GE Healthcare

Amersham ECL glycoprotein detection system

Sufficient for 50 solution labellings or 25 8 x 10 cm membrane labellings

Product Booklet

Codes: RPN2190
RPN2191

Page finder

1. Legal

http//www.gehealthcare.com/lifesciences

GE Healthcare UK Limited.
Amersham Place, Little Chalfont,
Buckinghamshire, HP7 9NA UK

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.

Warning: Potentially infectious material

Human blood products provided as components of this pack have been obtained from donors who were tested individually and who were found to be negative for the presence of Human Immunodeficiency Virus antibody (HIV-Ab)* as well as for Hepatitis B surface Antigen (HBsAg) using approved methods (enzyme-immunoassay).
As no test method can offer complete assurance that Hepatitis B virus, or Human Immunodeficiency Virus antibody (HIV-Ab)* or other infectious agents are absent, all human blood products should be considered potentially infectious. Handling, use, storage and disposal should be in accordance with the procedures defined by appropriate National biohazard safety guideline or regulation, where it exists (for example USA Centre for Disease Control/ National Institutes of Health manual 'Biosafety in Microbiological and Biomedical Laboratories', 2nd Edition, 1988).

Warning: Contains sodium metaperiodate, sodium metabisulphite and dimethylformamide.

We recommend that this product and components are 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. As all chemicals should be considered as potentially hazardous it is advisable when handling chemical reagents to wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In case of contact with skin or eyes wash immediately with water.

* HIV is the abbreviation used for HTLV-III and LAV.

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 glycoprotein detection module	**RPN 2190**
Component	
Sodium metaperiodate	2 g
Sodium metabisulphite	1 g
0.125 mM Biotin hydrazide The reagent is supplied ready for use in dimethylformamide.	0.25 ml
5.0 mM Biotin hydrazide The reagent is supplied ready for use in dimethylformamide.	0.25 ml
Membrane blocking agent	40 g
Streptavidin horseradish peroxidase conjugate A concentrated stock of streptavidin conjugated to horseradish peroxidase is supplied which must be diluted to the appropriate concentration before use with the four stated protocols.	2 x 0.5 ml
Transferrin A lyophilised material which requires reconstitution in 1 ml of distilled water. **Caution: Potentially infectious material, see safety warnings and precautions section on page 4.**	1 mg
Protocol summary cards	

ECL glycoprotein detection system	**RPN 2191**
ECL Western blotting reagents for 2000 cm^2 membrane	**RPN 2209**
Reagent 1	125 ml
Reagent 2	125 ml

4. Description

The ECL™ glycoprotein detection system allows labelling of a carbohydrate moiety with biotin and detection with streptavidin-horseradish peroxidase and enhanced chemiluminescence (1, 2). The principle of labelling and detection is outlined in figure 1.

The ECL glycoprotein detection system can be used to determine if a protein is glycosylated or not. In addition it is possible, using the appropriate protocol, to determine if a glycoprotein contains sialic acid residues (3). The labelling reaction can be performed in solution prior to Western blotting or after transfer to the membrane.

Cell surface proteins can be labelled with biotin via the carbohydrate group and the labelled protein used for immunoprecipitation. Glycosidase enzymes can be used together with the kit to obtain structural information about the carbohydrate groups attached to the protein.

Each batch of the system is tested by our quality control group to ensure the detection of at least 80 ng of transferrin by membrane labelling of total carbohydrate on Hybond™ PVDF.

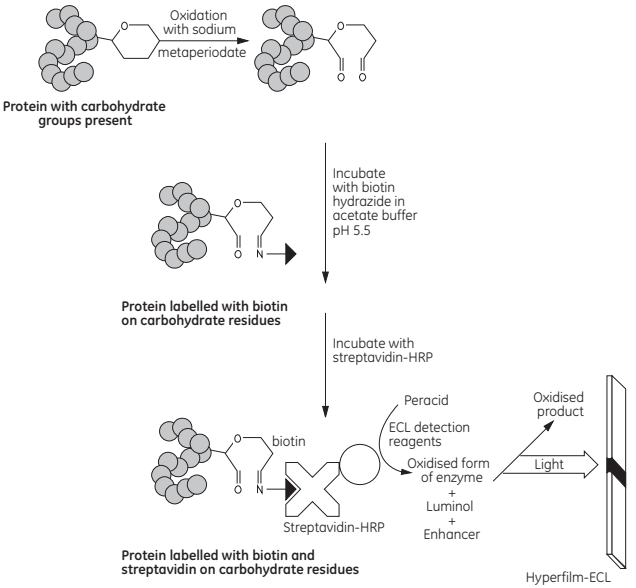

Figure 1. Principle of labelling and detection with the ECL glycoprotein detection system

