cytiva

Amersham ECL Plex

Western blotting system

Product booklet

Table of Contents

1 Introduction

Product codes

Amersham ECL Plex CyDye conjugated antibodies

PA45009–PA45012, PA43009, PA45010 and 28901106–28901111

Amersham Hybond LFP

10600091, 10600090, 10600060, 10600022, 10600040, 10600102

Amersham Protran Premium 0.2

10600004, 10600009, 10600014, 10600035, 10600049, 10600050, 10600066, 10600080, 10600081, 10600097

Amersham ECL Plex Fluorescent Rainbow markers

RPN850E and RPN851E

Blocking buffers

RPN418 and RPN412

Amersham ECL Plex combination packs

RPN998 and RPN999

Important

Read these instructions carefully before using the products.

Intended use

The products are intended for research use only, and shall not be used in any clinical or *in vitro* procedures for diagnostic purposes.

Safety

For use and handling of the products in a safe way, refer to the Safety Data Sheets.

Storage

On receipt store the lyophilized Amersham™ ECL Plex™ CyDye™ conjugated secondary antibodies at 2–8°C protected from light. Reconstitute in ultra pure water to a concentration of 1 µg/µl, and keep aliquots at -15°C to -30°C protected from light. Avoid repeated freezethaw cycles.

The membranes are provided in airtight re-sealable aluminium bags to prevent adsorption of moisture and other airborne contaminants. The membrane should be stored in the sealed bag at room temperature in a clean dry place.

The Amersham ECL Plex Fluorescent Rainbow™ markers should be stored at -15°C to -30°C. Avoid repeated freezethaw cycles.

Expiry

The reconstituted Amersham ECL Plex CyDye conjugated secondary antibodies are stable for at least 6 months when stored under recommended conditions.

The membranes are stable for at least 1 year before opening and once opened the performance is consistent for at least 3 months when stored under the recommended conditions.

The Amersham ECL Plex Fluorescent Rainbow markers is stable for at least 3 months when stored under the recommended conditions.

Formulation and packaging

The Amersham ECL Plex CyDye conjugated secondary antibodies are supplied in lighttight containers as a lyophilized solid in Phosphate-Buffered Saline (0.02 M Potassium Phosphate, 0.15 M NaCl) pH 7.2, containing 1% (w/v) Bovine Serum Albumin and 0.1% (w/v) Sodium Azide. The tubes containing the antibodies have color coded caps to facilitate discrimination between Cy™2, Cy3 and Cy5 antibody conjugates.

The membranes are packed in air-tight re-sealable aluminium bags.

The Amersham ECL Plex Fluorescent Rainbow markers is supplied in 30% glycerol and sample buffer containing mercaptoethanesulphonic acid (MESNA) as reducing agent.

Recommendations for use

Use 1.5–3 µl of Amersham ECL Plex Fluorescent Rainbow markers per 10 × 10 cm mini gel. Some bands of the Amersham ECL Plex Rainbow markers may appear weaker when scanned in the Cy3 channel. The Amersham ECL Plex Rainbow markers are not visible in the Cy2 channel. If your blot is only probed with Amersham ECL Plex Cy3 or Cy2 conjugated antibody, perform an additional scan in the Cy5 channel if needed.

The recommended dilution for the Amersham ECL Plex CyDye conjugated secondary antibodies (1 µg/µl) is 1/2500, but the optimal concentration (between 1/1250–1/4000) should be determined for each Western blotting experimental setup. For blocking and antibody incubations, use sufficient volume to cover the membranes (at least 0.3 ml/cm^2)

2 Important notes for working with fluorescence

Do not contaminate your blot with:

- Coomassie™ blue stain
- Ink from ball point pens
- Bromophenol blue
- Polyacrylamide gel fragments
- Triton™ X-100

Always use powder free gloves and clean forceps and trays.

