cytiva

Amersham ECL Western blotting detection reagents and analysis system

Product Booklet

Table of Contents

1 Introduction

Product codes

RPN2106/8/9

RPN2209

RPN2134

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.

You are reminded that certain components in the solutions may cause bleaching on contact with skin.

Note: *The protocol requires the use of Hydrochloric acid.*

WARNING
Hydrochloric Acid causes burns and is an irritant. Follow the manufacturer's safety data sheet relating to the safe handling and use of this material.

Storage

On receipt all components must be stored in a refrigerator at 2–8°C.

Expiry

The components of these products are stable until expiry when stored under the recommended conditions.

2 Components

RPN2106 ECL™ Western blotting detection reagents:

- Detection reagent 1 250 mL
- Detection reagent 2 250 mL
- Sufficient for 4000 cm^2 membrane

RPN2209 ECL Western blotting detection reagents:

- Detection reagent 1 125 mL
- Detection reagent 2 125 mL
- Sufficient for 2000 cm^2 membrane

RPN2109 ECL Western blotting detection reagents:

- Detection reagent 1 62.5 mL
- Detection reagent 2 62.5 mL
- Sufficient for 1000 cm^2 membrane

RPN2134 ECL Western blotting detection reagents:

- RPN2209 x 3
- Sufficient for 6000 cm^2 membrane

RPN2108 ECL Western Blotting Analysis System:

- Detection reagent 1 62.5 mL
- Detection reagent 2 62.5 mL
- Mouse IgG, Horseradish Peroxidase-linked whole antibody (from sheep), 100 µL
- Rabbit IgG, Horseradish Peroxidase-linked whole antibody (from donkey), 100 µL
- Blocking reagent, 5 g Sufficient for 10 blots 10 cm x 10 cm
- For the detection of either mouse or rabbit membrane bound primary antibodies.

Other materials required

Equipment

- Electrophoresis and blotting apparatus (for Western blots)
- Blotting membrane, recommend Hybond™ ECL (nitrocellulose) from Cytiva
- Orbital shaker
- Forceps with rounded, nonserrated tips
- X-ray film cassettes, recommend Hypercassette from Cytiva
- Timer
- Film, recommend Hyperfilm™ ECL, film developing facility and reagents from Cytiva

Reagents

- Tris base (Tris(Hydroxymethyl) Aminomethane)
- Sodium Chloride
- Hydrochloric Acid (1 M and 5 M)

- Tween™ 20
- Immunodetection reagents (if using RPN2106 and RPN2109)
- Distilled water
- Disodium Hydrogen
- Orthophosphate Anhydrous (Na_2HPO_4)
- Sodium Dihydrogen
- Orthophosphate ($NaH_2PO_4.2H_20$)

Buffers and working solutions

The chemical reagents required for these solutions are available from Cytiva and are detailed in the current catalogue.

Phosphate buffered saline (PBS) pH 7.5:

- 11.5 g Disodium Hydrogen Orthophosphate Anhydrous (80 mM)
- 2.96 g Sodium Dihydrogen Orthophosphate (20 mM)
- 5.84 g Sodium Chloride (100 mM)
- Dilute to 1000 mL with distilled water. Check pH.

Tris-buffered saline (TBS) pH 7.6

- 8 g Sodium Chloride
- 20 mL 1 M Tris HCl, pH 7.6
- Dilute to 1000 mL with distilled water. Check pH.

Diluent and wash buffer PBS Tween (PBS-T) and TBS Tween (TBS-T)

- Dilute required volume of Tween 20 in the corresponding buffer.
- A 0.1% Tween 20 concentration in PBS or TBS is suitable for most blotting applications.

Storage of buffers once prepared

All buffers must be stable for at least 3 months if prepared in advance and stored at room temperature, although storage in a refrigerator (2–8°C) may be necessary to avoid microbial spoilage.

Sodium Azide is not recommended for use as a bacteriocide.

Working solutions for ECL immunodetection Membrane blocking agent

Cytiva recommends the blocking reagent supplied (ECL Blocking Agent, RPN2125) or substitute with non-fat dried milk dissolved in PBS-T or TBS-T; 5 g per 100 mL (5%).

Immunodetection reagents

- Primary antibodies / HRP-linked secondary antibodies
- It is recommended that antibody dilutions are optimized to maximize signal and minimize background. When using the secondary antibodies supplied in RPN2108, a good starting dilution is 1:1000. See *Secondary antibodies, on page 28*. For details of the recommended ECL HRP antibodies see *Chapter 10 Related products, on page 42*.

Biotinylated antibody

It is recommended that the antibody dilution must be optimized to suit different blotting situations. See *Determination of optimum antibody concentration, on page 26*. The full range of biotinylated antibodies can be found in the current Cytiva catalogue.

