GE Healthcare

Amersham ECL Western blotting detection reagents and analysis system

Product Booklet

Codes: RPN2106/8/9
RPN2209
RPN2134

Page finder

1. Legal

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http://www.gelifesciences.com

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2. Handling

2.1. Safety warnings and precautions

Warning: ***For research use only.*** Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

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. Please follow the manufacturer's safety data sheet relating to the safe handling and use of this material.

2.2. Storage

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

2.3. Expiry

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

3. 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 × 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 × 10 cm

For the detection of either mouse or rabbit membrane bound primary antibodies.

3.1. Other materials required

Equipment
Electrophoresis and blotting apparatus (for Western blots)

Blotting membrane, recommend Hybond™ ECL (nitrocellulose) from GE Healthcare

Orbital shaker

Forceps with rounded, non-serrated tips

X-ray film cassettes, recommend Hypercassette™ from GE Healthcare

Timer

Film, recommend Hyperfilm™ ECL, film developing facility and reagents from GE Healthcare

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 GE Healthcare 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 should 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:
GE Healthcare 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 page 24. For details of the recommended ECL HRP antibodies see page 39.

Biotinylated antibody
It is recommended that the antibody dilution should be optimized to suit different blotting situations. See page 23. The full range of biotinylated antibodies can be found in the current GE Healthcare 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.

4. Description

4.1. Principles of ECL Western Blotting

ECL Western blotting from GE Healthcare is a light emitting non-radioactive 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 × 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, two-dimensional 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.

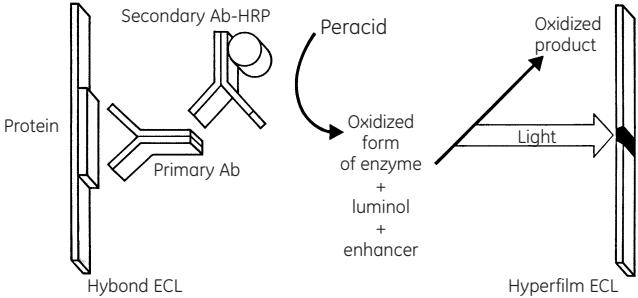


Figure 1. Principles of ECL Western blotting

4.2. 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.

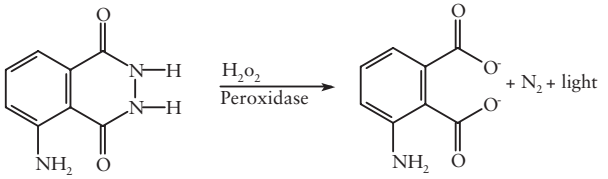


Figure 2.

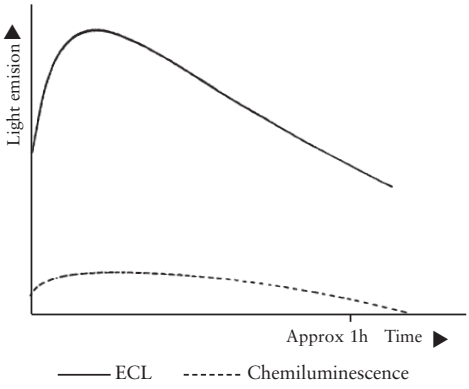


Figure 3. Graph of light emission versus time, showing the difference between chemiluminescence and ECL.

5. Critical parameters

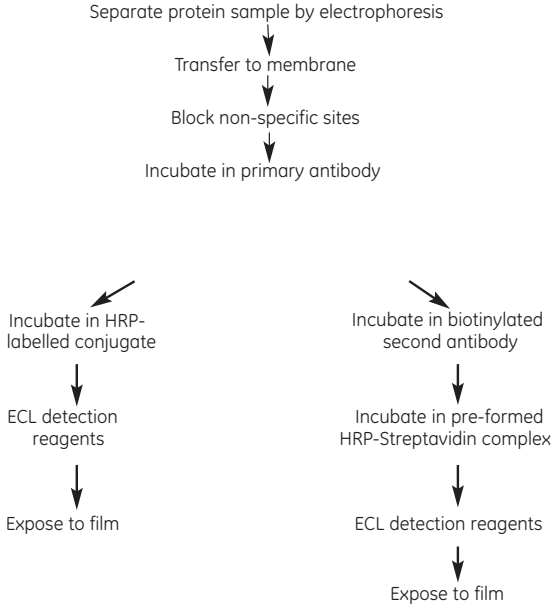
The following points are critical:

- 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 page 23 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.

