Calbiochem® & Novagen®

Protein Sample Preparation Tools for Signal Transduction Research & Proteomics

INSIDE:

EMD

ProteoExtract®
Subcellular Proteome Extraction Kit

The ProteoExtract® Subcellular Proteome Extraction Kit (S-PEK) is designed for reproducible extraction of subcellular proteomes from mammalian cells. Based on different solubilities of certain subcellular compartments, the S-PEK utilizes proprietary chemistries to yield four sub-proteome fractions which are enriched in cytosolic, membrane/organelle, nuclear, and cytoskeletal proteins. In the case of adherent cells, the procedure is performed directly in the tissue culture dish without the need for cell removal. For suspension grown cells, extraction starts with gentle sedimentation and washing of cells. For tissues, the isolation of viable cells is required before proceeding with the extraction protocol. (See ProteoExtract Tissue Dissociation Buffer Set, page 4). The especially mild S-PEK procedure results in non-denatured proteins suitable for many downstream applications.

Applications

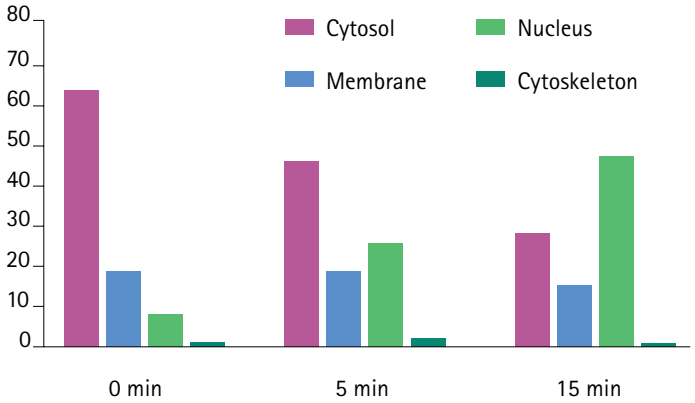
S-PEK fractions are particularly well suited for sensitive proteomics applications:

- Subcellular redistribution assays to monitor protein translocation
- Enzyme activity assays including reporter gene assays and kinase assays
- SELDI-profiling
- Non-denaturing gel electrophoresis
- Assaying protein expression levels using fluorescent labeled subcellular extracts, in microarrays

Product features

Kit configuration	All reagents included: extraction & wash buffers, protease inhibitor
Kit sizes	For 10 or 20 samples
Sample type	Mammalian adherent and suspension - grown cells mammalian tissue
Sample volume	10^6-10^7 cells 25-50 mg tissue scalable
Fractions	4 fractions: cytosolic, membrane/organelles, nuclear, and cytoskeletal
Downstream applications	1-& 2-D-GE, ELISA, SELDI, immunoblotting, activity assays, subcellular redistribution assays, arrays

Redistribution of NFκB upon stimulation of cells



Analysis of protein distribution profiles to characterize cellular changes, exemplified by a time-course analysis of NFκB redistribution in stimulated cells.

A431 cells were stimulated with 0.2 µg/ml TNFα, and stepwise extraction of cytosolic fraction (F1), organelle/membrane fraction (F2) nuclear fraction (F3) and cytoskeletal proteins (F4) was performed using the S-PEK procedure. Cell fractions were separated by SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was probed with anti-NFκB. The time-course analysis demonstrates a measurable translocation of NFκB from the cytoplasm to the nucleus as early as 5 min and a stronger response at 15 min.

Product	Size	Cat. No.	Price
ProteoExtract® Subcellular Proteome Extraction Kit	20 rxn	539790	$324
ProteoExtract® Subcellular Proteome Extraction Kit, Mini	10 rxn	539791	$185

Orders Phone 800 854 3417
Fax 800 776 0999
Web www.emdbiosciences.com/novagen

ProteoExtract®
Native Membrane Protein Extraction Kit

The ProteoExtract® Native Membrane Protein Extraction Kit (M-PEK) is designed for the isolation of native membrane proteins from mammalian cells and tissue. M-PEK extracts proteins based on the association of proteins with cellular membranes. The extremely mild extraction conditions yield a 3-5 fold enrichment of integral membrane and membrane-associated proteins. The simple two-step procedure allows processing of multiple samples in parallel. For tissues, the isolation of viable cells is required before proceeding with the extraction protocol. (See ProteoExtract Tissue Dissociation Buffer Set, page 4).