5. Critical parameters

- The periodate oxidation step must be performed in the dark.
- Certain substances, for example free amines, can interfere with the solution labelling protocol. If these cannot be avoided then the membrane labelling protocol should be used.
- Preferential labelling of sialic acid residues requires the periodate oxidation step to be performed on wet ice using reagents previously equilibrated in the ice.
- 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 (4), resulting in a decreased signal when detected by enhanced chemiluminescence.
- It is important that all electrophoresis and electroblotting equipment is scrupulously clean when detecting glycoproteins immobilised on the membrane.
- If membrane labelling is being performed on PVDF membrane it is important to use the reagent concentrations specified for both biotin hydrazide and streptavidin-HRP which are different from the protocol specified for nitrocellulose.
- 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.
- It is important not to use Tween™ 20 for any reagent dilutions or in the wash buffer because this will increase background.

6. Additional equipment and reagents required

Equipment

- Electrophoresis and blotting equipment for Western blotting
- Blotting membrane (Hybond PVDF is recommended, see related products)
- Orbital shaker
- Forceps with rounded, non-serrated tips
- X-ray film cassette(Hypercassette™ is recommended, see related products)
- Timer
- Autoradiography film (Hyperfilm™ ECL is recommended, see related products)
- Film developing facility and reagents
- SaranWrap™
- Aluminium foil

Reagents

- Distilled water
- Sodium acetate trihydrate
- Glacial acetic acid
- Phosphate buffered saline, pH 7.5
- Gel loading buffer
- Electrophoresis and blotting reagents

7. Use of the ECL glycoprotein detection system

7.1. Flow diagram

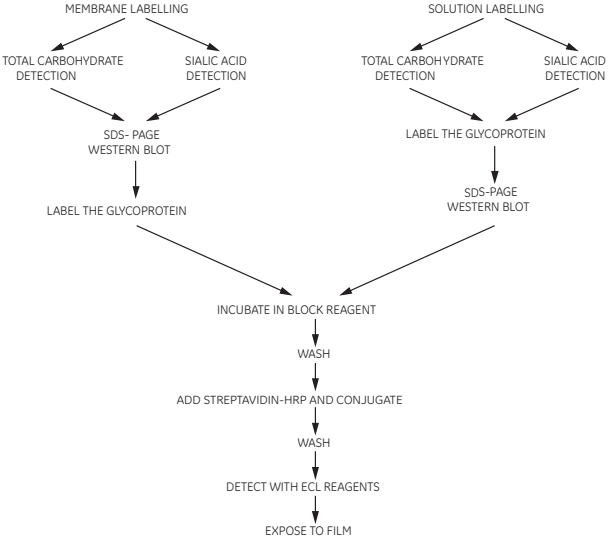

Figure 2. Flow diagram for the ECL glycoprotein detection system

7.2. Reagent preparation

Transferrin
Reconstitute with 1 ml of distilled water. Dispense into aliquots and store at -15°C to -30°C.

200 mM sodium acetate
Dissolve 27.21 g of sodium acetate trihydrate in 1 litre of distilled water.

200 mM acetic acid
Add 2 ml of glacial acetic acid to 172 ml of distilled water.

200 mM acetate buffer
220 ml of 200 mM sodium acetate + 30 ml of 200 mM acetic acid, check pH is 5.5.

100 mM acetate buffer
Dilute 200 mM acetate buffer 1:1 with distilled water.

The buffers above may be stored at 2–8°C for up to 1 month.

10 mM sodium metaperiodate reagent (for total carbohydrate labelling on the membrane)
42.78 mg dissolved in 20 ml of 100 mM acetate buffer. Prepare freshly just before use.

1 mM sodium metaperiodate reagent (for preferential sialic acid labelling on the membrane)
Dilute 10 mM sodium metaperiodate 1 in 10 with 100 mM acetate buffer. Prepare freshly just before use.

30 mM sodium metaperiodate reagent (for total carbohydrate labelling in solution)
6.42 mg dissolved in 1 ml of distilled water. Prepare freshly just before use.

3 mM sodium metaperiodate reagent (for preferential sialic acid labelling in solution)
Dilute 30 mM sodium metaperiodate 1 in 10 with distilled water. Prepare freshly just before use.

20 mM sodium metabisulphite
3.75 mg dissolved in 1 ml of 200 mM acetate buffer. Prepare freshly just before use.

4 mM sodium metabisulphite
Dilute the 20 mM sodium metabisulphite 1 in 5 in 200 mM acetate buffer. Prepare freshly just before use.

Membrane blocking agent
Add 1 g to 20 ml of PBS to give a 5% (w/v) solution. Prepare freshly just before use.

Streptavidin-HRP conjugate
Add an appropriate amount of streptavidin-HRP conjugate to 20 ml of PBS. 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.

