

PhosphoProtein Purification Handbook

For purification and analysis of phosphorylated proteins from eukaryotic cells

December 2002



© 2002 QIAGEN, all rights reserved.

QIAGEN Worldwide

QIAGEN Companies

Australia	QIAGEN Pty Ltd ABN 75 072 382 944	PO Box 25 • Clifton Hill • Victoria 3068 Orders 03-9489-3666 • Fax 03-9489-3888 • Technical 1-800-243-066
Canada	QIAGEN Inc.	2800 Argentia Road • Unit 7 • Mississauga • Ontario • L5N 8L2 Orders 800-572-9613 • Fax 800-713-5951 • Technical 800-DNA-PREP (800-362-7737)
France	QIAGEN S.A.	3 avenue du Canada • LP 809 • 91974 COURTABOEUF CEDEX Orders 01-60-920-920 • Fax 01-60-920-925 • Technical 01-60-920-930
Germany	QIAGEN GmbH	Max-Volmer-Straße 4 • 40724 Hilden Orders 02103-29-12000 • Fax 02103-29-22000 • Technical 02103-29-12400
Italy	QIAGEN S.p.A.	Via Grosio, 10/10 • 20151 Milano Orders 02-33430411 • Fax 02-33430426 • Technical 02-33430414
Japan www.qiagen.co.jp	QIAGEN K.K.	Forefront Tower II • 13-1, Kachidoki 3 Chome • Chuo-ku, Tokyo 104-0054 Telephone 03-5547-0811 • Fax 03-5547-0818 • Technical 03-5547-0811
Switzerland	QIAGEN AG	Auf dem Wolf 39 • 4052 Basel Orders 061-319-30-30 • Fax 061-319-30-33 • Technical 061-319-30-31
UK and Ireland	QIAGEN Ltd.	Boundary Court • Gatwick Road • Crawley • West Sussex, RH10 9AX Orders 01293-422-911 • Fax 01293-422-922 • Technical 01293-422-999
USA	QIAGEN Inc.	28159 Avenue Stanford • Valencia • CA 91355 Orders 800-426-8157 • Fax 800-718-2056 • Technical 800-DNA-PREP (800-362-7737)

www.qiagen.com

QIAGEN Distributors

Please see the last page for contact information for your local QIAGEN distributor.

Contents

Kit Contents	4
Storage Conditions	4
Product Use Limitations	4
Product Warranty and Satisfaction Guarantee	4
Technical Assistance	5
Safety Information	5
Introduction	6
The PhosphoProtein Purification Principle	9
Detecting Phosphorylated Proteins	9
■ PhosphoProtein Purification Protocol	11
Important notes before starting	11
Concentrating Protein Fractions Using Nanosep Ultrafiltration Columns	13
Immunodetection of Phosphorylated Proteins Using PhosphoSerine or PhosphoThreonine Antibodies (Chemiluminescent Method)	14
Troubleshooting Guide	16
Ordering Information	19
QIAGEN Distributors	23

Kit Contents

PhosphoProtein Purification Kit	Cat. No. 37101
PhosphoProtein Purification Columns	6
PhosphoProtein Lysis Buffer	2 x 120 ml
PhosphoProtein Elution Buffer	20 ml
CHAPS Stock Solution (10% [w/v])	7 ml
Benzonase [®] , endonuclease (DNase/RNase, >99% purity)	2000 Units (25 U/μl)
Protease inhibitor tablets	6
Nanosep [®] Ultrafiltration columns (10 kDa molecular weight cutoff)	6

Storage Conditions

PhosphoProtein Purification Columns, buffers, protease inhibitor tablets, and Benzonase should be stored at 2–8°C. They can be stored under these conditions for up to 12 months without any reduction in performance.

For longer storage Benzonase can be frozen at –20°C.

Nanosep ultrafiltration columns should be stored dry at room temperature.

Product Use Limitations

PhosphoProtein Purification Kits are developed, designed, and sold for research purposes only. They are not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see inside front cover).

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties regarding PhosphoProtein Purification Kits or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information please call one of the QIAGEN Technical Service Departments or local distributors (see inside front cover).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

PhosphoProtein Purification Columns contain ethanol. Flammable. Risk and safety phrases*: R10.

24-hour emergency information

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

* R10: Flammable.

Introduction

The complexity of the proteome derives not only from the number of individual proteins present in a cell, but also from their post-translational modifications. Additionally, splice-variants may be present, adding further to the complexity of the system. One of the most common post-translational modifications of proteins is phosphorylation of serine, threonine, and tyrosine residues. Phosphorylation of proteins plays a vital role in cell signaling, oncogenesis, apoptosis, and immune disorders. Around a third of all eukaryotic gene products can be post-translationally phosphorylated.