3 Components

Main components

- **Amersham ECL Plex CyDye conjugated antibodies -** PA45009–PA45012, PA43009, PA45010 and 28901106–20901111
- **Amersham Hybond LFP -** 10600091, 10600090, 10600060, 10600022, 10600040, 10600102
- **Amersham Protran -** 10600001, 10600094, 10600075, 10600063, 10600011, 10600043
- **Amersham ECL Plex Fluorescent Rainbow markers -** RPN850E and RPN851E
- **Amersham ECL Blocking buffers -** RPN418 and RPN412
- **Amersham ECL Plex combination packs -** RPN998 and RPN999

See *Chapter 12 Ordering information, on page 26* for a detailed description of the above products.

Other materials required

- SDS PAGE gels
- Electrophoresis system*
- Transfer Unit*
- Typhoon™ or other Fluorescent laser scanner* (for specific requirements, see *Fluorescent imaging systems on page 12*)
- Image analysis software*
- Power Supply*
- Hybond™ blotting paper*
- Methanol
- Tris*
- Glycine*
- Tween™ 20*
- SDS*
- DTT*
- Glycerol*
- Bromophenol blue*
- PBS (Phosphate-Buffered Saline)*
- Protein Quantification Kit*
- Trays/dishes
- Basic laboratory equipment
- Orbital shaker

* Products available from Cytiva, see *Chapter 12 Ordering information, on page 26*.

4 Quality control

Every batch of Amersham ECL Plex CyDye conjugated secondary antibody and Amersham Hybond LFP membrane is functionally tested in fluorescent Western blotting application (Amersham ECL Plex) to ensure minimal batch to batch variation. The antibody product was prepared from monospecific antiserum by immunoaffinity chromatography using mouse/rabbit IgG immobilized to agarose beads. This was followed by multiple solid phase adsorptions to eliminate cross reactivity. A single precipitin arc was observed against anti-goat serum, mouse/rabbit IgG and mouse/ rabbit serum when assayed by immunoelectrophoresis. No reaction was observed against calf, chicken, goat, guinea pig, hamster, horse, human, rabbit, mouse, and rat or sheep serum proteins. The Amersham ECL Plex Fluorescent Rainbow markers is assessed for color intensity and band integrity on a 4–20% gradient SDS-PAGE minigel.

5 Description

The Amersham ECL Plex Western blotting system is optimized for single protein detection as well as multiplex protein detection using Amersham ECL Plex CyDye conjugated secondary antibodies and Amersham Hybond-LFP (low fluorescent PVDF membrane), or Amersham Protran™ Premium (nitrocellulose membrane).

The optimized antibodies show high sensitivity, a very good dynamic range and low or no cross reactivity. The CyDye technology is safe and reliable and ensures accurate quantification. The system is optimized on the Typhoon scanner but is compatible with many fluorescent laser scanners and CCD cameras (see *Chapter 6 Compatibility,*).

The Amersham ECL Plex Fluorescent Rainbow markers is a mixture of individually colored proteins of defined size. Purified proteins are combined to produce bands of equal color intensity and even spacing when separated on a polyacrylamide gel. The visibility of the individual marker bands in the Cy3 or Cy5 can vary and depend on the primary antibodies used.

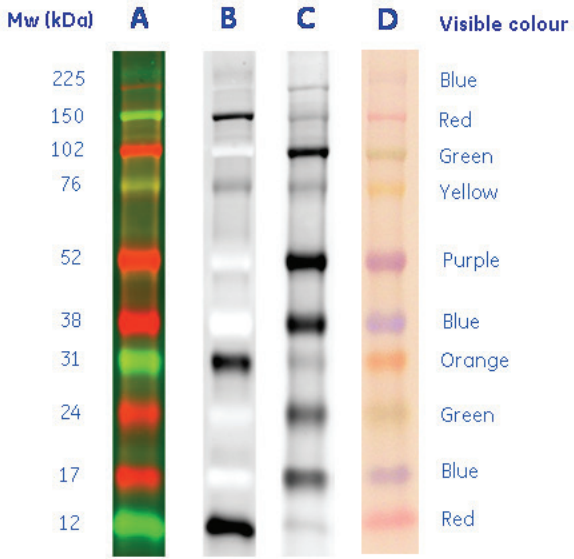

Fig 1. The Amersham ECL Plex Fluorescent Rainbow markers were separated on a 4–20% gradient polyacrylamide gel and transferred to a membrane. Cy5/Cy3 overlaid (A), Cy3 (B), Cy5 (C) and visual (D) images from the blot are shown.

