Amersham ECL Plex Western blotting detection system: fluorescent Cy™2, Cy3, and Cy5 conjugates for multiplex protein detection

Amersham ECL Plex Western blotting detection system has been developed for fluorescent detection in Western blotting. It complements the well-established Amersham ECL family of chemiluminescent Western blotting detection reagents (Amersham ECL, Amersham ECL Prime, and Amersham ECL Select™). Amersham ECL Plex uses direct fluorescent light detection in contrast to the alternative products, which are based on detection of chemiluminescent or chemifluorescent signals. The Amersham 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. In the multiplex application, two proteins can be detected in the same blot with minimal cross-reactivity between antibodies or dyes.

Amersham ECL Plex system has been complemented with improved ECL Plex goat-anti-mouse IgG-Cy3 and ECL Plex goat-anti-rabbit IgG-Cy3 antibodies. The 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 ECL Plex goat-anti-rabbit IgG-Cy2 and goat-anti-mouse IgG-Cy2 antibodies allow for multiplex applications using Typhoon™ FLA imagers.

Amersham ECL Plex offers:

- Compatibility with Typhoon FLA imagers, and other multipurpose imagers
- 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 broad dynamic range and high linearity: detects significant differences in protein levels with high accuracy. Rescanning of membranes is possible up to three months after experiment if stored protected from light
- Optimized system giving high degree of specificity: increased accuracy of results
- Simple protocol for fast analysis with nontoxic products

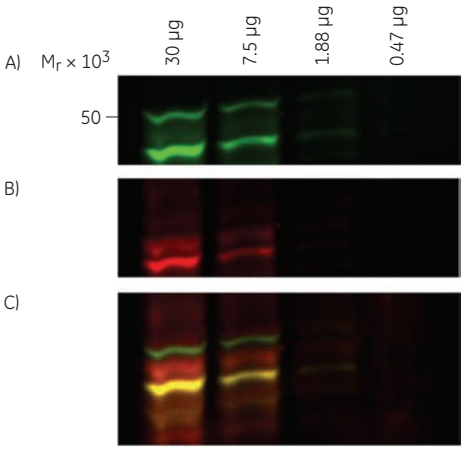


Fig 1. Color images of multiplex Western blot scans. Using a mixture of ECL Plex goat-α-mouse IgG-Cy3 and ECL Plex goat-α-rabbit IgG-Cy5 fluorescent antibodies, GSK3β and phospho-GSK3β (Ser 9) were simultaneously detected in one blot. Four-fold dilutions of human prostate carcinoma (PC-3U) cells from 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.

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 Amersham ECL Plex system.

Amersham 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 FLA series, this provides high-quality data for single or multiplex analyses (Fig 1).

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

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 Amersham 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 Prime 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 Amersham ECL Plex because different antibodies will perform differently under different blocking conditions.

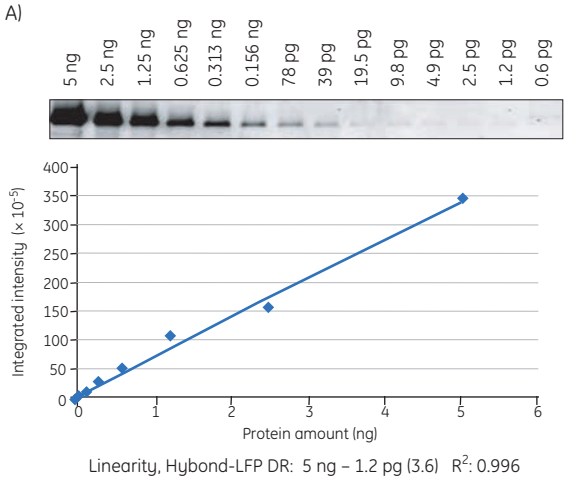
Table 1. Compatibility of blocking solutions with the Amersham ECL Plex system

Membrane	2% ECL Prime Blocking Reagent	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 Amersham ECL Plex secondary antibodies on Hybond-LFP membranes, scanned on the Typhoon imager.