The specific phosphorylation of serine, threonine, or tyrosine residues is the most common mechanism for the regulation of cellular protein activity. Kinases catalyze the addition of a phosphate moiety to the hydroxyl group of the respective amino acid. The activity of protein kinases is regulated by various intracellular key signals, e.g., the concentration of cyclic AMP or Ca^{2+} . Phosphatases catalyze the specific dephosphorylation of protein, allowing enzymes to switch between phosphorylated and dephosphorylated states. Reversible protein phosphorylation has been known for some years to control a wide range of cellular processes and activities such as transmembrane signaling, intracellular amplification of signals, and cell-cycle control. The analysis of such phosphorylated residues forms the core of signal-transduction studies.

During the investigation of signal-transduction paths, the phosphorylation profile of the entire cell or the phosphorylation status of amino acids in a defined protein can be examined.

If the phosphorylation status of a single protein is to be analyzed, both forms of the protein can be purified by conventional column chromatography or separated on a 2-D gel, and the extent of phosphorylation determined. The precise site of phosphorylation can be identified by mass spectroscopy. A common problem with such analyses is that the amount of protein that has undergone phosphorylation may be small compared to the unphosphorylated form. This can mean that after purification of a protein, the amount of phosphorylated material available is insufficient for analysis. In addition, in such analyses the signal from phosphorylated material must be measured against a high level of background signal that arises from unphosphorylated protein.

The phosphorylation profile of the entire cell can be investigated by *in vivo* ^{32}P labeling followed by 2-D electrophoresis and autoradiography. As an alternative to radiolabeling, western blots made from 2-D gels can be probed using antibodies specific for phosphorylated amino acids. Changes in the cell's complete phosphorylation profile can be detected in apoptotic and diseased cells, and also occur in different phases of the cell cycle and of growth, or under growth in differing media. In addition to the determination of a phosphorylation profile at a distinct time-point or under certain conditions, the kinetics of phosphorylation after the addition of an external stimulant can also be recorded.

Phosphorylation-profile analysis is also a cornerstone of the quickly growing field of proteomics — the analysis of complete complements of proteins. Proteomics includes not only the identification and quantification of proteins, but also the determination of their localization, modifications, interactions, activities, and, ultimately, their function.

In proteomics studies, sample preparation before 2-D gel analysis should be both gentle and standardized, in order to minimize protein degradation and aggregation. While the production of a phosphorylation profile that is reproducible and mirrors the actual profile found in the cell requires a sample preparation procedure that contains as few steps as possible, the complexity of the genome and the limitations of analysis methods require a fractionation before analysis. This reduces the complexity of the system and allows sensitive detection and analysis of proteins expressed at low levels.

As approximately 10% of the cell's protein mass is phosphorylated, a purification process that isolates phosphorylated proteins significantly reduces the complexity and increases the sensitivity of the analysis.

The PhosphoProtein Purification Kit enables a complete separation of the phosphorylated from the unphosphorylated cellular protein fraction (Figure 1). The affinity chromatography procedure, in which phosphorylated proteins are bound to a column while unphosphorylated proteins are recovered in the flow-through fraction, reduces complexity and greatly facilitates phosphorylation-profile studies. Both fractions retain full biological activity and can be further purified if desired.

Complete Separation of Unphosphorylated and Phosphorylated Proteins

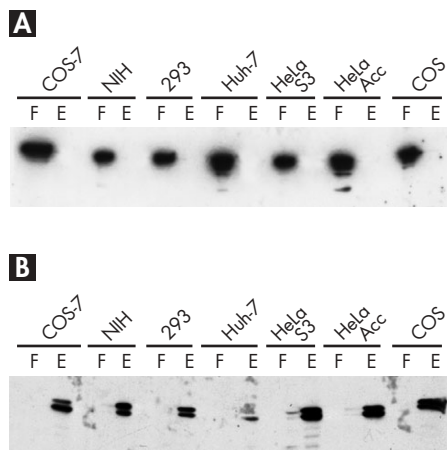
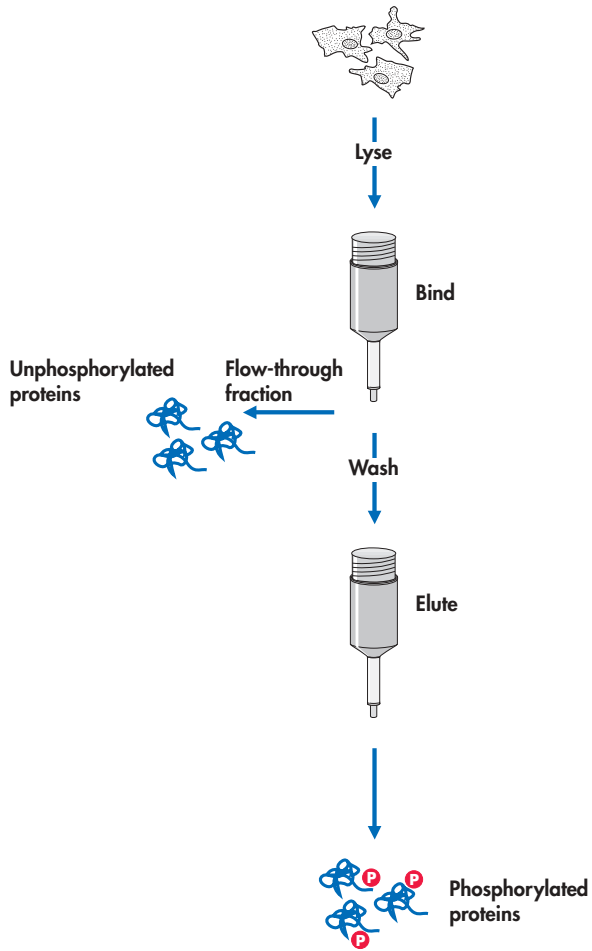