Storage of working solutions once prepared

All working strength solutions should be stable for one hour at room temperature. For longer periods it is recommended that they be kept in a refrigerator (2–8°C). For reproducible performance equilibrate to room temperature before use.

3 Description

Principles of ECL Western Blotting

ECL Western blotting fromCytiva is a light emitting nonradioactive method for detection of immobilized specific antigens, directly or indirectly with Horseradish Peroxidase (HRP) labelled antibodies.

- **High sensitivity non-radioactive detection system**

  At least 10 x more sensitive than colorimetric or radioactive detection systems.

- **High resolution**

  High contrast signal generated

- **Speed**

  Specific protein detection may be achieved in less than 1 minute.

- **Stable hard copy results on film**

Signal generated can be quantitated with a densitometer.

- **Detection of lower abundance protein in complex cell samples compared to colorimetric or radioactive systems**
- **Detection of antigen with a smaller amount of antibody or lower affinity antibody compared to colorimetric or radioactive systems**
- **Versatility**

  Detection of Western blotted proteins from one dimensional, twodimensional and agarose/acrylamide gels.

- **Optimized protocols**

  Reprobing; sequential reprobing of membranes with a variety of antibodies.

  Stripping and reprobing; the complete removal of primary and secondary antibodies from membranes, optimized to minimize loss of antigen.

  Determination of optimum antibody concentration.

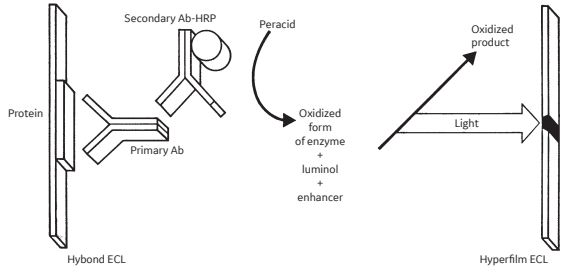


Fig 1. Principles of ECL Western blotting

Principles of ECL detection

Luminescence is defined as the emission of light resulting from the dissipation of energy from a substance in an excited state. In chemiluminescence the excitation is effected by a chemical reaction. The chemical reactions of cyclic Diacylhydrazides such as luminol have been widely used in chemical analysis (1, 2) and extensively studied (3, 4). One of the most clearly understood systems is the HRP/Hydrogen Peroxide catalyzed oxidation of luminol in alkaline conditions. Immediately following oxidation, the luminol is in an excited state which then decays to ground state via a light emitting pathway. Enhanced chemiluminescence (2) is achieved by performing the oxidation of luminol by the HRP in the presence of chemical enhancers such as phenols. This has the effect of increasing the light output approximately 1000 fold and extending the time of light emission. The light produced by this enhanced chemiluminescent reaction peaks after 5–

20 minutes and decays slowly thereafter with a half life of approximately 60 minutes. The maximum light emission is at a wavelength of 428 nm which can be detected by a short exposure to blue-light sensitive autoradiography film for example Hyperfilm ECL.

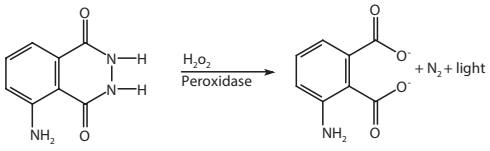


Fig 2.

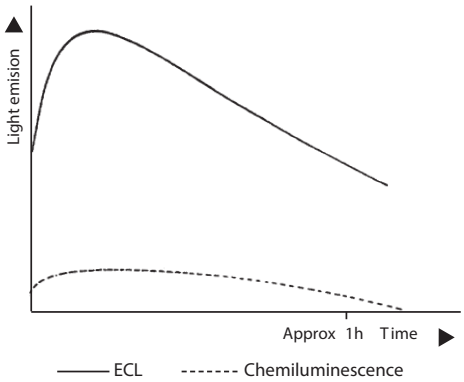


Fig 3. Graph of light emission versus time, showing the difference between chemiluminescence and ECL.