6 Compatibility

The Amersham ECL Plex Western blotting system is compatible with nitrocellulose and low fluorescent PVDF membranes, multiple blocking solutions and fluorescent laser scanners, imagers.

Membranes

Amersham Protran Premium and Amersham Hybond LFP membranes are optimized and recommended for use with the Amersham ECL Plex Western blotting system. For low abundant protein analysis, the Amersham Hybond LFP membrane is recommended. If stripping is required, the Amersham Hybond LFP membrane is the recommended choice.

Blocking solutions

Many blocking solutions are compatible (see Table 1 below), but the use of Amersham ECL Prime blocking agent in wash buffer (first choice) or 5% Bovine Serum Albumin in PBS/TBS is recommended. In case of unspecific detection, we recommend the use of 2% Amersham ECL Prime blocking agent in wash buffer to reduce the problems.

Table 1. Compatibility of blocking solutions.

Membrane	5% Amersham ECL blocking agent in wash buffer	2% Amersham ECL Prime blocking agent in wash buffer	5% BSA in PBS/TBS	10% Gelatine
Amersham Hybond LFP	++	++++	+++	-
Amersham Protran Premium	++	+++	+++	-

* This blocker can reduce unspecific detection

++++ = high performance

+++ = good performance

++ = acceptable performance

+ = poor performance

- = not compatible

Ratings are based on overall performance including level of auto fluorescence/background and unspecific detection as well signal intensity

Fluorescent imaging systems

Imaging systems with the capability of detecting Cy2, Cy3 and Cy5 are compatible with the Amersham ECL Plex Western blotting system; however, the Amersham ECL Plex CyDye coupled secondary antibodies have been optimized using Typhoon Imagers. Performance may vary depending on the imager used (see Table 2 below).

Table 2. Examples of the compatibility of Amersham ECL Plex CyDye conjugated antibodies with membranes and scanners.

Imager	Membrane	Amersham ECL Plex CyDye conjugated antibodies		
		Goat-α-mouse/ rabbit IgG-Cy2	Goat-α-mouse/ rabbit IgG-Cy3	Goat-α-mouse/ rabbit IgG-Cy5
Typhoon	Amersham Hybond LFP	+++(+)	++++	++++
	Amersham Protran Premium	++(+)	+++	+++
Biorad Molecular	Amersham Hybond LFP	NT	++++	++++
Imager FX Systems	Amersham Protran Premium	NT	++	++

Fuji	Amersham Hybond LFP	NT	++++	++++
FLA Imagers	Amersham Protran Premium	NT	++	++
Kodak™	Amersham Hybond LFP	+++	+++	+++
Image Station 2000MM	Amersham Protran Premium	++	++	++
Li-Cor	Amersham Hybond LFP	NA	NA	+++
Odyssey™	Amersham Protran Premium	NA	NA	++

++++ = high performance

+++ = good performance

++ = acceptable performance

+ = poor performance

NA = not compatible

NT = not tested

Ratings are based on overall performance including level of auto fluorescence/background and unspecific detection as well signal intensity

7 Protocols

Blotting protocol (wet electro transfer)

Any type of transfer system for Western blotting is possible to use, but we recommend the use of electro transfer/wet transfer (see other materials required/related products).