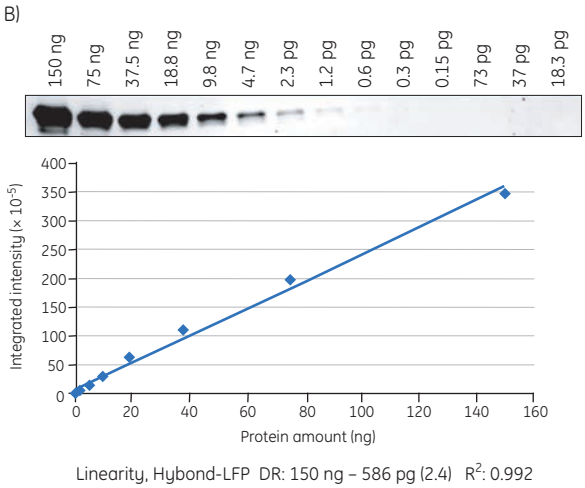


Fig 2. Amersham ECL Plex single protein detection on Hybond-LFP. A) human apotransferrin, 5 ng–0.6 pg (two-fold dilutions). Primary antibody: rabbit polyclonal anti-human transferrin; secondary antibody: ECL Plex goat-α-rabbit IgG-Cy3. B) bovine cardiac muscle actin 150 ng–18 pg (two-fold dilutions).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

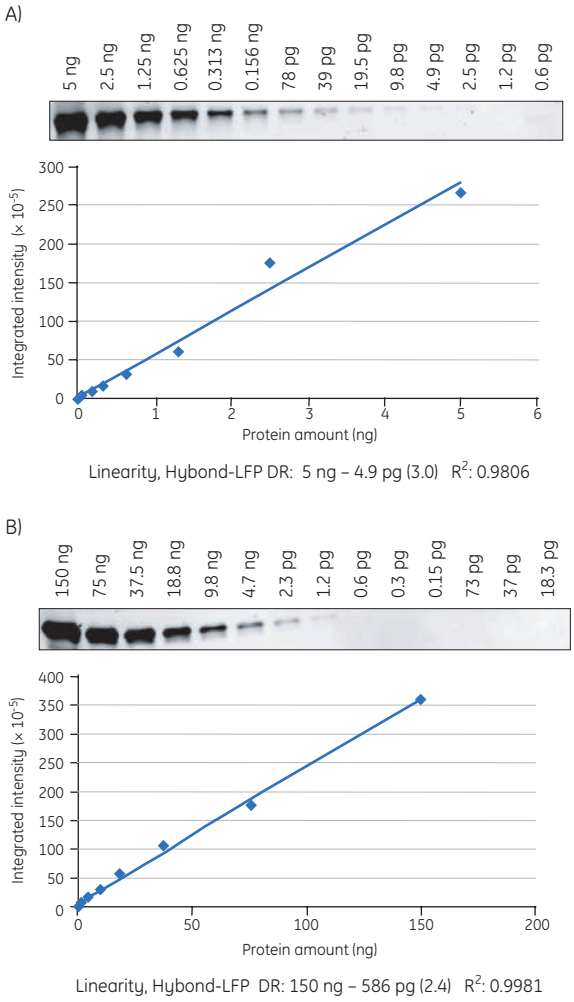


Fig 3. Amersham ECL Plex single-protein detection on Hybond-LFP. A) human apotransferrin, 5 ng–0.6 pg (two-fold dilutions). Primary antibody: rabbit polyclonal anti-human transferrin; secondary antibody: ECL Plex goat-α-rabbit IgG-Cy2. B) bovine cardiac muscle actin 150 ng–18 pg (two-fold dilutions).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

