GE Healthcare
Life Sciences

Amersham ECL Advance Western Blotting Detection Kit

Product Booklet

Code: RPN2135

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1. Legal

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2. Handling

2.1. Safety warnings and precautions

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

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

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 for at least 3 months when stored under the recommended conditions.

The ECL Advance reagents are sensitive to prolonged exposure to light. Long term storage of the individual reagents should be in the light tight containers in which they are provided.

3. Components

ECL Advance Solution A
Solution containing Tris buffer in 3.2% (v/v) Ethanol, 50 ml.

ECL Advance Solution B
Proprietary substrate in Tris buffer, 50 ml.

Sufficient for 1000 cm^2 membrane.

ECL Advance Blocking Agent
40 g reagent for use as block and antibody diluent.

To be used at 0.2 ml/cm^2 (20 ml per 10 cm x 10 cm blot).

3.1. Other materials required

Solutions required
The chemical reagents required for these solutions are available from GE Healthcare and are detailed in the current catalogue.

Phosphate buffered saline (PBS) pH7.5*
11.5 g di-Sodium Hydrogen Orthophosphate anhydrous (80 mM)
2.96 g Sodium Dihydrogen Orthophosphate (20 mM)
5.84 g Sodium Chloride (100 mM)

Dissolve in distilled water and make up total volume to 1000 ml. Check pH

Tris buffered saline (TBS) pH7.6*
8 g Sodium Chloride
20 ml 1 M Tris HCl, pH 7.6.
Dissolve in distilled water and make up total volume to 1000 ml. Check pH

Wash Buffer
PBS-Tween (PBS-T) or TBS-Tween (TBS-T)
Dilute the required amount of Tween™ 20 in the corresponding buffer.
A 0.1% (v/v) Tween 20 concentration is suitable for most blotting applications.

Blocking solution and antibody diluent
ECL Advance Blocking Agent (supplied with kit)

1. Shake the powdered block to ensure even distribution of components

2. Weigh out the appropriate amount of block for a 2% (w/v) solution, add PBS-T or TBS-T (see above), shake vigorously and stir for 15 minutes until all components are fully dissolved. Prepared blocking solution and antibody diluent can be stored at 2–8°C but should be used within 24 hours.

***Storage of buffers**

All buffers should be stable for at least 3 months if prepared in advance and stored at room temperature, although storage at 2–8°C may be necessary to avoid microbial spoilage. Do not use Sodium Azide as a bacteriocide.

Reagents required

Immunodetection reagents (for example, primary and secondary antibodies).

All antibodies should be diluted in the antibody diluent, as prepared on page 5.

The ECL Advance blocking agent should not be used as a diluent for Streptavidin HRP incubations.

4. Description

The ECL Advance™ Western blotting detection kit from GE Healthcare provides an extremely sensitive non-radioactive method for the detection of immobilised antigens using Horseradish Peroxidase (HRP) labelled antibodies.

This system builds on the combined strengths of GE Healthcare and Lumigen Inc., in developing high performance detection products. Using Lumigen® TMA-6, a proprietary new substrate for horseradish peroxidase, ECL Advance provides a more intense light output at 440 nm which can be readily imaged using CCD imagers as well as with film.

The intense and sensitive signal produced with these substrates enable reduced antibody concentrations to be employed. This is a benefit where antibody supply is scarce. The new membrane block controls background noise whilst retaining a high level of signal intensity. The system is fully compatible with both Hybond™ ECL (nitrocellulose) and Hybond-P (PVDF) membranes.

Horseradish Peroxidase present on a Western blot is detected by its reaction with Peroxide, an enhancer molecule and the novel chemiluminescent substrate, all of which are present in the substrate reagents provided.

The sensitivity increase over ECL Plus Western blotting can be up to 10 fold, depending on the immunodetection system being used.