6. Protocols

6.1. Flow diagram



6.2. 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 on page 21 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.

6.3. Electrophoresis and blotting

Protocol	Notes
1. Perform electrophoresis and blotting according to normal techniques. Protein should 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.	**1.** Hybond ECL should be pre-wetted in distilled water and equilibrated in transfer buffer for at least 10 minutes before blotting. **2.** Hybond-P PVDF should 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. **3.** ECL is also suitable for use with supported nitrocellulose such as Hybond-C Extra. This membrane should be prepared as for Hybond ECL.

6.4. Blocking the membrane

Protocol	Notes
1. Block non-specific binding sites by immersing the membrane in 5% non-fat dried milk, 0.1% (v/v) Tween 20 in PBS	**1.** The combination of non-fat dried milk and Tween should be suitable for most applications. Optimum Tween

Protocol	Notes
1. *Continued.* or TBS (PBS-T or TBS-T, see page 6) 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.	**1.** *Continued.* 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 (see page 6).	**2.** While washing prepare the diluted primary antibody (section 6.5., step 1)

6.5. Primary antibody incubation

Protocol	Notes
1. Dilute the primary antibody. in PBS-T or TBS-T. The dilution factor should be determined empirically for each antibody.	**1.** Optimization of the antibody dilution can be performed by dot blot analysis (see page 23).
2. Incubate the membrane in diluted primary antibody for 1 hour at room temperature on an orbital shaker.	**2.** Incubation times and temperatures may vary and should 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.	

Protocol	Notes
4. Wash the membrane for 3 × 5 minutes with fresh changes of wash buffer at room temperature.	**4.** While washing prepare the diluted secondary antibody (section 6.6., step 1).

6.6. Secondary antibody incubation

Protocol	Notes
1. Dilute the HRP labelled secondary antibody or biotinylated antibody in PBS-T or TBS-T. The dilution factor should be determined empirically for each antibody (see page 23).	**1.** 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.	**2.** Incubation times and temperatures may vary and should 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 × 5 minutes with fresh changes of wash buffer at room temperature.	**4.** If using HRP-labelled secondary antibody proceed directly to step 8 (detection) after this wash procedure.

Protocol	Notes
	4. *Continued.* If using a biotinylated antibody, while washing, prepare the diluted Streptavidin HRP conjugate or complex (section 6.7., step 1).

6.7. Streptavidin bridge incubation

Protocol	Notes
1. Dilute the streptavidin HRP conjugate or streptavidin-biotinylated HRP complex in PBS-T or TBS-T.	**1.** The dilution factor should be determined empirically (see pages 23–24).
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 × 5 minutes with fresh changes of wash buffer at room temperature.	

6.8. Detection

Protocol	Notes
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.	**1.** 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.	**2.** The reagents should 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.	
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.	**4.** Close the SaranWrap around the membrane to form an envelope or use an alternative, suitable detection pocket. Avoid using pressure on the membrane.

Protocol	Notes
5. Place the wrapped blots, protein side up, in an X-ray film cassette.	**5.** 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.	**6.** This stage should be carried out in a dark room, using red safelights. Do not move the film while it is being exposed.
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.	**7.** The detected blots can also be exposed to Polaroid™ film using the ECL mini-camera (RPN2069), which is specifically designed for blots generated from mini-gel apparatus. The ECL mini-camera is suitable for blots up to 52 × 77 mm. Images can also be aquired using a CCD camera such as Imagemaster™ VDS-CL (18-1130-55).

7. Additional information

7.1. 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.

Protocol	Notes
1. Wash the membrane for 2 × 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.	**2.** Refer to note in section 6.4., step 1 on page 14.
3. Repeat the immunodetection protocol, stages 6.5. to 6.8.	

7.2. 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 should be stored wet wrapped in SaranWrap in a refrigerator (2–8°C) after each immunodetection.

