GE Healthcare

Amersham ECL Protein Biotinylation Module

Sufficient for 5 x 2.5 mg solution labellings or 25 8 x 10 cm membrane labellings

Product Booklet

Codes: RPN2202
RPN2203

Page finder

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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.

2.2. Storage

Store at 2–8°C.

2.3. Stability

The kit components are stable for at least 3 months when stored under the recommended conditions.

3. Components of the system

ECL protein biotinylation module	RPN2202
Components	
Biotinylation reagent Biotinamidocaproate N-hydroxysuccinamide ester supplied ready to use in dimethylformamide.	2 x 0.5 ml
20 x sodium bicarbonate buffer, pH 8.6 Bicarbonate buffer which when diluted in distilled water gives a 40 mM solution. 20 x stock contains 0.1% Kathon™ CG as a preservative.	75 ml
Sephadex G-25 columns The disposable columns are supplied prepacked with Sephadex G-25 for the purification of samples up to 2.5 ml. Each column contains 9.1 ml of swollen gel in distilled water and 0.15% Kathon CG. These columns are suitable for the purification of proteins larger than 5 kDa.	5
Streptavidin-horseradish peroxidase conjugate 1500 x stock of streptavidin conjugated horseradish peroxidase.	0.5 ml
Membrane blocking reagent	40 g
ECL protein biotinylation system	RPN 2203
Components	
ECL protein biotinylation module	RPN 2202
ECL Western blotting detection reagents for 2000 cm^2 of membrane	RPN 2209
Reagent 1	125 ml
Reagent 2	125 ml

4. Description

The ECL protein biotinylation system combines the efficient labelling of proteins using biotin with the sensitivity of detection with streptavidin-horseradish peroxidase and enhanced chemiluminescence (1,2). The principle of the system is outlined in figure 1.

The ECL protein biotinylation system has a wide range of applications. Antibodies or other proteins labelled in solution with biotin can be used in a variety of applications such as immunodetection on Western blots, immunoprecipitation (3,4) and ligand(5) blotting. It is also possible to label cell surface proteins (3,4,6,7) with biotin prior to immunoprecipitation analysis, providing a sensitive alternative to ^{125}I lactoperoxidase labelling. Membrane biotinylation protocols can be used for total protein detection and give a sensitivity comparable to silver staining.

Each batch of the system is tested by our quality control group to ensure the detection of at least 20 pg of transferrin by solution biotinylation on Hybond™ ECL.

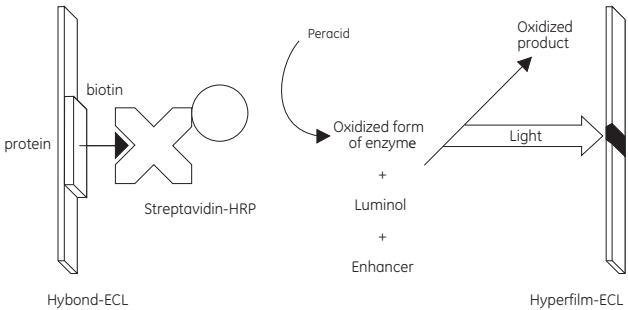

Figure 1. The principle of detection in the ECL protein biotinylation system.

5. Critical parameters

- Moisture will affect the performance of the biotinylation reagent. Always allow to equilibrate to room temperature before use. Do not leave open to the atmosphere.
- If the protein to be labelled is dissolved in amine containing buffers, for example Tris/HCl, these must be dialysed against phosphate buffered saline or bicarbonate buffer before use.
- Membrane block reagent should not be included in the streptavidin-HRP incubation. The binding of streptavidin to biotin is inhibited by the presence of endogenous biotin in milk (9), resulting in a decreased signal when detected by enhanced chemiluminescence.
- It is important that all electrophoresis and electroblotting equipment is scrupulously clean when performing biotinylation of proteins immobilised on the membrane, as all proteins present will be labelled.
- If membrane labelling is being performed on PVDF membrane it is important to use the reagent concentrations specified.
- For the cell surface labelling it is important that healthy, actively growing cells are used.
- With ECL detection there is no lag phase in the output of light. To achieve maximum sensitivity the blot should be exposed to film as soon as possible after the incubation in detection reagents.

6. Additional equipment and reagents required

Equipment

- Electrophoresis and blotting equipment for Western blots
- Blotting membrane (Hybond ECL is recommended, see related products
- Orbital shaker
- Roller mixer
- Forceps with rounded, non-serrated ips
- X-ray film cassettes (Hypercassette™ are recommended, see related products
- Timer
- Film (Hyperfilm ECL is recommended, see related products)
- Film developing facility and reagents
- SaranWrap™
- Adjustable pipettes

Reagents

- Phosphase buffered saline (PBS), pH7.5
- Phosphase buffered saline (PBS) containing 1% bovine serum albumin (BSA)
- Phosphate buffered saline (PBS) containing 0.1%(v/v) Tween™20 (PBS-T)
- Cell lysis buffer
- Protein A or G beads
- Distilled water
- Electrophoresis and blotting solutions.

7. Use of the ECL protein Biotin system

7.1. Flow diagram

Membrane biotinylation

Separate protein sample by electrophoresis

⇓

Transfer to membrane

⇓

Biotinylate protein

⇓

Block non-specific sites

⇓

Incubate with streptavidin-HRP conjugate

⇓

Detect with ECL reagents

⇓

Expose to film

Solution

Biotinylate protein sample in solution

⇓

Purify on G-25 Sephadex column

⇓

Use biotinylated probe in a range of applications

⇓

Incubate with streptavidin-HRP conjugate

⇓

Detect with ECL reagents

⇓

Expose to film

⇓

7.2. Preparation of reagents

Biotinylation reagent

This is supplied ready for use in dry dimethylformamide.

Caution: Moisture will affect the performance of this reagent. Always allow to equilibrate to room temperature prior to opening.