Step	Action
1	Separate the protein samples and molecular weight standards using SDS-PAGE electrophoresis.
2	Carefully remove the stacking gel and the Bromophenol Blue front and soak the gel in cold transfer buffer for 10–20 minutes.
3	Cut the membrane into suitable size and prepare it for blotting. Wet Amersham Protran Premium (nitrocellulose) membranes in ultra pure water for 20 seconds followed by 5 minutes in pre-chilled transfer buffer. Wet Amersham Hybond LFP (PVDF) membranes first in methanol for 20 seconds, then in ultra pure water also for 20 seconds and finally in cold transfer buffer for at least 5 minutes.
4	Place the electro cassette anodic side facing down in a tray filled 3 cm deep with pre chilled transfer buffer. Load the cassette starting at the anodic side with a foam sponge, followed by two Hybond blotting papers (pre-wetted in transfer buffer), the prepared membrane, the gel, and again two wet Hybond blotting papers and finally a second foam sponge. Make sure to remove any trapped air between filter papers, membrane and gel. Close the cassette and place the

Step	Action
	anodic side (+) in the same orientation as the following cassettes in the electro blotting tank filled with cold transfer buffer. Make sure to connect the + end of the cables of the lid of the transfer buffer tank to the + labelled sockets of the power supply.
5	Transfer at 25 V (maximum 400 mA) and 4°C with stirring for 2–2.5 hours.
6	Block the membrane in 2% Amersham ECL Prime blocking agent in wash buffer, shaking at 4°C over night or at room temperature for 1 hour.
7	After blocking, rinse the membrane twice in PBS-T and then wash 2 × 5 minutes. Keep the membrane in wash buffer or wash buffer without Tween 20 (for longer times than 3 days) until probed with primary and secondary antibodies (see sections *Single protein detection protocol on page 15* and *Multiplex detection protocol on page 17*) . Blotted membranes can be stored at 4°C in wash buffer without Tween 20 for up to one week before probing.

Single protein detection protocol

Step	Action
1	Dilute the primary antibody of mouse or rabbit origin to optimal concentration in wash buffer or blocking solution (see additional information).

Step	Action
2	Incubate a blocked membrane (protein side up) with the diluted primary antibody for 1.5 hours at room temperature, shaking.
3	Rinse the membrane twice in wash buffer, then wash the membrane for 2 × 5 minutes in wash buffer shaking at room temperature.
4	Dilute the Amersham ECL Plex CyDye conjugated secondary antibodies (1 µg/µl) to optimal concentration (between 1:1250 and 1:4000, we recommend 1:2500, see additional information) in wash buffer. Incubate the washed membrane protected from light for 1 hour at room temperature, shaking.
5	Rinse the membrane three times in wash buffer, followed by 4 × 5 minutes in wash buffer, shaking at room temperature and protected from light.
6	Rinse the membrane three times in wash buffer (without Tween 20).
7	Detect the secondary antibody signal by scanning the membrane using a fluorescent laser scanner. If Amersham Hybond LFP membrane is used, dry the membrane before scanning by placing it on Hybond blotting paper and incubate at 37°C to 40°C for 1 hour or at room temperature over night protected from light. If stripping is planned, remember to NOT dry the Amersham Hybond LFP membrane.

Multiplex detection protocol

Step	Action
1	Dilute the primary antibodies of mouse and rabbit origin to optimal concentrations in wash buffer or blocking solution (see additional information).
2	Incubate the blocked membrane (protein side up) with both diluted primary antibodies together for 1.5 hours shaking at room temperature. If primary antibodies are to be reused, it is possible to incubate the primary antibodies separately when multiplexing.
3	Rinse the membrane twice in wash buffer, then wash the membrane for 2 × 5 minutes in wash buffer at room temperature, shaking.
4	Dilute the Amersham ECL Plex CyDye conjugated secondary antibodies (1 µg/µl) to optimal concentration (between 1:1250 and 1:4000, we recommend 1:2500, see additional information) in wash buffer. Incubate the washed membrane protected from light for 1 hour at room temperature, shaking.
5	Rinse the membrane three times in wash buffer, followed by 4 × 5 minutes in the same buffer shaking at room temperature and protected from light.
6	Rinse the membrane three times in wash buffer (without Tween 20).