4 Critical parameters

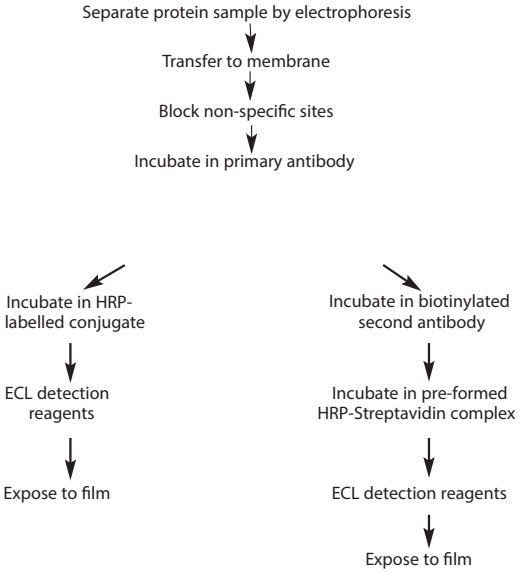
The following points are critical:

- It is essential to optimize both primary and secondary antibodies for results with high signal and low background due to the sensitive performance of the system. The high sensitivity means that much higher dilutions of antibodies are required than are used with other conventional systems such as colorimetric. See *Determination of optimum antibody concentration, on page 26* for details of optimization experiments that can be performed to determine the best concentrations of primary and secondary antibodies.
- It is necessary to work quickly once the membranes have been exposed to the detection reagents in order to capture the maximum signal.
- Wear powder-free gloves when handling detection reagents and film.
- Do not use Sodium Azide as a preservative for buffers to be used in immunodetection as it is an inhibitor of Horseradish Peroxidase.
- Proper blocking and washing of the membranes is critical for optimum results. It may be necessary to adjust blocking conditions for certain applications.
- Do not allow the membranes to dry out during the immunodetection procedure.
- When washing, the volume of wash buffer should be as large as possible; 4 mL of buffer per cm^2 of membrane is suggested. Brief rinses of the membrane in wash buffer before incubating will improve washing efficiency.

- If exposure times of less than 5 seconds are routinely required, it is recommended that the antibodies used are further diluted as it is difficult to perform such short exposures.
- Although the 'working mix' of the ECL reagents is stable for up to 1 hour, it is recommended that reagents are mixed immediately before use. In the event that mixed reagents need to be left longer than 1 hour before use, store at 2–8°C. For reproducible performance equilibrate to room temperature before use.

5 Protocol

Flow diagram



Detailed protocol and notes

The protocol outlined on the following pages has been developed in our laboratories to be the optimum for both sensitivity and convenience. A further rapid immunodetection protocol is outlined in *Rapid immunodetection protocol, on page 24* for situations where time is limiting. Users, however, may wish to adapt the protocols to suit their specific needs, and notes and a troubleshooting guide are provided to assist with this.

Electrophoresis and blotting

Step	Action
1	Perform electrophoresis and blotting according to normal techniques. Protein must be transferred to Hybond ECL or Hybond-P PVDF for optimum results. Blots may be used immediately or stored in a desiccator for up to 3 months. ***Note:*** *Hybond ECL must be prewetted in distilled water and equilibrated in transfer buffer for at least 10 minutes before blotting.* ***Note:*** *Hybond-P PVDF must be pre-wetted in 100% Methanol, washed in distilled water for 5 minutes and equilibrated in transfer buffer for at least 10 minutes before blotting.* ***Note:*** *ECL is also suitable for use with supported nitrocellulose such as Hybond-C Extra. This membrane must be prepared as for Hybond ECL.*

Blocking the membrane

Step	Action
1	Block non-specific binding sites by immersing the membrane in 5% non-fat dried milk, 0.1% (v/v) Tween 20 in PBS or TBS (PBS-T or TBS-T, see *Other materials required, on page 5*) for one hour at room temperature on an orbital shaker. Alternatively, membranes may be left in the blocking solution overnight in a refrigerator at 2–8°C, if more convenient. ***Note:*** *The combination of nonfat dried milk and Tween must be suitable for most applications. Optimum Tween concentrations will vary to suit specific experiments, but a 0.1% Tween 20 concentration is suitable for most blotting applications.*
2	Briefly rinse the membrane using two changes of wash buffer. ***Note:*** *While washing prepare the diluted primary antibody (Primary antibody incubation, on page 17, step 1)*

Primary antibody incubation

Step	Action
1	Dilute the primary antibody in PBS-T or TBS-T. The dilution factor must be determined empirically for each antibody. ***Note:*** *Optimization of the antibody dilution can be performed by dot blot analysis (see Determination of optimum antibody concentration, on page 26).*
2	Incubate the membrane in diluted primary antibody for 1 hour at room temperature on an orbital shaker. ***Note:*** *Incubation times and temperatures may vary and must be optimized for each antibody. The conditions indicated are recommended starting points.*
3	Briefly rinse the membrane with two changes of wash buffer and then wash the membrane in >4 mL/cm^2 of wash buffer for 15 minutes at room temperature.
4	Wash the membrane for 3 x 5 minutes with fresh changes of wash buffer at room temperature. ***Note:*** *While washing prepare the diluted secondary antibody (Secondary antibody incubation, on page 18, step 1).*

Secondary antibody incubation

Step	Action
1	Dilute the HRP labelled secondary antibody or biotinylated antibody in PBS-T or TBS-T. The dilution factor must be determined empirically for each antibody (see *Determination of optimum antibody concentration, on page 26*). ***Note:*** *Use either an appropriate HRP labelled secondary antibody or a biotinylated secondary antibody.*
2	Incubate the membrane in the diluted secondary antibody for 1 hour at room temperature on an orbital shaker. ***Note:*** *Incubation times and temperatures may vary and must be optimized for each antibody. The conditions indicated are recommended starting points.*
3	Briefly rinse the membrane with two changes of wash buffer and then wash the membrane in >4 mL/cm^2 of wash buffer for 15 minutes at room temperature.