Figure 1. Protein-specific immunodetection of **A** unphosphorylated HSP-60 protein, and **B** phosphorylated p44 and p42 mitogen-activated protein kinase (MAPK) proteins. **F** flow-through; **E** eluate fractions. The antibody used to detect MAPK recognizes an epitope containing phosphorylated residues at Thr202 and Tyr204 in the p44 (upper band) and p42 (lower band) MAPK amino acid sequences. The absence of unphosphorylated HSP-60 in the eluate fraction and the absence of phosphorylated MAPK in the flow-through fraction demonstrate the complete separation of phosphorylated proteins using the PhosphoProtein Purification Kit.

PhosphoProtein Purification Procedure



The PhosphoProtein Purification Principle

The PhosphoProtein Purification Kit is designed for the specific purification of phosphorylated proteins from complex cell lysates. Proteins that carry a phosphate group on any amino acid are bound with high specificity to a PhosphoProtein Purification Column, while proteins without phosphate groups do not bind to the column and can therefore be found in the column flow-through fraction (Figure 2).

Gentle lysis of the cells is carried out in a lysis buffer that contains the zwitterionic detergent CHAPS at a concentration of 0.25 % (w/v). In order to resolve protein-DNA complexes, Benzonase (a DNase/RNase) is added to the lysate. To prevent proteolytic degradation of the proteins in the lysate, a mixture of protease inhibitors is added. After lysis and a centrifugation step to clear the lysate, the protein content of the cells must be determined and adjusted to 0.1 mg/ml. This concentration adjustment is made to ensure that all phosphate groups are easily accessible during purification and are not hidden within protein complexes. Under the lysis buffer conditions used, no phosphatase activity is measurable, ensuring that the phosphorylated protein fraction that bind the PhosphoProtein Purification Column accurately reflects the *in vivo* phosphorylation profile. Subsequently, the lysate is added to the ready-to-use columns and the flow-through fraction containing non-phosphorylated proteins can be collected. The lysate passes through the column over approximately 50 minutes (flow rate = 0.5 ml/min), ensuring complete binding of phosphorylated proteins. After a wash step to remove any unphosphorylated proteins, the proteins carrying phosphate groups are eluted in a phosphate-buffered saline (PBS) buffer. Free phosphate in the elution buffer inhibits phosphatase activity, and therefore stabilizes the phosphorylation status of the eluted fraction during downstream processing and storage.

Starting material for one purification procedure is 10^7 cells. Using the provided lysis buffer approximately 2.5 mg of total protein can be obtained from this amount of cells. Normally, 7–15% of proteins from cells grown under standard conditions (i.e., without any induction of phosphorylation) carry one or more phosphate groups. Therefore, the expected yield from one PhosphoProtein Purification Column is 175–375 μg of phosphorylated protein (Table 1). Expected concentration of proteins in the eluate is around 0.2 $\mu\text{g}/\mu\text{l}$. The maximum binding capacity of the column is approximately 500 μg of phosphorylated protein.

Detecting Phosphorylated Proteins

QIAGEN offers PhosphoSerine and PhosphoThreonine Antibodies for immunodetection of phosphorylated proteins in blotting procedures. PhosphoSerine and PhosphoThreonine Antibodies recognize and bind to phosphorylated serine and threonine residues, irrespective of surrounding amino acids. The use of chemiluminescent detection in conjunction with QIAGEN® PhosphoProtein Antibodies is strongly recommended. Phosphorylated proteins are often present at very low concentrations, and therefore the sensitivity of detection should be maximized. The ECL™ system from Amersham BioSciences can be used in combination with HRP-conjugated anti-mouse secondary antibodies.