Applications

Extracted membrane proteins can be used for a variety of assays, including:

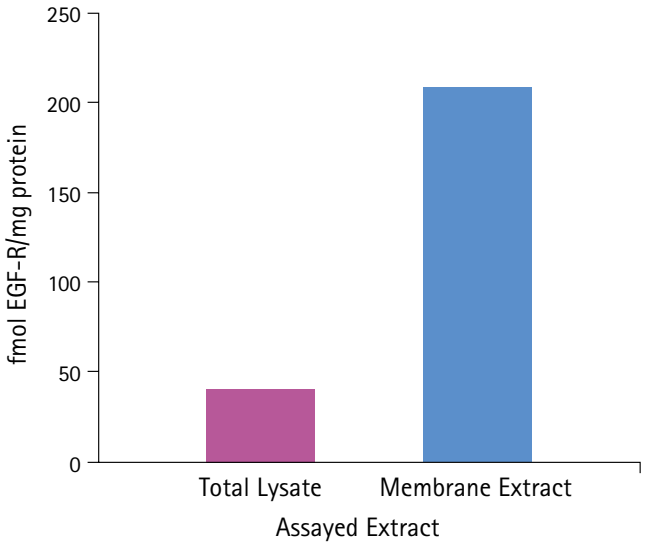
- Enzyme activity assays, including reporter gene assays and kinase assays
- (Non-denaturing) and denaturing gel electrophoresis, immunoblots and immunoassays
- Assaying post-translational modifications, such as phosphorylation
- SELDI-profiling of integral and membrane-associated proteins
- NHS ester labeling of membrane proteins

Product features

Kit configuration	Extraction & wash buffers, protease Inhibitor cocktail
Kit size	20 samples
Sample type	Adherent & suspension tissue culture cells, mammalian tissue*
Sample volume	2-5 x 10^6 cells 25-50 mg tissue scalable
Processing time	1-1.5 h from sample to membrane protein fraction
Downstream applications	1- & 2-D GE, co-immunoprecipitation, ELISA, immunoblotting, enzyme activity assays, SELDI

*requires treatment with tissue dissociation buffer set prior to extraction of membranes proteins

EGF-Receptor Enrichment



HEK293 cells were extracted with buffered 1% Triton® X-100 to generate a total lysate or extracted with M-PEK to yield a membrane fraction. Equal fractions of the fractions obtained were utilized to quantitate the concentration of EGF-receptor in the samples using a EGF-R ELISA Kit. Protein concentrations were determined and utilized to calculate the amount of EGF-R per mg protein in the total lysate and the membrane fraction, which are plotted in the depicted graph. The measurements demonstrate a 4.5-fold enrichment of the signaling receptor in the M-PEK extracted membrane fraction.

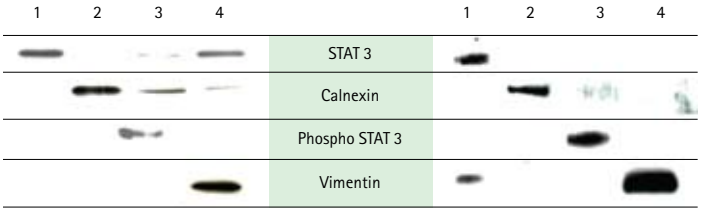
Product	Size	Cat. No.	Price
ProteoExtract® Native Membrane Protein Extraction Kit	20 rxn	444810	$291

ProteoExtract®
Tissue Dissociation Buffer Set

The ProteoExtract® Tissue Dissociation Buffer Set is a simple buffer system for the dissociation of fresh tissue. Fresh tissue is first minced into small pieces, washed with PBS, and incubated with Dissociation Buffer. Following the incubation, the tissue is strained using a tissue sieve. Due to the diversity of tissue samples, the optimal collagenase should be selected. (See ProteoExtract Collagenase Set, below).

The ProteoExtract Tissue Dissociation Buffer Set is an accessory product for following kits:

- ProteoExtract Subcellular Proteome Extraction Kit (S-PEK, Cat. No. 539790)
- ProteoExtract Subcellular Proteome Extraction Kit, Mini (S-PEK Mini, Cat. No. 539791)
- ProteoExtract Native Membrane Protein Extraction Kit (M-PEK, Cat. No. 444810).