Step	Action
4	Wash the membrane for 3 x 5 minutes with fresh changes of wash buffer at room temperature. ***Note:*** *If using HRP-labelled secondary antibody proceed directly to Detection, on page 20 after this wash procedure. If using a biotinylated antibody, while washing, prepare the diluted Streptavidin HRP conjugate or complex (Streptavidin bridge incubation, on page 19, step 1).*

Streptavidin bridge incubation

Step	Action
1	Dilute the streptavidin HRP conjugate or streptavidinbiotinylated HRP complex in PBS-T or TBS-T. ***Note:*** *The dilution factor should be determined empirically (see Determination of optimum antibody concentration, on page 26).*
2	Incubate the membrane in the dilution for 45–60 minutes at room temperature on an orbital shaker
3	Briefly rinse the membrane with two changes of wash buffer and then wash the membrane with >4 mL/cm^2 of wash buffer for 15 minutes at room temperature.
4	Wash the membrane for 3 x 5 minutes with fresh changes of wash buffer at room temperature.

Detection

Step	Action
1	Mix an equal volume of detection solution 1 with detection solution 2 allowing sufficient total volume to cover the membranes. The final volume required is 0.125 mL/cm^2 membrane. ***Note:*** *If the mixed reagent is not to be used immediately, store at 2–8°C. For reproducible performance equilibrate to room temperature before use.*
2	Drain the excess wash buffer from the washed membranes and place them, protein side up, on a Protocol sheet of SaranWrap™ or other suitable clean surface. Pipette the mixed detection reagent on to the membrane. ***Note:*** *The reagents must cover the entire surface of the membrane, held by surface tension on to the surface of the membrane.*
3	Incubate for 1 minute at room temperature.

Step	Action
4	Drain off excess detection reagent by holding the membrane gently with forceps and touching the edge against a tissue. Place the blots protein side down on to a fresh piece of SaranWrap, wrap up the blots and gently smooth out any air bubbles. ***Note:*** *Close the SaranWrap around the membrane to form an envelope or use an alternative, suitable detection pocket. Avoid using pressure on the membrane.*
5	Place the wrapped blots, protein side up, in an X-ray film cassette. ***Note:*** *Ensure that there is no free detection reagent in the film cassette; the film must not get wet.*
6	Place a sheet of autoradiography film (for example Hyperfilm ECL) on top of the membrane. Close the cassette and expose for 15 seconds. ***Note:*** *This stage should be carried out in a dark room, using red safelights. Do not move the film while it is being exposed.*

Step	Action
7	Remove the film and replace with a second sheet of unexposed film. Develop the first piece of film immediately, and on the basis of its appearance estimate how long to continue the exposure of the second piece of film. Second exposures can vary from 1 minute to 1 hour. ***Note:*** *The detected blots can also be exposed to Polaroid™ film using the ECL minicamera (RPN2069), which is specifically designed for blots generated from mini-gel apparatus. The ECL minicamera is suitable for blots up to 52 x 77 mm. Images can also be aquired using a CCD camera such as Imagemaster VDS-CL (18-1130-55).*

6 Additional information

Reprobing membranes

Following ECL detection it is possible to reprobe the membrane several times to either clarify or confirm results or when small or valuable samples are being analyzed (5). Sequential reprobing of membranes with a variety of antibodies is possible following the steps below. The membranes may be stored wet and wrapped in a refrigerator (2–8°C) after each immunodetection.

Step	Action
1	Wash the membrane for 2 x 10 minutes in TBS-T or PBS-T at room temperature using large volumes of wash buffer.
2	Block the membrane in 5% non-fat dried milk in PBS-T or TBS-T for 1 hour at room temperature. ***Note:*** *Refer to note on step 1 in Blocking the membrane, on page 16.*
3	Repeat the immunodetection protocol, from *Primary antibody incubation, on page 17* to *Detection, on page 20*.

Stripping and reprobing membranes

The complete removal of primary and secondary antibodies from the membrane is possible following the protocol outlined below. The membranes may be stripped of bound antibodies and reprobed several times. Membranes must be stored wet wrapped in SaranWrap in a refrigerator (2–8°C) after each immunodetection.