Table 1. Yields of phosphorylated proteins obtained using the PhosphoProtein Purification Kit

Cell type	Number of cells processed	Total protein in cell lysate (µg)	Protein loaded onto column (µg)	Protein in eluate (µg)	Percentage phosphorylated proteins
CHO	1.5 x 10 ⁷	3400	2500	300	12%
NIH 3T3	n.d.	2750	2500	165	7%
293	1.5 x 10 ⁷	3650	2500	200	8%
Cos-7	4.5 x 10 ⁶	1700	1700	120	7%
Huh-7	8.5 x 10 ⁶	2650	2500	235	9%
HT 29	n.d.	n.d.	2500	200	8%
LT 23	n.d.	n.d.	2500	275	11%
HeLa S3	1.8 x 10 ⁷	5950	2500	280	11%
HeLa Acc57	6.6 x 10 ⁶	2500	2500	235	9%

n.d.: not determined

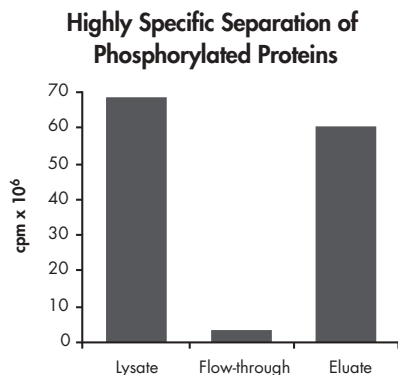


Figure 2. Non-stimulated Jurkat cells were radioactively labeled *in vivo* using ³²P. Cell lysate was processed using the PhosphoProtein Purification Kit and the radioactivity in each fraction measured. Data kindly provided by Gudrun Rehg and Sascha Dammeier, Byk Gulden, Konstanz, Germany.

PhosphoProtein Purification Protocol

Important notes before starting

- For most downstream applications, concentration of the separated protein fractions is required. The PhosphoProtein Purification Kit contains six Nanosep ultrafiltration devices that enable easy buffer exchange and concentration of dilute protein samples. The ultrafiltration columns have a molecular weight cutoff (MWCO) of 10 kDa. It is recommended that the most concentrated phosphorylated protein fraction (usually the third elution fraction) be concentrated by a factor of 10 for SDS-PAGE and subsequent Coomassie® staining or western blot analysis (i.e., the 500 µl elution volume should be reduced to a volume of 50 µl). For SDS-PAGE followed by silver staining, the eluates can be used without concentration.
- For 2-D gel analysis, both phosphorylated and unphosphorylated protein fractions must be concentrated and desalted. This can be achieved by reducing the volume of the eluate fraction to 50 µl by ultrafiltration; adding 450 µl 10 mM Tris-Cl, pH 7.0; reducing the volume to 50 µl once more by ultrafiltration; adding a further 450 µl 10 mM Tris-Cl, pH 7.0; and reducing the volume for a third time by ultrafiltration. The concentrated, desalted protein fraction can then be taken up in 2-D gel loading buffer and loaded onto the gel.
- Although lysis is carried out at 4°C the protein purification procedure is performed at room temperature. A protease inhibitor mix prevents proteolytic degradation of the proteins. Under the lysis buffer conditions used, no phosphatase activity is measurable, ensuring that the phosphorylated protein fraction that binds to the PhosphoProtein Purification Column accurately reflects the in vivo phosphorylation profile. Performing the purification at 4°C is not recommended as this significantly reduces the yield, and results in incomplete purification of phosphorylated proteins.
- Do not use phosphate buffer for washing the cells as this will interfere with binding of phosphorylated proteins to the column.

Procedure

1. **Add 875 µl CHAPS Stock Solution (10% [w/v]) to 35 ml of PhosphoProtein Lysis Buffer and 75 µl CHAPS Stock Solution (10% [w/v]) to 3 ml of PhosphoProtein Elution Buffer to yield a final concentration of 0.25 % (w/v) CHAPS in both buffers.**
2. **Add 1 Protease inhibitor tablet and 10 µl of Benzonase stock solution to a 5 ml aliquot of PhosphoProtein Lysis Buffer containing CHAPS prepared in step 1. Mix.**
3. **By gentle pipetting, resuspend a cell pellet corresponding to 10⁷ cells in the 5 ml of lysis buffer containing protease inhibitors and Benzonase prepared in step 2.**
4. **Incubate for 30 minutes at 4°C. Vortex briefly every 10 minutes.**
5. **After 30 minutes incubation, centrifuge the cell lysate at 10,000 x g and 4°C for 30 minutes.**

6. During centrifugation, prepare a PhosphoProtein Purification Column by detaching the top cap, breaking off the bottom closure, and allowing the storage buffer to flow out. Apply 4 ml PhosphoProtein Lysis Buffer containing 0.25% CHAPS (prepared in step 1) to equilibrate the column and allow the buffer to flow out.
7. Harvest the supernatant and determine the protein concentration.*
8. Take a volume of lysate containing approximately 2.5 mg of total protein, and adjust the protein concentration to 0.1 mg/ml by adding PhosphoProtein Lysis Buffer containing 0.25% CHAPS. This will yield a final volume of 25 ml of lysate.
9. Pour half the lysate (12.5 ml) into the upper reservoir of the PhosphoProtein Purification Column. When almost all the lysate has entered the gel bed, add the second half of the lysate and allow to pass through the column. This step will take approximately 50 min and allows enough time for complete binding of any phosphorylated protein to the affinity column. Collect the flow-through fraction if analysis of unphosphorylated proteins in the lysate is desired.