Subcellular Protein Extraction from Tissue Cells

Tissue dissociation of rat pancreatic tissue (panel A) and human pancreatic tissue (panel B) was performed using the ProteoExtract Tissue Dissociation Buffer Set. 1.3x 10^7 cells were used for fractionation following the ProteoExtract Subcellular Proteome Extraction Kit procedure (Cat. No. 539790). Proteins from the cytosolic fraction (lane 1), the membrane fraction (lane 2), the nuclear fraction (lane 3), and the cytoskeletal fraction (lane 4) were separated by SDS-PAGE, transferred to PVDF membrane, and probed with antibodies to the indicated markers.

Product features

Kit configuration	Dissociation buffer and protease inhibitor
Kit size	10 tissue samples
Sample type	Fresh mammalian tissue
Amount of sample	1 g tissue
Processing time	1-1.5 h
Downstream applications	Subcellular fractionation using S-PEK or M-PEK

Product	Size	Cat. No.	Price
ProteoExtract® Tissue Dissociation Buffer Set	10 rxn	539720	$80

NEW

ProteoExtract® Collagenase Set

ProteoExtract Collagenase Set is a collection of four types of crude preparations of collagenase from *Clostridium histolyticum* (Type I, II, III and IV) that are qualified for tissue dissociation. This kit is ideal for use with the ProteoExtract Tissue Dissociation Buffer Set and when working with a variety of sample types which often requires the optimal collagenase for proper dissociation. The kit includes 100 mg of each lyophilized enzyme.

Collagenase	Description	Activity	Storage
Type I	Generally recommended for adipocytes, epithelial, hepatocytes, lung, and adrenal cell isolation. Contains average amounts of collagenase, caseinase, clostripain, and trypsin.	≥ 125 Units per milligram dry weight	4° C
Type II	Generally used for cardiomyocytes, bone, cartilage, muscle, thyroid, and endothelial cell isolation. Contains higher protease contaminant levels, especially clostripain, and trypsin.	≥ 125 Units per milligram dry weight	4° C
Type III	Typically used for mammary and other soft tissues. Contains lower levels of contaminating proteases.	≥ 100 Units per milligram dry weight	4° C
Type IV	Typically used for pancreatic islets and other applications where membrane protein and receptor integrity is crucial. Contains especially low trypsin activity.	≥ 160 Units per milligram dry weight	4° C

Product	Size	Cat. No.	Price
ProteoExtract® Collagenase Set	1 Kit	71777-3	$130

Orders Phone 800 854 3417
Fax 800 776 0999
Web www.emdbiosciences.com/novagen

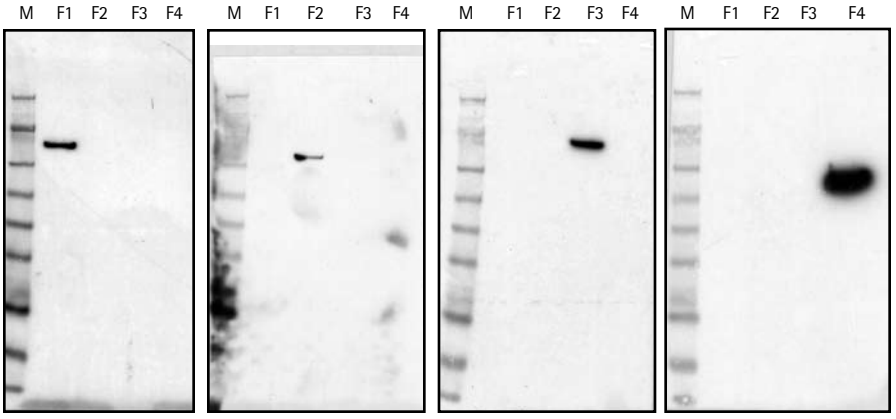
NEW

ProteoExtract®
S-PEK Antibody Control Kit

The ProteoExtract® S-PEK Antibody Control Kit is a set of four monoclonal antibodies that recognize proteins specific for each of the four sub-cellular fractions obtained with the ProteoExtract Subcellular Proteome Extraction Kit (S-PEK). These antibodies can be used in Western blot procedures to confirm proper sub-fractionation of cellular proteomes. The kit provides 15 µg/vial each of the following antibodies: Anti-HSP 90α, Anti-Vimentin, Anti-Calnexin, Anti-PARP-1. HSP90α protein is a marker for the cytosolic fraction, calnexin is a trans-membrane protein, PARP is a nuclear protein, and vimentin is an intermediate filament protein found in the cytoskeleton. Due to the species specificity of Anti-HSP 90 and Anti-Vimentin antibodies, we recommend to use this kit with cellular fractions obtained from human-derived cell lines and tissues.

