

PhosphoProtein Handbook

PhosphoProtein Purification Kit

PhosphoSerine Antibody Q5

PhosphoThreonine Antibody Q7

For purification and detection of phosphorylated proteins from eukaryotic cells and tissues



QIAGEN Sample and Assay Technologies

QIAGEN is the leading provider of innovative sample and assay technologies, enabling the isolation and detection of contents of any biological sample. Our advanced, high-quality products and services ensure success from sample to result.

QIAGEN sets standards in:

- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

Our mission is to enable you to achieve outstanding success and breakthroughs. For more information, visit www.qiagen.com.



Contents

Kit Contents	4
Storage	4
Product Use Limitations	4
Product Warranty and Satisfaction Guarantee	4
Quality Control	5
Technical Assistance	5
Safety Information	5
Introduction	6
Principle and Procedures	9
Detecting Phosphorylated Proteins	9
Protocols	
■ Phosphoprotein Purification from Cultured Cells	11
■ Phosphoprotein Purification from Tissues	13
■ Concentrating Protein Fractions Using Nanosep Ultrafiltration Columns	15
■ Immunodetection of Phosphorylated Proteins Using PhosphoSerine or PhosphoThreonine Antibodies (Chemiluminescent Method)	16
Troubleshooting Guide	18
Appendix A: Buffer Compositions	20
Ordering Information	21

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® Nuclease (DNase/RNase, >99% purity)	2000 Units (25 U/μl)
Protease inhibitor tablets	6
Nanosep® ultrafiltration columns (10 kDa molecular weight cutoff)	6

Storage

PhosphoProtein Purification Columns, buffers, protease inhibitor tablets, and Benzonase® Nuclease 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® Nuclease can be frozen at –20°C.

Nanosep ultrafiltration columns should be stored dry at room temperature (15–25°C).

Product Use Limitations

PhosphoProtein purification products and antibodies are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

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. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

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 back cover or visit www.qiagen.com).

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of PhosphoProtein products is tested against predetermined specifications to ensure consistent product quality.

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 sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding PhosphoProtein purification products 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 see our Technical Support center at www.qiagen.com/goto/TechSupportCenter or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

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.

The following risk and safety phrases apply to PhosphoProtein purification products:

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 2D 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 2D electrophoresis and autoradiography. As an alternative to radiolabeling, western blots made from 2D 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 2D 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.

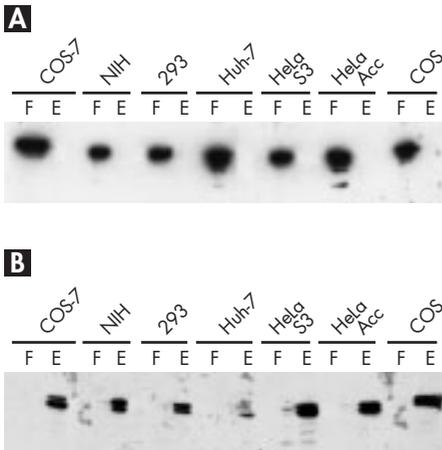
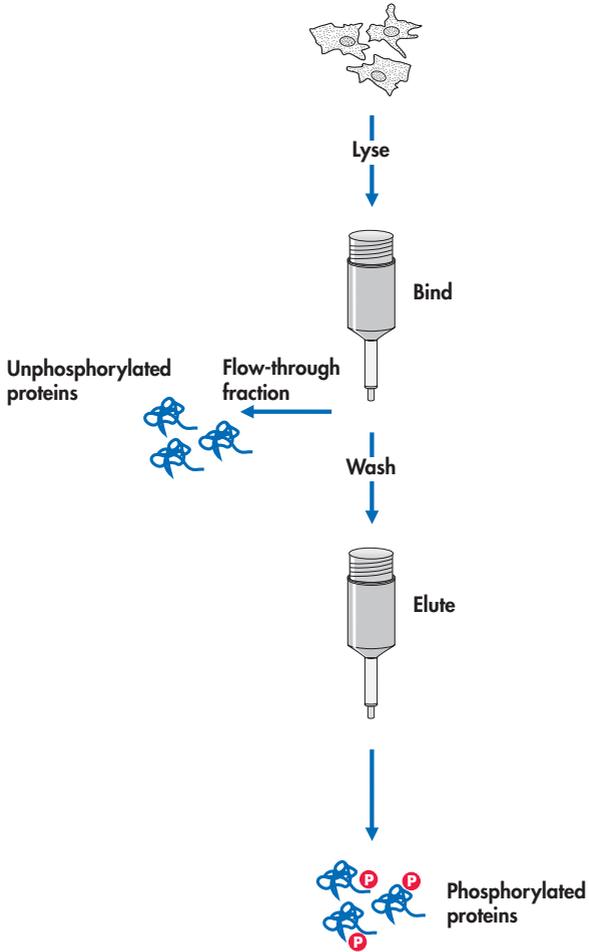