Bicarbonate buffer, pH 8.6

The sodium bicarbonate buffer (BB) is supplied at a concentration of 0.8 M. The concentrated buffer should be diluted 1 in 20 in distilled water for use as 40 mM solution.

This reagent should be freshly diluted for each use.

Prepare 5 ml for each 2.5 mg solution labelling or for cell surface labelling, and 60 ml for each 8 x 10 cm membrane labelling.

Sephadex G-25 columns

Before use, Sephadex G-25 columns should be equilibrated with 5 ml of PBS containing 1% BSA to block any protein binding sites, followed by 20 ml of PBS. Columns should be equilibrated just before use and should never be allowed to run dry.

Membrane blocking solution

Dissolve 1 g of blocking reagent per 20 ml of PBS containing 0.1%(v/v) Tween 20 (PBS-T). Prepare freshly on day of use.

Streptavidin-HRP conjugate

Add 12.5 µl per 20 ml of PBS-T. Prepare freshly just before use.

ECL detection reagents

Mix equal volumes of detection solution 1 with detection solution 2 to give sufficient reagent to cover the membrane (0.125 ml/cm^2 membrane). Prepare freshly just before use.

7.3. Protocol A: Biotinylation of antibodies and proteins in solution

The standard protocol is designed for labelling 2.5 mg amounts of protein. If labelling is to be performed on a significantly smaller scale, alternative purification columns will have to be used (see note 6).

Protocol	Notes
1. Determine the concentration of protein or antibody to be biotinylated	**1.** Methods such as UV absorbance (8) may be used.
2. Prepare a 40 mM working concentration of bicarbonate buffer (BB) by diluting 1 in 20 in distilled water (see preparation of reagents).	
3. Place the biotinylation reagent at room temperature, and ensure the vial has equilibrated to room temperature prior to opening.	**3.** Caution: moisture will affect the performance of the biotinylation reagent. Always allow to equilibrate to room temperature after use. Do not leave open to the atmosphere.
4. Dilute the protein to 1 mg/ml in the diluted bicarbonate buffer. The maximum volume suitable for loading on to the column is 2.5 ml, the minimum volume 2.0 ml. Add 40 µl of biotinylation reagent for each mg of protein.	**4a.** If the protein is dissolved in amine containing buffers, for example Tris/HCl, these must be dialysed against phosphate buffered saline or bicarbonate buffer before use. **4b.** If desired, labelling can be performed in PBS rather

Protocol	Notes
4. *Continued.* Incubate at room temperature for 1 hour with constant agitation.	**4b.** *Continued.* than bicarbonate buffer. However, labelling efficiency may be reduced by up to 10 fold.
5. Discard the buffer at the top of the Sephadex G25 column, and cut 1–2 mm off the tip seal. Equilibrate the column with 5 ml of PBS containing 1.0% bovine serum albumin (BSA) pH7.5 followed by 20 ml of PBS. Discard the column washings. Do not allow the column to run dry.	**5.** If the column is not to be used immediately following equilibration, apply the plastic stopper supplied to the tip of the column until required.
6. Allow the buffer level in the top of the column to fall to the level of the plastic sinter at the top of the gel bed. Apply the protein sample (in 2.0–2.5 ml) to the column. Allow the sample volume to enter the column before eluting with PSB and collecting fractions.	**6.** A minimum volume of 2 ml should be loaded on to the column. If a smaller volume biotinylation has been performed, either the sample volume should be made up to 2.0 ml with 40 mM bicarbonate buffer following biotinylation, or a smaller scale purification should be performed using, for example, a GE Healthcare NAP-5 column, or spin column (see additional information).

Protocol	Notes
7. Elute the sample in 5 ml of PBS. Collect fractions.	**7a.** A suitable fraction size is 1.0 ml. Smaller fraction sizes can be collected for higher resolution. Protein will not elute in the first 1 ml and should elute between 1.0 ml and 3.0 ml. **7b.** UV absorbance at 280 nm will verify the fraction number containing the eluted protein. The biotinylation reagent also gives an absorbance reading at 280 nm, this will not interfere with identification of fractions containing the biotinylated protein, but may interfere with accurate determination of protein concentration. Routinely a recovery of 90–95% is expected from Sephadex G-25 columns.
8. The biotinylated protein is now ready for use. Storage conditions are dependent on the characteristics of individual proteins. However, we recommend that biotinylated antibodies are stored 2–8°C in the presence	

Protocol	Notes
8. *Continued.* of 0.2% sodium azide. Biotinylated horseradish peroxidase should be stored in the presence of 0.01% hyamine and not sodium azide.	

7.4. Protocol B: Use of solution labelled biotinylated proteins in immunodetection

During immunodetection, sufficient solution should be used to adequately cover the membrane, and containers should be agitated gently on an orbital shaker. 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 before incubating in wash buffer will improve washing efficiency. All steps should be carried out at room temperature.

Protocol	Notes
1. Perform electrophoresis and Western blotting, or prepare dot/slot blots.	**1.** This protocol is optimised for use with Hybond ECL (nitrocellulose) membrane. Membranes may be used immediately or air dried and stored in a desiccator at 2–8°C for several weeks. Dot/slot blots must be dry before use.
2. Blocking the membrane Block the membrane by immersing in 5% blocking	**2a.** Use a fresh solution of blocking reagent each time.

