cytiva

Amersham
ECL Prime Western Blotting Detection Reagent

Product Booklet

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1 Introduction

Product codes

RPN2232

RPN2236

Important

Read these instructions carefully before using the products.

Intended use

Amersham™ECL™ Prime detection reagent is intended for chemiluminescent detection in Western blotting. The products are intended for research use only, and shall not be used in any clinical or *in vitro* procedures for diagnostic purposes.

Safety

For use and handling of the products in a safe way, refer to the Safety Data Sheets.

Quality control

Amersham ECL Prime detection reagent is manufactured in compliance with our ISO 9001 certified quality management system, and is in conformity with the acceptance criteria set up for the product.

Storage

On receipt, all components should be stored at 18–30°C. Amersham ECL Prime detection reagent is sensitive to prolonged exposure to light. Always store the individual reagents in the light-tight containers in which they are provided.

Expiry

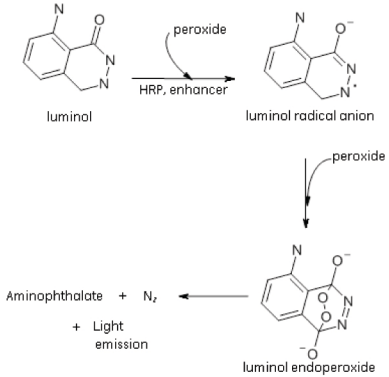
The components are stable for at least 3 months when stored under the recommended conditions. See expiry date on package.

2 Description

Amersham ECL Prime Western Blotting detection reagent from Cytiva provides high sensitivity for chemiluminescent detection of immobilized specific antigens conjugated to Horseradish Peroxidase (HRP) labeled antibodies.

Background

Chemiluminescence is defined as light emission produced in a multistep reaction whereby peroxidase catalyzes the oxidation of luminol. In the presence of chemical enhancers and catalysts, the light intensity and the duration of light emission is greatly increased in a process known as enhanced chemiluminescence (ECL). ECL based on horseradish peroxidase (HRP)-conjugated secondary antibodies is a sensitive detection method where the light emission is proportional to protein quantity. The multi-step reaction is illustrated below.



Design and features

Amersham ECL Prime detection reagent is designed to provide long signal duration (24 hours) and high sensitivity (picogram levels). The high intensity light output means that highly diluted antibodies can be used, and makes detection optimal using a CCD camera (e.g., ImageQuant™ LAS systems from Cytiva). The light output can also be detected using autoradiography film (e.g., Amersham Hyperfilm™ product range).

Membrane compatibility

Amersham ECL Prime detection reagent is optimized for use with Amersham Hybond™ PVDF membrane where the performance compared to standard chemiluminescent substrates is most enhanced, but is also compatible with Amersham Protran nitrocellulose membrane.

3 Required components

Kit components

The following components are included in the Amersham ECL Prime detection reagent kit.

RPN2232

Solution A: Luminol solution, 50 mL

Solution B: Peroxide solution, 50 mL

Sufficient for 1000 cm^2 membrane

RPN2236

Solution A: Luminol solution, 3 × 50 mL

Solution B: Peroxide solution, 3 × 50 mL

Sufficient for 3000 cm^2 membrane

Solutions

Required solutions are listed below.

- Phosphate buffered saline (PBS), pH 7.5
- Tris buffered saline (TBS), pH 7.6
- Dilution and wash buffer: PBS Tween™ (PBS-T) and TBS Tween (TBS-T).

A Tween 20 concentration of 0.1% is suitable for most blotting applications.

Membrane

Use a suitable protocol to separate proteins by electrophoresis and transfer them to a PVDF or nitrocellulose membrane.

Blocking reagents

Blocking reagents are typically diluted to 2% to 5% (v/v) in PBS-T or TBS-T buffer. The following blocking reagents are recommended:

- Amersham ECL Prime Blocking Agent
- Amersham ECL Blocking Agent
- Non-fat dry milk
- Bovine Serum Albumin (BSA)

Immunodetection reagents

- Primary antibody specific to the target protein(s)
- HRP conjugated secondary antibody specific to the primary antibody. See *Amersham ECL HRP-linked secondary antibodies, on page 28*.

Dilute the antibodies in PBS-T or TBS-T according to the recommendations in *Dilution ranges, on page 13*.