Figure 1 Complete separation of unphosphorylated and phosphorylated proteins. Protein-specific immunodetection of **A** unphosphorylated HSP-60 protein, and **B** phosphorylated p44 and p42 mitogen-activated protein kinase (MAPK) proteins. **F** flow-through; **E** eluate fractions. The antibody used 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



Principle and Procedures

PhosphoProtein purification products are 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 PhosphoProtein Purification resin, while proteins without phosphate groups do not bind to the resin and can therefore be found in the flow-through fraction (Figure 2, page 10).

Gentle lysis of samples 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 sample 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 binds to the PhosphoProtein Purification resin 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 resin relatively slowly, 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 or 30 mg tissue. 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, page 10). Expected concentration of proteins in the eluate is around 0.2 µg/µl. 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

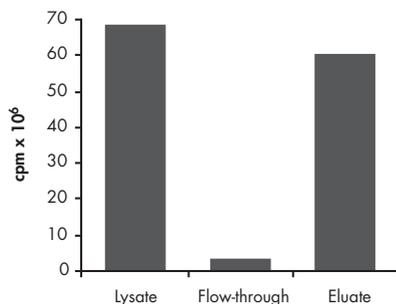


Figure 2. Highly specific separation of phosphorylated proteins. Non-stimulated Jurkat cells were radioactively labeled in vivo using ³²P. Cell lysate was processed using the PhosphoProtein Purification Kit and the radioactivity in each fraction measured. Data kindly provided by Gudrun Rehg and Sascha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

Protocol: PhosphoProtein Purification from Cultured Cells

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 2D 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 2D 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 cells as this will interfere with binding of phosphorylated proteins to the column.
- If required, a phosphatase inhibitor can be added to samples. We recommend using Phosphatase Inhibitor Cocktail A (cat. no. sc-45044) from Santa Cruz Biotechnology, Inc. (www.scbt.com) according to the manufacturer's instructions. Important: Small-molecule phosphatase inhibitors (e.g., vanadate, molybdate, or fluoride) should not be used with PhosphoProtein purification resins.

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® Nuclease 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 10^7 cells in the 5 ml of lysis buffer containing protease inhibitors and Benzonase® Nuclease prepared in step 2.
4. Incubate for 30 min at 4°C. Vortex briefly every 10 min.
5. After 30 min incubation, centrifuge the cell lysate at 10,000 x g and 4°C for 30 min.
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 (15–25°C).
10. Apply 6 ml of PhosphoProtein Lysis Buffer containing 0.25% CHAPS to wash the column, and allow to flow through.
11. Apply 500 µl 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.

Protocol: PhosphoProtein Purification from Tissues

This protocol is suitable for processing tissues. In comprehensive tests using rat tissues, optimal sample sizes per preparation were found to be 30 mg (liver, spleen, and brain).

Equipment and reagents to be supplied by the user

- TissueRuptor rotor-stator homogenizer

Important notes before starting

- Ensure that you are familiar with operating the TissueRuptor. Refer to the TissueRuptor User Manual for operating instructions.
- 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.
- If required, a phosphatase inhibitor can be added to samples. We recommend using Phosphatase Inhibitor Cocktail I from Sigma (cat. no. P2850) according to the manufacturer's instructions. Important: Small-molecule phosphatase inhibitors (e.g., vanadate, molybdate, or fluoride) should not be used with PhosphoProtein purification resins.

Procedure

1. Add 875 μ l CHAPS Stock Solution (10% [w/v]) to 35 ml of PhosphoProtein Lysis Buffer and 75 μ l 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 μ l of Benzonase® stock solution to a 5 ml aliquot of PhosphoProtein Lysis Buffer containing CHAPS prepared in step 1 and mix.
3. Using the TissueRuptor, homogenize the tissue sample in 350 μ l Lysis Buffer for 30 s at medium speed.
4. Dilute the tissue homogenate with 1500 μ l Lysis Buffer.
5. Incubate for 30 min at 4°C. Vortex briefly every 10 min.
6. After incubation, centrifuge the homogenate at 10,000 x g and 4°C for 30 min.