	Detection limit (pg)		Dynamic range* (orders of magnitude)	
Amersham ECL Plex secondary antibody	Typhoon	Storm	Typhoon	Storm
Goat-a-mouse IgG-Cy2	590	2300	2.4	1.8
Goat-a-rabbit IgG-Cy2	4.9	78	3.0	1.8
Goat-a-mouse IgG-Cy3	590	NA	2.4	NA
Goat-a-rabbit IgG-Cy3	1.2	NA	3.6	NA
Goat-a-mouse IgG-Cy5	590	1170	2.4	2.1
Goat-a-rabbit IgG-Cy5	1.2	2.5	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 Amersham 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 optimized antibody pairs. The ECL Plex goat-α-rabbit IgG-Cy3 is shown in comparison with a candidate antibody (Fig 4A and 4B, respectively). The 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 ECL Plex goat-α- rabbit IgG-Cy2 and goat-α-IgG-Cy2 (Fig 4E and 4F, respectively).

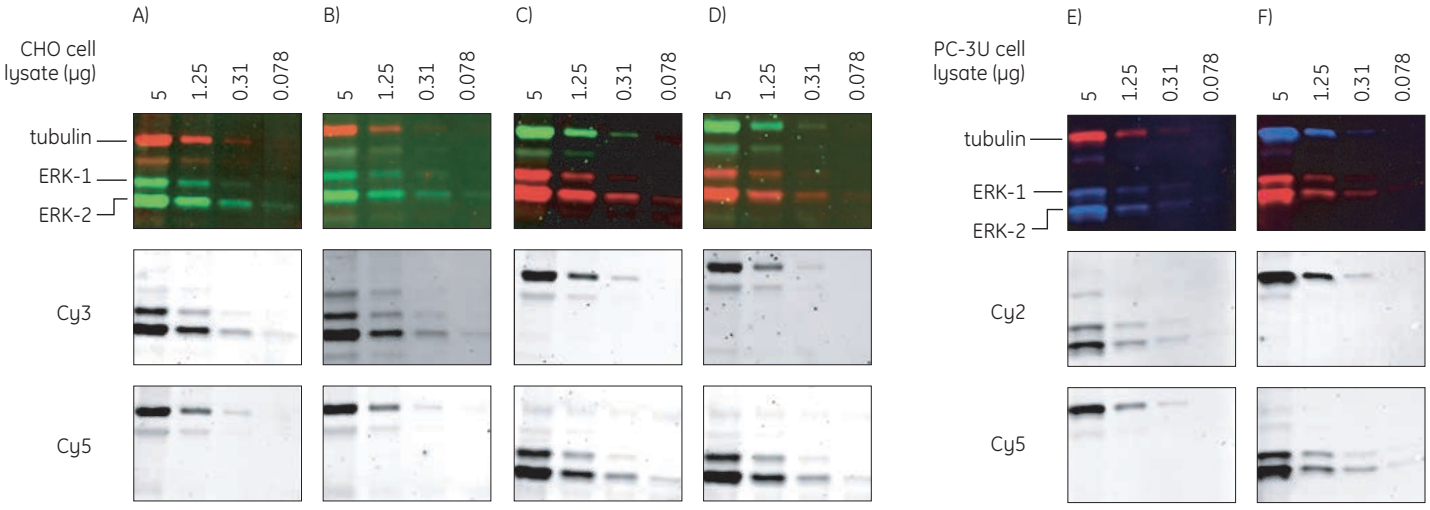


Fig 4. ECL Plex protein detection on Hybond-LFP: Four-fold dilutions of Chinese hamster ovary (CHO) cell lysate (A, B, C, and D) and human prostate carcinoma (PC-3U) cell lysate (E and F) ranging from 5 µg to 78 ng of protein were subjected to multiplex fluorescent Western blotting. Primary antibodies: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

