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1.0 Purpose

This SOP is used for the immunodetection of proteins that have been electroblotted to membranes from SDS-Page Tris-Glycine Gels or NuPAGE™/Bis-TRIS Gels (***SOP 22101 – SDS-Page Gel Electrophoresis Using Invitrogen Tris-Glycine Gels***, and ***SOP 22176 – SDS-Page Gel Electrophoresis Using the NuPAGE™/Bis-TRIS Gels***). This procedure contributes to the characterization and identification of the target protein.

Protocol #1 is to stain the blot transfer membranes to permit visualization of proteins on the membrane (total protein stain).

Protocol #2 is the immunodetection procedure used to identify specific proteins on a membrane using Alkaline Phosphatase (AP) Conjugates and a NBT (Nitro-Blue Tetrazolium Chloride)/BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt) based detection system.

Protocol #3 is the immunodetection procedure used to identify specific proteins on a membrane using Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP) labeled antibodies with a chemiluminescent detection system.

2.0 Scope

This assay will be performed by Process Analytics/Quality Control (PA/QC) Biopharmaceutical Quality Control (BQC) personnel.

3.0 Authority and Responsibility

3.1 The Director, PA/QC has the authority to define this procedure.

3.2 PA/QC is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BQA).

3.3 PA/QC personnel are responsible for the performance of this procedure.

3.4 PA/QC is responsible for reviewing the data and documenting the results of this procedure.

3.5 BQA is responsible for quality oversight of this procedure.

4.0 Materials and Equipment

4.1 Previously electroblotted membrane.

4.2 RODI water or "water of higher quality."

4.3 Coomassie Brilliant Blue R-250 (BDP PN 30250) or Brilliant Blue R Concentrate (BDP PN 30610), Methanol (BDP PN 30853) and Acetic Acid, Glacial (BDP PN 30860) or reagents of equivalent grade.

4.4 1M TrisHCl, pH 7.4, (BDP PN 30862), 5M NaCl (BDP PN 30863), Tween 20 (BDP PN 30633), BSA (Albumin, Bovine - BDP PN 30222), $MgC12 \cdot 6 H_2O$ (BDP PN 30926), and 1M TRIS-HCl, pH 8.0 (BDP PN 30864) or reagents of equivalent grade.

4.5 Alkaline Phosphatase Substrate Solution (1-Step NBT/BCIP solution – BDP PN 30431) containing Nitroblue Tetrazolium Chloride (NBT) and 5-Bromo-4-chloro-3-indolylphosphate p-toluene Salt (BCIP) or BDP approved equivalent.

4.6 Primary antibody and/or secondary antibody-alkaline phosphatase or Horseradish Peroxidase as appropriate for antigen detection.

4.7 Amersham ECL Advance Western Blotting Detection Kit (BDP PN 30618-1).

Kit Contents:

- ECL Advance Solution A, 50 mL.
- ECL Advance Solution B, 50 mL.
- ECL Advance Blocking Agent, 40 g.

4.8 WesternBreeze® Chemiluminescent Immunodetection Kits (Anti-Mouse BDP PN 30584-1), (Anti-Rabbit BDP PN 30632-1, Anti-Goat BDP PN 30799-1).

Kit Contents:

- Blocker/Diluent A
- Blocker/Diluent B
- Antibody Wash Solution (16X)
- Secondary Antibody Solution
- Chemiluminescent Substrate
- Chemiluminescent Substrate Enhancer

4.9 Shallow trays for incubation of membranes.

4.10 Orbital Shaker.

4.11 Digital Gel Imaging System (BDP MEF# 70770 or BDP approved equivalent).

NOTE: Gloves must be worn at all times during the procedure to prevent contamination of gels and membranes and to prevent exposure to irritants commonly used in electrophoresis and electro-transfer.

All plastic and glass boxes must be thoroughly cleaned before use to avoid staining artifacts. Membranes shall be handled only by the edges. Perform all steps at room temperature (unless otherwise described) and use gentle agitation. If a PVDF membrane is allowed to dry after transfer, wet for 5 seconds in 100% methanol and rinse with water before continuing.

5.0 Protocol 1 - Staining of Protein Immobilized on PVDF or Nylon Membranes

5.1 Staining Solutions

Coomassie Blue Stain: 0.025% (w/v) Coomassie brilliant blue R-250 in 40% methanol. (2.5g Coomassie Blue R-250, 500 mL Methanol, 500 mL H_2O)

If using Brilliant Blue R Concentrate (BDP PN 30610), mix with water to achieve a final volume of 1L.

Destain Solution: 30% methanol/10% acetic acid (600 mL Methanol, 200 mL

Acetic Acid, 1200 mL H_2O).

5.2 Remove the membrane from the transfer sandwich (***SOP 22179 - Western Transfer Blot***) and place it in an incubation dish.

5.3 Stain the membrane with Coomassie Blue Stain for 30 seconds to 5 minutes.

5.4 Destain the membrane with destain solution for 1 to 10 minutes. Watch as the membrane destains as it can be quickly over destained.

5.5 Once dried, the blot is taped to the Form 22101-02 (See **SOP 22101**) for 4-20% Tris-Glycine Gels or Form 22176-02 for 4-12% NuPAGE gels (See **SOP 22176**).

6.0 Protocol 2- NBT/BCIP Immunodetection of Protein Immobilized on Membranes

NOTE: Fill out the PA/QC BQC Immunoblot, NBT/BCIP Analysis Data Sheet, Form 22103-01, while performing this procedure.

6.1 Incubation Buffers

Immunoblot Buffer: - 20 mM Tris-HCl, 500 mM NaCl, pH 7.4 (40 mL of 1M Tris-HCl, 200 mL of 5M NaCl, 1760 mL H_2O).

Immunoblot Buffer with 0.05% Tween 20 – 40 mL of 1M Tris, pH 7.4, 200 mL of 5 M NaCl, 1mL Tween 20, 1760 mL H_2O.

Blocking Buffer: 3% (w/v) BSA in Immunoblot Buffer (1.2 grams of BSA in 40 mL Immunoblot Buffer).

Antibody Buffer: 1% (w/v) BSA in Immunoblot Buffer (0.4 grams BSA in 40 mL Immunoblot Buffer).