4 Western blotting optimization

Introduction

To achieve an optimal Western blotting result with high signal to noise ratio and best possible sensitivity and linearity, it is important to optimize the method and to select compatible products.

Consider the following:

- **Sample quality and loading amount** – It is important that the sample is of good quality and that detectable levels of target protein is present.
- **Membrane and blocking** – Select membranes and blocking agents compatible with sample and antibodies.
- **Primary and secondary antibodies** – Always select specific antibodies of high quality and optimize the antibody dilution.
- **Detection and imaging** – Select detection reagent according to your application need. A CCD imager offers high sensitivity and broad dynamic range and provide better quantification than X-ray film.

This chapter describes products recommended for use with Amersham ECL Prime detection reagent.

Molecular weight markers

Molecular weight markers are used to determine protein size. In addition, pre-stained markers allow confirmation of protein transfer and orientation (as the colored bands transfer to the membrane).

- Amersham Rainbow™ Markers are pre-stained multicolored markers for monitoring progress of protein electrophoresis, confirming transfer efficiency and determination of molecular weight of blotted proteins.

- Amersham ECL DualVue™ Markers are markers optimized for use with Amersham ECL, Amersham ECL Prime and Amersham ECL Select and contains a combination of pre-stained and tagged proteins markers. These markers enable monitoring of electrophoresis, confirming transfer efficiency and determination of molecular weight of blotted proteins without staining on gel and membrane, as well as in chemiluminescence detection.

Membranes

- **Amersham Hybond** are PVDF membranes with high protein binding capacity and mechanical strength, which makes them ideal for Western blotting applications where stripping and re-probing are needed. The membrane is optimal for use with Amersham ECL Prime and Amersham ECL Select detection reagents.
- **Amersham Protran** are nitrocellulose membranes compatible with all chemiluminescent Western blotting substrates. The main advantage is the normally low background.

Transfer

- **Wet transfer** is the most commonly used transfer method. It provides efficient transfer of small to large proteins.
- **Semi-dry transfer** is faster than wet transfer and consumes less buffer. Semidry transfer works well for most proteins but transfer may be less efficient for large proteins. It might have reduced sensitivity for very low abundance proteins.

Blocking

After protein transfer the membrane need to be incubated in a blocking solution to prevent non-specific binding of antibodies, which can cause background and non-specific protein bands on the blot. The blocking agent should be optimized for best results, no single blocking agent is optimal for all proteins and antibodies. Cytiva recommend the following blocking agents compatible with Amersham ECL, Amersham ECL Prime and Amersham ECL Select:

- Amersham ECL Prime Blocking Agent
- Amersham ECL Blocking Agent
- BSA Blocking Agent
- Non-fat dry milk

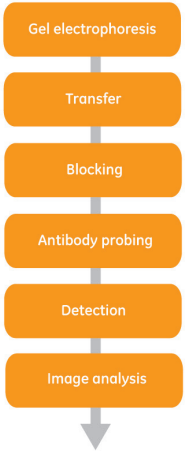
Western blotting handbook

More technical help, tips, and best practices can be found in the handbook Western Blotting Principles and Methods from Cytiva (product code 28999897).

5 Protocol

Protocol overview

Below is an overview of the Western blotting detection protocol.



Electrophoresis and transfer

Step	Action
1	Perform electrophoresis and transfer proteins to a suitable membrane according to standard protocols. Blots are preferably used immediately but may be stored in PBS-T or TBS-T at 2°C to 8°C. ***Note:*** *Amersham Hybond PVDF membranes should be prewetted in 100% methanol prior to equilibration in transfer buffer.*
2	Proceed with the next part of the protocol.

Blocking

Step	Action
1	Incubate the membrane in a suitable blocking solution on an orbital shaker for 1 hour at room temperature or overnight at 2°C to 8°C.
2	Briefly rinse the membrane with two changes of wash buffer. ***Note:*** *For preparation of wash buffer, see* *Solutions, on page 6**.*
3	Proceed with the next part of the protocol.

Antibody probing

Due to the improved sensitivity of the Amersham ECL Prime detection reagent, optimization of antibody concentrations is recommended to ensure the best results. The optimal dilution varies between antibodies depending on affinity and quality. Optimization of the antibody dilution can be performed by dot blot analysis (see *Determination of optimum antibody concentration, on page 20*).