Step	Action
1	Submerge the membrane in stripping buffer (100 mM 2-Mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) and Protocol incubate at 50°C for 30 minutes with occasional agitation. ***Note:*** *If more stringent conditions are required the incubation can be performed at 70°C or the incubation time increased.*
2	Wash the membrane for 2 x 10 minutes in PBS-T or TBS-T at room temperature using large volumes of wash buffer. ***Note:*** *Membranes may be incubated with ECL detection reagents and exposed to film to ensure removal of antibodies.*
3	Block the membrane by immersing in 5% Nonfat dried milk in PBS-T or TBS-T for 1 hour at room temperature.
4	Repeat the immunodetection protocol, from *Primary antibody incubation, on page 17* to *Detection, on page 20*.

Rapid immunodetection protocol

If time is short the following protocol allows the immunodetection using HRP-labelled antibodies to be completed in just over 2 hours, compared to 4 hours for the standard protocol. If desired, the protocol can be further shortened by also optimizing the primary antibody for a shortened incubation.

Step	Action
1	Block the membrane in 10% non-fat dried milk in PBS-T or TBS-T for 10 minutes at room temperature. ***Note:*** *This protocol has been optimized using 10% non-fat dried milk. Other blocking agents will need to be tested for their capacity to block effectively in a 10 minute incubation. The short block is suitable for both Nitrocellulose and PVDF membranes.*
2	Briefly rinse the membrane with Protocol two changes of wash buffer. ***Note:*** *While washing prepare the diluted primary antibody (step 3).*
3	Dilute the primary antibody in PBS-T or TBS-T. The dilution factor must be determined empirically for each antibody. ***Note:*** *Optimization of the antibody dilution can be performed by dot blot analysis, (see Determination of optimum antibody concentration, on page 26).*
4	Incubate the membrane in diluted primary antibody for 1 hour at room temperature on an orbital shaker. ***Note:*** *A further shortening of the immunodetection procedure is possible by increasing the primary antibody concentration, allowing a reduction in the incubation time without compromising sensitivity.*

Step	Action
5	Briefly rinse the membrane with three changes of wash buffer and then wash twice for 10 minutes in fresh changes of wash buffer, at room temperature. ***Note:*** *While washing, dilute the secondary antibody. In order to maintain the same sensitivity as obtained with the standard method, the secondary antibody must be used at a stronger concentration. As a guideline, increasing the concentration by four times must maintain the same sensitivity.*
6	Incubate the membrane in the diluted secondary antibody for 15 minutes at room temperature.
7	Briefly rinse the membrane with three changes of wash buffer and then wash twice for 10 minutes in fresh changes of wash buffer, at room temperature.
8	Perform the detection with ECL reagents as described in *Detection, on page 20*.

Determination of optimum antibody concentration

Due to the sensitivity of the ECL detection reagents, optimization of antibody concentrations is recommended to ensure the best results. In general, lower concentrations of both primary and secondary antibodies are required with ECL compared to colorimetric detection.

Outlined below are protocols for determining optimal antibody concentrations.

Primary antibodies

Dot blots are a quick and effective method of determining the optimum dilution of a primary antibody of unknown concentration.

Alternatively, a Western blot can be prepared and then cut into several strips. It must be noted that some antibodies may require alternative blocking and washing steps to the ones suggested below.

Step	Action
1	Spot a suitable amount of protein sample to a Nitrocellulose or PVDF membrane and allow to air dry. Prepare one blot for each primary antibody dilution to be tested.
2	Incubate in blocking solution for 1 hour at room temperature with agitation.
3	Rinse the membranes briefly with two changes of wash buffer.
4	Prepare several dilutions of primary antibody: e.g Nitrocellulose 1/100, 1/500, 1/1000, 1/1500; PVDF 1/500, 1/1000, 1/2500, 1/5000. Incubate 1 blot in each dilution for 1 hour at room temperature with agitation.
5	Rinse blots in two changes of wash buffer, then wash for 1 x 15 minutes and 3 x 5 minutes in fresh changes of wash buffer.

Step	Action
6	Dilute the secondary antibody (using only one concentration) and incubate the membranes for 1 hour at room temperature with agitation.
7	Wash as detailed in step 5.
8	Detect using ECL detection reagents as detailed in *Detection, on page 20*. of the protocol. The antibody dilution which gives the best signal with the minimum background must be selected.

Secondary antibodies

For a secondary antibody of unknown activity, a dot blot is also effective.