Gel loading buffer (4 x concentrate)

12.5 ml	1.0 M Tris-HCL, pH 6.8
4 g	SDS
20 ml	Glycerol
10 ml	Mercaptoethanol
0.5 ml	Saturated pyronin-Y solution

Make up to 50 ml with distilled water.
Store at room temperature.

7.3. Protocol notes

The protocols outlined on the following pages have been developed in our laboratories to give optimum signal and minimum background noise. The concentration of biotin hydrazide and the dilution of streptavidin-horseradish peroxidase conjugate required with total carbohydrate labelling on the membrane vary depending on whether a nitrocellulose or PVDF membrane is used. The sensitivity of PVDF membranes is greater than nitrocellulose and is therefore recommended for glycoprotein detection. Dot blots as well as SDS-PAGE/Western blots may be used.

Please note: The ECL detection system is extremely sensitive and for best results it is essential that the membrane is not damaged during the assay. If the membrane is exposed to elevated temperature or prolonged blotting, increased background may be seen particularly when the membrane labelling protocol is being used. It is recommended that the blotting time is kept to a minimum.

During the procedure, sufficient solution should be used to adequately cover the membrane and the 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. Although a 5 minute exposure to film is recommended, for high abundance material, a shorter time may be sufficient. Alternatively, if greater sensitivity is required, exposures longer than 5 minutes can be made although some increase in background is to be expected, particularly with the membrane labelling protocol for total carbohydrate.

The positive control (transferrin) may be used in order to become familiar with the kit and also to confirm performance for total carbohydrate labelling. As the level of detection sensitivity for each

glycoprotein will be different it is recommended that for test proteins, an initial concentration of 500 ng is used for membrane labelling to detect total carbohydrate and 100 ng for solution labelling to detect total carbohydrate. For sialic acid labelling, a protein such as fetuin is recommended and can be used at the same concentration.

It is important to include a negative control. A suitable negative control would be a sample of the test protein which has not been through the periodate oxidation step. The negative control must be processed separately from any periodate treated membrane throughout the assay. A variety of buffer constituents may interfere with the oxidation and labelling of the carbohydrate for example dithiothreitol, glucose, urea etc (see additional information). If any of these substances are known to be present in the protein sample then the solution labelling protocols should be avoided and the membrane labelling methods used. If the solution labelling protocols are being used and it is suspected that an interfering substance may be present in the sample it is suggested that an aliquot of transferrin (positive control) is diluted in the same buffer and the efficiency of transferrin labelling compared to a standard labelling reaction.

7.4. Total carbohydrate labelling on the membrane

During the procedure, sufficient solution should be used to cover the membrane adequately and the containers should be agitated gently on an orbital shaker. When washing, the volume of phosphate buffered saline (PBS) should be as large as possible; 4 ml of buffer per cm^2 of membrane is suggested. Brief rinses of the membrane in PBS before washing will improve efficiency.

It is advisable to run a negative control which uses the protein under test but replaces the periodate step with 100 mM acetate buffer, pH 5.5 only. The negative control should be processed separately from the rest of the membrane at all stages.

If nitrocellulose membranes are being used with this protocol it will be necessary to dilute the 5 mM biotin hydrazide 1 in 40 in dimethylformamide to obtain a sufficient amount to carry out 25 membrane labelling reactions.

Note: All incubations are performed with gentle agitation at room temperature

Protocol	Notes
1. Prepare a dot blot or SDS-PAGE/Western blot.	**1.1.** The detection limit for glycoproteins varies due to a variety of different factors including their carbohydrate content. (See additional information)
	1.2. Damage to the membrane leads to higher background and it is important not to extend blotting time unnecessarily. Optimum

Protocol	Notes
	1.2. *Continued.* transfer conditions for transferrin, using the Bio-Rad mini PROTEAN II apparatus are 80 volts for 90 minutes, with cooling. The transfer buffer should be pre-cooled to 2–8°C to minimise temperature rise.
	1.3. If the membrane is packed too tightly during transfer there will be damage to the membrane which appears as dark circular impressions after ECL detection. It is also essential that the pads are clean prior to use. It is recommended that pads are kept for this application only. To ensure the pads remain clean they should be soaked in a dilute detergent, for example 2% Decon™, overnight, then rinsed thoroughly in tap water and finally rinsed in distilled water. The transfer pads provided by BioRad are recommended.

Protocol	Notes
	1.4. The blots may be used immediately or air dried and stored desiccated at 2–8°C for several weeks.
	1.5. If a PVDF membrane is being used after drying, it must be soaked in methanol and thoroughly wetted before continuing. Refer to manufacturers wetting instructions.If nitrocellulose is used after drying it must be rehydrated by slowly sliding the membrane at a 45° angle into the buffer.
2. Incubate the membrane in PBS, for 10 minutes.	
3. Incubate the membrane for 20 minutes in a solution of 10 mM sodium metaperiodate dissolved in 100 mM acetate buffer, pH 5.5 (see reagent preparation). **Perform this reaction in the dark.**	**3.1.** The reaction must be performed in the dark. It is recommended that aluminium foil is used to cover the container.
	3.2. Vigorous mixing may cause damage to the membrane leading to increased background staining.
4. Briefly rinse the membrane twice in PBS then wash 3 times for 10 minutes with fresh changes of PBS.	**4.** As large a volume as possible of PBS should be used each time.

