ECL phosphorylation detection system

RPN 2220/1

STORAGE

Store at -15°C to -30°C
Store blocking reagent at 2-8°C

STABILITY

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

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.

Amersham Biosciences

COMPONENTS OF THE SYSTEM

ECL phosphorylation detection module
RPN 2220

Anti-phosphotyrosine-HRP conjugate **500µl**
Horseradish peroxidase conjugate of PY20 antibody clone supplied in 0.05M sodium phosphate buffer containing 0.1% chloroacetamide and 40% ethylene glycol.

Concentration: 50µg in 500µl

Origin: Clone PY20 is a mouse monoclonal generated by immunising Balb/c mice with the phosphotyrosine hapten conjugated to a carrier protein and fusing with NS-1. Resulting hybridomas were selected for reactivity with the phosphotyrosine hapten by ELISA.

Isotype: IgG2b

Species reactivity: Tested positive with human, mouse and rat. No other species tested.

Membrane blocking reagent **10g**

ECL phosphorylation detection system
RPN 2221

Components

ECL phosphorylation detection module	RPN 2220
ECL Western blotting detection reagents (sufficient for 2000cm^2 membrane)	RPN 2209

SAFETY WARNINGS AND PRECAUTIONS

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

Warning: Contains ethylene glycol and chloroacetamide solution. See safety data sheets on pages 30-33.

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 safety data sheets for specific advice).

Note the assay protocol requires the use of acrylamide.
Warning: acrylamide is toxic.
Note the assay protocol requires the use of SDS.
Warning: SDS is an irritant.
Note the assay protocol requires the use of mercaptoethanol.
Warning: mercaptoethanol is harmful.
Note the assay protocol requires the use of methanol.
Warning: methanol is toxic and inflammable.
Note the assay protocol requires the use of ammonium persulphate.
Warning: ammonium persulphate is harmful and an irritant.
Note the assay protocol requires the use of N,N,N',N'-tetramethylethylenediamine (TEMED).
Warning: TEMED is harmful and an irritant.

Please refer to the manufacturers safety data sheets relating to the safe handling and use of these materials.

DESCRIPTION

Protein phosphorylation[1] is a major mechanism by which protein function in eukaryotic cells is regulated. This important, reversible post translational event has two main functions: controlling the cellular responses to extracellular signals and control of cell cycle events. Antibodies, from various clones that recognise phosphorylated tyrosine residues have been extensively used in such studies. The PY20 clone[2] (IgG_{2b}) was analysed in comparison with other anti-phosphotyrosine antibodies and showed to have the highest affinity for phosphotyrosine with the least reactivity with other phosphoamino acids. This increase in affinity is due to a rare V-D-D-J-C joining[3] which was proven by Meek *et al.*[4]

The ECL™ phosphorylation detection system from Amersham Biosciences uses the horseradish peroxidase conjugate of the PY20 antibody clone to directly detect tyrosine-phosphorylated proteins without the need for secondary antibody reagents. Its use in combination with ECL reagents offers a highly sensitive system.

CRITICAL PARAMETERS

- The use of Tris buffered saline rather than phosphate buffered saline is recommended for maximum sensitivity.
- Use of milk based blocks should be avoided as they may cause high backgrounds due to their content of tyrosine phosphorylated proteins.
- 0.1% Tween™ 20 is recommended in blocking, antibody and wash buffers to avoid high backgrounds.
- The anti-phosphotyrosine-HRP conjugate may be initially viscous upon removal from the freezer. Pipetting accuracy may be aided by cutting off the end of the pipette tip.

ADDITIONAL EQUIPMENT AND SOLUTIONS REQUIRED

Equipment

Adjustable pipettes
Sterile pipette tips
Standard laboratory glassware
Orbital shaker
Polypropylene microcentrifuge tubes
Microcentrifuge
Boiling water bath
Gloves, preferably powder-free
Plastic boxes for incubating blots during immunodetection
Forceps with non-serrated tips (for handling blots)
Timer
SaranWrap™
Gel electrophoresis equipment (tanks, power units, etc)
Filter paper
Blotting membrane

Solutions

Buffer preparation
Buffers should be stable for at least 3 months unless otherwise stated.

