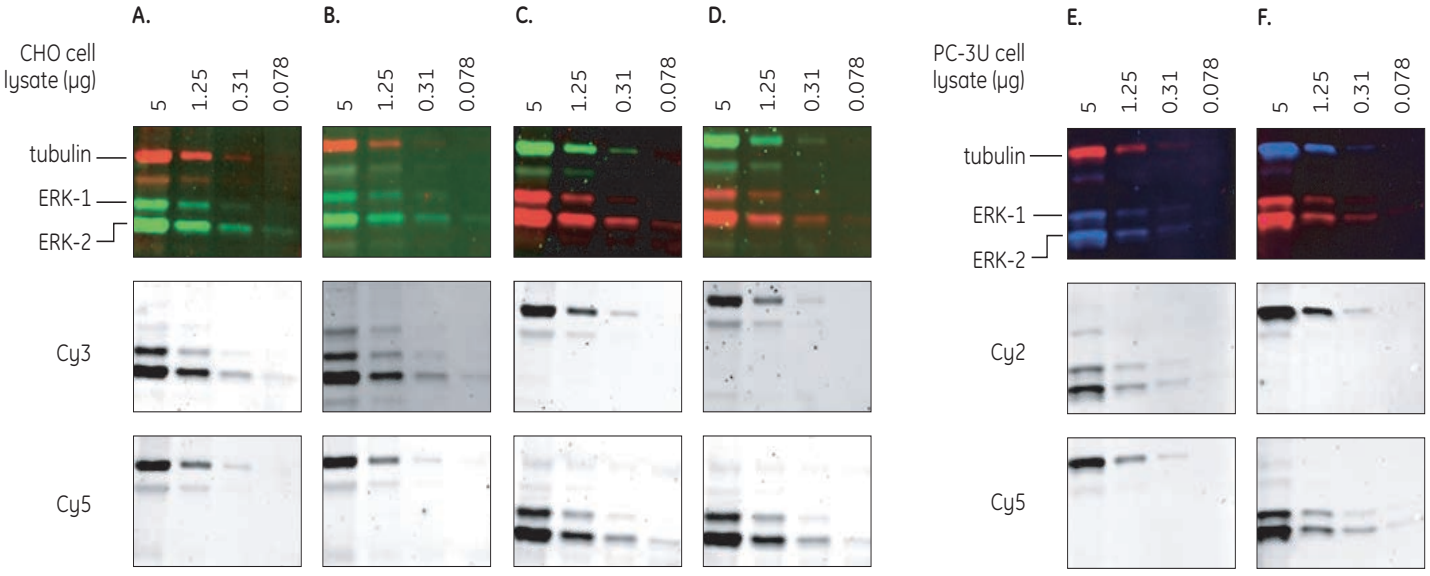

Fig 4. ECL Plex protein detection on Hybond-LFP: Four-fold dilutions of Chinese hamster ovary (CHO) cell lysate (A, B, C, and D) and human prostate carcinoma (PC-3U) cell lysate (E and F) ranging from 5 µg to 78 ng of protein were subjected to multiplex fluorescent Western blotting. Primary antibodies: mouse monoclonal anti-tubulin and rabbit polyclonal anti–ERK 1 and 2; secondary antibodies: ECL Plex goat-α-mouse IgG-Cy5 with new ECL Plex goat-α-rabbit IgG-Cy3 (A), candidate goat-α-rabbit IgG-Cy3 (B), and new ECL Plex goat-α-rabbit IgG-Cy2 (E); ECL Plex goat-α-rabbit IgG-Cy5 with improved ECL Plex goat-α-mouse IgG-Cy3 (C), original ECL Plex goat-α-mouse IgG-Cy3 (D), and new ECL Plex goat-α-mouse IgG-Cy2 (F).

Detection of post-translational modifications

Multiplex analysis is commonly used for the quantitation of one protein relative to a protein of known abundance (housekeeping protein), but can also be used for the detection and quantitation of post-translational modifications such as phosphorylation, provided that there are antibodies available. Detection of phosphorylated proteins is quite often difficult; one reason for this is that phosphorylation is a dynamic process in the living cell. Low background and high sensitivity are therefore very important in these kinds of studies.

Figure 5 shows TGF-β–induced phosphorylation of protein kinase B1 (Akt1) in human prostate cancer cells (PC-3U) cells. Figure 6 shows the inhibition of TGF-β–induced phosphorylation of Akt1.