Protocol	Notes
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.	**1.** 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 × 10 minutes in PBS-T or TBS-T at room temperature using large volumes of wash buffer.	**2.** 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% Non-fat dried milk in PBS-T or TBS-T for 1 hour at room temperature.	
4. Repeat the immunodetection protocol, stages 6.5. to 6.8.	

7.3. 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.

Protocol	Notes
1. Block the membrane in 10% non-fat dried milk in PBS-T or TBS-T for 10 minutes at room temperature.	**1.** This protocol has been optimized using 10% non-fat dried milk. Other blocking

Protocol	Notes
	1. *Continued.* 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. (see page 6).	**2.** While washing prepare the diluted primary antibody (step 3).
3. Dilute the primary antibody in PBS-T or TBS-T. The dilution factor should be determined empirically for each antibody.	**3.** Optimization of the antibody dilution can be performed by dot blot analysis, (see page 23).
4. Incubate the membrane in diluted primary antibody for 1 hour at room temperature on an orbital shaker.	**4.** 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.
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.	**5.** While washing, dilute the secondary antibody. In order to maintain the same sensitivity as obtained with the standard method, the secondary antibody should be used at a stronger concentration. As a

Protocol	Notes
	5. *Continued.* guideline, increasing the concentration by four times should 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 on page 18.	

7.4. 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 should be noted that some antibodies may require alternative blocking and washing steps to the ones suggested below.

1.1. 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.

1.2. Incubate in blocking solution for 1 hour at room temperature with agitation.

1.3. Rinse the membranes briefly with two changes of wash buffer.

1.4. 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.

1.5. Rinse blots in two changes of wash buffer, then wash for 1 × 15 minutes and 3 × 5 minutes in fresh changes of wash buffer.

1.6. Dilute the secondary antibody (using only one concentration) and incubate the membranes for 1 hour at room temperature with agitation.

1.7. Wash as detailed in step 1.5.

1.8. Detect using ECL detection reagents as detailed in section 6.8. of the protocol. The antibody dilution which gives the best signal with the minimum background should be selected.

Secondary antibodies

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

2.1. Prepare dot blots and block the membranes as detailed in 1.1. and 1.2.

2.2. Incubate in diluted primary antibody (using only one concentration) for 1 hour at room temperature with agitation.

2.3. Wash as detailed in step 1.5.

2.4. 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.

2.5. Wash as detailed in step 1.5.

2.6. Detect using ECL detection reagents as detailed in step 6.8. of the protocol. The antibody dilution which gives the best signal with minimum background should be selected.

7.5. 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.

1. 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 pages 26–27). 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.

2. 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 should be in the region of 1 msec.

3. 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 should be in the linear range of the film.
4. 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.

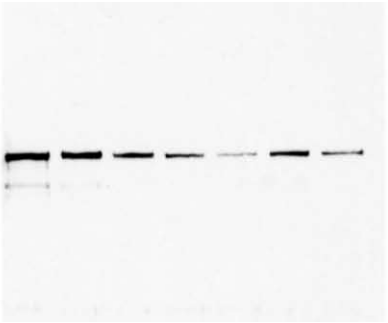


Figure 4. ECL detection of myosin standard curve and myosin test samples.

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

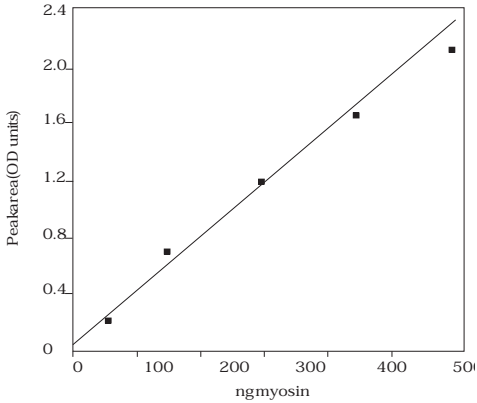


Figure 5. Peak area (OD units) against myosin concentration

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

7.6. 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.