Step	Action
1	Prepare dot blots and block the membranes as detailed step 1 and 2 in *Primary antibodies, on page 27*.
2	Incubate in diluted primary antibody (using only one concentration) for 1 hour at room temperature with agitation.
3	Wash as detailed in step 5 in *Primary antibodies, on page 27*
4	Prepare several dilutions of secondary antibody: e.g. nitrocellulose 1/1000, 1/2500, 1/5000, 1/10 000; PVDF 1/2500, 1/5000, 1/10 000, 1/15 000. Incubate 1 blot in each dilution for 1 hour at room temperature with agitation.

Step	Action
5	Wash as detailed in step 5 in *Primary antibodies, on page 27*
6	Detect using ECL detection reagents as detailed in *Detection, on page 20*. The antibody dilution which gives the best signal with minimum background must be selected.

Quantification of proteins on ECL Western blots

It has been demonstrated (17) that Hyperfilm ECL exhibits a linear response to the light produced from enhanced chemiluminescence. This relationship can be used for the accurate quantification of proteins of ECL Western blots, using densitometry. The range over which the film response is linear can be extended by pre-flashing the film prior to exposure, making quantification of lower levels of protein, in particular, more accurate. Outlined below are guidelines to enable quantification of unknown levels of protein.

- The sample containing the protein to be quantified plus a set of standards (known amounts of the same antigen) are used to prepare a Western blot. It is suggested that at least 5 different standard dilutions are used. The dilution range should not be greater than one order of magnitude (see *Example, on page 30*). It is important that the concentration of the protein to be quantified lies within the standard range. To ensure this, it may be worth running more than one dilution of the protein.

- If desired, the film to be used can be pre-flashed. This is performed using a modified flash unit such as Sensitize RPN2051 that has been calibrated (by adjusting its distance from the film), to raise the film optical density 0.1 to 0.2 OD units above that of the standard film. The flash duration must be in the region of 1 msec.
- The Western blot is detected using standard protocols and then exposed to film. For quantification to be accurate, it is important that the light produced is in the linear range of the film. This can be achieved by making several exposures of different lengths of time. If the standard of lowest concentration is only just visible on the film, then the light from the rest of the standards must be in the linear range of the film.
- The films can then be scanned using a densitometer, and a graph of peak area against protein concentration plotted. The concentration of the protein being quantified can then be read off this graph, taking into account any dilutions made.

Example

A dilution series of myosin (chicken gizzard) was prepared containing 600 ng, 450 ng, 300 ng, 150 ng and 60 ng per 10 µL of loading buffer. Two further test samples in the range 60–600 ng were also prepared. Samples were electrophoresed and blotted on to Hybond ECL. Immunodetection was performed using anti-myosin at a 1:20 dilution, anti-mouse Ig-HRP at a 1:3000 dilution and ECL detection reagents.

A series of exposures to Hyperfilm ECL were made and the film on which the lowest concentration of myosin was just detectable was used for densitometric analysis. The film was scanned using a densitometer and a graph was plotted of peak area (OD units) against myosin concentration. The concentrations of the two test samples were then estimated from the standard curve.

Fig 4. ECL detection of myosin standard curve and myosin test samples. From left to right: myosin standards 600 ng, 450 ng, 300 ng, 150 ng, 60 ng, myosin test samples 1,2. 15 second exposure to Hyperfilm ECL.

Table 1. Peak area (OD units) for myosin standards and test samples.

Myosin samples		Peak area (OD units)
Standards	600 ng	2.075
	450 ng	1.620
	300 ng	1.149
	150 ng	0.692
	60 ng	0.200
Test samples	1	0.865
	2	0.476

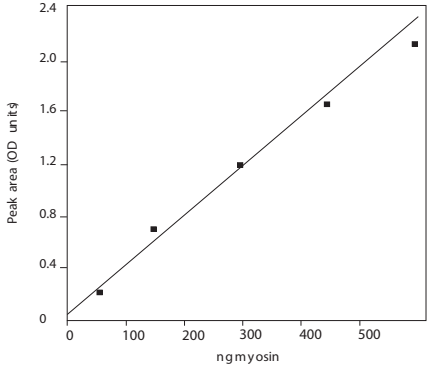


Fig 5. Peak area (OD units) against myosin concentration

Table 2. Comparison of calculated with actual concentration for the myosin test samples.

Test sample	Actual concentration (ng)	Calculated concentration (ng)
1	240 ng	235
2	120 ng	125

Use of ECL protein molecular weight markers

The ECL protein molecular weight markers (RPN2107) are a mixture of six different proteins labelled with biotin for use in Western blotting following electrophoresis on a Polyacrylamide gel prepared by the method of Laemmli (6). Incubation of the blot with Streptavidin Horseradish Peroxidase followed by detection with the ECL Western blotting system will result in a ladder of bands of approximately equal intensity.