1M Tris.HCl pH 7.5
Dissolve 121.1g of Tris base in 900ml of distilled water. Adjust the pH to 7.5 using concentrated HCl . Make up the total volume to 1000ml with distilled water. Store at 2-8°C.

Tris buffered saline (TBS) pH 7.5
10ml 1M Tris.HCl pH 7.5 (10mM)
5.8g sodium chloride (100mM)
Dilute to 1000ml with distilled water - check pH.
Store at 2-8°C.

TBS Tween (TBS-T)
Use for wash buffer, block diluent and antibody diluent. Add 1ml of Tween 20 to 1000ml of TBS.
Store at room temperature.

Blocking buffer (1% BSA)
Add 0.2g of block reagent to 20ml of TBS-T. Prepare freshly on day of use.

USE OF THE ECL PHOSPHORYLATION DETECTION SYSTEM

Immunodetection

During immunodetection, sufficient solution should be used to adequately cover the membrane. The containers should be agitated gently on a mixer platform. When washing, the volume of wash buffer should be as large as possible; 4ml of buffer per cm^2 of membrane is suggested. Brief rinses of the membrane before incubating in wash buffer will improve washing efficiency. All steps should be carried out at room temperature.

Protocol	Notes
1) Performing electrophoresis and blotting Separate the proteins using SDS-PAGE electrophoresis and electroblot on to Hybond™ PVDF or nitrocellulose membrane as recommended by the equipment manufacturers.	**1)** If using PVDF membrane it should be pre-wetted in methanol for 5 seconds, rinsed in water for 5 minutes to remove the methanol, then equilibrated in transfer buffer for 10-15 minutes.
2) Blocking the membrane Remove the membranes from the apparatus and trim the edges to prevent a background outline. Non-specific binding sites are blocked by immersing the membrane in blocking buffer (see page 8)	**2.1)** The use of milk based block reagents may give elevated background due to the presence of tyrosine phosphorylated proteins. **2.2)** Omission of Tween 20 will lead to reduced signal: noise.

Protocol	Notes
for one hour on an orbital shaker at room temperature. **3) Washing** Briefly rinse the membrane twice with fresh changes of TBS-T to remove excess block.	**2.3**) Alternatively, membranes may be left in the blocking solution overnight at 2-8°C.
4) Dilution of the anti-phosphotyrosine-HRP conjugate. During the blocking step dilute the conjugate 1:1000 in TBS-T.	**4**) 1:1000 is the recommended dilution of conjugate but this concentration can be increased or decreased to suit the application. The inclusion of 1% block in the conjugate incubation is not normally necessary but may improve signal: noise in some circumstances.
5) Incubation Incubate the membrane in the diluted conjugate for 1 hour on an orbital shaker at room temperature.	**5**) The presence of high salt levels will inhibit the binding of the antibody[3].

6) Washing Briefly rinse the membrane twice with fresh changes of TBS-T, then wash 3 times - once for 15 minutes and twice for 5 minutes with fresh changes of washing buffer on an orbital shaker at room temperature.	

Detection with ECL reagents

Read through this whole section before proceeding. It is necessary to work quickly once the membranes have been exposed to the detection solution. All steps can be carried out in a dark room; it is only necessary to switch off the light after step 5). Equipment needed are an X-ray film cassette, a roll of SaranWrap, a timer and autoradiography film such as Hyperfilm™ ECL RPN 2103.

The use of gloves is strongly recommended from this stage to prevent hand contact on film or detection reagents. If possible wear powder-free gloves as the powder can inhibit the ECL detection reagents leading to blank patches on the film.