5. Critical parameters

- Read the entire protocol thoroughly before using the kit.
- ECL Advance will provide an increase in sensitivity over ECL Plus on both nitrocellulose and PVDF membranes.
- ECL Advance is an extremely sensitive system. For results showing the best signal to noise ratio, it is essential to optimise the concentrations of both primary and secondary antibodies. Higher dilutions of antibodies are likely to be required when using ECL Advance in place of ECL Plus Western blotting reagents.
- During immunodetection, a sufficient volume of solution should be used to adequately cover the membrane. Containers should be agitated gently on a mixer platform.
- The use of ECL Advance Blocking Agent is strongly recommended for membrane blocking and all antibody diluents in order to reduce non-specific binding and background noise resulting from the high sensitivity of this reagent. **Ensure the powdered blocking agent is shaken thoroughly before use.**

NOTE: The ECL Advance blocking agent should not be used as a diluent for streptavidin HRP incubations.

- When washing, the volume of wash buffer should be as large as possible; 4 ml of buffer per cm^2 of membrane is suggested. Brief rinses of the membrane in wash buffer before incubating will improve washing efficiency.
- If exposure times of less than 5 seconds are routinely required, it is recommended that the antibodies used are further diluted as it is difficult to perform such short exposures.
- The 'working mix' of ECL Advance reagents is stable for up to 1 month stored at 2–8°C. Therefore, the reagents can be mixed and stored before use or mixed immediately before use. If mixing

and storing the reagents, the container used should be similar to those provided. If the container is not light tight, a covering of foil should be used and the container should be stored at 2–8°C in the dark.

- Accidental freezing of the substrates will not cause degradation. In the event that freezing occurs, thaw the solutions, mix well and store as recommended.

6. Protocol

6.1. Electrophoresis and blotting

Protocol	Notes
1. Perform electrophoresis and blotting according to usual techniques. Proteins should be transferred to Hybond-P PVDF or Hybond ECL for optimum results. Blots may be used immediately or stored in a desiccator at 2–8°C for up to 3 months.	**1.** Hybond-P PVDF should be pre-wetted in 100% (v/v) Methanol, washed in distilled water for 5 minutes and equilibrated in transfer buffer for at least 10 minutes before blotting. Hybond ECL should be pre-wetted in distilled water and equilibrated in transfer buffer for at least 10 minutes before blotting. ECL Advance is also suitable for use with supported nitrocellulose such as Hybond C-Extra, this membrane should be prepared as for Hybond ECL.

6.2. Blocking the membrane

Protocol	Notes
1. Prepare sufficient blocking solution and antibody diluent (as described on page 5) for use as block and antibody diluent.	**1.** The combination of ECL Advance Blocking Agent and Tween 20 should be sufficient for most applications. Optimum Tween 20 concentrations will vary to suit specific experiments, but 0.1% (v/v) Tween 20 is suitable for most blotting applications.
2. Block non-specific binding sites by immersing the membrane in blocking solution (see 6.2 step 1), for 1 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.	
3. Briefly rinse the membrane with two changes of wash buffer. (see page 5).	

6.3. Primary antibody incubation

Protocol	Notes
1. Dilute the primary antibody in antibody diluent (see 6.2 step 1). The dilution factor should be determined empirically for each antibody.	**1.** Optimisation of the antibody dilution can be performed by dot blot analysis, (see page 18).
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 optimised for each antibody. The conditions indicated are recommended starting points.
3. Briefly rinse the membrane with two changes of wash buffer and then wash the membrane in >4 ml/cm^2 of wash buffer for 15 minutes at room temperature.	
4. Wash the membrane for 3 x 5 minutes with fresh changes of wash buffer at room temperature.	**4.** While washing prepare the diluted secondary antibody (see 6.4. step 1).

6.4. Secondary antibody incubation

Protocol	Notes
1. Dilute the HRP labelled secondary antibody or biotinylated antibody in antibody diluent (see 6.2. step 1). **If it is necessary to dilute the antibody in 2 lots of diluent, the first dilution can be made using PBS-T or TBS-T (up to 1:1000), but the final dilution should be made in antibody diluent.** The dilution factor can be determined empirically for each antibody (see pages 18–20).	**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 optimised for each antibody. The conditions indicated are recommended starting points.
3. Briefly rinse the membrane with two changes of wash buffer and then wash the membrane in >4 ml/cm^2 of wash buffer for 15 minutes at room temperature.	