Alkaline Phosphatase Buffer: 200 mL Tris-HCl pH 8.0, 40 mL of 5M NaCl, 2.03g of $MgCl2 \cdot 6 H_2O$, bring up to 2L with water. Adjust pH to 9.5 with Sodium Hydroxide. (Final concentration of the buffer will be 0.1 M Tris-HCl pH 8.0, 0.1 M NaCl, 5 mM $MgCl2 \cdot 6 H_2O$.)

NOTE: Record buffer and solution preparation in the QC solution logbook per ***SOP 22702 - Solutions Used in Process Analytics***. Also, record each buffer used along with the corresponding QC solution number on the BQC Immunoblot, NBT/BCIP Analysis Data Sheet, Form 22103-01.

6.2 Remove the membrane from the transfer sandwich (***SOP 22179 - Western Transfer Blot***) and place it in incubation dish.

6.3 Wash membrane 2 times for 10 minutes each with Immunoblot Buffer.

6.4 Block sheet with 3% (w/v) BSA (1.2 g) in Immunoblot Buffer (40 mL) for at least 3 hours. The membrane can be left overnight in this step.

6.5 Incubate with the primary antibody (diluted appropriately with Immunoblot Buffer and 1% BSA) for 2 to 18 hours with shaking. (First antibody dilution is determined empirically but is usually 1:100 to 1:1000 for polyclonal antibodies, 1:10 to 1:100 for hybridoma supernatants, and >1:1000 for murine ascites fluid.) Commercial antibodies generally have a recommended dilution on the Certificate of Analysis.

EX: 1:2,000 dilution = 20 µL of antibody in 40 mL Immunoblot Buffer with 0.4 grams of BSA.

1:20,000 = 2 µL of antibody in 40 mL Immunoblot Buffer with 0.4 grams of BSA.

6.6 Remove the primary antibody solution and wash membrane 3 times for 20 to 60 minutes with Immunoblot Buffer with 0.05% Tween 20.

6.7 Wash the membrane for 10 to 20 minutes with Immunoblot Buffer.

NOTE: If only one antibody (i.e., primary antibody is Alkaline Phosphatase labeled) is required skip to step 6.11.

6.8 Incubate with the second antibody (for example, Goat-anti-rabbit IgG-alkaline phosphatase conjugate) appropriately diluted with 20 mM TRIS-HCl, 500 mM NaCl, 1% BSA, pH 7.5 for at least 2 hours. The membrane can be left overnight in this step.

6.9 Wash the membrane 3 times for 20 to 60 minutes with Immunoblot Buffer with 0.05% Tween 20.

6.10 Wash 10 to 20 minutes with Immunoblot Buffer.

6.11 Wash the membrane with Alkaline Phosphatase (AP) Buffer for 10 to 20 minutes.

6.12 Add ~ 20 mL of Alkaline Phosphatase Substrate Solution (1-Step NBT/BCIP Solution).

6.13 Incubate the membrane with the substrate solution until the color reaction occurs. The reaction can be terminated by washing with water.

6.14 Allow the membrane body to dry on a paper towel.

6.15 Once dried, the blot is taped to the NuPAGE Gel Sheet in **SOP 22176** Form Number 22176-02 or 4-20% Tris-Glycine Gel (See Blue Pre-Stained Standard Form Number 22101-02 (see **SOP 22101**).

NOTE: The above protocol is a general procedure employing a primary antibody, for specific detection of the antigen, and a secondary antibody-alkaline phosphatase conjugate, which will bind the primary antibody and allow color development. There are several permutations that will work with equal efficiency and can be used. For example, if the antigen on the membrane is a murine monoclonal antibody, then no secondary antibody is required and the mouse antibody (i.e., antigen) can be detected directly with goat anti-mouse-alkaline phosphatase.

IT IS IMPERATIVE THAT THE PA/QC IMMUNOBLOT, NBT/BCIP ANALYSIS DATA SHEET, FORM 22103-01 (ATTACHMENT I) BE FILLED OUT WITH DETAILS OF THE PROCEDURE USED. ATTACH RESULTS TO FORM 22002-01 (SOP 22002).

7.0 Protocol 3 – Chemiluminescent Immunodetection of Protein Immobilized on Membranes

NOTE: Fill out the PA/QC Immunoblot, Chemiluminescent Analysis Data Sheet, Form 22103-02, while performing this procedure. All incubation steps are performed at room temperature on an orbital shaker unless stated otherwise.

7.1 Incubation Buffers

Immunoblot Buffer with 0.05% Tween 20 – 40 mL of 1M Tris, pH 7.4, 200 mL of 5 M NaCl, 1mL Tween 20, qs to 2L with H_2O.

Blocking Solution/Antibody Diluent: 100 mL of Blocking Solution is prepared by dissolving 2 grams of ECL Advance Blocking Agent in 100 mL of Immunoblot Buffer with 0.05% Tween 20.

WesternBreeze® Chemiluminescent Western Blot Immunodetection Kits – contains all required buffers and detection components other than a primary antibody. See Attachment 2 for protocol specifics. Record all incubations, washes, antibodies and detection reagents on Form 22103-02.

NOTE: Record buffer and solution preparation in the PA/QC solution logbook per ***SOP 22702 - Solutions Used in Process Analytics***. Alternatively, single use solution preparations not requiring storage (i.e. 2mL Diluent A + 3 mL Diluent B + 5mL Ultrapure H_2O, Kit BDP lot R123199010, exp. 03/18/2026) can be recorded directly on the PA/QC Immunoblot, Chemiluminescent Analysis Data Sheet, Form 22103-02. Record each buffer used along with the corresponding PA/QC solution number on the PA/QC Immunoblot, Chemiluminescent Analysis Data Sheet, Form 22103-02.

7.2 Remove the membrane from the transfer sandwich (***SOP 22179 - Western Transfer Blot***) and place it in an incubation dish containing Immunoblot Buffer with 0.05% Tween 20. Wash the membrane for 10 minutes.

7.3 Immerse the membrane in blocking solution for 1 hour. Alternatively, membranes may be left in blocking solution overnight at 2°-8°C, if more convenient.

7.4 Dilute the primary antibody in antibody diluent. The dilution factor should be determined empirically for each antibody (see recommendation in section 6.5). Commercial antibodies generally have a suggested dilution range listed on the Certificate of Analysis or product data sheet. Record dilution on Form 22103-02.

Incubate membrane in primary antibody for 1-3 hours at room temperature or overnight at 2°-8ºC.

7.5 Wash the membrane with a total of four changes of wash buffer. Typically, 1 wash of 15 minutes followed by 3 washes of 5 minutes each.