Protocol	Notes
2. *Continued.* reagent in PBS-T (see reagent preparation) for 1 hour.	**2b.** Alternatively, membranes may be left in blocking solution overnight at 2–8°C if more convenient. **2c.** The recommended combination of Tween and blocking reagent should be suitable for most protein blotting work.
3. Rinsing Discard the blocking solution and rinse the membrane twice in PBS-T.	**3.** Dilute the primary antibody. Dilution of the primary antibody required to give optimum results will vary and should be determined for each antibody
4. Incubation Incubate the membrane in primary antibody for 1 hour.	**4.** Incubation times and temperatures will vary, and should be optimised for each antibody.
5. Washing Rinse the membrane twice in PBS-T, then wash once for 15 minutes, and twice for 5 minutes with fresh changes of PBS-T.	
6. Incubation During the washing step dilute the streptavidin-HRP (1 in 1500 in PBS-T) if a biotinylated primary antibody has been used, or	**6.** It is strongly advised that blocking reagent should not be included in the streptavidin-HRP incubation. The binding of streptavidin to biotin is inhibited in the

Protocol	Notes
6. *Continued.* the biotinylated secondary antibody has been used. Incubate for 1 hour. If a biotinylated secondary antibody has been used proceed to step 8.	**6.** *Continued.* presence of endogenous biotin in milk, resulting in a much decreased signal when detecting with enhanced chemiluminescence (9).
7. Washing If a biotinylated primary antibody has been used, rinse twice in PBS-T, then wash 3 times for 15 minutes with fresh changes of PBS-T. Proceed to ECL detection (page 29).	
8. Washing If a biotinylated secondary antibody has been used rinse the membrane twice in PBS-T, then wash once for 15 minutes and twice for 5 minutes with fresh changes of PBS-T.	
9. Incubation Dilute the streptavidin-HRP as in step 6. Incubate for 1 hour.	**9.** See note 6.
10. Rinse and wash as in step 7.	
11. Proceed to ECL detection (page 29).	

7.5. Protocol C: Biotinylation of membrane bound proteins

During biotinylation and immunodetection, sufficient solution should be used to adequately cover the membrane and containers should be agitated gently on an orbital shaker. 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 before incubating in wash buffer will improve washing efficiency. All steps should be carried out at room temperature.

Protocol	Notes
1. Perform electrophoresis and Western blotting, or prepare dot/slots blots.	**1a.** It is particularly important to ensure that gel apparatus and Western blotting pads etc. are clean, as with membrane biotinylation all proteins on the membrane will be detected.
	1b. This protocol is optimised for use with Hybond ECL (nitrocellulose), if PVDF membranes are to be used different concentrations of reagents are required. Guidelines for concentrations of reagents required for use with PVDF are given in the appropriate notes sections. Membranes may be used immediately or air dried and stored in

Protocol	Notes
	1b. *Continued.* a desiccator at 2–8°C for several weeks.
	1c. Dot blots must be dry before biotinylation is commenced.
	1d. If GE Healthcare rainbow molecular weight markers (RPN 755, RPN 756) are on the membrane, it is recommended that this track is removed prior to biotinylation, as following biotinylation the high concentration of protein present in the markers produces an excessive signal when detected with ECL reagents, which may obscure other samples on the membrane.
2. Prepare a 40 nM working concentration of bicarbonate buffer (BB) by diluting 1 in 20 in distilled water (see reagent preparation). Wash the membrane once in diluted BB for 10 minutes.	**2.** There is sufficient BB to use 30 ml for washing each 8 x 10 cm membrane.
3. Place the biotinylation reagent at room temperature, and ensure the	**3.** Caution: moisture will affect the performance of the biotinylation reagent. Always allow to equilibrate

Protocol	Notes
3. *Continued.* vial has equilibrated to room temperature prior to opening.	**3.** *Continued.* to room temperature before use. Do not leave open to the atmosphere. Sufficient BB and biotinylation reagent are provided for up to 30 ml of diluted biotinylation reagent to be used, for each 8 x 10 cm membrane.
4. Prepare the reagent for biotinylation by diluting 5 µl of the biotinylation reagent into each 10 ml of BB.	**4.** For PVDF membranes dilute an aliquot of the biotinylation reagent 1 in 200 in dimethylformamide. Use the freshly diluted biotinylation reagent, diluting 2 µl into each 10 ml of BB.
5. Biotinylation Discard the buffer and incubate the membrane in the diluted biotinylation reagent for 15 minutes.	
6. Washing Discard the biotinylation reagent. Rinse the membrane twice with PBS-T. Wash the membrane for 3 x 5 minutes with fresh changes of PBS-T.	
7. Blocking Block the membrane by immersing in 5% blocking	**7a.** Use a fresh solution of blocking reagent each time. **7b.** Alternatively, membranes may be left in blocking

Protocol	Notes
7. *Continued.* reagent (see reagent preparation) in PBS-T, for 1 hour.	**7b.** *Continued.* solution overnight at 2–8°C if more convenient. **7c.** It is strongly advised that block should not be included in the streptavidin-HRP incubation. The binding of streptavidin to biotin is inhibited in the presence of endogenous biotin in milk, resulting in a much decreased signal when detected with enhanced chemiluminescence.
8. Rinsing During the blocking stage dilute the streptavidin-HRP 1 in 1500 in PBS-T. Discard the blocking solution and rinse the membrane twice in PBS-T.	**8.** For PVDF membranes dilute the streptavidin-HRP 1 in 10 000 to 1 in 20 000 in PBS-T.
9. Incubation Add the diluted conjugate to the membrane and incubate for 1 hour.	
10. Washing Discard the diluted conjugate and rinse twice in PBS-T. Wash 3 times for 15 minutes using fresh changes of PBS-T.	
11. Proceed to ECL detection (page 29)	

7.6. Protocol D: Detection of cell surface proteins using biotinylation and immunoprecipitation

Only healthy, actively growing cells should be used for labelling. Cells that are over confluent should not be used. Only guidelines can be given for cell lysis and immunoprecipitation as the optimal conditions will depend on the protein being analysed. Cells can be biotinylated as monolayers in tissue culture flasks or in suspension. For low abundance antigens, cell labelling in suspension is recommended, as the cells can be concentrated into a smaller volume. During labelling, cell lysis and immunoprecipitation, all solutions should be pre-cooled on ice and equipment to be used should be placed in the cold room.