Step	Action
7	Detect the multiplexed secondary antibody signals by scanning the membrane using a fluorescent laser scanner or imager. If Amersham Hybond LFP membrane is used, dry the membrane before scanning by placing it on Hybond blotting paper and incubate at 37°C to 40°C for 1 hour or at room temperature over night protected from light. If stripping is planned, remember to NOT dry the Amersham Hybond LFP membrane.

Buffers and solutions

- Sample loading buffer: 120 mM Tris pH 6.8, 20% Glycerol, 4% SDS, 200 mM DTT and trace amount of Bromophenol blue.
- Running buffer: 25 mM Tris, 192 mM Glycine and 0.1% SDS.
- Ultrapure water.
- Transfer buffer: 25 mM Tris, 192 mM Glycine, 20% Methanol.
- Blocking solution: 2% Amersham ECL Prime blocking agent in wash buffer.
- Wash buffer: PBS pH 7.4, 0.1% Tween (PBS-T) or TBS pH 7.4–8, 0.1% Tween (TBS-T). TBS-T is recommended for detection of phosphorylated proteins.
- PBS pH 7.4 or TBS pH 7.4–8. TBS is recommended for detection of phosphorylated proteins.

8 Scanning

We recommend the scanning of dried membranes. However, Amersham Protran Premium membranes (nitrocellulose) can become fragile when dried. If this is the case, we recommend scanning the membrane while wet. Scanning dried membranes usually results in a more even background and a higher signal to noise ratio.

Note: *Always place the membranes with the protein side facing down on the scanner bed.*

- **Dried membranes:**

  Place a low fluorescent glass plate on top of the membranes when scanning dried membranes. Make sure to label the dried Amersham Hybond LFP membranes by cutting corners or using a pencil, because they can easily lose their position on the scanner bed during handling.

- **Wet membranes:**

  To minimize the formation of air bubbles when scanning wet membranes, a small amount of water should be placed on the scanner bed prior to applying the membrane (protein side down). Also, roll an empty 10 ml pipette or similar across the membrane on the scanner bed to remove any trapped air.

For the Typhoon scanner use the Cy3 and Cy5 channels and a PMT value between approximately 500 and 700 V for wet membranes and 300 to 500 V for dry membranes.

- It is important to clean the Typhoon scanner bed after use, to avoid contaminating the membranes. We recommend that the glass platen is first cleaned with 10% hydrogen peroxide followed by 75% ethanol and finally distilled water.

9 Quantification

For accurate quantification of the proteins of interest, the image analysis software called ImageQuant™ TL from Cytiva is recommended. Other software programs are also compatible.

10 Additional information

Determination of optimum antibody concentration

Primary antibody

Step	Action
1	Make a serial dilution of the protein that the primary antibody is directed against.
2	Perform dot blot or perform full Western blotting procedure. Block the membranes.
3	Wash and incubate the membranes with several dilutions of the primary antibody, for example 1:100, 1:500, 1:1000 and 1:5000.
4	Wash and incubate the membranes with the secondary antibody in the same dilution for all dilutions of primary antibody.

Step	Action
5	Select the optimal dilution as the maximal signal to noise ratio.

Secondary antibody

Step	Action
1	Make a serial dilution of the protein that the primary antibody is directed against.
2	Perform dot blot or perform full Western blotting procedure. Block the membranes.
3	Wash and incubate the membranes with the optimal dilution of the primary antibody.
4	Wash and incubate the membranes with several dilutions of the secondary antibody, for example 1:1250, 1:1500, 1:2500, 1:3500 and 1:4000.
5	Select the optimal dilution as the maximal signal to noise ratio.

Storage of probed membranes

The membrane can be stored in dried condition between two blotting papers wrapped in foil at ambient temperature for at least 3 months. Remember that the Amersham Protran Premium membrane may become fragile when dry.