NOTE: If only one antibody is required (i.e., primary antibody is HRP labeled) skip to step 7.8.

7.6 Dilute the HRP labeled secondary antibody in antibody diluent. If it is necessary to dilute the antibody in more than one step (example 1:10 followed by a 1:1,000), the first dilution can be made using Immunoblot Buffer with 0.05% Tween 20 (up to 1:1,000), but the final dilution should be made in antibody diluent. The dilution factor should be determined empirically for each antibody.

Incubate the membrane in the diluted secondary antibody for 1-3 hours at room temperature or overnight at 2°-8ºC.

7.7 Wash the membrane with a total of four changes of wash buffer. Typically, 1 wash of 15 minutes followed by 3 washes of 5 minutes each. During the last wash, remove the detection reagents (ECL Advance Solution A and B) from 2°-8ºC storage and equilibrate at room temperature.

7.8 Mix equal volumes of ECL Advance, Solution A and B. Prepare an adequate amount of the mixture to cover the entire surface of the membrane.

7.9 Incubate for 5 minutes at room temperature on an orbital shaker.

7.10 Detected blot images can be captured using an imaging system capable of detecting light output at ~440 nm (ex. Kodak 400 Image System). If using the Kodak 400 Image System, follow ***SOP 22120 - Digital Gel Imaging Using the Kodak 400 Image System***.

7.11 Attach detected western blot image printout to the corresponding gel lane assignment sheet: Form 22101-02 (See **SOP 22101**) for 4-20% Tris-Glycine Gels or Form 22176-02 for 4-12% NuPAGE gels (See **SOP 22176**).

NOTE: The above protocol is a general procedure employing a primary antibody, for specific detection of the antigen, and a secondary antibody-HRP labeled, which will bind the primary antibody and allow for chemiluminescence. There are several permutations that will work with equal efficiency and can be used. For example, if the antigen on the membrane is a murine monoclonal antibody, then no primary antibody is required and the mouse antibody (i.e., antigen) can be detected directly with goat anti-mouse-HRP.

IT IS IMPERATIVE THAT THE PA/QC IMMUNOBLOT, CHEMILUMINESCENT ANALYSIS DATA SHEET, FORM 22103-02 BE FILLED OUT WITH DETAILS OF THE PROCEDURE USED. ATTACH RESULTS TO FORM 22002-01 (SOP 22002).

8.0 References

SOP 22002 *Request for Quality Control Testing.*

SOP 22179 *Western Transfer Blot.*

SOP 22101 *SDS-Page Gel Electrophoresis Using Tris-Glycine Gels.*

SOP 22176 *SDS-Page Gel Electrophoresis Using the NuPAGE™/Bis-TRIS Gels.*

SOP 22702 *Solutions Used in Process Analytics.*

SOP 22120 *Digital Gel Imaging Using the Kodak 400 Image System*

Form 22103-1 Immunoblot Analysis Data Sheet

Form 22103-2 PA/QC Immunoblot, Chemiluminescent Analysis Data Sheet

Invitrogen Western Transfer Apparatus Instructions (Revised Protocol 7-95). Towbin, H. et al, Proc. Natl. Acad. Sci. U.S.A. 76: 4350-4354.

Current Protocols in Immunology.

Electrophoresis Chapter 10 in Current Protocols in Protein Science, 1995.

9.0 Attachments

9.1 **Attachment 1** Amersham ECL Western blotting detection reagents and analysis system

9.2 **Attachment 2** WesternBreeze Chemiluminescent Western Blot Immunodetection Kit

ATTACHMENT 1

GE Healthcare

Amersham ECL Western blotting detection reagents and analysis system

Product Booklet

Codes: RPN2106/8/9
RPN2209
RPN2134

Attachment 1 (continued)

Page finder

Attachment 1 (continued)

1. Legal

http://www.gelifesciences.com

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

Attachment 1 (continued)

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.

Attachment 1 (continued)

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

Attachment 1 (continued)

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.

Attachment 1 (continued)

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.

Attachment 1 (continued)

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.

Attachment 1 (continued)

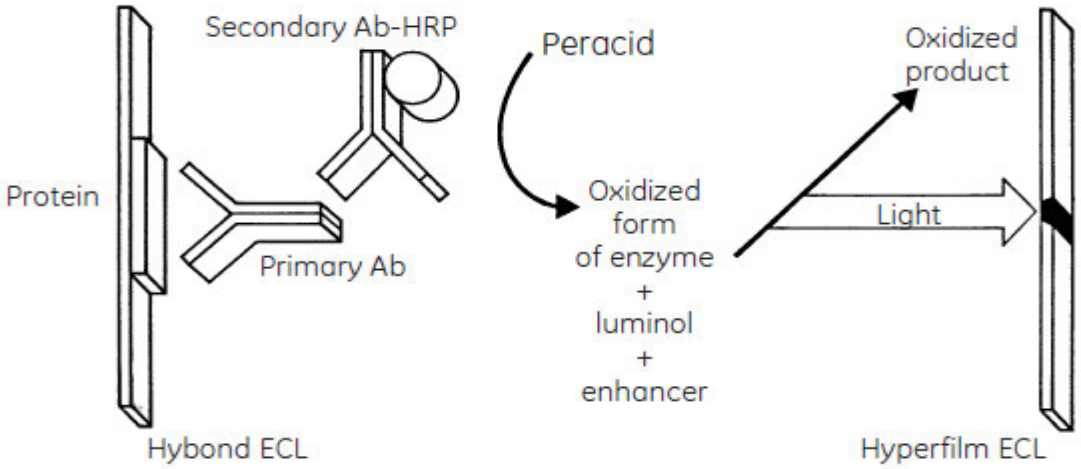


Figure 1. Principles of ECL Western blotting

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

Attachment 1 (continued)

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.

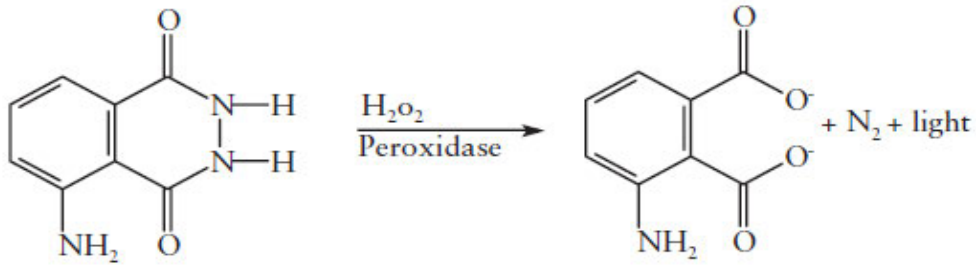


Figure 2.