Antibody	Epitope	Species Reactivity	Applications
Anti-Hsp90α Mouse mAb (EMD-17D7)	Recognizes the ~90 kDa Hsp90α (heat shock protein 90α) protein in HeLa cells. Does not recognize Hsp90β.	human	IB, IP, ELISA
Anti-Calnexin Mouse mAb (TO-5)	Synthetic peptide corresponding to amino acids 509-524 of human calnexin	human	IB
Anti-Vimentin/LN6 (Ab-1) Mouse mAb (V-9)	Recognizes the ~58 kDa vimentin protein. Does not cross-react with closely related intermediate filament proteins including desmin and GFA.	chicken, human, porcine, rat, not mouse	IB, IF, PS, FS
Anti-PARP-1 (Ab-2) Mouse mAb (C-2-10)	Recognizes the ~116 kDa full-length PARP protein and the ~85 kDa cleaved fragment of PARP in etopside-treated HL-60 cells	hamster, human, monkey, mouse, rat, not chicken	IB, IF

ELISA: enzyme-linked immunosorbent assay, FS: Frozen Sections, PS: Paraffin Sections IB: immunoblotting, IP: immunoprecipitation, IF: Immunofluorescence



A - HSP90α
B - Calnexin
C - PARP-1
D - Vimentin

Sub-cellular fractions from suspension SAOS-2 cells were prepared according to the S-PEK protocol and analyzed by Western blot. For each blot, 2.0 µg of total protein from S-PEK fractions F1, F2, F3, and F4 was run on an 8-12% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were blocked with PBST containing 5% (w/v) non-fat dry milk for 30 minutes at room temperature and then incubated in a solution containing the primary antibody for one hour at room temperature. After three washes with PBST, blots were incubated with a goat anti-mouse HRP-conjugated secondary antibody at a 1:10,000 dilution for 30 min at room temperature. Blots were washed three times with PBS-T and developed using a chemiluminescent substrate. For all blots, lane 1: molecular weight markers; lane 2: S-PEK fraction F1 (cytoplasmic protein); lane 3: S-PEK fraction F2 (membrane/organelle proteins); lane 4: S-PEK fraction F3 (nuclear proteins); lane 5: S-PEK fraction F4 (cytoskeletal proteins).

1° Antibody	Concentration (µg/mL)	M.W. of Antigen (kDa)
HSP90α	1.0	90
Calnexin	1.0	68
PARP1	1.0	116
Vimentin	0.1	58

Product	Size	Cat. No.	Price
ProteoExtract® S-PEK Antibody Control Kit	1 Kit	71771-3	$295

Technical Support
Phone 800 628 8470
E-mail novagen@emdchemicals.com

NEW

ProteoExtract® Transmembrane Protein Extraction Kit

The ProteoExtract® Transmembrane Protein Extraction Kit (TM-PEK) provides a novel detergent-free chemistry that enables mild and efficient extraction of transmembrane proteins from mammalian cells and tissues. GPCRs (G-protein coupled receptors) or 7-TMs (seven transmembrane passing proteins) are the largest protein family known, which are involved in all types of stimulus-response pathways, from intercellular communication to physiological sensing. The GPCR family of receptors is well-established as the premier target family for drug development and the new TM-PEK is a unique tool to study the function of these drug target proteins *in vitro*. Extracted and enriched protein classes comprise small to very large proteins, proteins with single pass to several transmembrane passing domains and even large protein complexes.