Protocol	Notes
5. Nitrocellulose membranes: Add 40 µl of 0.125 mM biotin hydrazide to 20 ml of 100 mM acetate buffer pH 5.5. **PVDF membranes:** Add 4 µl of 0.125 mM biotin hydrazide to 20 ml of 100 mM acetate buffer, pH 5.5. Incubate the membrane in the appropriate solution for 60 minutes.	**5.1.** If large amounts of protein are present on the membrane (for example, greater than 200 ng of transferrin) the reaction time may be shortened to 30 minutes. **5.2.** Use the appropriate concentration of biotin hydrazide for the type of membrane: if too much is used for PVDF, an increased background results and if too little is used for nitrocellulose, a reduction in sensitivity occurs.
6. Wash as in step 4.	**6.** See note 4.
7. Prepare 20 ml of 5% membrane blocking agent in PBS. Incubate the membrane in this solution for 60 minutes. Alternatively it is possible to incubate the membrane in this solution overnight at 2–8°C.	**7.1.** A shorter incubation time of 30 minutes may be used when larger amounts of protein are present and shorter film exposure times are envisaged. **7.2.** The overnight incubation may be used for convenience.
8. Wash as in step 4.	**8.** See note 4.

Protocol	Notes
9. Nitrocellulose membranes: Prepare 20 ml of a 1 in 1000 dilution of the streptavidin horseradish peroxidase conjugate in PBS. **PVDF membranes:** Prepare 20 ml of a 1 in 6000 dilution of the streptavidin horseradish peroxidase in PBS. Incubate the membrane in this solution for 30 minutes.	**9.** Ensure that the correct dilution is selected depending upon which membrane is used. If an increased concentration is selected for PVDF membranes background will be increased.
10. Wash as in step 4.	**10.** See note 4.
11. Proceed to ECL detection on page 31.	

7.5. Preferential sialic acid labelling on the membrane

During the procedure, sufficient solution should be used to cover the membrane adequately and the containers should be agitated gently on an orbital shaker. When washing, the volume of phosphate buffered saline (PBS) should be as large as possible; 4 ml of buffer per cm^2 of membrane is suggested. Brief rinses of the membrane in PBS before washing will improve efficiency.

It is advisable to run a negative control which uses the protein under test, but replaces the periodate with 100 mM acetate buffer pH 5.5 only. The negative control should be processed separately from the rest of the membrane at all stages.

Note: All incubations are performed with gentle agitation.

Protocol	Notes
1. Prepare a dot blot or SDS-PAGE/Western blot.	**1.1.** The detection limit for glycoproteins varies due to a variety of different factors including their carbohydrate content (see additional information). **1.2.** Damage to the membrane leads to higher background and it is important not to extend blotting time unnecessarily. Optimum transfer conditions using the Bio-Rad mini-PROTEAN II apparatus are 80 volts for 90 minutes, with cooling. The transfer buffer should be pre-cooled to 2–8°C to minimise temperature rise. **1.3.** If the membrane is packed too tightly during transfer there will be damage to the membrane which appears as dark circular impressions after ECL detection. It is also essential that the pads are clean prior to use. It is recommended that pads are kept for this application only. To ensure that pads remain clean they should be soaked in a dilute

Protocol	Notes
	1.3. *Continued.* detergent, for example 2% Decon, overnight, then rinsed thoroughly in tap water and finally rinsed in distilled water. After the pads have dried they must be kept free from contamination. The transfer pads provided by BioRad are recommended.
	1.4. The blots may be used immediately or air dried and stored desiccated at 2–8°C for several weeks.
	1.5. If a PVDF membrane is being used after drying, it must be soaked in methanol and thoroughly wetted before continuing. Refer to manufacturers wetting instructions. If nitrocellulose is used after drying it must be rehydrated by slowly sliding the membrane at a 45° angle into the buffer.