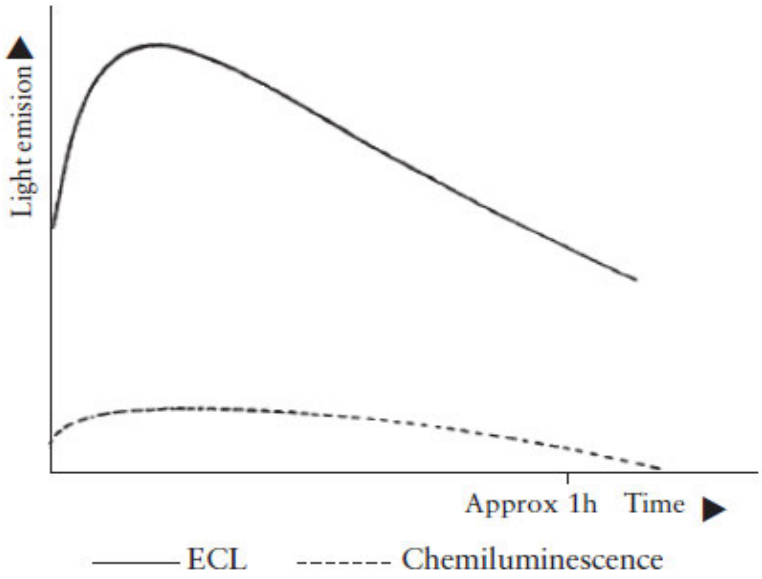


Figure 3. Graph of light emission versus time, showing the difference between chemiluminescence and ECL.

Attachment 1 (continued)

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.

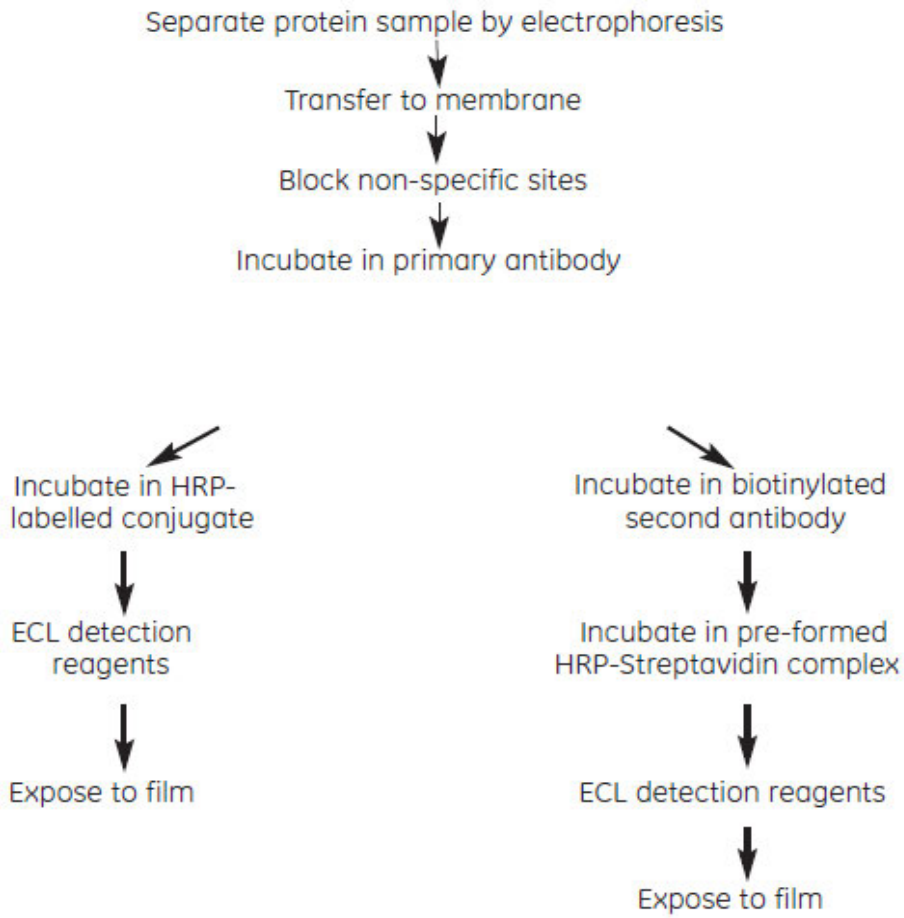
Attachment 1 (continued)

- 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.

Attachment 1 (continued)

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.

Attachment 1 (continued)

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

Attachment 1 (continued)

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/cm² of wash buffer for 15 minutes at room temperature.	

Attachment 1 (continued)

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/cm² 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.

Attachment 1 (continued)

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.	

Attachment 1 (continued)

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/cm² 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).

Attachment 1 (continued)

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.

Attachment 1 (continued)

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

Attachment 1 (continued)

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

Attachment 1 (continued)

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.

Attachment 1 (continued)

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.

Attachment 1 (continued)

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

Attachment 1 (continued)

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.

Attachment 1 (continued)

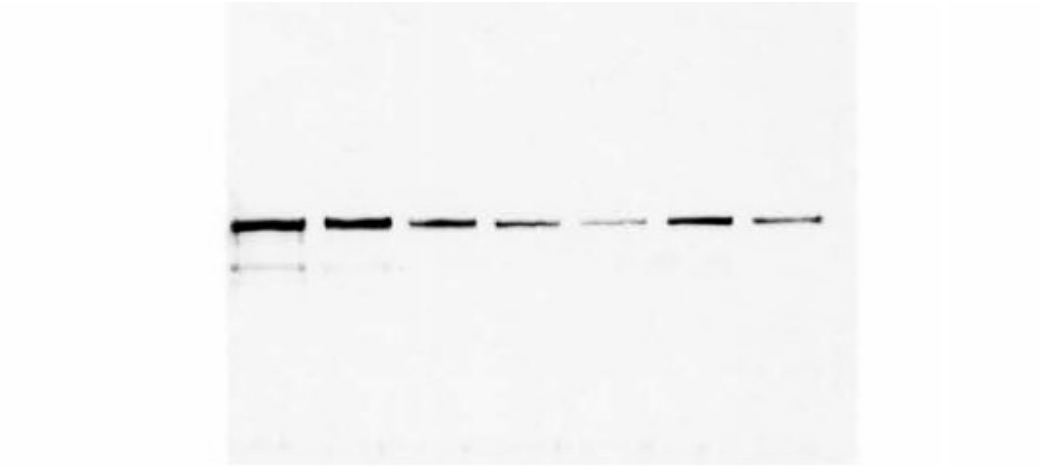


Figure 4. ECL detection of myosin standard curve and myosin test samples.