7. 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.
8. Harvest the supernatant and determine the protein concentration (e.g., using the Bradford method).
9. 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.
10. 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.

Important: This and all subsequent steps should be performed at room temperature (15–25°C).

11. Apply 6 ml of PhosphoProtein Lysis Buffer containing 0.25% CHAPS to wash the column, and allow to flow through.
12. Apply 500 μ l PhosphoProtein Elution Buffer containing 0.25% CHAPS to the column, and collect the eluted fraction.
13. Repeat step 12 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. 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 μ l elution volume should be reduced to a volume of 50 μ l). For SDS-PAGE followed by silver staining, the eluates can be used without concentration.

For 2D 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 μ l by ultrafiltration; adding 450 μ l 10 mM Tris-Cl, pH 7.0; reducing the volume to 50 μ l once more by ultrafiltration; adding a further 450 μ l 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 2D gel loading buffer and loaded onto the gel.

Protocol: 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 min.
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 min (Figure 3).

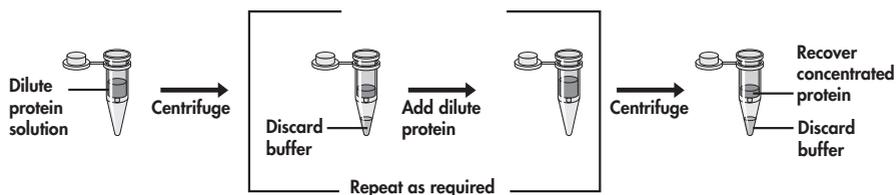


Figure 3 Concentration of dilute protein solutions using the NanoSep ultrafiltration column.

Protocol: 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 buffer:	10 mM Tris-Cl; 150 mM NaCl; 0.1% (v/v) Tween® 20, 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 (15–25°C).**

2. **Incubate for 1 hour in blocking buffer at room temperature (15–25°C).**

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 (15–25°C).**

4. **Wash membrane for 10 minutes with TBS buffer at room temperature (15–25°C).**

5. **Incubate membrane with PhosphoProtein Antibody solution (1/100 –1/200 dilution of antibody in TBS-Tween buffer) at 4°C overnight.**

Membrane can be sealed in plastic bags.

Note: To avoid cross-reactions, do not use blocking buffer to dilute PhosphoSerine or PhosphoThreonine antibodies.

6. **Wash twice for 10 minutes each time in TBS-Tween/Triton buffer at room temperature (15–25°C).**

7. **Wash for 10 minutes in TBS buffer at room temperature (15–25°C).**

8. **Incubate with secondary antibody solution for 1 hour at room temperature (15–25°C).**

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 (15–25°C).**

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

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

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

a) Lysate is too concentrated

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

b) 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.

c) 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.

d) 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 2D gel

Salt present in sample

For 2D gel analysis, protein fractions must be concentrated and desalted. Use a Nanosep ultrafiltration column to perform buffer exchange and concentration (see page 15).

High background on membrane using PhosphoSerine or PhosphoThreonine Antibodies

a) Blocking buffer used to dilute PhosphoProtein Antibodies

Do not use blocking buffer for dilution of PhosphoProtein Antibodies.

b) 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)

Appendix A: Buffer Compositions

PhosphoProtein Binding Buffer (1 liter):

25 mM MES	4.88 g	2-Morpholino-ethanesulfonic acid monohydrate (MW 137.99 g/mol)
1 M NaCl	58.44 g	Sodium chloride (MW 58.44 g/mol)
0.25% (w/v) CHAPS	2.50 g	CHAPS

Adjust pH to 6.0 using NaOH and sterile filter (0.2 or 0.45 μ m).

PhosphoProtein Elution Buffer (1 liter):

50 mM Potassium phosphate	7.24 g	di-potassium hydrogen phosphate (K_2HPO_4 , MW 174.2 g/mol)
	1.15 g	Potassium dihydrogen phosphate (KH_2PO_4 , MW 136.1 g/mol)
50 mM NaCl	2.92 g	Sodium chloride (MW 58.44 g/mol)

pH should be 7.5. Sterile filter (0.2 or 0.45 μ m) before use.