Labelling of primary antibodies

Cy3 and Cy5 labelled primary antibodies could also be prepared (using CyDye antibody labelling kits or CyDye-esters: see under related products), but the sensitivity is likely to be lower than using indirect detection (i.e. unlabelled primary antibody with Amersham ECL Plex CyDye conjugated secondary antibodies).

Protocol for membrane stripping

Following fluorescent detection using the Amersham ECL Plex Western blotting system it is possible to re-probe the Amersham Hybond LFP (PVDF) membrane several times. If stripping is planned for, remember to NOT dry the membranes. When scanning wet Amersham Hybond LFP membranes the background will be higher and can appear uneven (due to partial dryness). The membranes can be stored in PBS at 2°C to 8°C for up to 3 months.

It is not recommended to strip and re-probe Amersham Protran Premium (nitrocellulose) membranes.

Protocol for membrane stripping:

Incubate the membrane in 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.8 shaking for 30 minutes at 50°C. Wash the membrane 2 × 10 minutes in wash buffer shaking at room temperature. Block and re-probe the membrane according to recommended protocols.

Western blotting handbook

More technical help, tips, and best practices can be found in the handbook Western Blotting Principles and Methods from Cytiva (code no. 28999897).

11 Tips and troubleshooting when using the Amersham ECL Plex fluorescent Western blotting system

Handling of membranes

- Always use powder free gloves. Powder from the gloves can give rise to contamination on fluorescent images of membranes.
- Do not use a ball point pen on the membrane, since it can contaminate other membranes in the same tray and is visible upon fluorescent scanning. If you need to write something on the membrane use a pencil.
- Do not touch membranes probed with the secondary antibody with gloved hands. This could leave marks on the fluorescent image. Always use forceps (slide forceps is recommended) when handling the membranes.
- Make sure to always keep the protein side of the membranes up throughout the protocol. Remember to scan the membranes with protein side facing down on the scanner bed.
- Make markings by cutting, for example, corners on the membrane to avoid mixing up when handling them. This is also very useful in a multi-membrane scan to be able to discriminate them from each other during image analysis.

Problems with high/uneven background and unspecific detection

- Make sure to remove the stacking gel before transfer. Also, make sure to remove any small pieces of gel after transfer before blocking the membrane. Any remaining gel pieces can give rise to black spots on fluorescent images of the membranes.
- Remove all bromophenol blue (BFB) in the front from the SDS-PAGE gel before transfer. BFB on the membrane can give fluorescent signals.
- Make sure that your trays are clean and not contaminated by for example Coomassie blue stain, which can cause background problems.
- If you have problems with detection of many unspecific protein bands, we recommend using Amersham Hybond LFP membrane. The use of Amersham ECL Prime blocking agent may further reduce unspecific detection. This problem is often dependent on the primary antibody.
- A common reason for uneven background is that the membrane is only partly dried. Make sure that the membrane is completely dry when scanned. If the Amersham Protran Premium membranes are scanned wet, it is important to ensure that the membrane has not partially dried during handling.

- It can be advantageous to incubate the primary antibodies diluted in blocking solution, to reduce unspecific binding and increase signal intensity. For some primary antibodies diluted in blocking solution, a lower concentration of antibodies can result in a much stronger signal compared to when diluted in wash buffer (even at a higher concentration). For other primary antibodies there is no difference in signal intensity when diluted in blocking solution.

Detection of low abundant proteins

- Use Amersham ECL Plex Cy5 conjugated secondary antibody for the protein you expect to have the lowest concentration in your sample. The Amersham ECL Plex Cy5 secondary antibody is slightly more sensitive than the Amersham ECL Plex Cy3 secondary antibody and the Amersham ECL Plex Cy2 secondary antibody.
- When detecting low abundant proteins, the signal can for a few target proteins become stronger if the species of primary antibody is switched in addition to careful optimization of antibody dilution. Sometimes the signal for the protein of interest can become stronger using an anti-mouse instead of an anti-rabbit primary antibody and vice versa.
- For improved detection of weak signals using the Amersham Hybond LFP membrane, skip the blocking step and instead dry the membrane directly after transfer. Dry the membrane for at least 2 hours at ambient temperature. Another tip for increasing weak signals is to use PBS 0.1% Triton X-100 instead of PBS 0.1% Tween 20 throughout the protocol. However, Triton is NOT compatible with the Cy3 channel (high background).