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

Attachment 1 (continued)

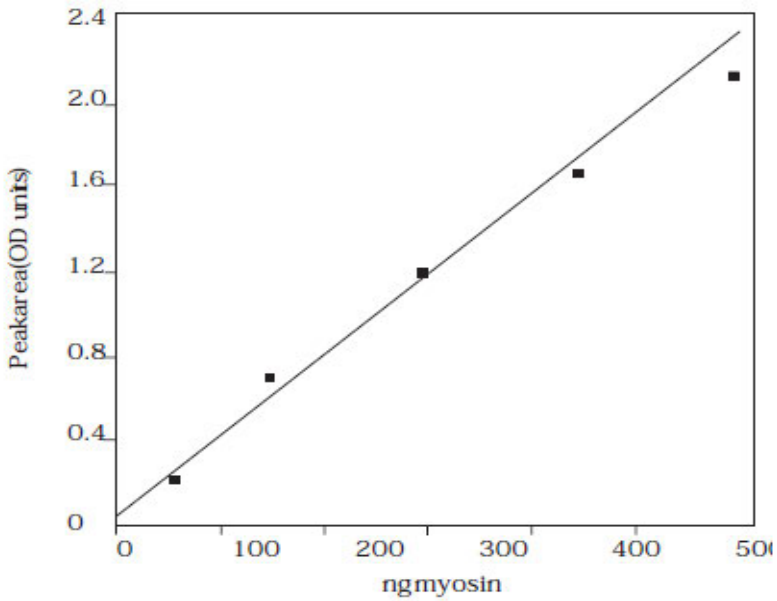


Figure 5. Peak area (OD units) against myosin concentration

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

Attachment 1 (continued)

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

Attachment 1 (continued)

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,

Attachment 1 (continued)

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.

Attachment 1 (continued)

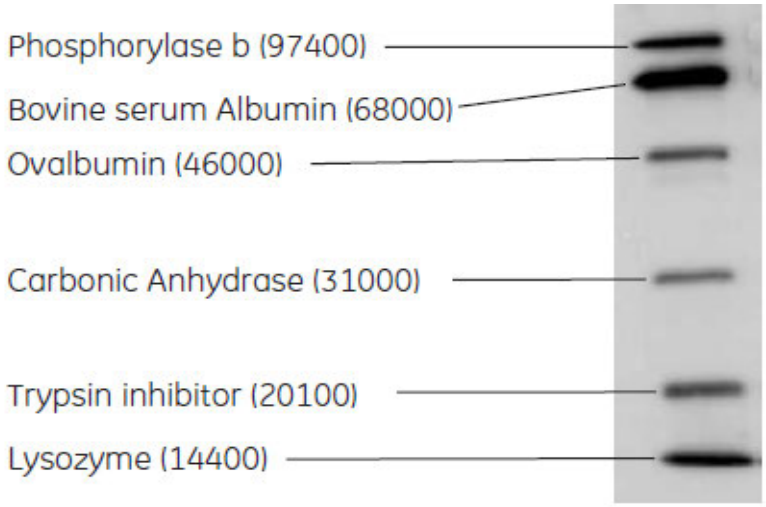


Figure 6. Profile of ECL protein molecular weight markers.

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.

Attachment 1 (continued)

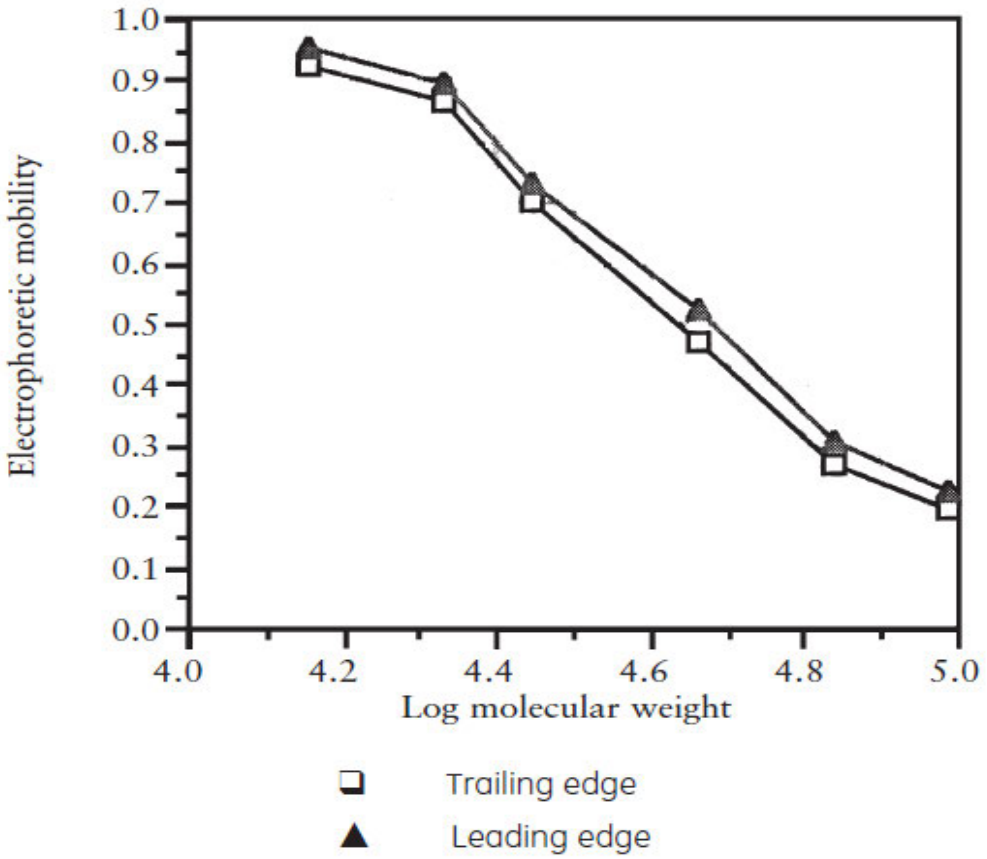


Figure 7. ECL protein molecular weight markers calibration line.