Protocol	Notes
1) Mix equal volumes of detection solution 1 and detection solution 2 to give sufficient reagent to cover the membrane. 2) Drain the excess wash buffer from the membrane and place it on SaranWrap protein side up. Add the detection reagents to the protein side of the membrane so that the reagents are held by surface tension on	1) The final volume required is 0.125ml/cm^2 membrane.

the surface of the membrane; do not allow the surface of the membrane to become uncovered. 3) Incubate for precisely 1 minute at room temperature, without agitation.	
4) Drain off excess detection reagents and wrap membrane in a fresh piece of SaranWrap™. Gently smooth out air pockets.	**4**) Drain off excess detection reagent by holding the membrane vertically and touching the edge of the membrane against tissue paper. Gently place the membrane, protein side down, on to SaranWrap. Close the SaranWrap to form an envelope avoiding pressure on the membrane.
5) Place the membrane protein side up, in the film cassette. Work as quickly as possible; minimise the delay between incubating the membrane in detection reagent and exposing it to the film (next step).	**5**) Ensure that there is no free detection reagent in the film cassette; the film must not get wet.

Protocol	Notes
6) Switch off the lights and carefully place a sheet of autoradiography film, such as Hyperfilm ECL, on top of the membrane, close the cassette and expose for 1 minute.	6) Do this in a dark-room, using red safelights. Do not move the film while it is being exposed.
7) Remove film, immediately replace with a fresh piece of unexposed film, and reclose the cassette.	7) Develop the first piece of film immediately, and on the basis of its appearance estimate how long to continue the exposure of the second piece of film - this will depend on the amount of target protein on the membrane. In general, exposure times will vary from 1-15 minutes for high target applications and 15-90 minutes for lower target applications. If background is high the blot may be rewashed twice for 10 minutes with wash buffer and redetected following steps 1 - 7 with slight loss of sensitivity.

RELATED PROTOCOLS

Controls

Positive control
EGF stimulated A431 cells cultured in DMEM can be used as a positive control.
Stimulate the cell culture for 20 minutes with 50ng/ml EGF.

Negative control
Prepare an A431 cell lysate without EGF stimulation.

Suggested cell culture media for A431 cells[5]
500ml DMEM
5ml Penicillin/streptomycin (stock at 5000 units penicillin, 5mg streptomycin/ml)
5ml L-Glutamine (stock at 200mM)
50ml Foetal bovine serum

Lysis buffer

A variety of different lysis buffers can be used for the solubilisation of cellular antigens. Factors that affect the efficiency of solubilisation include concentration and type of detergent, ionic strength and pH of the buffer, and the presence or absence of chelating agents. Buffers using more than one type of detergent may be efficient at solubilisation[6] but may cause denaturation resulting in less immunoreactivity in immunoprecipitation.

The inclusion of protease inhibitors is strongly recommended. To cover all types of proteases a cocktail of inhibitors is normally used: for example, aprotinin and phenylmethyl-

sulphonylfluoride (PMSF) for serine proteases, EDTA for metalloproteases and leupeptin for serine proteases and thiolproteases. As many of these inhibitors have very short half lives once added to the buffers, the lysis solutions need to be prepared freshly. Concentrated stocks of protease inhibitors can be prepared and stored at -15°C to -30°C[7].

For the analysis of phosphorylation on proteins, it is also important to include phosphatase inhibitors such as sodium orthovanadate and sodium fluoride.

The following buffer is a good starting point for preparation of cellular lysates:
50mM Tris. HCl pH 7.5
1% NP40
0.25% Sodium deoxycholate
150mM NaCl
1mM EGTA
1mM PMSF
1µg/ml aprotinin
1µg/ml leupeptin
1µg/ml pepstatin
1mM Na_3VO_4
1mM NaF

Stripping and reprobing

The complete removal of antibodies from membranes is possible following the method outlined below. The membranes may be stripped of bound antibodies and reprobed several times. Prior to stripping, membranes should be stored wet wrapped in SaranWrap at 2-8°C after immunodetection.

Protocol	Notes
1) Submerge the membrane in freshly prepared stripping buffer (100mM 2-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5mM Tris.HCl pH6.7) and incubate at 50°C for 30 minutes with occasional agitation.	**1**) If more stringent conditions are required the incubation can be performed at higher temperatures up to a maximum of 70°C.
2) Wash the membrane for 2x10 minutes in TBS-T at room temperature using large volumes of wash buffer.	**2**) Membranes may be incubated with the ECL detection reagents and exposed to film to ensure removal of antibodies.
3) Block the membrane by immersing in 1% blocking reagent /TBS-T for 1 hour at room temperature.	
4) Perform immunodetection using procedures as described on page 9.	