Step	Action
1	Remove 1 µL of markers and add to 9 µL of gel loading buffer (containing 5% 2-β-Mercaptoethanol). ***Note:*** *Prepare dilution freshly, do not store the markers in loading buffer.*
2	Heat to 100°C for 4 minutes. Samples may be loaded on to the gel immediately, or stored temporarily on ice. ***Note:*** *Do not subject the markers to more than one denaturation.*
3	Load 10 µL per well. ***Note:*** *A 10 µL loading is sufficient to produce clearly visible bands after a 15 second exposure using overnight blotting in Towbin buffer (9) and standard ECL Western blotting immunodetection protocols.*

Step	Action
4	Following electrophoresis and transfer to nitrocellulose membranes, membranes are processed by standard immunodetection protocols as outlined in the main protocol section. If the protocol used is not a Biotin-Streptavidin system then Streptavidin-HRP (RPN1231) is added (1:1500) in the final antibody incubation. ***Note:*** *It is strongly advised that milk should not be included in the Streptavidin-HRP incubation. The binding of Streptavidin to Biotin is inhibited due to the presence of endogenous Biotin in the milk, resulting in a much decreased signal when detected by enhanced chemiluminescence.* ***Note:*** *If cross reactivity is observed between the Streptavidin-HRP and the protein samples on the blot, it is suggested that the lane containing the markers is removed and incubated in Streptavidin-HRP separately. The strip can then be re-aligned with the rest of the membrane for ECL detection.*
5	The membranes are then washed and detected using ECL reagents as detailed in *Detection, on page 20*.

Step	Action
6	The volume of markers required to give optimum results will depend on the electroblotting and immunodetection conditions used and the length of exposure to film required. The exact loading will have to be determined for each application. ***Note:*** *The loading recommended, will give clearly visible bands after a 15 second exposure. If the bands take longer to appear, the probable cause is inefficient transfer to membrane. This is most likely to be a problem with large gels.* *Transfer must be overnight for tank blotting, and greater than 1 hour for semi-dry blotting. There should be good contact between the gel and the membrane during transfer. For tank blots the use of extra Scotch-brite pads and additional securing of the transfer cassettes, with rubber bands, will improve transfer.* ***Note:*** *Conversely, if the bands produced are too intense or a longer exposure would be more convenient, it is suggested that a higher dilution of markers is used.*

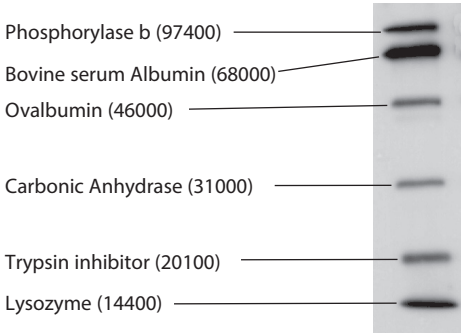


Fig 6. Profile of ECL protein molecular weight markers.

1 µg sample ECL protein molecular weight markers diluted with 9 µL of loading buffer and run on a 12% Polyacrylamide gel for 1 hour at 150 volts, followed by electroblotting on to Hybond ECL overnight at 30 volts. Processing of the blot was outlined in the ECL Western blotting protocol, using Streptavidin-HRP (RPN1231,1:1500 dilution) and ECL Western blotting detection reagents. The light emission was captured using Hyperfilm ECL for a 15 second exposure.

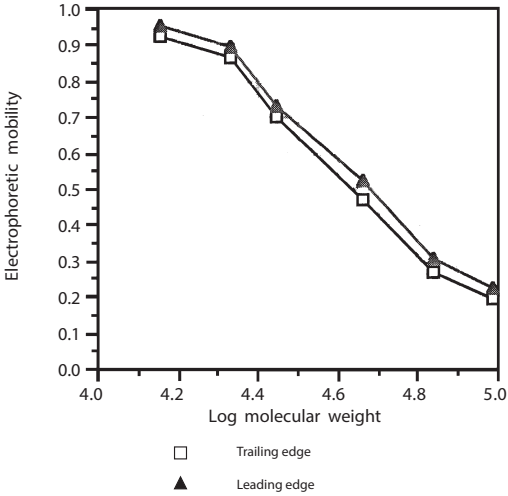


Fig 7. ECL protein molecular weight markers calibration line.

7 Troubleshooting guide

Problem: No signal

Possible causes and solutions

- Check that transfer equipment is working properly and that the correct procedure has been followed.
- Check protein transfer by staining the gel and/or membrane.

- Some antigens may be affected by the treatments required for electrophoresis.
- Target protein degradation may occur if the blots are stored incorrectly.
- ECL detection reagents may have become contaminated.
- Incorrect storage of the ECL detection reagents may cause a loss of signal.