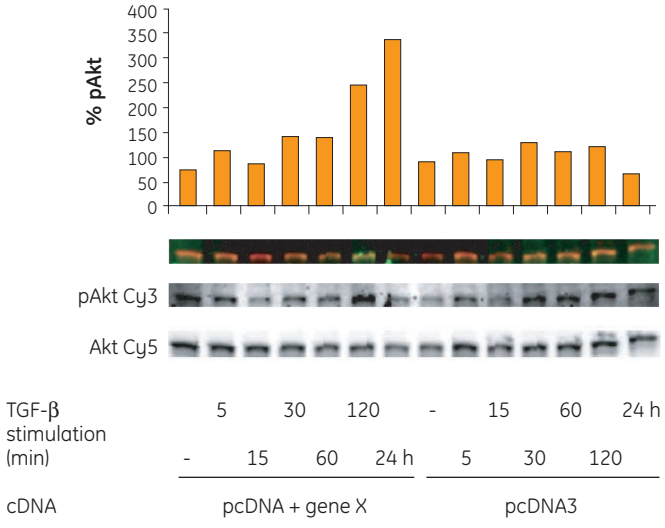

Fig 5. Detection of TGF-β–induced phosphorylation of protein kinase B1 (Akt1) in human prostate cancer (PC-3U) cells. PC-3U cells transfected with plasmid containing gene X (pcDNA3 + gene X) or empty plasmid (pcDNA3) were starved for 24 h, and then stimulated with TGF-β for different lengths of time. 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.*

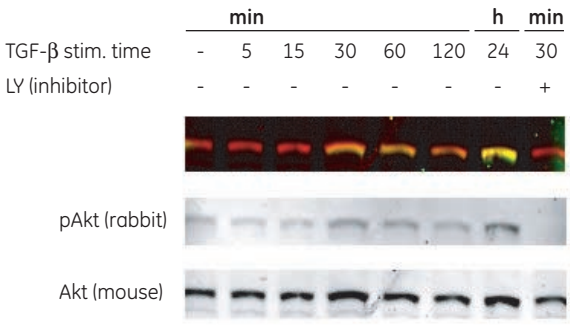

Fig 6. Detection of TGF-β–induced phosphorylation of protein kinase B1 (Akt1) in human prostate cancer (PC-3U) cells. PC-3U cells starved for 12 h, pretreated with or without phosphatidylinositol-3 kinase inhibitor LY for 1 h, and then stimulated with TGF-β for different lengths of time. Protein extracts were separated on SDS-PAGE gels and then blotted onto 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.*

ECL Plex technical data

		λ_{max} (nm)	
		excitation	emission
CyDye characteristics	Cy2	489	506
	Cy3	550	570
	Cy5	649	670

Sensitivity	1.2 pg potential in model system (in the same model system, ECL Advance shows similar sensitivity; ECL Plus detects ~5 pg; and ECL detects ~10 pg)
Primary antibody dilution range	1:100–1:5000
ECL Plex secondary antibody dilution range	1:1250–1:4000
Emission duration (on membrane)	> 3 months, protected from light
Recommended membrane	Hybond-LFP (highest sensitivity), Hybond ECL
Recommended detection method	Fluorescence imager compatible with Cy2, Cy3, and Cy5 dyes
Recommended use	High sensitivity, multiplexing, linear quantitation

Ordering information

ECL Plex products

ECL Plex Western blotting combination pack: RPN998

Cy3, Cy5, Hybond ECL

Combination pack optimized for ECL Plex Western blotting, includes Hybond ECL (nitrocellulose membrane). Contains the following components, sufficient for at least 1000 cm^2 of membrane:

ECL Plex goat-α-mouse IgG-Cy3, 150 µg ***IMPROVED***

ECL Plex goat-α-rabbit IgG-Cy5, 150 µg

ECL Plex Fluorescent Rainbow™ Markers, full-range, 120 µl

Hybond ECL, 10 x 10 cm, 10 sheets

ECL Plex Western blotting combination pack: RPN999

Cy3, Cy5, Hybond-LFP

Combination pack optimized for ECL Plex Western blotting, includes Hybond-LFP (low-fluorescent PVDF membrane). Contains the following components, sufficient for at least 1000 cm^2 of membrane:

ECL Plex goat-α-mouse IgG-Cy3, 150 µg ***IMPROVED***

ECL Plex goat-α-rabbit IgG-Cy5, 150 µg

ECL Plex Fluorescent Rainbow Markers, full-range, 120 µl

Hybond-LFP, 20 x 20 cm, 3 sheets

ECL Plex Cy2-, Cy3-, and Cy5-conjugated antibodies

ECL Plex goat-α-mouse IgG-Cy2, 150 µg ***NEW*** 28-9011-08
Sufficient for at least 1000 cm^2 of membrane

ECL Plex goat-α-mouse IgG-Cy2, 600 µg ***NEW*** 28-9011-09
Sufficient for at least 4000 cm^2 of membrane

ECL Plex goat-α-rabbit IgG-Cy2, 150 µg ***NEW*** 28-9011-10
Sufficient for at least 1000 cm^2 of membrane

ECL Plex goat-α-rabbit IgG-Cy2, 600 µg ***NEW*** 28-9011-11
Sufficient for at least 4000 cm^2 of membrane

ECL Plex goat-α-mouse IgG-Cy3, 150 µg ***IMPROVED*** PA43009
Sufficient for at least 1000 cm^2 of membrane

ECL Plex goat-α-mouse IgG-Cy3, 600 µg ***IMPROVED*** PA43010
Sufficient for at least 4000 cm^2 of membrane

ECL Plex goat-α-rabbit IgG-Cy3, 150 µg ***NEW*** 28-9011-06
Sufficient for at least 1000 cm^2 of membrane

ECL Plex goat-α-rabbit IgG-Cy3, 600 µg ***NEW*** 28-9011-07
Sufficient for at least 4000 cm^2 of membrane

ECL Plex goat-α-mouse IgG-Cy5, 150 µg PA45009
Sufficient for at least 1000 cm^2 of membrane

ECL Plex goat-α-mouse IgG-Cy5, 600 µg PA45010
Sufficient for at least 4000 cm^2 of membrane

ECL Plex goat-α-rabbit IgG-Cy5, 150 µg PA45011
Sufficient for at least 1000 cm^2 of membrane

ECL Plex goat-α-rabbit IgG-Cy5, 600 µg PA45012
Sufficient for at least 4000 cm^2 of membrane

Hybond-LFP

Low-fluorescent PVDF membrane, 0.2-µm pore size.
Optimized for use with the ECL Plex Western Blotting System.

Hybond-LFP, 20 x 20 cm, 3 sheets RPN2020LFP3

Hybond-LFP, 20 x 20 cm, 10 sheets RPN2020LFP

Hybond-LFP, 14 x 16 cm, 15 sheets RPN1416LFP

Hybond-LFP, 30 cm x 3 m, 1 roll RPN303LFP

Hybond ECL

Low-fluorescent nitrocellulose membrane, 0.45-µm pore size.
Optimized for use with the ECL Plex Western Blotting System.

Hybond ECL, 20 x 20 cm, 10 sheets RPN2020D

Hybond ECL, 7 x 8 cm, 50 sheets RPN78D

Hybond ECL, 20 cm x 3 m, 1 roll RPN203D

Hybond ECL, 30 cm x 3 m, 1 roll RPN303D

Hybond ECL, 30 cm x 3 m, 1 roll RPN3032D*

*0.2-µm pore size

ECL Plex Fluorescent Rainbow Markers

Full-range, defined molecular weight standards 10–250 kDa.
Optimized for use with the ECL Plex Western Blotting System.
Supplied in gel loading buffer.

ECL Plex Fluorescent Rainbow Markers, 120 µl RPN850

ECL Plex Fluorescent Rainbow Markers, 500 µl RPN851

Blocking buffer

Optimized for use with the ECL Plex Western Blotting System

ECL Advance Blocking Reagent, 20 g RPN418

BSA, 25 g RPN412

Related products

ECL Western blotting reagents

ECL Western Blotting System RPN2108

ECL Plus Western Blotting Detection Reagents RPN2132

ECL Advance Western Blotting Detection Kit RPN2135

CyDye Antibody Labeling Kits, using bis-Reactive CyDye

Cy2 Ab Labeling Kit PA32000

Cy3 Ab Labeling Kit PA33000

Cy5 Ab Labeling Kit PA35000

CyDye Value Packs (bis-Reactive NHS esters)