- If you are detecting low abundant proteins, use a smaller amount of Amersham ECL Plex Rainbow Markers (1.5 µl) or leave an empty lane between the markers and the sample. The markers can otherwise appear too strong and even disturb the analysis of the sample lane.
- TBS and TBS-T is recommended for use with antibodies against phosphorylated proteins. The phosphorate in PBS buffer can interfere and reduce the antibody binding.

12 Ordering information

ECL Plex products

Amersham ECL Plex Cy3 and Cy5 conjugated antibodies	**Code No.**
Amersham ECL Plex goat-α-mouse IgG, Cy5, 150 µg Recommended for at least 1000 cm^2 membrane	PA45009
Amersham ECL Plex goat-α-mouse IgG, Cy5, 600 µg Recommended for at least 4000 cm^2 membrane	PA45010
Amersham ECL Plex goat-α-rabbit IgG, Cy5, 150 µg Recommended for at least 1000 cm^2membrane	PA45011
Amersham ECL Plex goat-α-rabbit IgG, Cy5, 600 µg Recommended for at least 4000 cm^2 membrane	PA45012
Amersham ECL Plex goat-α-mouse IgG-Cy3, 150 µg Recommended for at least 1000 cm^2 membrane	PA43009
Amersham ECL Plex goat-α-mouse IgG-Cy3, 600 µg Recommended for at least 4000 cm^2 membrane	PA43010
Amersham ECL Plex goat-α-rabbit IgG-Cy3, 150 µg Recommended for at least 1000 cm^2 membrane	28901106

Amersham ECL Plex goat-α-rabbit IgG-Cy3, 600 µg Recommended for at least 4000 cm^2 membrane	28901107
Amersham ECL Plex goat-α-mouse IgG-Cy2, 150 µg Recommended for at least 1000 cm^2 membrane	28901108
Amersham ECL Plex goat-α-mouse IgG-Cy2, 600 µg Recommended for at least 4000 cm^2 membrane	28901109
Amersham ECL Plex goat-α-rabbit IgG-Cy2, 150 µg Recommended for at least 1000 cm^2 membrane	28901110
Amersham ECL Plex goat-α-rabbit IgG-Cy2, 600 µg Recommended for at least 4000 cm^2 membrane	28901111

Amersham Protran Premium, Low fluorescent nitrocellulose membrane. Optimized for use with Amersham ECL Plex Western Blotting System.	**Code No.**
Amersham Protran Premium 0.2 NC (20 × 20 cm) 10 sheets	10600050
Amersham Protran Premium 0.2 NC (8 × 9 cm) 25 sheets	10600097
Amersham Protran 0.2 NC 300 mm × 4 m 1 roll	10600004

Amersham Hybond LFP, Low fluorescent PVDF membrane, 0.2 µm pore size. Optimized for use with Amersham ECL Plex Western Blotting System.	**Code No.**
Amersham Hybond LFP, 20 × 20 cm 10 sheets	10600060
Amersham Hybond LFP, 10 × 10 cm 10 sheets	10600091

Amersham Hybond LFP, 8 × 9 cm	10600102
25 sheets	
Amersham Hybond LFP, 254 mm × 4 m	10600022
1 roll	

Amersham ECL Plex Fluorescent Rainbow markers; full-range. Optimized for use with ECL Plex Western Blotting system. Supplied in gel loading buffer. Defined molecular weight standards from 12 000 to 225 000 kD.	**Code No.**
Amersham ECL Plex Fluorescent Rainbow markers;	RPN850E
full range, 120 µl	
Amersham Plex Fluorescent Rainbow markers;	RPN851E
full range, 500 µl	