Protocol	Notes
Labelling of cells as monolayers	
1. Prepare a 40 mM working concentration of bicarbonate buffer (BB) by diluting 1 in 20 in distilled water. Allow to chill on ice for 5 minutes.	**1.** If desired, cell surface labelling can be performed using PBS, however, labelling efficiency may be reduced by up to 10 fold.
2. Remove media from the flask and rinse cells twice with ice cold PBS.	
3. Add 3–5 ml of BB (depending on cell density) per 75 cm^2 flask.	**3.** If the antigen is not present on the cells at a very high concentration it may be better to label the cells in suspension as the resulting

Protocol	Notes
	3. *Continued.* lysate will be more concentrated.
4. Add 40 µl of biotinylation reagent (pre-warmed to room temperature prior to opening) per ml of BB and mix gently.	**4.** Caution: moisture will affect the performance of the biotinylation reagent. Always allow to equilibrate to room temperature before use. Do not leave open to the atmosphere.
5. Incubate the flask on an orbital shaker, for 30 minutes at 2–8°C, ensuring the cells remain covered in buffer during this time. During the incubation prepare the cell lysis buffer and place on ice to cool.	**5.** The best cell lysis buffer to use will depend on the protein being studied. Conditions used should be as gentle as possible to retain the antibody binding sites but harsh enough to ensure quantitative release of the antigen. A suggested lysis buffer is: 250 mM Nacl 25 mM Tris-HCl, pH7.5 5 mM EDTA, pH8.0 0.1–1%(v/v) NP-40 2 µg/ml aprotinin 100 µg/ml PMSF
6. Remove the biotinylation buffer and wash the cell monolayer twice with ice-cold PBS.	

Protocol	Notes
7. Add 3–5 ml of ice-cold lysis buffer and incubate flash on an orbital shaker, for 20 minutes at 2–8°C.	
8. Remove lysate and transfer to microcentrifuge tubes. Spin in a microcentrifuge at 13 000 rpm for 10 minutes at 4°C.	**8.** If large amounts of lysate are being analysed samples can be spun in a high speed centrifuge at 12 000 x g for 20 minutes.
9. Remove supernatants to fresh microcentrifuge tubes and store on ice prior to immunoprecipitation.	**9.** Supernatants can be stored at -70°C if necessary, although the freeze-thawing may cause some protein degradation.
Labelling of cells in suspension	
1. Prepare a 40 mM working concentration of bicarbonate buffer (BB) by diluting 1 in 20 in distilled water. Allow to chill on ice for 15 minutes.	**1.** Cell surface labelling can be performed using PBS if desired, however, labelling efficiency may be reduced by up to 10 fold.
2. Trypsinize the cells, if necessary, to produce a single cell suspension. Dilute to 25 ml with ice-cold PBS and spin at 400–800 xg in a bench top centrifuge for 4 minutes at 4°C. Wash the cell pellet in this way twice more.	

Protocol	Notes
3. After the final wash resuspend the cells in ice-cold BB at a concentration of approximately $5x10^6$/ml and transfer to microcentrifuge tubes.	**3.** Cells can be resuspended at concentrations of up to 1×10^7/ml but the volume of biotinylation reagent used should not be increased above 40 µl/ml.
4. Add 40 µl of biotinylation reagent (prewarmed to room temperature prior to opening) per ml of cells. Incubate the microcentrifuge tubes on a roller mixer for 30 minutes at 2–8°C.	**4.** Labelling times should not be extended as there is the risk of cell damage.
5. Spin the cells in a microcentrifuge at 2000 rpm for 2 minutes at 4°C. Remove the supernatant and wash the cells twice by resuspending in PBC and spinning.	
6. To the washed cell pellet, add 1 ml of ice-cold lysis buffer, mix well and incubate on a roller mixer for 20 minutes at 2–8°C.	**6.** See note 5 on page 23 on recommended lysis buffer. Smaller volumes of lysis buffer can be added for low abundance antigens.
7. Spin the lysate in a microcentrifuge at 13 000 rpm for 10 minutes at 4°C.	

Protocol	Notes
8. Remove supernatant to a fresh tube and store on ice prior to immunoprecipitation.	**8.** Supernatants can be stored at -70°C if necessary, although the freeze-thawing may cause some protein degradation.
Immunoprecipitation	
1. Prepare an appropriate amount of immobilised protein A or G beads by washing in lysis buffer for 10 minutes on a roller mixer and spinning at 13 000 rpm for 1 minute. Repeat twice more, then resuspend to the original volume.	**1.** The decision whether to use protein A or G will depend on the species and subclass of immunoprecipitating antibody.
2. To 1 ml of cell lysate in a microcentrifuge tube, add a suitable amount of the immunoprecipitating antibody and 20–40 ml of immobilised protein A or G beads.	**2.** The amount of lysate to be used will vary depending on the prevalence of the antigen. This will have to be determined empirically. The amount of immunoprecipitating antibody will also have to be optimised. For a 1 ml immunoprecipitation the addition of a volume of antibody corresponding to 1.5 µg is a good starting point.

Protocol	Notes
3. Incubate the tube on a roller mixer for 2–3 hours at 2–8°C.	**3.** Longer incubations can be performed but seldom show any advantages. For high affinity antibodies the incubation time can be reduced.
4. Spin the tube in a microcentrifuge at 13 000 rpm for 1 minute at 4°C. Remove the supernatant and wash the pellet by resuspending in 1 ml of ice-cold lysis buffer and placing on a roller mixer for 5 minutes at 2–8°C. Repeat this wash step twice more.	**4.** Care should be taken not to accidentally remove protein A/G beads during the supernatant removal. Many protease inhibitors are only active for a few hours so it is advisable to add fresh inhibitors to the lysis buffer after 2–3 hours.
5. After the final spin remove as much as possible of the supernatant and resuspend the pellet in 20–100 µl of SDS-PAGE loading buffer.	**5.** The exact volume in which to resuspend the pellet will have to be determined empirically.
6. Elute the antigen by boiling for 4 minutes followed by a brief microcentrifuge spin before gel loading.	
Detection of biotinylated proteins (See introductory paragraph.Use of solution labelled biotinylated proteins in immunodetection on page 15.)	