Dilution ranges

The following dilution ranges are recommended:

Antibody	Dilution range from 1 mg/mL stock solution
Primary	1:1000 - 1:30 000
Secondary	1:50 000 - 1:200 000

The table below shows suggested starting dilutions for primary antibodies with different levels of affinity.

Type of antibody	Primary antibody dilution	Secondary antibody dilution
High affinity primary antibodies	1:5000	1:50 000
Medium to low affinity primary antibodies	1:3000	1:30 000

Primary antibody incubation

Step	Action
1	Dilute the primary antibody in PBS-T or TBS-T.

Step	Action
2	Incubate the membrane in the primary antibody solution on an orbital shaker for 1 hour at room temperature or overnight at 2°C to 8°C.
3	Briefly rinse the membrane with two changes of wash buffer.
4	Wash the membrane 4 to 6 times in wash buffer for 5 minutes each at room temperature on an orbital shaker. ***Note:*** *Exposure to X-ray film requires 6 wash steps, to avoid background.*

Secondary antibody incubation

Step	Action
1	Dilute the secondary antibody (HRP conjugated or biotinylated antibody) in PBS-T or TBS-T. ***Note:*** *Increase the light output by building a three layer sandwich using biotinylated secondary antibodies and HRP conjugated streptavidin.*
2	Incubate the membrane in the secondary antibody solution for 1 hour at room temperature on an orbital shaker.
3	Briefly rinse the membrane with two changes of wash buffer.

Step	Action
4	Wash the membrane 4 to 6 times in wash buffer for 5 minutes each at room temperature on an orbital shaker. ***Note:*** *Exposure to film requires 6 wash steps, to avoid background.*

If using an HRP conjugated secondary antibody, proceed directly to *Detection, on page 16*.

If using a biotinylated antibody, proceed with the streptavidin bridge incubation protocol which follows.

Streptavidin bridge incubation

Step	Action
1	Dilute the Streptavidin HRP conjugate or Streptavidin biotinylated HRP complex in PBS-T or TBS-T.
2	Incubate the membrane in the diluted solution for 1 hour at room temperature on an orbital shaker.
3	Briefly rinse the membrane with two changes of wash buffer.
4	Wash the membrane by suspending it in enough wash buffer to cover the membrane and agitate for 5 minutes at room temperature. Replace wash buffer at least 4 to 6 times.

Detection

Step	Action
1	Mix detection solutions A (luminol) and B (peroxide) in a ratio of 1:1 to a working solution. The final volume of detection reagent required is 0.1 mL/cm^2 membrane. ***Note:*** *If the mixed reagent is not to be used immediately, protect it from exposure to the light.*
2	Drain excess wash buffer from the washed membrane and place it protein side up in a suitable box or on a sheet of plastic wrap or other suitable clean surface. Add detection reagent onto the membrane and make sure it completely covers the membrane.
3	Incubate for 5 minutes at room temperature.
4	Drain off excess detection reagent by holding the membrane edge gently against a tissue.
5	Proceed with the next part of the protocol.

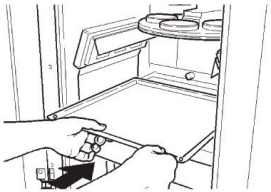
Image analysis

Two protocols for image analysis are described, one for CCD camera based imaging and another using X-ray film.

CCD camera

Step	Action
1	Place the blot, protein side up on a sample tray.

Step	Action
	Note: *The blot can be placed on a piece of plastic wrap, protein side up, to facilitate easy movement of the film on the sample tray.*
2	Place the sample tray in the CCD camera compartment and select suitable exposure time and/or function.



Note:
Use the automatic exposure function or select exposure time manually. Recommended starting exposure time is 60 seconds. Increase or decrease exposure time depending on the obtained signal intensity.

X-ray film

Step	Action
1	Place the blot with protein side down on to a fresh piece of plastic wrap, wrap the blots and gently smooth out any air bubbles.
2	Place the wrapped blot with protein side up in an X-ray film cassette. ***Note:*** *Make sure there is no free detection reagent in the cassette; the film must not get wet.*
3	Place a sheet of X-ray film (Amersham Hyperfilm product range) on top of the membrane. Close the cassette and allow exposure. Suitable exposure start time is 1 minute. ***Note:*** *This stage should be carried out in a dark room using red safe lights. Do not move the film while it is being exposed.*
4	Develop the film immediately and, on the basis of the signal intensity obtained, estimate exposure time for a second sheet of film.