Protocol	Notes
4. Wash the membrane for 3 x 5 minutes with fresh changes of wash buffer at room temperature.	**4.** If using an HRP labelled secondary antibody proceeddirectly to 6.6. (detection) after this wash procedure. If using a biotinylated antibody, while washing, prepare the diluted Streptavidin HRP conjugate or complex (6.5. step 1)

6.5. 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 18–20).
2. Incubate the membrane in the dilution for 15 minutes at room temperature on an orbital shaker.	
3. Briefly rinse the membrane with two changes of wash buffer and then wash the membrane in >4 ml/cm^2 of wash buffer for 15 minutes at room temperature.	
4. Wash the membrane for 3 x 5 minutes with fresh changes of wash buffer at room temperature.	

6.6. Detection

Protocol	Notes
1. Remove the detection reagents from storage at 2–8°C and allow to equilibrate to room temperature before opening.	
2. Mix detection solutions A and B in a ratio of 1:1 (for example, 2 ml solution A + 2 ml solution B) or use premixed solution. The final volume of detection reagent required is 0.1 ml/cm^2.	**2.** If the mixed reagent is not to be used immediately protect it from exposure to the light either by wrapping in foil or storing in a dark place.
3. Drain the excess wash buffer from the washed membranes and place **protein side up** on a sheet of SaranWrap™ or other suitable clean surface. Pipette the mixed detection reagent on to the membrane.	**3.** The reagents should cover the entire surface of the membrane, held by surface tension on to the surface of the membrane.
4. Incubate for 5 minutes at room temperature.	
5. 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	**5.** Close the SaranWrap around the membrane to form an envelope or use an alternative, suitable detection pocket. Avoid applying pressure on to the membrane.

Protocol	Notes
5. *Continued.* SaranWrap, wrap up the blots and gently smooth out any air bubbles.	
6. Place the wrapped blots, protein side up, in an X-ray film cassette.	**6.** Ensure there is no free detection reagent in the cassette, as the film must not get wet.
7. Place a sheet of autoradiography film (for example, Hyperfilm™ECL) on top of the membrane. Close the cassette and expose for 15 seconds.	**7.** This stage should be carried out in a dark room using red safe lights. Do not move the film while it is being exposed.
8. 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.	**8.** 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 x 77 mm. Images can also be acquired using a CCD camera such as the Imagemaster™ VDS-CL (18-1130-55).

7. Additional information

7.1. Stripping and reprobing membranes

The complete removal of primary and secondary antibodies from the membrane is possible following the protocol outlined below. The membranes may be stripped of bound antibodies and reprobed several times. Membranes should be stored wet wrapped in SaranWrap in a refrigerator (2–8°C) after each immunodetection.

Protocol	Notes
1. Submerge the membrane in stripping buffer (100 mM 2-Mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HC1 pH 6.7) and 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 incubate for a longer time.
2. Wash the membrane for 2 x 10 minutes in PBS-T or TBS-T at room temperature using large volumes of wash buffer.	**2.** Membranes may be incubated with ECL Advance detection reagents and exposed to film to ensure removal of antibodies.
3. Block the membrane in blocking solution (see page 5) for 1 hour at room temperature.	
4. Repeat the immunodetection protocol, main protocol stages 3 to 6.	

7.2. Determination of optimum antibody concentration

Due to the improved sensitivity of the ECL Advance detection reagents, optimisation of antibody concentrations is recommended to ensure the best results. In general, lower concentrations of both primary and secondary antibodies are required with ECL Advance compared to ECL Plus Western blotting reagents.

Outlined below are protocols for determining optimal antibody concentrations.

Protocol	Notes
1. Primary antibodies Dot blots are a quick and effective method of determining the optimum dilution of a primary antibody of unknown concentration. Alternatively, a Western blot can be prepared and then cut into several strips. It should be noted that some antibodies may require alternative blocking and washing steps to the following suggestions.	**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. **2.** Incubate in blocking solution (see page 5) for 1 hour at room temperature with agitation. **3.** Rinse the membranes briefly with two changes of wash buffer. **4.** Prepare several dilutions of primary antibody in antibody diluent: e.g. For both nitrocellulose and PVDF membranes: 1/10 000, 1/25 000, 1/50 000,