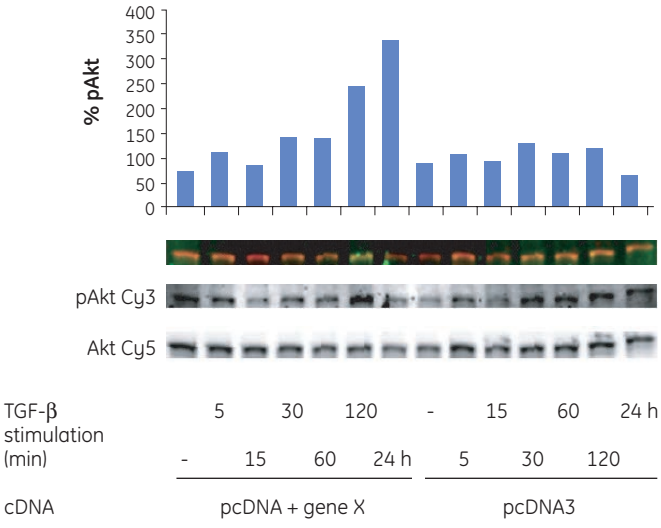


Fig 5. Detection of TGF-β–induced phosphorylation of protein kinase B1 (Akt1) in human prostate cancer (PC-3U) cells. PC-3U cells transfected with plasmid containing gene X (pcDNA3 + gene X) or empty plasmid (pcDNA3) were starved for 24 h, and then stimulated with TGF-β for different lengths of time.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

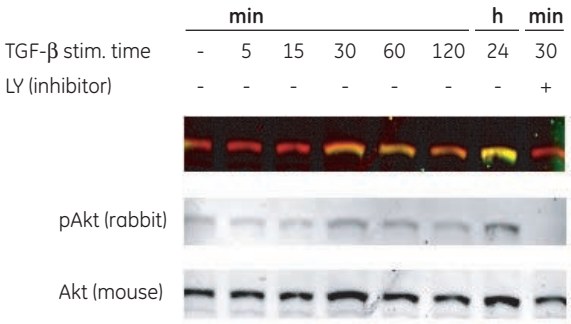


Fig 6. Detection of TGF-β–induced phosphorylation of protein kinase B1 (Akt1) in human prostate cancer (PC-3U) cells. PC-3U cells starved for 12 h, pretreated with or without phosphatidylinositol-3 kinase inhibitor LY for 1 h, and then stimulated with TGF-β for different lengths of time. Protein extracts 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 ECL Plex goat-α-rabbit IgG-Cy3. *Data courtesy of Dr. Marene Landström and Anders Marcusson, Ludwig Institute, Uppsala, Sweden.*

Technical specifications

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

Sensitivity	1.2 pg potential in model system
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

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).

Ordering information

Product	Code number
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 cm² of membrane:	
ECL Plex goat-a-mouse IgG-Cy3, 150 µg ECL Plex goat-a-rabbit IgG-Cy5, 150 µg ECL Plex Fluorescent Rainbow™ Markers, full-range, 120 µl Hybond ECL, 10 × 10 cm, 10 sheets	
Amersham ECL Plex Western blotting combination pack	RPN998
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 cm² of membrane:	
ECL Plex goat-a-mouse IgG-Cy3, 150 µg ECL Plex goat-a-rabbit IgG-Cy5, 150 µg ECL Plex Fluorescent Rainbow Markers, full-range, 120 µl Hybond-LFP, 20 × 20 cm, 3 sheets	
Amersham ECL Plex Western blotting combination pack	RPN999
Amersham ECL Plex Cy2-, Cy3-, and Cy5-conjugated antibodies	
Amersham ECL Plex goat-a-mouse IgG-Cy2, 150 µg Sufficient for at least 1000 cm² of membrane	28-9011-08
Amersham ECL Plex goat-a-mouse IgG-Cy2, 600 µg Sufficient for at least 4000 cm² of membrane	28-9011-09
Amersham ECL Plex goat-a-rabbit IgG-Cy2, 150 µg Sufficient for at least 1000 cm² of membrane	28-9011-10
Amersham ECL Plex goat-a-rabbit IgG-Cy2, 600 µg Sufficient for at least 4000 cm² of membrane	28-9011-11
Amersham ECL Plex goat-a-mouse IgG-Cy3, 150 µg Sufficient for at least 1000 cm² of membrane	PA43009
Amersham ECL Plex goat-a-mouse IgG-Cy3, 600 µg Sufficient for at least 4000 cm² of membrane	PA43010
Amersham ECL Plex goat-a-rabbit IgG-Cy3, 150 µg Sufficient for at least 1000 cm² of membrane	28-9011-06
Amersham ECL Plex goat-a-rabbit IgG-Cy3, 600 µg Sufficient for at least 4000 cm² of membrane	28-9011-07
Amersham ECL Plex goat-a-mouse IgG-Cy5, 150 µg Sufficient for at least 1000 cm² of membrane	PA45009
Amersham ECL Plex goat-a-mouse IgG-Cy5, 600 µg Sufficient for at least 4000 cm² of membrane	PA45010
Amersham ECL Plex goat-a-rabbit IgG-Cy5, 150 µg Sufficient for at least 1000 cm² of membrane	PA45011
Amersham ECL Plex goat-a-rabbit IgG-Cy5, 600 µg Sufficient for at least 4000 cm² of membrane	PA45012