6 Additional information

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 and reprobed several times.

Step	Action
1	Place the membrane in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) and incubate at 50°C for 30 minutes with occasional agitation. ***Note:*** *If more stringent conditions are required, the incubation can be performed at 70°C or for a longer time.*
2	Wash the membrane for 3 × 10 minutes in PBS-T or TBS-T at room temperature using large volumes of wash buffer. The membrane may be used immediately or stored in PBS-T or TBS-T at 2 to 8°C. ***Note:*** *Membranes may be incubated with Amersham ECL Prime detection reagent and exposed to film or imaged by CCD camera to ensure removal of antibodies.*
3	Block the membrane in a suitable blocking solution for 1 hour at room temperature.
4	Repeat the immunodetection protocol, from *Antibody probing, on page 13* to *Image analysis, on page 16*.

Determination of optimum antibody concentration

Due to the high sensitivity of the Amersham ECL Prime detection reagent, optimization of antibody concentrations is recommended to ensure the best results. In general, lower concentrations of both primary and secondary antibodies are required with Amersham ECL Prime detection reagent compared to standard chemiluminescent substrates. Outlined below are protocols for determining optimal antibody concentrations.

Dilution ranges

The following dilution ranges are recommended:

Antibody	Dilution range from 1 mg/mL stock solution
Primary	1:1000 - 1:30 000
Secondary	1:50 000 - 1:200 000

Primary antibody optimization

Dot blotting is a quick and effective method of determining the optimum dilution of a primary antibody. 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 as follows.

Step	Action
1	Spot different amounts of protein sample, preferably a dilution series, on to a PVDF or nitrocellulose membrane and allow to air dry. Alternatively, use a dot blot or slot blot manifold. Make one sample series for each dilution to be tested.

Step	Action
	Note: *PVDF membranes must be pre-wetted in methanol.*
2	Incubate in blocking solution for 1 hour at room temperature with agitation.
3	Rinse the membranes briefly with two changes of wash buffer.
4	Cut the membrane to get each sample series on a separate membrane strip.
5	Prepare different primary antibody solutions within the recommended antibody range. Incubate each membrane strip in antibody solution for 1 hour at room temperature with agitation.
6	Briefly rinse the membrane with two changes of wash buffer. Wash the membrane by suspending it in wash buffer and agitate for 5 minutes in room temperature. Replace wash buffer at least 4 to 6 times.
7	Dilute the secondary antibody (using only one concentration) and incubate the membranes for 1 hour at room temperature with agitation.
8	Rinse blots in two changes of wash buffer, then wash 4 to 6 times in fresh changes of wash buffer.

Step	Action
9	Detect using Amersham ECL Prime detection reagent detailed in *Detection, on page 16*. The antibody dilution which gives the best signal with the minimum background should be selected.

Secondary antibody optimization

Step	Action
1	Spot different amounts of protein sample, preferably a dilution series, on to a PVDF or nitrocellulose membrane and allow to air dry. Alternatively, use a dot blot or slot blot manifold. Make one sample series for each dilution to be tested. ***Note:*** *PVDF membranes must be pre-wetted in methanol.*
2	Incubate in blocking solution for 1 hour at room temperature with agitation.
3	Incubate in diluted primary antibody (optimized concentration) for 1 hour at room temperature with agitation.
4	Briefly rinse the membrane with two changes of wash buffer. Wash the membrane by suspending it in wash buffer and agitate for 5 minutes in room temperature. Replace wash buffer at least 4 to 6 times.
5	Cut the membrane to get each sample series on a separate membrane strip.

Step	Action
6	Prepare different secondary antibody solutions within the recommended antibody range. Incubate each membrane strip in antibody solution for 1 hour at room temperature with agitation.
7	Briefly rinse the membrane with two changes of wash buffer. Wash the membrane by suspending it in wash buffer and agitate for 5 minutes in room temperature. Replace wash buffer at least 4 to 6 times.
8	Detect using Amersham ECL Prime detection reagent detailed in *Detection, on page 16*. The antibody dilution which gives the best signal with minimum background should be selected.