Protocol	Notes
2. Incubate the membrane in PBS (equilibrated to approximately 0°C in wet ice), for 10 minutes.	
3. Incubate the membrane for 20 minutes on wet ice (0°C), in a 20 ml solution of ice cold 1 mM sodium metaperiodate in 100 mM acetate buffer pH 5.5. **Perform this reaction in the dark.**	**3.1.** The reaction can be carried out in a sealed container on wet ice. **3.2.** The reaction must be performed in the dark. It is recommended that aluminium foil is used to cover the container. **3.3.** Vigorous mixing may cause damage to the membrane leading to increased background staining.
4. Briefly rinse the membrane twice in ice cold PBS then wash 3 times for 10 minutes with fresh changes of PBS at 0°C. **This step can be performed in the light. All subsequent steps are performed at room temperature in the light.**	**4.** As large a volume as possible of PBS should be used each time.
5. Nitrocellulose and PVDF membranes: Add 4 µl of 5.0 mM biotin hydrazide to 20 ml of 100 mM acetate buffer pH 5.5.	**5.** If large amounts of protein are present on the membrane (for example, greater than 200 ng of transferrin) the

Protocol	Notes
5. *Continued.* Incubate the membrane in this solution for 60 minutes.	**5.** *Continued.* reaction time may be shortened to 30 minutes.
6. Briefly rinse the membrane twice in PBS then wash 3 times for 10 minutes with fresh changes of PBS.	**6.** See note 4.
7. Prepare 20 ml of 5% membrane blocking agent in PBS. Incubate the membrane in this solution for 60 minutes. Alternatively it is possible to incubate the membrane in this solution overnight at 2–8°C.	**7.1.** A shorter incubation time of 30 minutes may be used when larger amounts of protein are present and shorter exposure times are envisaged. **7.2.** The overnight incubation may be used for convenience.
8. Wash as in step 6.	**8.** See note 4.
9. Nitrocellulose and PVDF membranes: Prepare 20 ml of a 1 in 1000 dilution of the streptavidin horseradish peroxidase conjugate in PBS. Incubate the membrane in this solution for 30 minutes.	
10. Wash as in step 6.	**10.** See note 4.
11. Proceed to ECL detection on page 31.	

7.6. Total carbohydrate labelling in solution

During the membrane processing steps, sufficient solution should be used to cover the membrane adequately and the containers should be agitated gently on an orbital shaker. When washing, the volume of phosphate buffered saline (PBS) should be as large as possible; 4 ml of buffer per cm^2 of membrane is suggested. Brief rinses of the membrane in PBS before washing will improve efficiency.

It is advisable to run a negative control which uses the protein under test but replaces the periodate step with water.

Note: All incubations are performed at room temperature.

Protocol	Notes
1. Dissolve 0.01–10 µg of sample in 20 µl of 100 mM acetate buffer pH 5.5. Dilute aqueous samples 1:1 (10 µl + 10 µl) in 200 mM acetate buffer pH 5.5.	**1.1.** If a sample is already in a buffer the pH must be between 5 and 6 after it has been diluted in the acetate buffer. **1.2.** This labelling method gives greater sensitivity since all generated aldehyde groups are available for biotinylation. It is also less prone to elevated background because the membrane is not exposed to the periodate and hydrazide reagents which can cause non-specific interactions.

Protocol	Notes
2. Add 10 µl of 30 mM sodium metaperiodate in distilled water to the sample, mix and incubate for 20 minutes. **Perform this reaction in the dark.**	**2.** This reaction must be carried out in the dark. It is recommended that aluminium foil is used to cover the reaction vessel.
3. Add 10 µl of 20 mM sodium metabisulphite in 200 mM acetate buffer pH 5.5, mix and incubate for 5 minutes.	**3.** The metabisulphite is required to remove excess periodate reagent.
4. Add 5 µl of 5 mM biotin hydrazide, mix and incubate for 60 minutes.	
5. Prepare a dot blot, or, if preparing a Western blot, add 15 µl of 4 x gel running buffer, mix, heat at 100°C for 4 minutes and load on to a polyacrylamide gel.	**5.** Background staining is less of a problem but it is still recommended to keep blotting time to a minimum.
6. After Western blotting, the membrane may be used immediately or air dried and stored desiccated at 2–8°C for several weeks.	**6.** If PVDF membrane is being used after drying, it must be soaked in methanol and thoroughly wetted before continuing. Refer to manufacturers wetting instructions.If nitrocellulose is used after drying, it must be rehydrated by slowly sliding the membrane at a 45° angle into the buffer.

Protocol	Notes
7. Prepare 20 ml of 5% membrane blocking agent in PBS. Incubate the membrane in this solution for 60 minutes. Alternatively, it is possible to incubate in this solution overnight at 2–8°C.	**7.1.** A shorter incubation time of 30 minutes may be used when larger amounts of protein are present and shorter exposure times are envisaged. **7.2.** The overnight incubation may be used for convenience.
8. Briefly rinse the membrane twice in PBS then wash 3 times for 10 minutes with fresh changes of PBS.	**8.** As large a volume as possible of PBS should be used each time.
9. **Nitrocellulose and PVDF membranes:** Prepare 20 ml of a 1 in 1000 dilution of the streptavidin horseradish peroxidase conjugate in PBS. Incubate the membrane in this solution for 30 minutes.	
10. Wash as in step 8.	**10.** See note 8.
11. Proceed to ECL detection on page 31.	