Attachment 1 (continued)

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.

Attachment 1 (continued)

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.

Attachment 1 (continued)

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.

Attachment 1 (continued)

10. References

1. Isaccson, U. and Watermark, G., *Anal. Chim. Acta.* **68**, 339–362 (1974).

2. Whitehead, T. P. *et al., Clin. Chem.* **25**, 1531–1546 (1979).

3. Rosewell, D. F. and White, E. H., *Methods in Enzymology.* **57**, Deluca, M. A. (Ed). Academic Press, New York, pp.409–423 (1978).

4. Motsenbocker, M. A., *J. Biolum. Chemilum.* **2**, 9–16 (1968).

5. Kaufmann, S. H. *et al., Anal. Biochem.* **161**, 89–95 (1987).

6. Laemmli U.K., *Nature.* **227**, pp.680-685 1970.

7. Hoffman, W. L. and Jump, A. A., *Anal. Biochem.* **181**, 318–320 (1989).

8. Towbin, H. and Gordon, J., *J. Immunol. Meth.* **72**, 313–340 (1984).

Related references (electrophoresis and blotting)

9. Gershoni, J. M. and Palade, G.E., *Anal. Biochem.* **131**, 1–15 (1983).

10. Peluso, R.W. and Rosenberg, G.H., *Anal. Biochem.* **162**, 389–398 (1987).

11. Towbin, H. *et al., Proc. Natl. Acad. Sci. (USA)* **76**, 4350–4354 (1979).

12. Gershoni, J. M. and Palade, G.E., *Anal. Biochem.* **124**, 396–405 (1982).

13. Burnette, W. M., *Anal. Biochem.* **112**, 195–203 (1981).

14. Bittner, M. *et al., Anal. Biochem*, **102**, 457–471 (1980).

15. Lin, W. and Kasamatsu, H., *Anal. Biochem.* **128**, 302–311 (1983).

16. Andrews, A. T., *Electrophoresis: Theory, Techniques and Biochemical and Clinical Applications*, Second Edition in Monographs on Physical Biochemistry, edited by A.R. Peacock and W. R. Harrington, Oxford Science Publications, (1986).

17. Johnstone, A. and Thorpe, R., *Immunochemistry in Practice.*, Blackwell Science Publications, (1982).

18. Life Science. **7**, 12–13, Amersham International plc, (1992).

Attachment 1 (continued)

11. Related products

SDS-PAGE electrophoresis chemicals

See the complete range in the catalogue

Low-Range Rainbow™ MW Markers, natural, 45, 30, 20.1, 14.3, 6.5, 3.5 and 2.5 kDa	RPN755
High-Range Rainbow MW Markers, natural, 220, 97, 66, 45, 30, 20.1 and 14.3 kDa	RPN756
Full-Range Rainbow MW Markers, recombinant, 250, 160, 105, 75, 50, 35, 30, 25, 15 and 10 kDa	RPN800
ECL Western Blotting MW Markers, biotinylated. 97, 66, 45, 30, 20.1 and 14.3 kDa	RPN2107
Hybond ECL Membrane (Nitrocellulose, pore size 0.45 µm) 20 × 20 cm, pack of 10 sheets	RPN2020D
Hybond ECL Membrane (Nitrocellulose, pore size 0.2 µm) 30 cm × 3 m, 1 roll	RPN3032D
Hybond-P Membrane (PVDF, pore size 0.45 µm) 20 × 20 cm, pack of 10 sheets	RPN2020F
Hybond-P Membrane (PVDF, pore size 0.45 µm) 20 cm × 3 m, 1 roll	RPN203F
Hybond-C Extra Membrane (Supported nitrocellulose, pore size 0.45 µm) 20 × 20 cm, pack of 10 sheets	RPN2020E
Hybond Blotting Paper 20 × 20 cm, pack of 100 sheets	RPN6101M
ECL Blocking Agent, 40 g	RPN2125
Mouse IgG, Horseradish Peroxidase-linked Whole Antibody (from Sheep), 1 ml and 100 µl	NA931
Human IgG, Horseradish Peroxidase-linked Whole Antibody (from Sheep), 1 ml	NA933

Attachment 1 (continued)

Rabbit IgG, Horseradish Peroxidase-linked Whole Antibody (from Donkey), 1 ml and 100 µl	NA934
Rat IgG, Horseradish Peroxidase-linked Whole Antibody (from Goat), 1 ml	NA935
Mouse IgG, Horseradish Peroxidase-linked Whole Antibody (from Sheep), general purpose screening reagent, 1 ml	NXA931
Mouse IgG, Horseradish Peroxidase-linked F(ab´)$_2$ Fragment (from Sheep), 1 ml	NA9310
Human IgG, Horseradish Peroxidase-linked F(ab´)$_2$ Fragment (from sheep), 1 ml	NA9330
Rabbit IgG, Horseradish Peroxidase-linked F(ab´)$_2$ Fragment (from Donkey), 1 ml	NA9340
Rat IgG, Horseradish Peroxidase-linked F(ab´)$_2$ Fragment (from Goat), 1 ml	NA9350
Mouse IgG, Biotinylated Whole Antibody (from Sheep), 2 ml	RPN1001
Human IgG, Biotinylated Whole Antibody (from Sheep), 2 ml	RPN1003
Rabbit IgG, Biotinylated Whole Antibody (from Donkey), 2 ml	RPN1004
Rat IgG, Biotinylated Whole Antibody (from Goat), 2 ml	RPN1005
Immunoprecipitation Starter Pack	17-6002-35
Streptavidin Biotinylated Horseradish Peroxidase Complex	RPN1051
Streptavidin Horseradish Peroxidase Conjugate	RPN1231
Anti-GST, Horseradish Peroxidase Conjugate	RPN1236

Attachment 1 (continued)