Troubleshooting guide

Problems	Possible causes / remedies
No signal	• Non-detectable amounts of target protein. • Primary antibody is not binding to target protein, which may be due to bad quality and/or unspecific primary antibody. • Incorrect species of secondary antibody has been used. • PVDF membrane not pre-wetted in methanol. • Check that transfer equipment is working properly and that the correct procedure has been followed. • Check protein transfer by staining the membrane and/or gel. • Confirm transfer efficiency by using pre-stained Rainbow marker. • Some proteins may be affected by the treatments required for electrophoresis. • Detection reagents do not function properly. – To test the detection reagent activity, in a darkroom prepare 1 to 2 mL of detection reagent working solution in a clear test tube. Add 1 µL of undiluted HRP-conjugated antibody solution. The solution should immediately emit a visible blue light that fades during the next several minutes. • Incorrect storage of Amersham ECL Prime detection reagent may cause a loss of signal. Bacterial growth inhibit the reagent.
Weak signal	• Transfer efficiency may have been poor. • Insufficient protein was loaded on to the gel. • The concentration of primary and secondary antibodies could be too low; optimization is required. • Bad quality and/or unspecific primary antibody. • Exposure time may have been too short.

Problems	Possible causes / remedies
Excessive, diffuse signal or rapidly fading signal	• Too much protein was loaded on to the gel. • The concentrations of primary and secondary antibodies could be too high; optimization is required.
White (negative) bands on the film	• Negative bands generally occur when protein target is in excess and antibody concentrations are too high. The effect is caused by substrate depletion. - Load less amount of protein. - Dilute both primary and secondary antibody further.
Uneven, spotted background	• Areas of the blot may have dried during some of the incubations. • Incorrect handling can lead to contamination on the blots and/or membrane damage which may cause non-specific signal. • The blocking agent is not completely dissolved in the buffer. • Insufficient washing. - Add additional washing steps.

Problems	Possible causes / remedies
High backgrounds	• Too high concentrations of primary and/or secondary antibodies; optimization is required. • Insufficient washing. - Use sufficient amount of wash buffer and add additional washing steps. • Transfer and incubation buffers may have become contaminated and require replacing. - Always use fresh solutions • Insufficient blocking. • The blocking agent used was not freshly prepared, was too diluted or was incompatible with the application. • The level of Tween used in the blocking agent was not sufficient for the application performed. • The membrane was allowed to dry during some of the incubations. • Poor gel quality. • Unspecific and bad quality of antibodies. • The film detection of the signal was allowed to over expose. • The level of signal is so high that the film has become completely overloaded. • Non compatible products.

7 Related products

This chapter presents a subset of related products. For more information, refer to *cytiva.com*.

Blotting equipment

Product	Quantity	Product code
Amersham Protran Supported 0.2 NC (20 × 20 cm)	25 sheets	10600053
Amersham Protran Supported 0.2 NC (8 × 9 cm)	25 sheets	10600099
Amersham Hybond LFP 0.2 PVDF (20 × 20 cm)	10 sheets	10600060
Amersham Hybond LFP 0.2 PVDF (8 × 9 cm)	25 sheets	10600102
Amersham Hybond 0.2 PVDF (20 × 20 cm)	25 sheets	10600057
Amersham Hybond 0.2 PVDF (8 × 9 cm)	25 sheets	10600101

Amersham ECL HRP-linked secondary antibodies

Product	Quantity	Product code
Amersham ECL Mouse IgG, HRP-Linked Whole Ab (from sheep)	1 mL	NA931-1ML
Amersham ECL Human IgG, HRP-Linked Whole Ab (from sheep)	1 mL	NA933-1ML
Amersham ECL Rabbit IgG, HRP-Linked Whole Ab (from donkey)	1 mL	NA934-1ML
Amersham ECL Mouse IgG, HRP-Linked $F(ab)_2$ fragment (from sheep)	1 mL	NA9310-1ML
Amersham ECL Rabbit IgG, HRP-Linked $F(ab)_2$ fragment (from donkey)	1 mL	NA9340-1ML
Streptavidin- Horseradish Peroxidase Conjugate	100 µL	RPN1231-100UL

Detection reagents

Product	Quantity	Product code
Amersham ECL Prime Western Blotting Detection Reagent	for 1000 cm^2	RPN2232
Amersham ECL Prime Western Blotting Detection Reagent	for 3000 cm^2	RPN2236

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