Note: This and all subsequent steps should be performed at room temperature.

10. Apply 6 ml of PhosphoProtein Lysis Buffer containing 0.25% CHAPS to wash the column, and allow to flow through.
11. Apply 500 μ l PhosphoProtein Elution Buffer containing 0.25% CHAPS to the column, and collect the eluted fraction.
12. Repeat step 11 four times. Determine protein concentration in all eluate fractions (e.g., using the Bradford method) to determine the most concentrated fraction. The highest concentrations of phosphorylated proteins should be found in elution fractions 3 and 4.

* Protein concentration can be quickly and easily determined using the Bradford method. Bradford assay dye reagent is available commercially, e.g., Bio-Rad Protein Assay Dye Reagent Concentrate, cat. no. 500-0006.

Concentrating Protein Fractions Using Nanosep Ultrafiltration Columns

1. Place 500 μl of the protein fraction into the sample reservoir of the Nanosep ultrafiltration column.
2. Centrifuge the device at 10,000 $\times g$ for up to 10 minutes.
3. After concentration, the protein sample can be pipetted from the retentate chamber. Concentrating samples down to a volume of 50 μl is recommended. If samples are reduced to a volume $<50 \mu\text{l}$, add buffer to a total volume of 50 μl to optimize yields.

To concentrate volumes larger than 500 μl (e.g., to combine phosphorylated protein eluates), after centrifugation of an initial aliquot, discard the flow-through fraction, add dilute sample to the concentrated protein in the retentate chamber up to a total volume of 500 μl , and centrifuge again at 10,000 $\times g$ for up to 10 minutes (Figure 3).

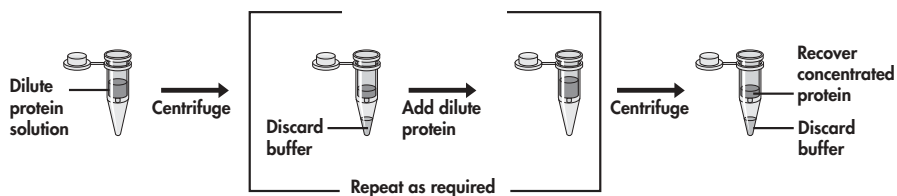


Figure 3. Concentration of dilute protein solutions using the NanoSep ultrafiltration column.

Immunodetection of Phosphorylated Proteins Using PhosphoSerine or PhosphoThreonine Antibodies (Chemiluminescent Method)

Reagents to be supplied by the user

TBS buffer:	10 mM Tris-Cl; 150 mM NaCl, pH 7.5
TBS-Tween/Triton buffer:	20 mM Tris-Cl; 500 mM NaCl; 0.05% (v/v) Tween® 20; 0.2% (v/v) Triton® X-100, pH 7.5
Blocking buffer:	5% (w/v) BSA; 0.1% (v/v) Tween 20 in TBS buffer
Secondary antibody dilution buffer:	10% (w/v) skim milk powder; 0.1% (v/v) Tween 20 in TBS buffer

PhosphoSerine Antibody stock solution

PhosphoSerine Antibodies should be stored lyophilized until they are to be used. They can be stored lyophilized for 6 months at 2–8°C. In solution they can be stored for up to 6 months in aliquots at –20°C. Avoid repeated freezing and thawing. Dissolve the lyophilized antibody (100 µg) in 1 ml water per vial (final concentration 0.1 mg/ml). PhosphoSerine Antibody is a mixture of two mouse monoclonal antibodies recognizing a broad spectrum of serine-phosphorylated proteins. These antibodies belong to isotypes IgG1 and IgM and are recognized by secondary antibodies that react with mouse antibodies from both isotypes.

PhosphoThreonine Antibody stock solution

PhosphoThreonine Antibodies should be stored lyophilized until they are to be used. They can be stored lyophilized for 6 months at 2–8°C. In solution they can be stored for up to 6 months in aliquots at –20°C. Avoid repeated freezing and thawing. Dissolve the lyophilized antibody (100 µg) in 1 ml water per vial (final concentration 0.1 mg/ml). PhosphoThreonine Antibody is a monoclonal antibody from mouse that recognizes a broad spectrum of threonine-phosphorylated proteins. This antibody belongs to isotype IgG1 and is recognized by secondary antibodies that react with mouse antibodies from this isotype.