Protocol	Notes
1. Perform electrophoresis and Western blotting.	**1.** This protocol is optimised for use with Hybond ECL (nitrocellulose) membrane. PVDF membrane may also be used immediately or air dried and stored in a desiccator at 2–8°C for several weeks.
2. Blotting Block the membrane by immersing in 5% blocking agent in PBS-T (see reagent preparation) for 1 hour.	**2.** Use a fresh solution of block reagent each time.
3. Rinsing During the blocking stage dilute the streptavidin-HRP 1 in 1500 in PBS-T. Discard the blocking solution and rinse the membrane twice in PBS-T.	
4. Incubation Add the diluted conjugate to the membrane and incubate for 1 hour.	**4.** It is strongly advised that milk should not be included in the streptavidin-HRP incubation. The binding of streptavidin to biotin is inhibited in the presence of endogenous biotin in milk, resulting in a much decreased signal when

Protocol	Notes
	4. *Continued.* detecting with enhanced chemiluminescence.
5. Washing Discard the diluted conjugate and rinse twice in PBS-T. Wash 3 times for 15 minutes using fresh changes of PBS-T.	
6. Proceed to ECL detection.	

7.7. Protocol E: Detection using ECL reagents

Read through this whole section before proceeding. It is necessary to work quickly once the membranes have been exposed to the detection solution. All steps can be carried out in a dark room; it is necessary to switch off the light only after step 5. Equipment needed are an X-ray film cassette, a roll of SaranWrap, a timer and autoradiography film; Hyperfilm ECL is recommended.

If possible, wear powder free gloves as the powder can inhibit the ECL detection reagents leading to blank patches on the film.

Protocol	Notes
1. Mix equal volumes of detection solution 1 with detection solution 2 to give sufficient reagent to cover the membrane.	**1.** The final volume required is 0.125 ml/cm^2 membrane.

Protocol

2. Drain the excess buffer from the washed membrane(s) and place on a piece of SaranWrap protein side up. Add the detection reagents to the protein side of the membranes so that the reagents are held by surface tension on the surface of the membranes. Do not allow the surface of the membranes to become uncovered.

3. Incubate for precisely 1 minute at room temperature without agitation.

4. Drain off excess detection reagent and wrap membranes in SaranWrap. Gently smooth out air pockets.

5. Place the membranes, protein side up, in the film cassette. Work as quickly as possible; minimise the delay between incubating the membranes in the detection

Notes

4. Drain off excess detection reagent by holding the membrane vertically and touching the edge of the membrane against tissue paper. Gently place the membrane protein side down, on to SaranWrap to form an envelope avoiding pressure on the membrane.

5. Ensure that there is no free detection reagent in the film

Protocol	Notes
5. *Continued.* reagent and exposing them to film (next step).	cassette; the film must not get wet.
6. Switch off the lights and carefully place a sheet of autoradiography film (Hyperfilm ECL) on top of the membranes, close the cassette and expose for 1 minute.	**6.** Do this in the dark room, using red safe lights. Do not move the film while it is being exposed.
7. Remove film, immediately replace with a fresh piece of unexposed film, and re-close film cassette.	**7.** 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 5 minutes up to one hour, this will depend on the amount of target protein on the membrane. If the background is high, the membrane may be rewashed twice for 10 minutes with wash buffer and redetected following steps 1–7 with slight loss of sensitivity.

8. Additional information

8.1. Small scale spin column purification

If biotinylation has been carried out in a volume smaller than 2.0 ml, and further dilution of the protein for purification on the supplied G-25 column is not desirable, then purification may be carried out using a G-25 spin column (reagents not supplied).

Protocol	Notes
1. Prepare the Sephadex G-25 by mixing the resin with an excess volume of PBS. Allow to stand overnight at 2–8°C.	
2. Stopper the end of a 1 ml plastic syringe with glass wool and fill with preswelled Sephadex G-25.	
3. Centrifuge the syringe column upright in a 10 ml centrifuge tube at 1600 xg for 4 minutes.	
4. Discard eluate, refill and respin the column as before.	
5. Repeat until the Sephadex is within 1 cm of the top of the syringe.	
6. Immediately prior to use, calibrate the column by adding a volume of PBS to the top of the column identical to the volume of	**6.** Volumes of 50–250 µl can be purified on 1 ml spin columns. If necessary dilute the protein to provide an adequate volume.

Protocol	Notes
6. *Continued.* biotinylated protein to be purified.	
7. Place the column upright in a centrifuge tube, with a microcentrifuge tube or similar below the column to collect the eluent.	
8. Centrifuge the column at 1600 x g for 4 minutes. Accurately measure the volume eluted.	
9. Repeat steps 6 to 8 until the volume eluted equals the volume added. Once equilibrated, the column should be used immediately.	**9.** This may take 3–4 attempts, until the Sephadex has settled and sample volumes are fully recovered from the column.
10. Load the biotinylated protein on to the column, place a clean microcentrifuge tube below the column to collect the purified protein. Centrifuge at 1600 x g for 4 minutes.	**10.** The percentage of protein recovery from spin columns can be lower than from the larger G-25 Sephadex columns provided, depending on the protein and amount loaded, in some instances recovery may be as low as 60%.
11. The biotinylated protein is now ready for use. Storage conditions are dependent on the characteristics of	

Protocol	Notes
11. *Continued.* individual proteins. However, we recommend that biotinylated antibodies are stored at 2–8°C in the presence of 0.2% sodium azide. Biotinylated horseradish peroxidase should be stored in the presence of 0.01% hyamine and not sodium azide.	

9. Troubleshooting guides

9.1. Troubleshooting guide for protocol A: Biotinylation of proteins in solution

Problem: No biotinylation

Possible cause	Action
1. Protein in Tris or NH^2 containing buffer	**1.** Dialyse protein into bicarbonate buffer.
2. Incorrect pH	**2.** Dialyse protein into bicarbonate buffer.
3. Inaccessible NH^2 groups	**3.** Add detergent for example 0.1% Tween 20, Triton, SDS.
4. Decomposition of biotinylation reagent	**4.** Use new vial of biotinylation reagent. Ensure biotinylation reagent is equilibrated to room temperature prior to opening. Do not leave open to the atmosphere.