Applications

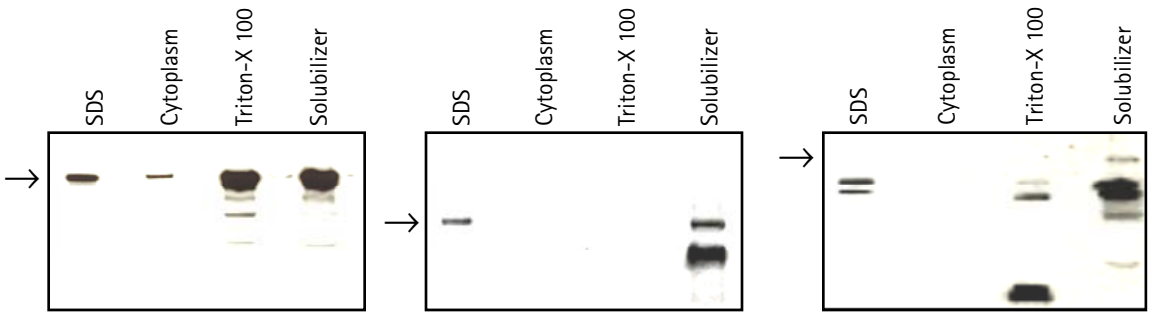
Fraction of enriched membrane proteins can be used for a variety of assays including:

- Enzyme activity assays including kinase assays
- Non-denaturing gel electrophoresis
- Immunoblots and immunoassays
- Tryptic in-gel digest followed by MS analysis

Product features

Kit configuration	Extraction buffers, two alternative solubilizing reagents, protease inhibitor cocktail
Kit size	40 samples
Sample type	Tissue culture cells, mammalian tissues
Amount of sample	2-5 x 10^6 cells 25-50 mg tissue scalable
Processing time	1 - 1.5 h from sample to membrane protein fraction
Downstream applications	1 & 2D PAGE, immunoprecipitation, ELISA, immunoblotting, enzyme activity assays, MS

Extraction of transmembrane proteins



Transmembrane proteins were extracted from MDA-MB 468 (human breast cancer) using the TM-PEK two-step procedure. In the first step, three identical pools of 1 × 10^7 cells were treated with the TM-PEK extraction buffer 1, which recovers proteins from the cytosolic fraction. The insoluble material (i.e., the membrane fraction), was then treated with TM-PEK extraction buffer 2A (TM-PEK 2A), 0.5% SDS, or 0.5% Triton® X-100. One-tenth of the total volume from each extraction (equivalent to 1 × 10^6 cells) was run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were blocked and incubated with the primary antibody. Blots were developed using an HRP-conjugated secondary antibody and a chemiluminescent substrate.

SDS is a harsh, total extraction of membrane proteins and serves as positive control. Results show that TM-PEK 2A recovers EGFR, a single TM protein, with efficiency comparable to Triton X-100. However, TM-2A extracts Frizzled-4 and CELSR-3, both seven transmembrane proteins, with far greater efficiency than does Triton X-100 (and SDS).

Product	Size	Cat. No.	Price
ProteoExtract® Transmembrane Protein Extraction Kit	20 rxn	71772-3	$320

Orders Phone 800 854 3417
Fax 800 776 0999
Web www.emdbiosciences.com/novagen

NEW ProteoExtract® Phosphopeptide Enrichment TiO_2 Kit & Phosphopeptide Enrichment SCIMAC Kit

In eukaryotic cells, post-translational modifications of proteins such as phosphorylation and dephosphorylation are involved in numerous metabolic pathways and in the transmission of signals that control proliferation, differentiation, and apoptosis. The dis-regulation of the tightly controlled balance between phosphorylation and dephosphorylation may lead to serious pathological conditions. Determining the site of phosphorylation is therefore important to understanding essential signaling pathways and to gain insight into the molecular basis of diseases. The identification of phosphorylation sites is routinely accomplished by mass spectrometry (MS). Due to the high complexity of cellular proteome fractions there is a general need for specific and efficient enrichment strategies of phosphorylated peptides prior to MS. Efficient enrichment strategies are necessary to compensate for the low stoichiometry of phosphopeptides relative to their unphosphorylated counterparts and for poor ionization and ion suppression effects inherent to MS analysis.

Of the different chemical- and affinity-based methods for phosphopeptide capture, no one method is sufficient for enrichment of the entire phosphoproteome. The two different kits introduced here enrich for different, partially overlapping segments of the phosphoproteome, which is especially useful when working with complex samples. As a result of different binding mechanisms, maximum subsets of phosphopeptides are captured, independent of peptide properties like amino acid sequence, additional posttranslational modifications, and conformational features.