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
TissueRuptor	Handheld rotor–stator homogenizer, 5 TissueRuptor Disposable Probes	9001271* 9001272† 9001273‡ 9001274§
TissueRuptor Disposable Probes (25)	25 nonsterile plastic disposable probes for use with the TissueRuptor	990890

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

* 120 V, 60 Hz (for North America and Japan)

† 235 V, 50/60 Hz (for Europe, excluding UK and Ireland)

‡ 235 V, 50/60 Hz (for UK and Ireland)

§ 235 V, 50/60 Hz (for Australia)

Notes

Trademarks: QIAGEN® (QIAGEN Group); Benzonase® (Merck KGaA, Germany); ECL™ (Amersham Biosciences); Nanosep® (Pall Corporation); Triton® (Rohm & Haas Company); Tween® (ICI Americas Inc.).

Benzonase® Nuclease is supplied by Merck KGaA and its Affiliates. Benzonase® is a registered trademark of Merck KGaA, 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.

Limited License Agreement

Use of this product signifies the agreement of any purchaser or user of PhosphoProtein purification products and antibodies to the following terms:

1. PhosphoProtein purification products and antibodies may be used solely in accordance with the *PhosphoProtein Handbook* and for use with components contained in the Kit only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this Kit with any components not included within this Kit except as described in the *PhosphoProtein Handbook* and additional protocols available at www.qiagen.com.
2. Other than expressly stated licenses, QIAGEN makes no warranty that this Kit and/or its use(s) do not infringe the rights of third-parties.
3. This Kit and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the Kit agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the Kit and/or its components.

For updated license terms, see www.qiagen.com.

© 2002–2011 QIAGEN, all rights reserved.

www.qiagen.com

Australia = Orders 1-800-243-800 = Fax 03-9840-9888 = Technical 1-800-243-066

Austria = Orders 0800-28-10-10 = Fax 0800/28-10-19 = Technical 0800-28-10-11

Belgium = Orders 0800-79612 = Fax 0800-79611 = Technical 0800-79556

Brazil = Orders 0800-557779 = Fax 55-11-5079-4001 = Technical 0800-557779

Canada = Orders 800-572-9613 = Fax 800-713-5951 = Technical 800-DNA-PREP (800-362-7737)

China = Orders 86-21-3865-3865 = Fax 86-21-3865-3965 = Technical 800-988-0325

Denmark = Orders 80-885945 = Fax 80-885944 = Technical 80-885942

Finland = Orders 0800-914416 = Fax 0800-914415 = Technical 0800-914413

France = Orders 01-60-920-926 = Fax 01-60-920-925 = Technical 01-60-920-930 = Offers 01-60-920-928

Germany = Orders 02103-29-12000 = Fax 02103-29-22000 = Technical 02103-29-12400

Hong Kong = Orders 800 933 965 = Fax 800 930 439 = Technical 800 930 425

Ireland = Orders 1800 555 049 = Fax 1800 555 048 = Technical 1800 555 061

Italy = Orders 800-789-544 = Fax 02-334304-826 = Technical 800-787980

Japan = Telephone 03-6890-7300 = Fax 03-5547-0818 = Technical 03-6890-7300

Korea (South) = Orders 080-000-7146 = Fax 02-2626-5703 = Technical 080-000-7145

Luxembourg = Orders 8002-2076 = Fax 8002-2073 = Technical 8002-2067

Mexico = Orders 01-800-7742-639 = Fax 01-800-1122-330 = Technical 01-800-7742-639

The Netherlands = Orders 0800-0229592 = Fax 0800-0229593 = Technical 0800-0229602

Norway = Orders 800-18859 = Fax 800-18817 = Technical 800-18712

Singapore = Orders 1800-742-4362 = Fax 65-6854-8184 = Technical 1800-742-4368

Spain = Orders 91-630-7050 = Fax 91-630-5145 = Technical 91-630-7050

Sweden = Orders 020-790282 = Fax 020-790582 = Technical 020-798328

Switzerland = Orders 055-254-22-11 = Fax 055-254-22-13 = Technical 055-254-22-12

UK = Orders 01293-422-911 = Fax 01293-422-922 = Technical 01293-422-999

USA = Orders 800-426-8157 = Fax 800-718-2056 = Technical 800-DNA-PREP (800-362-7737)