ADDITIONAL INFORMATION

Troubleshooting guide

Problem	Possible cause	Remedy
1. No signal	**1.1**) No transfer of proteins during Western blotting	**1.1**) Re-evaluate blotting procedure: • stain gels with dye to check transfer efficiency. • stain membrane with protein stain to check transfer efficiency. • optimise gel acrylamide concentration, time for transfer and current using molecular weight markers covering the molecular weight range expected to be blotted, or use a biotinylated positive control, for example, ECL biotinylated markers. • check that the gel and membrane make proper contact during blotting. • check that gel and blotting

		membrane are correctly orientated with respect to the anode. • check that excess temperatures are not reached during electroblotting producing bubbles, or gel/membrane distortion, etc.
	1.2) Protein degradation on storage of blots prior to detection	**1.2**) Use fresh blots.
	1.3) No retention of proteins on membranes	**1.3.1**) Assess no transfer of proteins during blotting as 1.1). **1.3.2**) Use fresh supply of membrane to ensure proper hydration.
	1.4) Detection system	**1.4.1**) Check that the detection reagents are being stored correctly and used as recommended. **1.4.2**) Check that the detection reagents are working: pre-mix small quantities of detection reagents 1 and

Problem	Possible cause	Remedy
		2 (0.5ml each) and in the dark add 1µl anti pTyr-HRP. Visible blue light should be produced.
2) Weak signal	**2.1**) See 1) above **2.2**) Insufficient protein loaded on gel	**2.1**) See 1) above. **2.2.1**) Load more protein on gel. **2.2.2**) Increase the concentration and/or incubation time of the anti pTyr- HRP.
	2.3) Inhibition of antibody binding by high salt levels	**2.3**) Use the buffer recommended in the protocol.
	2.4) Removal of phosphate groups on tyrosine residues by endogenous protein phosphatases	**2.4**) Use phosphatase inhibitors in the lysis buffer unless lysate is prepared and run immediately on the gel.

	2.5) Low level of signal	**2.5.1**) Expose film for an extended period (1-2 hours). **2.5.2**) Pre-flashing the film will increase its sensitivity to the signal and linearise its response. This does, however, require care as increased backgrounds may result. Pre-flashing involves hypersensitising the film just before use by pre-exposure to a short flash of light (approximately 1msec). Conventional photographic flash units are suitable when attenuated with a diffuser and Kodak™ Wratten™ 6B filter, to give a flash of the required intensity to increase the 540nm absorbance of the developed film to 0.15 above that of the unexposed film. **2.5.3**) Poor protein transfer on to membrane.

Problem	Possible cause	Remedy
3) Excessive diffuse signal	**3.1**) Overloading of protein	**3.1**) Load less protein on gel.
	3.2) Improper gel conditions	**3.2**) Optimise gel, electrophoresis and blotting conditions: • increase acrylamide concentration of gel • check gel and buffer recipes • check that no bubbles interfere with transfer from gel to membrane.
4) Uneven/spotted blot	**4.1**) Improper blotting technique	**4.1**) See 1) above.
	4.2) Unevenly hydrated membrane	**4.2.1**) Use new/fresh membranes. **4.2.2**) Make sure that the membrane is fully covered and wetted during incubations.
	4.3) Fingerprints and/or keratin contamination	**4.3**) Avoid touching membrane. Use gloves and blunt forceps.