ECL Plus™ Western Blotting Detection Reagents (sufficient for 1000 cm² membrane)	RPN2132
ECL Plus Western Blotting Detection Reagents (sufficient for 3000 cm² membrane)	RPN2133
ECL PLus Western Blotting Reagent Pack **Does not contain detection reagents** Anti-mouse IgG, HRP-linked whole antibody (from sheep), 100 µl Anti-rabbit IgG, HRP-linked whole antibody (from donkey), 100 µl Blocking reagent, 5 g Sufficient for 10 blots, 10 × 10 cm	RPN2124
ECL Glycoprotein Detection Module 25 membrane reactions *Order ECL Detection Reagents separately*	RPN2190
ECL Protein Biotinylation Module *Order ECL Detection Reagents separately*	RPN2202
ECL Protein Biotinylation System Sufficient for 2000 cm² membrane	RPN 2203
ECL Phosphorylation Module Sufficient for 25 blots *Order ECL Detection Reagents separately*	RPN2220
GST Western Blotting Detection Kit Sufficient for 2000cm² membrane	RPN1237
Hypercassette	
18 × 24 cm	RPN11642
30 × 40 cm	RPN11644
10 × 12 inches	RPN11650
5 × 7 inches	RPN11648

Attachment 1(continued)

Hypertorch™, red Darkroom Torch	RPN1620
Sensitize Pre-flash Unit	RPN2051
Hyperfilm ECL	
Pack of 25 films, 18 × 24cm	RPN2103K
Pack of 25 films, 30 × 40 cm	RPN2104K
Pack of 25 films, 10 × 12 inches	RPN1681K
Pack of 25 films, 5 × 7inches	RPN1674K
Hyperprocessor™ Automatic Film Processor *(Not available in all countries)*	
220/240 V	RPN1700
110/120 V	RPN1700A
ECL Mini-camera	RPN2069
Imagemaster VDS-CL, CCD Camera	18-1130-55

For further details see the GE Healthcare catalogue or contact your local office.

Attachment 1 (continued)

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http://www.gelifesciences.com

GE imagination at work

28955347

RPN2106PL AD 06/2009

Attachment 1 (continued)

Amersham
Western blotting protocol summary
Product protocol card

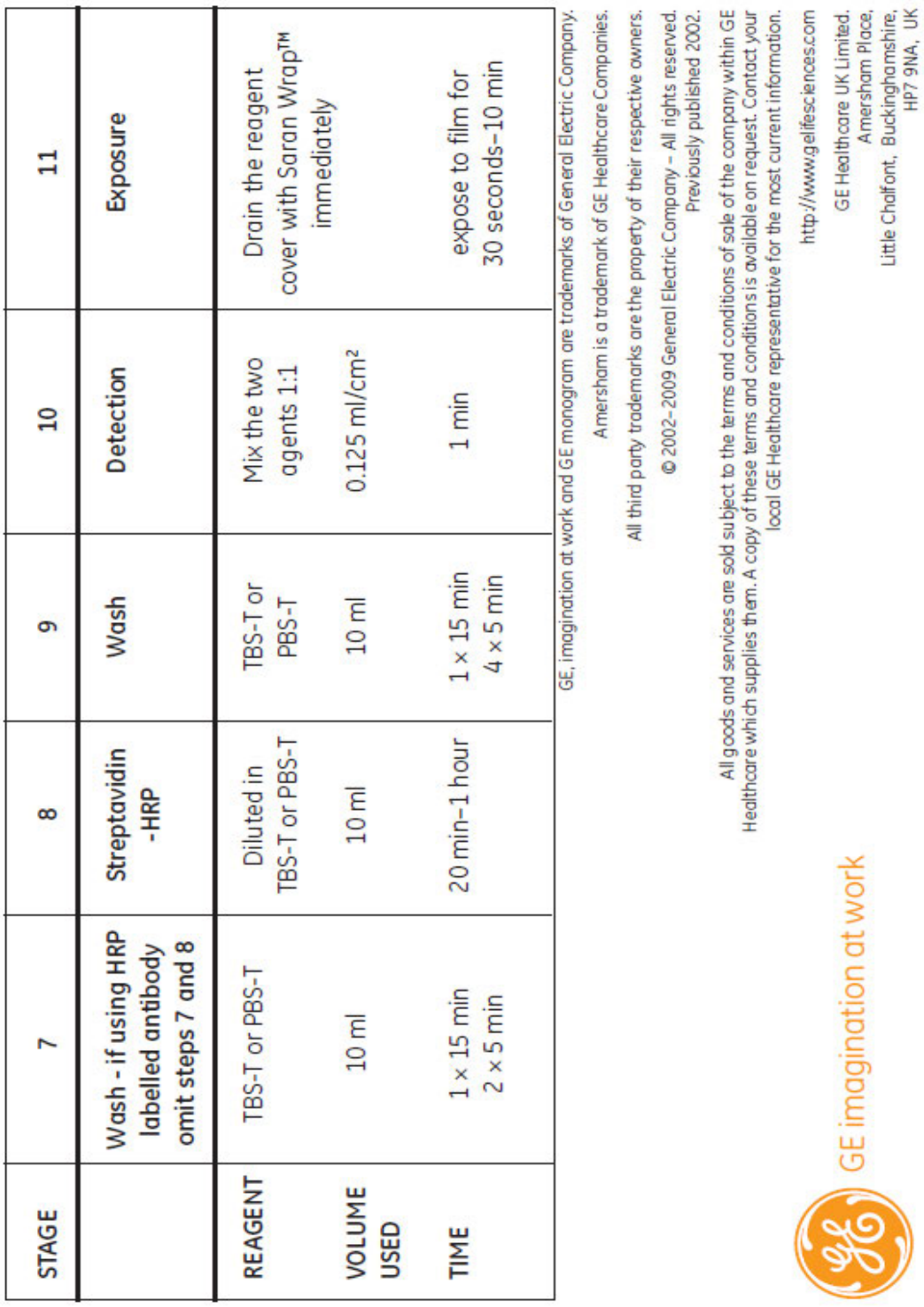
RPN2106/8/9, RPN2209, RPN2134

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 x 15 min 2 x 15 min	1 hour	1 x 15 min 2 x 5 min	20 min–1 hour

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.

RPN2106PC AD 06/2009

Attachment 1 (continued)



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 x 15 min 2 x 5 min	20 min–1 hour	1 x 15 min 4 x 5 min	1 min	expose to film for 30 seconds–10 min

Attachment 2

Protocol Pub. No. MAN0007885 Rev.1.0

WesternBreeze® Chemiluminescent Western Blot Immunodetection Kit

invitrogen by life technologies

Print Options

Package Contents

Catalog Numbers
WB7104 Anti-Mouse
WB7106 Anti-Rabbit
WB7108 Anti-Goat

Contents list

Storage Conditions

- Store all solutions at 4°C.
- Once diluted, use the solutions the same day.
- All solutions are proprietary formulations and contain chlorobutanol as a preservative.