Protocol	Notes
1. Remove 1 µl of markers and add to 9 µl of gel loading buffer (containing 5% 2-β-Mercaptoethanol).	**1.** 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.	**2.** Do not subject the markers to more than one denaturation.
3. Load 10 µl per well.	**3.** 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.
4. Following electrophoresis and transfer to nitrocellulose membranes, membranes are processed by standard	**4.1.** It is strongly advised that milk should not be included in the Streptavidin-HRP incubation. The binding of

Protocol	Notes
4. *Continued.* 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.	**4.1.** *Continued.* 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. **4.2.** 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 on page 18.	
6. The volume of markers required to give optimum results will depend on the electroblotting and immunodetection conditions	**6.1.** The loading recommended, will give clearly visible bands after a 15 second exposure. If the bands take longer to appear,

Protocol	Notes
6. *Continued.* used and the length of exposure to film required. The exact loading will have to be determined for each application.	**6.1.** *Continued.* the probable cause is inefficient transfer to membrane. This is most likely to be a problem with large gels. Transfer should 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. **6.2.** 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.

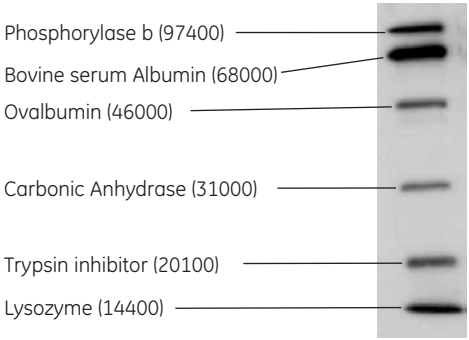


Figure 6. Profile of ECL protein molecular weight markers.

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.

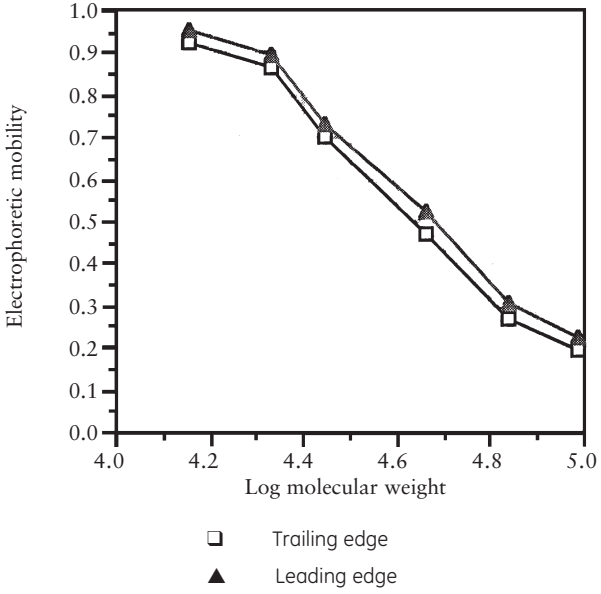


Figure 7. ECL protein molecular weight markers calibration line.

8. Troubleshooting guide

Problem: No signal
Possible causes and solutions

1. Check that transfer equipment is working properly and that the correct procedure has been followed.

2. Check protein transfer by staining the gel and/or membrane.

3. Some antigens may be affected by the treatments required for electrophoresis.

4. Target protein degradation may occur if the blots are stored incorrectly.

5. ECL detection reagents may have become contaminated.

6. Incorrect storage of the ECL detection reagents may cause a loss of signal.

Problem: Weak signal
Possible causes and solutions

1. Transfer efficiency may have been poor.

2. Insufficient protein was loaded on to the gel.

3. The concentration of primary and secondary antibodies could be too low; optimization is required.

4. Film exposure time may have been too short.

Problem: Excessive diffuse signal
Possible causes and solutions

1. Too much protein was loaded onto the gel.

2. Electrophoresis and transfer protocols may need optimization.

3. 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

1. 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

1. Blotting technique requires optimization.

2. Areas of the blot may have dried during some of the incubations.

3. 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

1. The concentrations of primary and secondary antibodies could be too high; optimization is required.

2. Contamination can be transferred to the blots from electrophoresis and related equipment used in blot preparation.

3. Transfer and incubation buffers may have become contaminated and require replacing.

4. The blocking agent used was not freshly prepared, was too dilute or was incompatible with the application.

5. The level of Tween used in the blocking agent was not sufficient for the application performed.

6. The membrane was allowed to dry during some of the incubations.

Problem: High backgrounds *Continued.*
Possible causes and solutions

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

9. Quality control

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

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Amersham
Western blotting protocol summary
Product protocol card

RPN2106/8/9, RPN2209, RPN2134

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

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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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