Immunodetection of phosphorylated proteins

The use of chemiluminescent detection in conjunction with PhosphoProtein Antibodies is recommended. Phosphorylated proteins are often present at very low concentrations, and therefore the sensitivity of detection should be maximized. The ECL system from Amersham BioSciences can be used in combination with HRP-conjugated secondary antibodies. Please refer to manufacturer's recommendations.

Procedure

1. **After western blotting, wash membrane twice for 10 minutes each time with TBS buffer at room temperature.**

2. **Incubate for 1 hour in blocking buffer at room temperature.**

Note: Do not use blocking buffer containing milk powder when using PhosphoSerine or PhosphoThreonine Antibodies for detection. This will cause an intense background signal because of serine- and threonine- phosphorylated proteins present in the milk powder.

3. **Wash membrane twice for 10 minutes each time in TBS-Tween/Triton buffer at room temperature.**

4. **Wash membrane for 10 minutes with TBS buffer at room temperature.**

5. **Incubate in PhosphoProtein Antibody solution (1/100–1/200 dilution of antibody stock solution in blocking buffer) at 4°C overnight.**

Membrane can be sealed in plastic bags.

Note: Do not use blocking buffer containing milk powder when using PhosphoSerine or PhosphoThreonine Antibodies for detection. This will cause an intense background signal because of serine- and threonine- phosphorylated proteins present in the milk powder.

6. **Wash twice for 10 minutes each time in TBS-Tween/Triton buffer at room temperature.**

7. **Wash for 10 minutes in TBS buffer at room temperature.**

8. **Incubate with secondary antibody solution for 1 hour at room temperature.**

Rabbit anti-mouse IgG/IgM HRP-conjugate from Jackson ImmunoResearch (Cat.No. 315-035-048) yields good results with PhosphoSerine and PhosphoThreonine Antibodies. Goat anti-mouse IgG/HRP-conjugate from Jackson Immunoresearch (Cat. No. 115-035-003) yields good results with PhosphoThreonine Antibodies. Dilute according to the manufacturer's recommendations. 10% skim milk powder in TBS is used for incubation with secondary antibody. Milk powder is needed to reduce background signal because BSA does not block sufficiently for the very sensitive chemiluminescent detection method.

9. **Wash 4 times for 10 minutes each time in TBS-Tween/Triton buffer at room temperature.**

10. **Perform chemiluminescent detection reaction and expose to X-ray film according to the manufacturer's recommendations.**

Troubleshooting Guide

Comments and suggestions

Flow-through contains phosphorylated proteins

Lysate is too concentrated

Ensure that protein concentration in the lysate is adjusted to 0.1 mg/ml. This concentration adjustment is made to ensure that all phosphate groups are easily accessible during purification and are not hidden within protein complexes.

Eluate contains unphosphorylated proteins

Lysate is too concentrated

Ensure that protein concentration in the lysate is adjusted to 0.1 mg/ml. This concentration adjustment is made to ensure that protein complexes are reduced and unphosphorylated proteins are not copurified due to interaction with phosphorylated proteins.

Yield in eluate is lower than expected

Lysate is too concentrated

Ensure that protein concentration in the lysate is adjusted to 0.1 mg/ml.

Lysate passed too quickly through the column

Flow-through should not pass through the column at a flow rate greater than 0.5 ml/min to allow for complete binding.

Lysis was carried out in the cell culture vessel

Lysis should be carried out using harvested cell pellets, not directly on the plate. This ensures that protein complexes are well resolved and phosphate groups are freely accessible.

Cell culture-medium components interfere with binding

Wash cells before harvesting in HEPES-based buffer to prevent possible interference of medium components with column binding. Do not use phosphate buffer for washing the cells as this will interfere with binding of phosphorylated proteins to the column.

No signals on western blot or on Coomassie stained gel when analyzing the eluate

Protein concentration is too low

As the protein concentration in the eluate is quite low, it is recommended that the most concentrated phosphorylated protein fraction (usually the third elution fraction) be concentrated by a factor of 10 for SDS-PAGE and subsequent Coomassie staining or western blot analysis (i.e., the 500 μ l elution volume should be reduced to a volume of 50 μ l). Perform a Bradford protein determination of the eluate to ensure that sufficient protein is present for your application. For SDS-PAGE followed by silver staining, the eluates can be used without concentration.

Smearing in 2-D gel

Salt present in sample

For 2-D gel analysis, protein fractions must be concentrated and desalted. Use a Nanosep ultrafiltration column to perform buffer exchange and concentration (see page 13).

High background on membrane using PhosphoSerine or PhosphoThreonine Antibodies

Blocking buffer contained milk powder

Do not use blocking buffer containing milk powder for blocking of the membrane or dilution of PhosphoProtein Antibodies. This will cause an intense background signal because of threonine- and serine-phosphorylated proteins present in the milk powder.