Product	Code number
Hybond-LFP	
Low-fluorescent PVDF membrane, 0.2-µm pore size. Optimized for use with the ECL Plex Western blotting system.	
Hybond-LFP, 20 × 20 cm, 3 sheets	RPN2020LFP3
Hybond-LFP, 20 × 20 cm, 10 sheets	RPN2020LFP
Hybond-LFP, 14 × 16 cm, 15 sheets	RPN1416LFP
Hybond-LFP, 26.5 cm × 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 × 20 cm, 10 sheets	RPN2020D
Hybond ECL, 7 × 8 cm, 50 sheets	RPN78D
Hybond ECL, 20 cm × 3 m, 1 roll	RPN203D
Hybond ECL, 30 cm × 3 m, 1 roll	RPN303D
Hybond ECL, 30 cm × 3 m, 1 roll	RPN3032D*
*0.2-µm pore size	
Amersham ECL Plex Fluorescent Rainbow Markers	
Visible marker bands on gels and membranes, as well as fluorescent images using Cy3 and Cy5 channels. M_r 12 000 to 225 000	
Amersham ECL Plex Fluorescent Rainbow Markers, 120 µl	RPN850E
Amersham ECL Plex Fluorescent Rainbow Markers, 500 µl	RPN851E
Blocking buffer	
Optimized for use with the ECL Plex Western blotting system	
ECL Prime Blocking Reagent	RPN418

Related products	Code number
Amersham ECL Western blotting reagents	
Amersham ECL Western blotting system	RPN2108
Amersham ECL Prime Western blotting detection reagent	RPN2232
Amersham ECL Select Western blotting detection reagent	RPN2235
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)	
Cy3.5 Bis NHS ester, 5 mg	PA13500
Cy5.5 Bis NHS ester, 5 mg	PA15500
Cy7 Bis NHS ester, 5 mg	PA17000
CyDye Monoclonal Antibody Labeling Kits, using mono-Reactive CyDye	
Cy2 mAb Labeling Kit	PA32001
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

Related products	Code number
Gel electrophoresis and transfer equipment	
Amersham ECL Gel Box	28-9906-08
Amersham ECL Gel, 10%, 10 well	28-9898-04
Amersham ECL Gel, 12%, 10 well	28-9898-05
Amersham ECL Gel, 4-12%, 10 well	28-9898-06
Amersham ECL Gel, 4-20%, 10 well	28-9901-54
Amersham ECL Gel, 8-16%, 10 well	28-9898-07
TE 70 PWR ECL Semi-Dry Transfer Unit	11-0013-41
TE 77 PWR ECL Semi-Dry Transfer Unit	11-0013-42
TE 22 Mini Tank Transfer Unit	80-6204-26
Cooled Vertical Unit	
ECL Multiprobe	11-0033-95
ECL Multiprobe XL	11-0033-96
EPS 301 Power Supply	18-1130-01
Scanner and image analysis software	
Typhoon 9500	29-0040-80
ImageQuant™ TL	28-9236-62
Other	
Deep Purple™ Total Protein Stain, 5 ml (makes 1 l)	RPN6305
Deep Purple Total Protein Stain, 25 ml (makes 5 l)	RPN6306

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