Problem: Weak signal

Possible causes and solutions

- Transfer efficiency may have been poor.
- Insufficient protein was loaded on to the gel.
- The concentration of primary and secondary antibodies could be too low; optimization is required.
- Film exposure time may have been too short.

Problem: Excessive diffuse signal

Possible causes and solutions

- Too much protein was loaded onto the gel.
- Electrophoresis and transfer protocols may need optimization.
- The concentrations of primary and secondary antibodies could be too high; optimization is required.

Problem: White (negative) bands on the film

Possible causes and solutions

Negative bands generally occur when protein target is in excess and antibody concentrations are too high. The effect is caused by substrate depletion. To rectify this either, reduce the amount of target loaded, use lower antibody concentrations or a combination of both.

Problem: Uneven, spotted backgrounds

Possible causes and solutions

- Blotting technique requires optimization.
- Areas of the blot may have dried during some of the incubations.
- Incorrect handling can lead to contamination on the blots and/or membrane damage, which may cause non-specific signal.

Problem: High backgrounds

Possible causes and solutions

- The concentrations of primary and secondary antibodies could be too high; optimization is required.
- Contamination can be transferred to the blots from electrophoresis and related equipment used in blot preparation.
- Transfer and incubation buffers may have become contaminated and require replacing.
- The blocking agent used was not freshly prepared, was too dilute or was incompatible with the application.
- The level of Tween used in the blocking agent was not sufficient for the application performed.
- The membrane was allowed to dry during some of the incubations.

- The type of membrane used was not compatible with nonradioactive systems.
- The post antibody washes were not performed for a sufficient period of time or were not performed in a high enough volume.
- There was insufficient Tween in the post antibody washes.
- Insufficient changes of post antibody washes were used.
- The film detection of the signal was allowed to over expose.
- The level of signal is so high that the film has become completely overloaded.

8 Quality control

Every batch of ECL detection reagents is functionally tested in a Western blotting application to ensure minimal batch to batch variability.

9 References

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10 Related products

SDS-PAGE electrophoresis chemicals See the complete range in the catalogue	
Low-Range Rainbow MW Markers, natural, 45, 30, 20.1, 14.3, 6.5, 3.5 and 2.5 kDa	RPN755
High-Range Rainbow MW Markers, natural, 220, 97, 66, 45, 30, 20.1 and 14.3 kDa	RPN756
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Hybond-P Membrane (PVDF, pore size 0.45 µm) 20 cm x 3 m, 1 roll	RPN203F
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Rat IgG, Horseradish Peroxidase-linked Whole Antibody (from Goat), 1 mL	NA935

SDS-PAGE electrophoresis chemicals See the complete range in the catalogue	
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Rabbit IgG, Horseradish Peroxidase-linked F(ab´)$_2$ Fragment (from Donkey), 1 mL	NA9340-1ML
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Human IgG, Biotinylated Whole Antibody (from Sheep), 2 mL	RPN1003
Rabbit IgG, Biotinylated Whole Antibody (from Donkey), 2 mL	RPN1004
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ECL Plus Western blotting detection reagents (sufficient for 3000 cm$_2$ membrane)	RPN2133

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Anti-rabbit IgG, HRP-linked whole antibody (from donkey), 100 µL	
Blocking reagent, 5 g	
Sufficient for 10 blots, 10 x 10 cm	
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ECL Mini-camera	RPN2069
Imagemaster VDS-CL, CCD Camera	18113055

11 Cue cards

STAGE	1	2	3	4	5	6
	Electrophoresis and blotting	**Block**	**Wash**	**Primary antibody**	**Wash**	**Biotinylated antibody or HRP labelled antibody**
REAGENT		5% blocking reagent in TBS-T or PBS-T	TBS-T or PBS-T	Diluted in TBS-T or PBS-T	TBS-T or PBS-T	Diluted in TBS-T or PBS-T
VOLUME USED		10 ml	10 ml	10 ml	10 ml	10 ml
TIME	Usual electrophoresis and blotting times	1 hour	1 × 15 min 2 × 15 min	1 hour	1 × 15 min 2 × 5 min	20 min–1 hour

STAGE	7	8	9	10	11
	Wash - if using HRP labelled antibody omit steps 7 and 8	**Streptavidin -HRP**	**Wash**	**Detection**	**Exposure**
REAGENT	TBS-T or PBS-T	Diluted in TBS-T or PBS-T	TBS-T or PBS-T	Mix the two agents 1:1	Drain the reagent cover with Saran Wrap™ immediately
VOLUME USED	10 ml	10 ml	10 ml	0.125 ml/cm²	
TIME	1 × 15 min 2 × 5 min	20 min–1 hour	1 × 15 min 4 × 5 min	1 min	expose to film for 30 seconds–10 min

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