TiO_2 Kit

The TiO_2 Kit uses a novel titanium dioxide material to enable identification of large numbers of phosphorylated species from complex protein mixtures. The titanium dioxide is highly selective for phosphorylated peptides in the presence of abundant non-phosphorylated peptides. The protocol and buffers are optimized to produce high yields of the phosphopeptides. Enrichment and selectivity for phosphopeptides is further improved by using a 2.5-DHB "displacer" concentration that is directly compatible with LC-MS and MALDI-MS analysis. The efficiency and specificity of phosphopeptide capturing by the TiO_2 kit is shown in the Figure C (next page). After enrichment, the predominant signals derive from phosphopeptide ions (marked with arrows) and the majority of non-phosphorylated peptides were removed. Only monophosphorylated phosphopeptides were detectable under these conditions.

SCIMAC Kit

The SCIMAC Kit utilizes two sequential batch chromatography steps. First, samples are applied to a strong cation exchange (SCX) resin. Non-acidic peptides bind to the SCX resin, allowing highly acidic peptides that interfere with downstream purification and analysis to be discarded with the supermatant. The eluted sample from the cation exchange step is applied to the MagPrep® Phosphobind Resin, a unique Zr^{2+}-charged IMAC resin, which selectively captures phosphorylated peptides. The efficiency and specificity of phosphopeptide capturing by the SCIMAC Kit is shown in Figure B (next page).

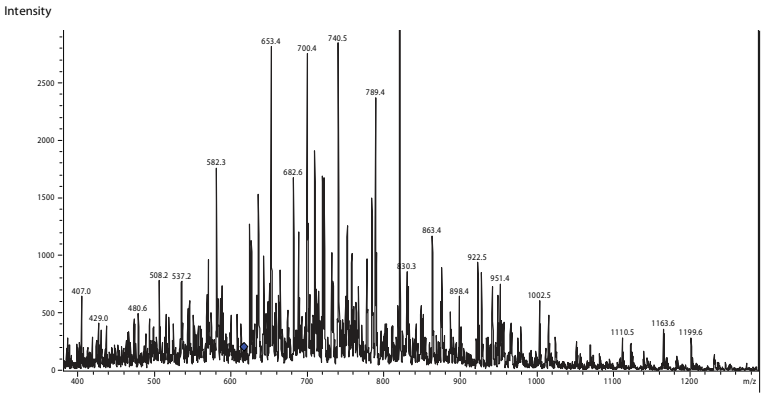
Product features

Kit configurations	All reagents included: binding, wash and elution buffers, resins
Kit size	For 100 samples
Sample type	Proteolytic digests of protein samples
Amount of sample	2.5 nanomole phosphopeptides; scalable
Processing time	0.5 - 1 h from sample binding to elution
Downstream applications	LC/ESI-MS or MALDI-MS

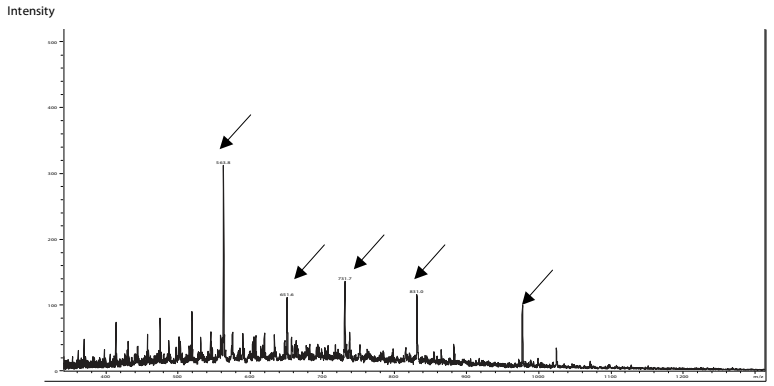
Product	Size	Cat. No.	Price
ProteoExtract® Phosphopeptide Enrichment SCIMAC Kit	100 rxn	539723	$275
ProteoExtract® Phosphopeptide Enrichment TiO_2 Kit	100 rxn	539722	$285

Selective and sensitive enrichment of phosphopeptides from complex mixtures using either SCX/IMAC or Titanium dioxide