7.7. Preferential sialic acid labelling in solution

During the membrane processing steps sufficient solution should be used to cover the membrane adequately and the containers should be agitated gently on an orbital shaker. When washing, the volume of phosphate buffered saline (PBS) 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 PBS will improve washing efficiency.

It is advisable to run a negative control which uses the protein under test, but replaces the periodate step with water.

Protocol	Notes
1. Dissolve 0.01–10 µg of sample in 20 µl of 100 mM acetate buffer pH 5.5. Dilute aqueous samples 1:1 in 200 mM acetate buffer pH 5.5. Equilibrate on wet ice.	**1.1.** If a sample is already in a buffer, the pH must be between 5 and 6 after it has been diluted in the acetate buffer. **1.2.** This labelling method gives greater senstivity since all aldehyde groups generated are available for biotinylation. It is also less prone to elevated background because the membrane is not exposed to the periodate and hydrazide reagents which can cause non-specific interactions.

Protocol	Notes
2. Add 10 µl of ice cold 3 mM sodium metaperiodate in distilled water, mix and incubate for 20 minutes. **Perform this reaction on wet ice in the dark.**	**2.** This reaction must be carried out in the dark. It is recommended that aluminium foil is used to cover the reaction vial.
3. Add 10 µl of 4 mM sodium metabisulphite in 200 mM acetate buffer pH 5.5, mix and incubate for 5 minutes at room temperature. **This and all subsequent steps are performed at room temperature in the light.**	**3.** This reagent is required to remove excess periodate reagent.
4. Add 5 µl of 5 mM biotin hydrazide, mix and incubate for 60 minutes.	
5. Prepare a dot blot, or, if preparing a Western blot add 15 µl of 4 × gel running buffer, mix, heat at 100°C for 4 minutes and load on to a polyacrylamide gel.	**5.** Background staining is less of a problem but it is still recommended to keep blotting time to a minimum.
6. After Western blotting, the membrane may be used immediately or air-dried and stored desiccated at 2–8°C for several weeks.	**6.** If PVDF membrane is being used after drying, it must be soaked in methanol and thoroughly wetted before continuing. Refer to manufacturer's wetting instructions.

Protocol	Notes
	6. *Continued.* If nitrocellulose is used after drying it must be rehydrated by slowly sliding the membrane at a 45° angle into the buffer.
7. Prepare 20 ml of 5% membrane blocking agent in PBS. Incubate the membrane in this solution for 60 minutes. Alternatively it is possible to incubate in this solution overnight at 2–8°C.	**7.1.** A shorter incubation time of 30 minutes may be used when larger amounts of protein are present and shorter exposure times are envisaged. **7.2.** The overnight incubation may be used for convenience.
8. Briefly rinse the membrane twice in PBS then wash 3 times for 10 minutes with fresh changes of PBS.	**8.** As large a volume as possible of PBS should be used each time.
9. Nitrocellulose and PVDF membranes: Prepare 20 ml of a 1 in 1000 dilution of the streptavidin horseradish peroxidase conjugate in PBS. Incubate the membrane in this solution for 30 minutes.	
10. Wash as in step 8.	**10.** See note 8.
11. Proceed to ECL detection on page 31.	

7.8. Detection using ECL reagents

Read through the 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 only necessary to switch out the light after step 5. Equipment needed are an X-ray film cassette, a roll of SaranWrap (other 'clingfilms' may be suitable), a timer and autoradiography film; Hyperfilm ECL (RPN 2103) is recommended.

The use of gloves is strongly recommended from this stage onwards to prevent hand contact on film or detection reagents. 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 to cover the membrane.	**1.** The final volume required is 0.125 ml/cm^2 membrane.
2. Drain excess buffer from the washed membranes and place them on a piece of SaranWrap, protein side up. Add the detection reagents to the protein side of the membrane, so that the reagents are held by surface tension on the surface of the membrane. Do not allow the surface of the membranes to become uncovered.	

Protocol	Notes
3. Incubate for precisely 1 minute at room temperature, without agitation.	
4. Drain off excess detection reagent and wrap the membranes in SaranWrap. Gently smooth out any air pockets.	**4.** Drain off excess detection reagent by holding the membrane vertically and touching the edge against tissue paper. Gently place the membrane, protein side down, on to SaranWrap. Close the SaranWrap to form an envelope avoiding pressure on the membrane.
5. Place the membranes, protein side up, in the film cassette. Work as quickly as possible: minimise the delay between incubating the membranes in detection reagent and exposing them to the film (next step).	**5.** Ensure there is no free detection reagent in the film 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, using red safelights. Do not move the film while it is being exposed.