Problem: No protein in column fraction

Possible cause	Action
1. Non-specific adsorption to column	**1.** Include a carrier protein in the elution buffer, for example 0.1–0.2% bovine serum albumin.
2. Non-specific adsorption to reaction vial	**2.** Wash vial with PBS and Tween 20, and check the UV absorbance of the washings at 280 nm.

9.2. Troubleshooting guide for Protocol B: Use of solution biotinylated proteins in immunodetection

Problem: No signal

Possible cause	Action
1. No transfer of proteins during Western blotting	**1.1.** Re-evaluate blotting procedure: stain gels (10) with dye, or membranes with protein stain to check transfer efficiency.
	1.2. Optimise gel acrylamide concentration, time of transfer and current, use molecular weight markers covering the molecular weight range expected to be blotted (molecular weight and Stoke's radius both affect transfer).
	1.3. Check that gel and membrane make proper contact during blotting.
	1.4. Check that gel and membrane are correctly orientated with respect to the anode (10).
	1.5. Check that excess temperatures are not reached during electroblotting producing bubbles or gel membrane distortions.
2. Protein degradation on storage of membrane prior to detection	**2.** Use fresh blots.

Problem: No signal *continued.*

Possible cause	Action
3. No biotinylation	**3.1.** Check for successful labelling of antibody or other protein by preparing dot blots of the protein and detecting with streptavidin-HRP.
	3.2. Decomposition of biotinylation reagent. Use new vial of biotinylation reagent. Ensure biotinylation reagent is equilibrated to room temperature prior to opening. Do not leave open to the atmosphere.
4. No retention of proteins on membrane	**4.1.** Assess no transfer of proteins during Western blotting as above.
	4.2. Where dot/slot blots are used ensure the proteins are dry on the membrane before the detection commences.
	4.3. Use fresh supply of membranes to ensure proper hydration.
5. Detection system	**5.1.** Check that the detection reagents are being stored correctly.
	5.2. Check detection reagents are working: premix small quantities of detection reagent 1 and detection reagent 2 (0.5 ml of each) and in the dark room add 1 µl of streptavidin-HRP. Visible light should be produced.

Problem: Weak signal

Possible cause	Action
1. See 'No signal'	**1.** See 'No signal' page 36.
2. Insufficient protein loaded on gel	**2.** Load more protein on the gel.
3. Low level of signal	**3.** Preflashing the film will increase its sensitivity to signal and linearise its response. This does, however, require care as increased backgrounds may result. Pre-flashing involves hypersensitising the film just before use by pre-exposure to a short flash of light (approximately 1 msec). Conventional photographic flash units are suitable when attenuated with a diffuser and *KODAK WRATTEN* 6B filter, to give a flash of the required intensity to increase the 540 nm absorbance of the developed film to 0.15 above the unexposed film.
4. Inclusion of blocking agent in streptavidin-HRP	**4.** Ensure blocking agent is not included in the streptavidin-HRP incubation. The binding of streptavidin to biotin is inhibited in the presence of endogenous biotin in milk, resulting in a much decreased signal when detecting with enhanced chemiluminescence.

Problem: Excessive diffuse signal

Possible cause	Action
1. Overloading of protein	**1.** Load less protein on gel.
2. Improper gel conditions	**2.** Optimise gel electrophoresis and blotting conditions: Increase acrylamide concentration of gel. Check gel and buffer recipes. Check that no bubbles interfere with transfer from gel to membrane.
3. Antibody concentrations too high	**3.** Reduce antibody concentrations.

Problem: Uneven/spotted membrane

Possible cause	Action
1. Improper blotting technique	**1.** See 'No signal' page 36.
2. Unevenly hydrated membrane	**2.** Use new fresh membranes. Ensure that membrane is fully covered and wetted during incubations.
3. Fingerprint and/or keratin contamination	**3.** Avoid touching membrane. Use gloves and blunt forceps.

Problem: High backgrounds

Possible cause	Action
1. Antibody concentrations too high	**1.** Reduce, optimise antibody concentration.
2. Contaminated blotting equipment	**2.** Clean or replace all equipment.
3. Contaminated buffers	**3.** Ensure all buffers are freshly prepared.
4. Inadequate blocking	**4.1.** Check that blocking agent has been prepared correctly and a freshly prepared solution has been used. **4.2.** Increase Tween concentration (Caution: Tween may reduce the binding of antibodies, particularly of low affinity primary antibodies).
5. Problems with membranes	**5.1.** Check that membranes are completely immersed in all solutions especially during washing, and that membranes hydrate thoroughly. **5.2.** Use a fresh supply of membranes. Use high quality membranes: Hybond ECL (RPN 2020D or RPN 82D) are the recommended membranes. **5.3.** Damage to the membrane can cause non-specific binding of the immunodetection reagents. Handle

Problem: High backgrounds *continued.*

Possible cause	Action
	5.3. *continued.* membranes carefully with gloved hands and blunt forceps. Use clean forceps to handle membranes after washing.
6. Inadequate washing	**6.1.** Increase washing times and volumes of wash buffers.
	6.2. Increase concentration of Tween in washing/ and or blocking solutions.
7. Detection reagents	**7.1.** Rewash membranes twice for 10 minutes in wash buffer and repeat detection steps.
	7.2. Excess detection reagents on membranes. Drain well by absorbing the excess on tissue paper before placing membranes in film cassettes.
8. Over exposure	**8.** Expose the film for a minimum period (an initial 15 second exposure may be all that is required). If exposure time is too short to be convenient, reduce antibody concentrations. Leave the membranes in the cassette for 5–10 minutes before re-exposing.