Secondary antibody dilution buffer does not provide sufficient blocking

Use 10% skim milk powder for incubation with secondary antibody. Milk powder is needed to reduce background signal because BSA does not block sufficiently for the very sensitive chemiluminescent detection method.

Limited signals using PhosphoSerine Antibody

Secondary antibody does not recognize both antibody isotypes

PhosphoSerine Antibody is a mixture of two mouse monoclonal antibodies that belong to isotypes IgG1 and IgM and must be used in combination with secondary antibodies that react with mouse antibodies from both isotypes (e.g., Rabbit anti-mouse IgG/IgM HRP-conjugate from Jackson ImmunoResearch (Cat.No. 315-035-048)).

Trademarks

Patented or patent-pending technology and/or registered or registration-pending trademarks of the QIAGEN Group: QIAGEN®.

Benzonase endonuclease is a registered trademark of Merck KGaA, Germany.

ECL is a trademark of Amersham Biosciences.

Nanosep is a registered trademark of Pall Corporation.

Triton is a registered trademark of Rohm & Haas Company.

Tween is a registered trademark of ICI Americas Inc.

Benzonase endonuclease is covered by several patents, i.e. the US patent No. 5.173,418 and EP No. 0229866. Nycomed Pharma A/S, Denmark claims worldwide patent rights to Benzonase endonuclease which are licensed exclusively to MERCK KdaA, Darmstadt (Germany).

Registered names, trademarks, etc. used in this document, even when not specifically marked as such, are not to be considered unprotected by law.



Ordering Information

Product	Contents	Cat. No.
PhosphoProtein Purification Kit (6)	6 PhosphoProtein Purification Columns; buffers; reagents, 6 Nanosep Ultrafiltration Columns	37101
PhosphoThreonine Antibody Q7 (100 µg)	100 µg anti-phosphothreonine antibody (isotype mouse IgG1, for 200 ml working solution)	37420
PhosphoSerine Antibody Q5 (100 µg)	100 µg mixture of anti-phosphoserine antibodies (isotypes mouse IgG1 and IgM, for 200 ml working solution)	37430

Notes

Notes

Notes



QIAGEN Companies

Please see the inside front cover for contact information for your local QIAGEN office.

QIAGEN Distributors

Argentina

Tecnolab S.A.
Charlone 144 - C1427BXD
Capital Federal
Tel: (011) 4555 0010
Fax: (011) 4553 3331
E-mail: info@tecnolab.com.ar
Web site: www.tecnolab.com.ar

Austria

VWR International GmbH
Zimbasgasse 5
1147 Wien
Austria
Tel: (01) 576 00 0
Fax: (01) 576 00 350
E-mail: merck-wien@merckeurolab.at
Web site: www.vwr.com

Belgium/Luxemburg

Westburg b.v.
P.O. Box 214
3830 AE Leusden
The Netherlands
Tel: 0800-1-9815
Fax: (31) 33-4951222
E-mail: info@westburg.nl
Web site: www.westburg.nl

Brazil

Uniscience do Brasil
Av. Cândido Portinari, 933/937
05114-001 São Paulo - SP
Brazil
Tel: 011 3622 2320
Fax: 011 3622 2323
E-mail: info@uniscience.com
Web site: www.uniscience.com

China

Gene Company Limited
Unit A, 8/F., Shell Industrial Building
12 Lee Chung Street
Chai Wan, Hong Kong, P.R.C.
Tel: (852)2896-6283
Fax: (852)2515-9371
E-mail:
Hong Kong: info@genehk.com
Beijing: gene@public2.bta.net.cn
Shanghai: gene@public.sta.net.cn
Chengdu: gene@public.cd.sc.cn
Guangzhou:
gzrtao@public.guangzhou.gd.cn

Cyprus

Scientronics Ltd
34, Zenonos Sozou Str
1075 Nicosia
Tel: 02-357 22 765416
Fax: 02-357 22 764614
E-mail:
a.sarpetsas@bioltronics.com.cy

Czech Republic

BIO-CONSULT spol. s.r.o.
Božejovická 145
142 01 Praha-Libuš
Tel/Fax: (420) 2 417 29 792
E-mail: bio-cons@login.cz
Web site: www.bio-consult.cz

Denmark

VWR International A/S
Roskildevej 16
2620 Albertslund
Tel: 43 86 87 88
Fax: 43 86 87 90
E-mail: info@dk.vwr.com
Web site: www.vwr.com

Egypt

Clinilab
P.O. Box 12 El-Manial
4, 1160 St., El-Ehahad Square
Riham Tower, El-Maadi
Cairo
Tel: 52 57 212
Fax: 52 57 210
E-mail: Clinilab@link.net