Amersham ECL Plex Western blotting combination pack;	**Code No.**
Cy3, Cy5, Hybond-ECL	RPN998
Combination pack optimized for ECL Plex Western blotting including Amersham Protran Premium (nitrocellulose membrane). Contains the following components, sufficient for at least 1000 cm^2 of membrane:	
Amersham ECL Plex goat-α-mouse IgG, Cy3, 150 µg	
Amersham ECL Plex goat-α-rabbit IgG, Cy5, 150 µg	
Amersham ECL Plex Fluorescent Rainbow markers, full-range 120 µl	
Amersham Protran, 10 × 10 cm, 10 sheets	

ECL Plex Western blotting combination pack;	**Code No.**
Cy3, Cy5, Hybond-LFP	RPN999
Combination pack optimized for ECL Plex Western blotting including Amersham Hybond LFP (low fluorescent PVDF membrane). Contains the following components, sufficient for at least 1000 cm^2 of membrane:	

Amersham ECL Plex goat-α-mouse IgG, Cy3, 150 µg
Amersham ECL Plex goat-α-rabbit IgG, Cy5, 150 µg
Amersham ECL Plex Fluorescent Rainbow markers, full-range 120 µl
Amersham Hybond LFP, 20 × 20 cm, 3 sheets

Blocking buffer, optimized for use with ECL Plex Western Blotting system	**Code No.**
Amersham ECL Prime Blocking agent, 20 g	RPN418
Bovine Serum Albumin, 25 g	RPN412

Antibody labeling kit, using bis-Reactive CyDye:	**Code No.**
Cy2 Ab labelling kit	PA32000
Cy3 Ab labelling kit	PA33000
Cy5 Ab labelling kit	PA35000

Bis-Reactive CyDye NHS esters:	**Code No.**
Cy5.5 NHS ester	PA15500
Cy7 NHS ester	PA17000

Monoclonal antibody labeling kit, using mono-Reactive CyDye:	**Code No.**
Cy3 Ab labelling kit	PA33001
Cy5 Ab labelling kit	PA35001

Mono-Reactive CyDye NHS esters:	**Code No.**
Cy3.5 NHS ester	PA13605

Cy5.5 NHS ester	PA15605
Cy7 NHS ester	PA17105

13 Amersham ECL Plex Western blotting system Product protocol card

PA43009

Step	Action
1	Run an SDS-PAGE gel loaded with with your samples diluted in sample loading buffer and heated to 96°C for 5 minutes. Apply 1.5–3 µl Amersham ECL Plex Rainbow markers without pre-heating.
2	Blot the gel onto a Amersham Hybond LFP or Amersham Protran™ Premium membrane using electro transfer (wet transfer) at 25 V for approximately 2.5 hours.
3	Block the membrane in 2% Amersham ECL Prime blocking agent in wash buffer for 1 hour at RT or overnight at 4°C, shaking.
4	Rinse the membrane twice followed by 2 × 5 minutes in wash buffer at RT, shaking.
5	Incubate the membrane with an optimized dilution (in wash buffer or blocking solution) of primary antibodies for at least 1.5 h at RT or at overnight at 4°C, shaking.

Step	Action
6	Rinse the membrane twice followed by 2 × 5 minutes in wash buffer at RT, shaking.
7	Incubate the membrane with an optimized dilution (between 1:1250 and 1:4000. 1:2500 dilution is a recommended starting point) of Amersham ECL Plex CyDye-conjugated secondary antibodies (1 µg/µl) for 1 h at RT, shaking.
8	Rinse the membrane three times followed by 4 × 5 minutes in wash buffer at RT, shaking.
9	Rinse the membrane three times in PBS or TBS without Tween 20.
10	Dry the membrane for 1 h at 37–40°C. Do not dry the Amersham Hybond LFP membrane if stripping and re-probing is planned for.
11	Scan the membrane using a compatible imager (Typhoon Imager) and analyse the protein levels using suitable software (ImageQuant TL).

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