Protocol	Notes
	4. *Continued.* 1/75 000, 1/100 000. Incubate 1 blot in each dilution for 1 hour at room temperature with agitation.
	5. Rinse blots in two changes of wash buffer, then wash for 1 x 15 minutes and 3 x 5 minutes in fresh changes of wash buffer.
	6. Dilute the secondary antibody (using only one concentration) in antibody diluent and incubate the membranes for 1 hour at room temperature with agitation.
	7. Wash as detailed in step 5.
	8. Detect using ECL Advance detection reagents as detailed in section 6.6. of the main protocol. The antibody dilution which gives the best signal with the minimum background should be selected.
2. Secondary antibodies	**1.** Prepare dot blots and block the membranes as detailed in steps 1 and 2.

Protocol	Notes
	2. Dilute the primary antibody (using only one concentration) in antibody diluent and incubate the membranes for 1 hour at room temperature with agitation. **3.** Wash as detailed in step 5 of protocol 1. **4.** Prepare several dilutions of secondary antibody in antibody diluent: e.g. For both nitrocellulose and PVDF membranes: 1/50 000 1/100 000, 1/250 000, 1/500 000 Incubate 1 blot in each dilution for 1 hour at room temperature with agitation. **5.** Wash as detailed in step 5 of protocol 1. **6.** Detect using ECL Advance detection reagents as detailed in 6.6. of the main protocol. The antibody dilution which gives the best signal with minimum background should be selected.

Protocol	Notes
3. Streptavidin bridge incubation	**1.** Optimisation of the streptavidin bridge incubation can be performed following the primary/secondary antibody optimisation protocols. It is most likely that all steps will require the use of higher dilutions. Suggested starting concentrations are 1:100 000 – 1:500 000. Alternatively less protein target can be used.

8. Troubleshooting guide

Problems	Possible causes and solutions
1. No signal	**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 Advance detection reagents may have become contaminated.
	6. Incorrect storage of the ECL Advance detection reagents may cause a loss of signal.
2. Weak signal	**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.
3. Excessive diffuse signal	**1.** Too much protein was loaded onto the gel.
	2. Electrophoresis and transfer protocols may need optimization.

Problems	Possible causes and solutions
3. Excessive diffuse signal *continued.*	**3.** The concentrations of primary and secondary antibodies could be too high; optimization is required.
4. White (negative) bands on the film	**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.
5. Uneven, spotted backgrounds	**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.
6. High backgrounds	**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.

Problems	Possible causes and solutions
6. High backgrounds *continued*	**5.** The level of Tween used in the blocking agent was not sufficient for the application performed. **6.** The membranes were blocked for an insufficient time. **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. **13.** The membrane was allowed to dry during one of the incubations.

9. Quality control

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

10. Related products

GE Healthcare offers a comprehensive range of Western blotting reagents and hardware all with proven compatibility to ensure reproducible high quality results. For a complete listing of products available see the current GE Healthcare catalogue or visit our web site at www.gehealthcare.com/lifesciences

ECL Western Blotting Detection Reagents For 4000 cm^2 Membrane	RPN2106
ECL Plus Western Blotting Detection Reagents For 1000 cm^2 Membrane Other pack sizes and detection reagents also available	RPN2132
ECL DualVue™ Western Blotting Markers	RPN810
Low-Range Rainbow™ MW Markers	RPN755
High-Range Rainbow MW Markers	RPN756
Full-Range Rainbow MW Markers (Recombinant)	RPN800
ECL Western Blotting MW Markers	RPN2107
Hybond-P Membrane (PVDF, Pore Size 0.45 µm)	RPN2020F
Hybond-ECL Membrane (Nitrocellulose, Pore Size 0.45 µm)	RPN303D
Other membrane sizes also available Hyperfilm ECL 18 x 24, pack of 25 films Other film sizes are also available	RPN2103K
Streptavidin-Biotinylated Horesradish Peroxidase Complex	RPN1051
Streptavidin Horseradish Peroxidase Conjugate	RPN1231
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
Rabbit IgG, Horseradish Peroxidase-linked Whole Antibody (from Donkey), 1 ml and 100 µl	NA934

11. References

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28992069

RPN2135PL AF 02-2011