9.3. Troubleshooting guide for Protocol C: Biotinylation of membrane bound proteins

Problem: No signal

Possible cause	Action
1. No transfer of proteins during Western blotting	**1.1.** Re-evaluate blotting procedure: stain gels (10) with dye, or membranes with protein stain to check transfer efficiency.
	1.2. Optimise gel acrylamide concentration, time of transfer and current, use molecular weight markers covering the molecular weight range expected to be blotted (molecular weight and Stoke's radius both affect transfer).
	1.3. Check that gel and membrane make proper contact during blotting.
	1.4. Check that gel and membrane are correctly orientated with respect to the anode (10).
	1.5. Check that excess temperatures are not reached during electroblotting producing bubbles or gel membrane distortions.
2. Protein degradation on storage of membrane prior to detection	**2.** Use fresh membranes.

Problem: No signal *continued.*

Possible cause	Action
3. Decomposition of biotinylation reagent	**3.** Use new vial of biotinylation reagent. Ensure biotinylation reagent is equilibrated to room temperature prior to opening. Do not leave open to the atmosphere.
4. No retention of proteins on membrane	**4.1.** Assess the transfer of proteins during Western blotting as above. **4.2.** Where dot/slot blots are used, ensure the proteins are dry on the membrane before the detection commences. **4.3.** Use fresh supply of membranes to ensure proper hydration.
5. Detection system	**5.1.** Check that the detection reagents are being stored correctly. **5.2.** Check detection reagents are working: premix small quantities of detection reagent 1 and detection reagent 2 (0.5 ml of each) and in the dark room add 1 µl of streptavidin-HRP. Visible light should be produced.

Problem: Weak signal

Possible cause	Action
1. See 'No signal' page 42	**1.** See 'No signal' page 42.
2. Insufficient protein loaded on gel	**2.** Load more protein on the gel.
3. Low level of signal	**3.** Preflashing the film will increase its sensitivity to signal and linearise its response. This does, however, require care as increased backgrounds may result. Pre flashing involves hypersensitising the film just before use by pre-exposure to a short flash of light (approximately 1 msec). Conventional photographic flash units are suitable when attenuated with a diffuser and *KODAK WRATTEN* 6B filter, to give a flash of the required intensity to increase the 540 nm absorbance of the developed film to 0.15 above the unexposed film.
4. Inclusion of blocking agent in streptavidin-HRP	**4.** Ensure blocking agent is not included in the streptavidin-HRP incubation. The binding of streptavidin to biotin is inhibited in the presence of endogenous biotin in milk, resulting in a much decreased signal when detecting with enhanced chemiluminescence.

Problem: Excessive diffuse signal

Possible cause	Action
1. Overloading of protein	**1.** Load less protein on gel.
2. Improper gel conditions	**2.** Optimise gel electrophoresis and blotting conditions: Increase acrylamide concentration of gel. Check gel and buffer recipes. Check that no bubbles interfere with transfer from gel to membrane.

Problem: Uneven spotted background

Possible cause	Action
1. Contaminated electrophoresis apparatus/blotting pads	**1.** Ensure that gel apparatus and Western blotting pads etc. are cleaned thoroughly, as with membrane biotinylation all proteins on the membrane will be detected.
2. Improper blotting technique	**2.** See 'No signal' page 42.
3. Unevenly hydrated membrane	**3.** Use new fresh membranes. Ensure that membrane is fully covered and wetted during incubations.
4. Fingerprint and/or keratin contamination	**4.** Avoid touching membrane. Use gloves and blunt forceps.

Problem: High background

Possible cause	Action
1. Contaminated electrophoresis apparatus/blotting pads	**1.** Ensure that gel apparatus and Western blotting pads etc. are cleaned thoroughly in detergent and rinsed well with water, as with membrane biotinylation all proteins on the membrane will be detected.
2. Improper blotting technique	**2.** Ensure no membrane damage is caused during blotting by high temperature or pressure of blotting pads. Optimisation of blotting conditions specifically for membrane labelling may be required.
3. Contaminated buffers	**3.** Ensure all buffers are freshly prepared.
4. Inadequate blocking	**4.1.** Check that blocking agent has been prepared correctly and a freshly prepared solution has been used.
	4.2. Increase Tween concentration.
5. Problems with membranes	**5.1.** Check that membranes are completely immersed in all solutions especially during washing, and that membranes hydrate thoroughly.
	5.2. Use a fresh supply of membranes. Use high quality membranes: Hybond ECL (RPN 2020D or RPN 82D) are the recommended membranes.

Problem 5: High background *continued.*

Possible cause	Action
5. Problems with membranes *continued.*	**5.3.** Damage to the membrane can cause non-specific binding of the biotinylation and streptavidin-HRP reagents. Handle membranes carefully with gloved hands and blunt forceps. Use clean forceps to handle membranes after washing.
6. Inadequate washing	**6.** Increase washing times and volumes of wash buffers. Increase concentration of Tween in washing and/or blocking solutions.
7. Detection reagents	**7.1.** Rewash membranes twice for 10 minutes in wash buffer and repeat detection steps.
	7.2. Excess detection reagents on membranes. Drain well by absorbing the excess on tissue paper before placing membranes in film cassettes.
8. Over exposure	**8.** Expose the film for a minimum period (an initial 15 second exposure may be all that is required). If exposure time is too short to be convenient, reduce antibody concentrations. Leave the membranes in the cassette for 5–10 minutes before re-exposing.
9. Too high a concentration of streptavidin-HRP	**9.** Reduce the concentration of streptavidin-HRP used.

Problem 6: Anomalous bands at 54 k Da and 68 k Da and vertical streaks.

Possible cause	Action
1. Artifacts arising from reducing agent in loading buffer	**1.** These artifacts, caused by 2-mercaptoethanol (2-ME) or DTT from the loading buffer, are observed with other gel/membrane staining methods (11). If problematic, TCEP-HCl (Pierce) at 12-200 mM can be used in place of 2-ME in the loading buffer. Alternatively, after boiling samples, quench 2-ME by addition of 2-fold molar excess of n-ethylmaleimide (1 M stock in DMF) and incubate at room temperature for 5 minutes (a doublet of low molecular weight bands (<20 kDa) may sometimes be observed).