Cy5.5 Bis NHS ester, 5 mg PA15500

Cy7 Bis NHS ester, 5 mg PA17000

CyDye Monoclonal Antibody Labeling Kits, using mono-Reactive CyDye

Cy3 mAb Labeling Kit	PA33001
Cy5 mAb Labeling Kit	PA35001

CyDye Value Packs (mono-Reactive NHS esters)

Cy3.5 NHS ester, 1 mg	PA13601
Cy5.5 NHS ester, 1 mg	PA15601
Cy7 NHS ester, 1 mg	PA17101

Gel electrophoresis and transfer equipment

TE 70 PWR ECL Semi-Dry Transfer Unit	11-0013-41
TE 77 PWR ECL Semi-Dry Transfer Unit	11-0013-42
TE 70 ECL Semi-Dry Transfer Unit	80-6210-34
TE 77 ECL Semi-Dry Transfer Unit	80-6211-86
TE62 Transfer Unit	80-6209-58
miniVE Vertical Electrophoresis system	80-6418-77
miniVE blot module	80-6418-96
SE260 Mini-Vertical Unit for two slab gels	80-6149-35
SE600 Ruby™ Standard Dual Cooled Vertical Unit	80-6479-57
ECL Multiprobe	11-0033-95
ECL Multiprobe XL	11-0033-96
EPS 301 Power Supply	18-1130-01

Scanner and image analysis software

Typhoon 9410 and ImageQuant™ TL	63-0055-80
Ettan DIGE Imager	63-0056-42
Ettan DIGE Imager cassette, with low-fluorescent glass for naked gels	11-0027-33
Storm 860 and ImageQuant TL	63-0035-63

Protein stain

Deep Purple™ Total Protein Stain, 5 ml (makes 1 l)	RPN6305
Deep Purple Total Protein Stain, 25 ml (makes 5 l)	RPN6306

References

1. Data file: ECL Plex Western Blotting Detection System: Multiplex protein detection based on direct fluorescent CyDye-labeled conjugates, GE Healthcare, 28-4015-39, Edition AA (2005).
2. Application note: Multiplex protein detection using the ECL Plex fluorescent Western blotting system, GE Healthcare, 28-4015-40, Edition AB (2005).
3. Application note: Multiplex protein detection with the ECL Plex fluorescent Western blotting system using the Ettan DIGE Imager, GE Healthcare, 28-4041-97, Edition AB (2005).

Asia Pacific: Tel: +65 6275 1830 Fax: +65 6275 1829 **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: 02 416 82 06 **Canada** Tel: 1 800 463 5800 Fax: 1 800 567 1008 **Central, East, & South East Europe** Tel +43 1 972720 Fax: +43 1 97272 2750 **Denmark** Tel: 45 16 2400 Fax: 45 16 2424 **Eire** Tel: 01494 544000 Fax: 0044 1494 542010 **Finland & Baltics** Tel: +358-(0)9-512 39 40 Fax: +358 (0)9 512 39 439 **France** Tel: 01 6935 6700 Fax: 01 6941 9677 **Germany** Tel: (089) 96281 660 Fax: (089) 96281 620 **Greater China** Tel: +852 2100 6300 Fax: +852 2100 6338 **Italy** Tel: 02 27322 1 Fax: 02 27302 212 **Japan** Tel: +81 3 5331 9336 Fax: +81 3 5331 9370 **Latin America** Tel: +55 11 3933 7300 Fax: +55 11 3933 7304 **Middle East & Africa** Tel: +30 210 9600 687 Fax: +30 210 9600 693 **Netherlands** Tel: 0800 82 82 82 1 Fax: 0800 82 82 82 4 **Norway** Tel: 815 65 555 Fax: 815 65 666 **Portugal** Tel: 21 417 7035 Fax: 21 417 3184 **Russia & other C.I.S. & N.I.S** Tel: +7 (495) 956 5177 Fax: +7 (495) 956 5176 **Spain** Tel: 93 594 49 50 Fax: 93 594 49 55 **Sweden** Tel: 018 612 1990 Fax: 018 612 1910 **Switzerland** Tel: 0848 8028 12 Fax: 0848 8028 13 **UK** Tel: 0800 616928 Fax: 0800 616927 **USA** Tel: +1 800 526 3593 Fax: +1 877 295 8102

www.gehealthcare.com/ecl
GE Healthcare UK Limited
Amersham Place
Little Chalfont
Buckinghamshire
HP7 9NA
UK

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