Protocol	Notes
7. Remove the film, immediately replace with a fresh piece of unexposed film, and re-close the cassette.	**7.** Develop the first piece of film immediately, and on the basis of its appearance decide how long to expose the second piece of film. A typical second exposure would be for 2–5 minutes for total carbohydrate membrane labelling and 5–15 minutes for the other three protocols.

8. Additional information

8.1. Guidelines on sensitivity

The detection limit will vary between different glycoproteins depending on the particular protein being analysed and its carbohydrate content. Optimised blotting conditions are essential to obtain maximum sensitivity. As already indicated a short blotting protocol is required for total carbohydrate labelling on the membrane, this may not give maximum transfer but will give the best achievable sensitivity (see note 1.2 on page 16 for details). Greater sensitivity can be achieved using PVDF membranes and these are therefore recommended for this application. The following sensitivities have been achieved with the Bio-Rad mini PROTEAN II system using SDS-PAGE and blotting on to nitrocellulose membranes (10 µl sample size):

Protein	Protocol			
	Total carbohydrate (on membrane)	Preferential sialic acid (on membrane)	Total carbohydrate (in solution)	Preferential sialic acid (in solution)
Transferrin	8 ng	-	1 ng	-
Fetuin	8 ng	10 ng	4 ng	1 ng
Ovalbumin	16 ng	-	20 ng	-
Ribonuclease-B	-	-	8 ng	-

Solution labelling protocols will provide the lowest background signal because the membrane is not exposed to periodate or hydrazide reagents which can cause non-specific interactions.
If possible solution labelling should be used unless substances known to interfere with the oxidation and labelling are present in the protein sample.

8.2. Substances which can interfere with solution labelling

A variety of substances can interfere with solution oxidation and labelling. If these substances are known to be present then the appropriate membrane labelling protocol should be selected. These substances include precipitants, reducing agents, periodate sensitive compounds such as glucose and galactose, free amines including Tris and transition metal ions.

If there is any doubt about a potential contaminant being present in a sample buffer it is suggested that an aliquot of transferrin (positive control) is diluted in the same buffer and the efficiency of transferrin labelling compared to a standard labelling reaction.

8.3. Troubleshooting guide

Problem: No signal

Possible cause	Remedy
1. No transfer of proteins during Western blotting	**1.1.** Stain gels with Coomassie blue stain to check transfer efficiency. **1.2.** Optimise gel acrylamide concentration, time of transfer and current (if electroblotting) using molecular weight markers covering the range expected to be blotted (molecular weight and Stoke's radius both affect transfer). **1.3.** If long transfer times are required it is essential to control temperature as elevated temperatures cause high background.

Possible cause	Remedy
	1.4. Check that the gel and the membrane are making proper contact during blotting.
	1.5. Check that the gel and membrane are correctly orientated with respect to the anode.
	1.6. Check that no bubbles are present between the gel and the membrane preventing transfer.
2. Glycoprotein degradation on storage of blots prior to detection	**2.** Prepare fresh blots.
3. No retention of glycoproteins on the membrane	**3.1.** Assess transfer of proteins during Western blotting as in 1.1 on page 35.
	3.2. Ensure the correct protocol has been followed to hydrate a PVDF membrane if used.
	3.3. If either nitrocellulose or PVDF has been used, use a fresh supply to ensure proper hydration.

Problem: Weak signal

Possible cause	Remedy
1. See 'No signal', page 35	**1.** See 'No signal', page 35.
2. Insufficient glycoprotein loaded on gel	**2.** Load more sample. Make sure the membrane is fully covered during all incubations.
3. Low level of signal	**3.** Expose film for extended period; background may limit this time when using protocol for total carbohydrate labelling on the membrane.
4. Carbohydrate labelling has not worked	**4.** Check labelling reagents are working by performing labelling on a dot blot using the transferrin control protein.
5. Substances present in protein sample buffer which interfere with solution labelling (see additional information)	**5.** Use membrane labelling protocols.

Problem: Excessive diffuse signal

Possible cause	Remedy
1. Overloading of glycoprotein	**1.** Load less sample on the gel.
2. Improper gel conditions	**2.** Optimise gel, electrophoresis and blotting conditions 2.1. Increase acrylamide concentration of gel. Check gel and buffer recipes. 2.2. Check that no bubbles interfere with transfer from gel to membrane.

Problem: Uneven/speckled blot

Possible cause	Remedy
1. Improper blotting technique	**1.** See 'No signal', page 35.
2. Unevenly hydrated membrane	**2.1.** Use new/fresh membranes. **2.2.** Make sure the membrane is fully covered during all incubations. **2.3.** PVDF membranes must be rehydrated using methanol. If any part of the membrane is allowed to dry out during the protocol, dark areas or blank patches may appear on the final film exposure. Avoid touching the membrane. Use gloves and blunt ended forceps.
3. Fingerprints and/or keratin contamination	**3.** Avoid touching the membrane. Use gloves and blunt ended forceps.