Finland

VWR International Oy
Niityrinne 7
02270 Espoo
Tel: (09) 804 551
Fax: (09) 8045 5200
E-mail: info@fi.vwr.com
Web site: www.vwr.com

Greece

BioAnalytica S.A.
11, Laskareos Str.
11471 Athens
Tel: (210)-640 03 18
Fax: (210)-646 27 48
E-mail: bioanalyt@hol.gr

India

Genetix
C-88, Kirti Nagar
Lower Ground Floor
New Delhi-110 015
Tel: (011)-542 1714
or (011)-515 9346
Fax: (011)-546 7637
E-mail: genetix@nda.vsnl.net.in

Israel

IRSLAB [Israel] Ltd.
1, Habursekai St. Kiriat Ha'asakim
Beer Sheva 84899
Tel: 08-6650813/4
or 1-800 20 22 20 (toll free)
Fax: 08-6650934
E-mail: info@westburg.co.il
Web site: www.westburg.co.il

Korea

IRS Laboratories, Inc.
SongBuk P.O. Box 61
Seoul, 136-600
Tel: (02) 924-86 97
Fax: (02) 924-86 96
E-mail: webmaster@rslab.co.kr
Web site: www.rslab.co.kr

Malaysia

RESEARCH BIOLABS SDN. BHD.
11-A, Jalan BK 5A/2
Bandar Kinrara
47100 Puchong, Selangor Darul Ehsan
Tel: (603)-8070 3101
Fax: (603)-8070 5101
E-mail: biolabs@tm.net.my
Web site: www.researchbiolabs.com

Mexico

Quimica Valaner S.A. de C.V.
Jalapa 77, Col Roma
Mexico D.F. 06700
Tel: (55) 55 25 57 25
Fax: (55) 55 25 56 25
E-mail: qvalaner@infosel.net.mx

The Netherlands

Westburg b.v.
P.O. Box 214
3830 AE Leusden
Tel: (033)-4950094
Fax: (033)-4951222
E-mail: info@westburg.nl
Web site: www.westburg.nl

New Zealand

Biolab Scientific Ltd.
244 Bush Road
Albany, Auckland
Tel: (09)9806700
or 0800933966
Fax: (09)9806788
E-mail: info@biolab.co.nz
Web site: www.biolab.co.nz

Norway

VWR International AS
Kakkelovnskroken 1
P.B. 45, Kalbakken,
0901 Oslo
Tel: 22 90 00 00
Fax: 22 90 00 40
E-mail: info@no.vwr.com
Web site: www.vwr.com

Poland

Syngen Biotech Sp.z.o.o.
ul. Legionicka 62 A
54-204 Wrocław
Tel: (071) 351 41 06
or 0601 70 60 07
Fax: (071) 351 04 88
E-mail: info@syngen.com.pl
Web site: www.syngen.com.pl

Portugal

IZASA PORTUGAL, LDA
Rua do Proletariado, 1 - Quinta do
Paizinho
2795-648 Carnaxide
Tel: (21) 424 7312
Fax: (21) 417 2674

Singapore

Research Biolabs Pte Ltd
211 Henderson Road #14-01
Henderson Industrial Estate
Singapore 159552
Tel: 2731066
Fax: 2734914
E-mail: biolabs@singnet.com.sg

Slovak Republic

BIO-CONSULT Slovakia spol. s.r.o.
Ružová dolina 6
SK-821 08 Bratislava 2
Tel/Fax: (02) 5022 1336
E-mail: bio-cons@post.sk
Web site: www.bio-consult.cz

South Africa

Southern Cross Biotechnology (Pty) Ltd
P.O. Box 23681
Claremont 7735
Cape Town
Tel: (021) 671 5166
Fax: (021) 671 7734
E-mail: info@scb.co.za
Web site: www.scb.co.za

Spain

IZASA, S.A.
Aragón, 90
08015 Barcelona
Tel: (93) 902.20.30.90
Fax: (93) 902.22.33.66
E-mail: suministros@izasa.es

Sweden

VWR International AB
Fagerstagatan 18A
163 94 Stockholm
Tel: (08) 621 34 00
Fax: (08) 760 45 20
E-mail: info@se.vwr.com
Web site: www.vwr.com

Taiwan

TAIGEN Bioscience Corporation
3F, No. 306, Section 4
Chen-Der Road
111 Taipei
Taiwan, R.O.C.
Tel: (02) 2880 2913
Fax: (02) 2880 2916
E-mail: taigen@ms10.hinet.net

Thailand

Theera Trading Co. Ltd.
64 Charan Sanit Wong Road
(Charan 13) Bangkokyay
Bangkok 10600
Tel: (02) 412-5672
Fax: (02) 412-3244
E-mail: theetrad@samart.co.th

All other countries

QIAGEN GmbH, Germany