Required Materials

List of Materials

Timing

Preparation and immunodetection: ~3 hours

Selection Guide

Western Blotting Kits
Go online to view related products.

Product Description

- WesternBreeze® Chemiluminescent Kits detect proteins that have been immobilized on membranes (nitrocellulose or PVDF) following western transfer or bound directly from solution (dot blots).
- Detection is accomplished with a ready-to-use CDP-Star® chemiluminescent substrate for alkaline phosphatase.
- Protein bands can be captured either by X-ray film or a CDP-Star® compatible imaging system.

Important Guidelines

- Avoid touching the working surface of the membrane, even with gloves.
- Use ultra-filtered water, free from alkaline phosphatase activity. Do not allow the membrane to dry out after adding the chemiluminescent substrate.
- Use a rotary shaker platform rotating at 1 revolution/second for all washing, blocking, and incubating steps.
- Add solutions to the trays slowly, at the membrane edge, to avoid bubbles forming under the membrane. Decant from the same corner of the dish to ensure complete removal of previous solutions.

Online Resources

Visit our product pages for additional information and protocols. For support, visit www.lifetechnologies.com/support.

For Research Use Only. Not for use in diagnostic procedures.

Protocol Outline

This process involves the following phases:

A. Prepare membrane and solutions.
B. Incubate membrane with Primary and then Secondary Antibodies.
C. Add and incubate substrates.
D. Complete detection.

Western Blotting Protocol

See page 2 for quantities and guidelines to prepare the antibodies and solutions.

See page 3 to view a typical immunodetection procedure for use with small membranes (60 cm²).

Preparing the Membrane

For Westerns or Western dot blots, wash membrane twice for 5 minutes in 20 mL of water to remove gel, transfer buffer, and weakly bound proteins. Then, proceed with immunodetection.
For water washed and dried membranes:

- NC Membranes: Proceed with immunodetection.
- PVDF membranes: Re-wet membrane in methanol followed by two 20-mL water washes for 5 minutes.

Scaling up for Large Membranes

To blot standard size gels (~200 cm²), scale up the required solution volumes × 3.3. Use a tray that closely matches the dimensions of the membrane for the most efficient use of the solutions.

Limited Product Warranty and Disclaimer Details

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Attachment 2 (continued)

WesternBreeze® Chemiluminescent Western Blot Immunodetection Kit Protocol Rev. 1.0

Preparing Solutions for Nitrocellulose and PVDF Membranes

The Blocking Solution is used for blocking and as a Primary Antibody Diluent for NC membranes. For western blots from Novex® or other mini gels, prepare solutions as described in table below for ~60 cm^2 membrane.

Steps	Procedure Details			
	For NC Membrane		For PVDF Membrane	
Prepare Blocking Solution	Ultra filtered Water	14 mL	Ultra filtered Water	5 mL
	Blocker/Diluent (Part A)	4 mL	Blocker/Diluent (Part A)	2 mL
	Blocker/Diluent (Part B)	2 mL	Blocker/Diluent (Part B)	3 mL
	Total Volume	20 mL	Total Volume	10 mL
Prepare Primary Antibody Diluent	For NC Membrane		For PVDF Membrane	
	Dilute your Primary Antibody according to the manufacturers recommendations into 10 mL of NC Blocking Solution (see above). Typically, commercial Primary Antibody preparations are diluted 1:1000 to 1:5000 to a concentration of about 1 to 0.2 µg/mL.		Ultra filtered Water	7 mL
			Blocker/Diluent (Part A)	2 mL
			Blocker/Diluent (Part B)	1 mL
			Total Volume	10 mL
			Dilute your Primary Antibody into this diluent according to manufacturer's recommendations.	
Prepare Antibody Wash	For NC Membrane		For PVDF Membrane	
	Ultra-filtered Water	150 mL	Ultra-filtered Water	150 mL
	Antibody Wash Solution (16X)	10 mL	Antibody Wash Solution (16X)	10 mL
	Total Volume	160 mL	Total Volume	160 mL
Prepare Chemiluminescent Substrate	For NC Membrane		For PVDF Membrane	
	Chemiluminescent Substrate	2.375 mL	Use the CDP-Star directly from the bottle. DO NOT add the Chemiluminescent Substrate enhancer (Nitro-Block-II enhancer).	
	Chemiluminescent Substrate Enhancer	0.125 mL		
	Total Volume	2.5 mL	Total Volume	2.5 mL

For support, visit www.lifetechnologies.com/support.

Attachment 2 (continued)

WesternBreeze® Chemiluminescent Western Blot Immunodetection Kit Protocol Rev. 1.0

Immunodetecting with the Chemiluminescent Western Blot Kit

The table below describes the procedure for immunodetection using small membranes (60 cm²).

Timeline	Steps	Procedure Details
Day 1, 1	**Block membrane**	Use 10 mL of blocking solution in the dish provided. Incubate 30 minutes on a rotary shaker (1 revolution/second). Decant the Blocking Solution.
2	**Wash membrane with water**	Rinse the membrane with 20 mL of water for 5 minutes, then decant. Repeat once.
3	**Incubate with Primary Antibody**	Incubate the membrane with 10 mL of Primary Antibody Solution for 1 hour, then decant.
4	**Wash membrane with Antibody Wash**	Wash membrane for 5 minutes with 20 mL of prepared Antibody Wash, then decant. Repeat 3 times.
5	**Incubate with Secondary Antibody**	Incubate the membrane in 10 mL of Secondary Antibody Solution for 30 minutes, then decant.
6	**Wash membrane with Antibody Wash**	Wash the membrane for 5 minutes with 20 mL of Antibody Wash, then decant. Repeat 3 times.
7	**Wash membrane with water**	Rinse the membrane with 20 mL of water for 2 minutes, then decant. Repeat twice.
8	**Add substrate**	Place the membrane on sheet of transparency plastic. Do not allow membrane to dry out. Evenly apply 2.5 mL of Chemiluminescent Substrate Solution to the membrane without touching the surface (remember to add Chemiluminescent Substrate Enhancer for NC membranes).
9	**Incubate with substrate**	Allow reaction to develop for 5 minutes at room temperature.
10	**Detect**	Blot excess Chemiluminescent Substrate from membrane using filter paper in the kit. Do not allow membrane to dry out. Cover membrane with another piece of transparency plastic. Expose x-ray film. for one second to several minutes, or use another imaging device.

10 June 2013

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