5) High backgrounds	**5.1**) Contaminated blotting equipment	**5.1**) Clean or replace equipment.
	5.2) Contaminated buffers	**5.2**) Ensure all buffers are freshly prepared and filtered.
	5.3) Inadequate blocking	**5.3.1**) Check that blocking solution has been made up properly. **5.3.2**) Use a freshly prepared solution of blocking agent. **5.3.3**) Increase incubation time of blocking incubation.
	5.4) Problems with membranes	**5.4.1**) Check that membranes are completely immersed in all solutions especially during washing, and that membranes hydrate thoroughly. **5.4.2**) Use a fresh supply of membranes. **5.4.3**) Use high quality membranes: Hybond PVDF or Hybond ECL are recommended.

Problem	Possible cause	Remedy
		5.4.4) Damage to the membrane can cause non-specific binding of the detection reagents. Handle blots carefully with gloved hands and blunt non-serrated forceps. **5.4.5**) Use clean forceps to handle blots after washing.
	5.5) Detection reagents	**5.5.1**) Rewash blots twice for 10 minutes in wash buffer and repeat detection steps. **5.5.2**) Excess detection reagents in blots. Drain well by absorbing the excess on tissue paper before placing blots in film cassettes.
	5.6) Inadequate washing	**5.6.1**) Increase wash times and volumes of wash buffer. **5.6.2**) Add Tween 20 to reagents if not already added.

		5.6.3) Increase concentration of Tween 20 in the blocking solution.
	5.7) Over exposure	**5.7.1**) Expose the film for a minimum period (an initial 1 minute exposure may be all that is required). **5.7.2**) Leave blots in the cassette for 5-10 minutes before re-exposing to film.

Related products

ECL glycoprotein detection module (sufficient for 25 8x10cm membrane labellings or 50 solution labellings)	RPN 2190
ECL glycoprotein detection system (contains RPN 2190 and RPN 2209)	RPN 2191
ECL protein biotinylation module (sufficient for 25 8x10cm membrane labellings or 5x2.5mg solution labellings)	RPN 2202
ECL protein biotinylation system (contains RPN 2202 and RPN 2209)	RPN 2203
ECL Western blotting detection reagents	
Sufficient for 4000cm^2 membrane	RPN 2106
Sufficient for 2000cm^2 membrane	RPN 2209
Sufficient for 1000cm^2 membrane	RPN 2109
ECL Western blotting analysis system For the detection of either mouse or rabbit membrane bound primary antibodies. Sufficient for 1000cm^2 membrane.	RPN 2108
ECL protein molecular weight markers (molecular weight range 14400-97400Da)	RPN 2107
ECL protein molecular weight markers with streptavidin-horseradish peroxidase and blocking reagent	RPN 2280
Rainbow™ coloured protein molecular weight markers (molecular weight range 2350-46000Da)	RPN 755

Rainbow coloured protein molecular weight markers (molecular weight range 14300-220000Da)		RPN 756
Streptavidin-horseradish peroxidase conjugate		RPN 1231
Horseradish peroxidase-labelled second antibody conjugates		
Mouse Ig, HRP-linked whole antibody (from sheep)		NA 931
Rabbit Ig, HRP-linked whole antibody (from donkey)		NA 934
Rat Ig, HRP-linked whole antibody (from sheep)		NA 932
Human Ig, HRP-linked whole antibody (from sheep)		NA 933
Hybond ECL		
High quality nitrocellulose membrane, 20x20cm, 10 sheets		RPN 2020D
Hybond PVDF		
Pack of 10 PVDF membranes	20x20cm	RPN 2020P
Roll of PVDF membrane	20cmx3m	RPN 203P
Hyperfilm ECL		
Pack of 25 films	18x24cm	RPN 2103
	30x40cm	RPN 2104
	10x12in	RPN 1681
	5x7in	RPN 1674
ECL mini-camera		RPN 2069
Sensitize™ pre-flash unit		RPN 2051

Hypercassette™

18x24cm	RPN 1642
30x40cm	RPN 1644
5x7in	RPN 1648

References

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3. RUFF-JAMIESON, S., CAMPOS-GONZALEZ, R and GLENNEY, J.R., *J. Biol. Chem.,* **266**, p.6607, 1991.
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7. HARLOW, E. and LANE, D, *Antibodies. A Laboratory Manual*, Cold Spring Harbor 1988.