9.4. Troubleshooting guide for Protocol D: Detection of cell surface proteins using biotinylation and immunoprecipitation

Problem 1: Cell lysis

Possible cause	Action
1. Cells damaged before labelling	**1.** Trypsinize monolayer cells for a minimum length of time and examine viability after washing.
2. Cells damaged during labelling	**2.** Cell lines vary in robustness and should be centrifuged and resuspended with care. Reducing the labelling time, decreasing the amount of biotinylation reagent and/or using PBS rather than bicarbonate buffer may be necessary for some cell lines.

Problem 2: No signal

Possible cause	Action
1. Western blotting/ detection system problems	**1.** See troubleshooting guide for protocol B, 1.1) page 36.
2. No biotinylation has occurred	**2.** Check by running biotinylated whole cell lysate alongside unlabelled lysate and detecting on a Western blot. Perform control solution labelling to check biotinylation reagent is active.

Problem 2: No signal *continued.*

Possible cause	Action
3. No antigen immuno-precipitated	**3.** Ensure cell lysis and immunoprecipitation conditions are optimised for the antigen. Check antibody will still bind to the protein when it is biotinylated. Reducing the amount of biotinylation reagent may help or for glycoproteins labelling via carbohydrate residues can be performed (see Tech Tips available page 55).
4. Immuno-precipitated protein has degraded	**4.** Ensure active protease inhibitors are present in the lysis buffer during immunoprecipitation and washing.

Problem 3: Weak signal

Possible cause	Action
1. Too little antigen present to be detected	**1.** Optimise cell lysis and immunoprecipitation conditions. Increase the density of cells present in the lysate and examine antibody and protein A/G levels. Load more sample on to gel.
2. Inclusion of blocking agent in streptavidin-HRP incubation	**2.** Ensure blocking agent is not included in the streptavidin-HRP incubation. The binding of streptavidin to biotin is inhibited in the presence of endogenous biotin in milk, resulting in a much decreased signal when detecting with enhanced chemiluminescence.

Problem 4: Non-specific bands

Possible cause	Action
1. Non-specific binding of biotinylated proteins	**1.** Increase washing time after immunoprecipitation. Preclear lysate with control serum or an irrelevant antibody before immunoprecipitation. Preblock protein A/G beads with unlabelled cell lysate before use.
2. Too much sample loaded on to gel	**2.** Reduce amount of sample loaded and examine difference in intensity between target bands and non-specific bands.

Problem 5: High backgrounds

Possible cause	Action
1. See high background section on pages 40-41.	

10. References

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11. Related products

ECL Western blotting detection reagents (for 4000 cm^2 membrane)	RPN 2106
ECL Western blotting detection reagents (for 2000 cm^2 membrane)	RPN 2209
ECL Western blotting detection reagents (for 1000 cm^2 membrane)	RPN 2109
ECL Western blotting analysis system (for the detection of membrane bound rabbit and mouse primary antibodies on 1000 cm^2 membrane)	RPN 2108
ECL protein molecular weight markers (sufficient for 25 loadings)	RPN 2107
Streptavidin-horseradish peroxidase conjugate	RPN 1231
ECL *in vitro* translation labelling reagents (rabbit reticulocyte lysate) (sufficient for 20 standard 50 µl reactions)	RPN 2194
ECL *in vitro* translation streptavidin-HRP and blocking reagent (sufficient for 2000 cm^2 membrane)	RPN 2195
ECL *in vitro* translation module (rabbit reticulocyte lysate) (contains RPN 2194 and RPN 2195)	RPN 2196
ECL *in vitro* translation system (rabbit reticulocyte lysate) (contains RPN 2196 and RPN 2209)	RPN 2197
ECL cell-free labelling module (sufficient for 40 standard 50 µl reactions)	RPN 2199
ECL cell-free labelling system (contains RPN 2199 and RPN 2209)	RPN 2200

ECL glycoprotein detection module (sufficient for 25 8 x 10 cm membrane labellings or 50 solution labellings)	RPN 2190
ECL glycoprotein detection system (contains RPN 2190 and RPN 2209)	RPN 2191
Hybond ECL High quality nitrocellulose, recommended for use with ECL.	
Pack of 10 nitrocellulose membranes, 20 x 20 cm	RPN 2020D
Pack of 50 nitrocellulose discs, 82 mm diameter	RPN 82D
Hybond-PVDF	
Pack of 10 PVDF membranes, 20 x 20 cm	RPN 2020P
Roll of PVDF membrane, 20 cm x 3 m	RPN 203P
Hyperfilm ECL	
Pack of 25 films, 18 x 24 cm	RPN 2103
Pack of 25 films, 30 x 40 cm	RPN 2104
Pack of 25 films, 10 x 12 in	RPN 1681
Pack of 25 films, 5 x -7 in	RPN 1674
ECL mini-camera A camera luminometer (using polaroid film, not supplied) specifically designed for ECL Western blots, generated on mini-gel apparatus. Five sample boats are supplied. (For blots up to 5.2 x 7.7 cm)	RPN 2069
Sensitize™ pre-flash unit	RPN 2051
Hypercassette	
Hypercassette, 18 x 24 cm	RPN 1642
Hypercassette, 30 x 40 cm	RPN 1644
Hypercassette, 5 x 7 in	RPN 1648

GE Healthcare also supply a range of ECL products for nucleic acid labelling and detection. For details please contact your nearest local GE Healthcare office.

11.1. Tech Tips available relevant to the ECL protein biotinylation system

140 Use of ECL glycoprotein detection system and ECL protein biotinylation system to detect total protein and sialic acid groups on glycoproteins after treatment with neuraminidase.

145 Use of the ECL protein biotinylation system – stripping and reprobing of membranes biotinylated for total protein detection.

146 Use of ECL protein biotinylation system for biotinylation of primary antibodies for the detection of immunoprecipitates.

147 The ECL protein biotinylation system – detection of cell surface antigens using biotinylation, immunoprecipitation and detection with ECL, and comparison with ^{125}I and iodogen labelling.

149 The ECL glycoprotein detection system – detection of cell surface antigens using biotinylation and immunoprecipitation.

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