Problem: High backgrounds

Possible cause	Remedy
1. Contaminated blotting equipment	**1.** Clean or replace all equipment.
2. Contaminated buffers	**2.** Ensure all buffers are freshly prepared and filtered.
3. Extended blotting time	**3.** Reduce blotting time and/or use transfer buffer cooled to 4°C. Transfer under cooling conditions.
4. Problems with membrane	**4.1.** Check that the membranes are completely immersed in buffer throughout the entire protocol.
	4.2. Use a fresh supply of membranes.
	4.3. Use high quality membranes: Hybond PVDF (RPN2020P) is the recommended membrane: Hybond ECL (RPN2020D) may also be used.
	4.4. Damage to membranes can cause non-specific binding. Handle blots with care using blunt non-serrated forceps.
	4.5. Use clean forceps to handle membranes after washing.
5. Detection reagents	**5.** Excess detection reagents in blots. Drain well by absorbing the excess on tissue paper before placing blots in film cassette.
6. Over exposure	**6.** If the signal is too high at 1 minute then a reduced exposure time should be chosen. If it is not possible to obtain a clear signal then less sample should be assayed.

Problem: High backgrounds *Continued.*

Possible cause	Remedy
7. Blotting pads	**7.** New blotting pads should be washed before use to remove any contaminants which may cause background or interfere with the detection. Ensure all pads are washed after use and kept free of contamination.
8. Tween 20 used in PBS wash buffer.	**8.** Use of detergents in these protocols should be avoided.

8.4. Related products

ECL Western blotting detection reagents RPN2106
(for 4000 cm^2 membrane)

(ECL Western blotting detection reagents RPN2209
(for 2000 cm^2 membrane)

ECL Western blotting detection reagents RPN2109
(for 1000 cm^2 membrane)

ECL Western blotting analysis system RPN2108
(for the detection of either mouse or rabbit membrane bound primary antibodies on 1000 cm^2 membrane)

ECL protein molecular weight markers RPN2107
(sufficient for 25 loadings)

Streptavidin-horseradish peroxidase conjugate RPN1231

ECL *in vitro* translation labelling reagents
(rabbit reticulocyte lysate) RPN2194
(sufficient for 20 standard 50 µl reactions)

ECL *in vitro* translation streptavidin-HRP and blocking reagent (sufficient for 2000 cm^2 membrane)	RPN2195
ECL *in vitro* translation module (rabbit reticulocyte lysate) (contains RPN2194 and RPN2195)	RPN2196
ECL *in vitro* translation system (rabbit reticulocyte lysate) (contains RPN2196 and RPN2209)	RPN2197
ECL cell-free labelling module (sufficient for 40 standard 50 µl reactions)	RPN2199
ECL cell-free labelling system (contains RPN2199 and RPN 2209)	RPN2200
ECL protein biotinylation module (sufficient for 25 8 x 10 cm membrane labellings or 5 2.5 mg solution labellings	RPN2202
ECL protein biotinylation system (contains RPN2202 and RPN2209)	RPN2203
Hybond ECL High quality nitrocellulose, recommended for use with ECL	
Pack of 10 nitrocellulose membranes, 20 x 20 cm	RPN2020D
Hybond PVDF	
Pack of 10 PVDF membranes, 20 x 20 cm	RPN2020P
Roll of PVDF membrane, 20 cm x 3 m	RPN203P
Hyperfilm ECL	
Pack of 25 films, 18 x 24 cm	RPN2103
Pack of 25 films, 30 x 40 cm	RPN2104

Pack of 25 films, 10 x 12 inches	RPN1681
Pack of 25 films, 5 x 7 inches	RPN1674

ECL mini-camera RPN2069

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).

Sensitize™ pre-flash unit RPN2051

Hypercassette

Hypercassette, 18 x 24 cm	RPN1642
Hypercassette, 30 X 40 cm	RPN1644
Hypercassette, 5 x 7 inches	RPN1648

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

8.5. Background references

1. WHITEHEAD, T.P. *et al., Clin. Chem.,* **25**, pp.1531-1546, 1979.

2. ROSEWELL, D.F. AND WHITE, E.H., *Meth. Enzymol.*, **57**, pp.409-423, 1978.

3. MURRAY, M.C., BHAVANDAN, V.P. AND DAVIDSON, E.A., *Carbohy. Res.,* **186**, pp255-265, 1989.

4. HOFFMAN, W.L. and JUMP, A.A., *Anal, Biochem.*, **181**, pp.318-320, 1989.

Tech tips available relevant to the ECL glycodetection kit

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

141 Use of the ECL glycoprotein detection system to detect glycoproteins after treatment with peptide-N-glycosidase F.

142 Use of the ECL glycoprotein detection system to detect glycoproteins after treatment with endoglycosidase H.

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

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