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Safety data sheet SDS239/AA Date of issue Jul 1998

Amersham Biosciences UK Limited Amersham Place Little Chalfont Buckinghamshire England HP7 9NA Telephone: (01494) 544000.

Product name:	40% ethylene glycol.	CAS No: 107-21-1
	R: 22	Harmful if swallowed
	S: (2)	(keep out of the reach of children)

Composition: Colourless viscous liquid.

Hazards identification: Harmful if swallowed.

First aid measures: In case of contact immediately flush eyes or skin with copious amounts of water. Remove contaminated clothing and shoes. Ensure adequate flushing of eyes by separating the eyelids with fingers. If inhaled remove to fresh air. In severe cases seek medical attention.

Fire-fighting measures: Water spray, foam, dry powder or carbon dioxide.

Accidental release: Wear suitable protective clothing. If local regulations permit, mop up with plenty of water and run to waste, diluting greatly with running water. Otherwise absorb on an inert absorbent, transfer to container and arrange removal by disposal company. Ventilate area to dispel residual vapour.

Handling and storage: Wear suitable protective clothing including laboratory overalls,

	safety glasses and gloves. Do not get in eyes or on skin or on clothing. Do not breathe vapour. Wash thoroughly after handling. Keep out of the reach of children.
Personal protection:	See above instructions for handling and storage.
Physical and chemical properties:	Melting point: -12°C Boiling point: 197°C Specific gravity: 1.11
Stability and reactivity:	Can react vigorously with oxidising agents. Can form products with perchloric acid. Can react violently with phosphorus pentasulphide.
Toxicological information:	LD_{50}: 4700mg/kg oral, rat.
Ecological information:	Not applicable.
Disposal considerations:	Dispose of through a chemical waste route.
Transport information:	No special precautions applicable.
Regulatory information:	The information contained in this safety data sheet is based on published sources and is believed to be correct. It should be used as a guide only. It is the responsibility of the user of this product to carry out an assessment of work-place risks, as may be required under national legislation.

For further information contact your local office. See page 29.

Safety data sheet SDS245/AA Date of issue Jul 1998

Amersham Biosciences UK Limited Amersham Place Little Chalfont Buckinghamshire England HP7 9NA Telephone: (01494) 544000.

Product name:	Chloroacetamide	CAS No: 79-07-02
	R: 20/21/22	Harmful by inhalation, in contact with skin and if swallowed.
	S: 22-37, 39-44	Do not breathe dust, gas/fumes/vapour/ spray, avoid contact with skin and eyes. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice, take off immediately all contaminated clothing. Wear eye/face protection. If you feel unwell seek medical advice.
Composition:	Solution (≥0.1%, <1%) (w/v).	
Hazards identification:	Chloroacetamide at this concentration is considered harmful by inhalation, in contact with skin and if swallowed.	
First aid measures:	Eye contact:	Wash eyes with water for at least 15 minutes.
	Skin contact:	Wash immediately with plenty of water and treat the skin with skin-disinfectant.
	Ingestion:	Requires medical treatment.
Fire-fighting measures:	No restriction.	

Accidental release:	Take up solids dry, avoiding formation of dust. Dilute liquids with water and absorb.
Handling and storage:	Liquid does not burn, solid burns. Open and handle container carefully.
Personal protection:	Protective glasses and rubber gloves.
Physical and chemical properties:	Formula weight: 95.51.
Stability and reactivity:	Avoid strong oxidising agents, strong acids, strong bases and strong reducing agents.
Toxicological information:	LD_{50}: 155mg/kg oral.
Ecological information:	Not available.
Disposal considerations:	Used reagent can be disposed of in waste water in accordance with local regulations.
Transport information:	No restriction.
Regulatory information:	The information contained in this safety data sheet is based upon published sources and is believed to be correct. It should be used as a guide only. It is the responsibility of the user of this product to carry out an assessment of workplace risks, as may be required under national legislation.

For further information contact your local office. See page 29.

This kit is protected by the following patents:

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and is sold under licence from the patent holder (British Technology Group Ltd)

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RPN2220Pl/AA