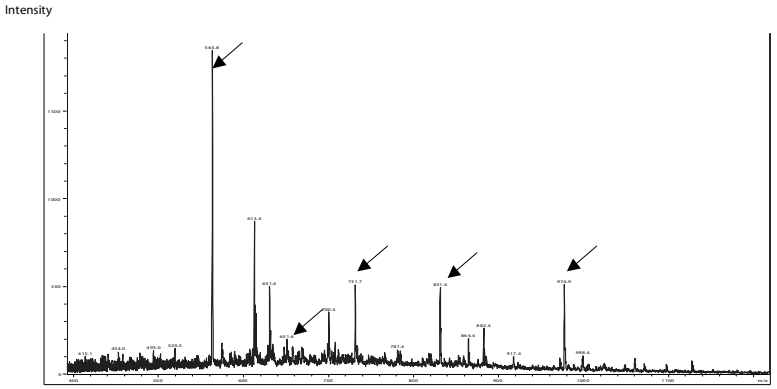
A



B



C



Spiked Phosphopeptides:

1 50pM Angiotensin ~ 6 fmol/ 125µl, ~ 7pg/125µl
2 67pM Calcineurin substrate ~ 8 fmol/ 125µl, ~ 18pg/125µl
3 140pM α-Casein ~ 18 fmol/ 125µl, ~ 425pg/125µl

Titanium dioxide and the combination of SCX and IMAC allow for a specific and sensitive phosphopeptide enrichment with reduced background from complex mixtures. A complex peptide mixture derived from a tryptic digest of porcine liver extract was spiked with alpha-casein and two synthetic phosphopeptides (concentrations see above) and subsequently processed using optimized materials and protocols for strong cation exchange chromatography (SCX) and IMAC or titanium dioxide. Mass spectrometry analysis was performed using an ESI-LC/MS equipment operated in positive mode. Only monophosphorylated phosphopeptides were detectable under these conditions. A: Unprocessed sample B: Enrichment of phosphopeptides using SCX in combination with IMAC. C: Enrichment of phosphopeptides using titanium dioxide. Arrows indicate phosphopeptides.

Application Notes

Fractionate RNA and protein from the same sample to study signal transduction events in siRNA experiments

Researchers are increasingly interested in both the expression level of a specific mRNA and the expression level of the corresponding protein isoform(s). Procedures that allow the combined isolation of RNA and protein from the same experimental sample reduce effort and costs, and simplify the interpretation of experimental results. Although such methods have been described previously, they often involve hazardous chemicals, tend to be denaturing, and are cumbersome due to high sample viscosity. The resulting protein fraction tends to be complex, making it difficult to detect low abundance proteins.

To overcome these limitations, the procedure of the ProteoExtract® Subcellular Proteome Extraction Kit (S-PEK) was successfully adapted to enable parallel, quantitative extraction of both RNA and protein from a single sample. This was effectively achieved by adapting the original extraction buffers of the kit in the following way: The ionic strength of Extraction Buffer (EB) I was increased by supplementing with 285 mM NaCl. Benzonase® Nuclease was added to EB IV rather than to EB III. EB III was supplemented with 3 mM $MgCl_2$.

The increased ionic strength of EB I results in the release of bound polysomes from cellular structures such as the cytoskeleton and ensures that the largest RNA pool (>80%) is collected intact in the first fraction. The extra salt might lead to slight differences in the extraction selectivity compared to the conventional subcellular extraction method. This phenomenon seems to be protein- and cell-type dependent, as no fraction shift for c-Jun was observed in DU-145 cells, whereas in HEK 293 cells the majority of c-Jun was found in the cytosolic fraction rather than in the nucleus. Although the adapted procedure requires a division of the extracted subcellular fractions for subsequent RNA and protein processing, the amount of protein and RNA obtained from 1 x10^6 cells are usually sufficient for various applications, including Northern, dot and slot blot analysis, RT-PCR, and quantitative real-time RT-PCR for RNA analysis and SDS-PAGE, immunoblotting or enzyme assays for protein analysis. Following sequential extraction with the adapted buffer formulations, samples to be used for RNA analysis were mixed immediately with the same volume of a cocktail for RNA precipitation, consisting of 5 M guanidine thiocyanate in ethanol. After precipitation, the RNA can be purified using conventional methods such as oligo-dT enrichment or chaotropic adsorption to silica surfaces. The quality and quantity of RNA extracted by the "modified S-PEK procedure" is comparable to that of RNA extracted by conventional chaotropic methods (data not shown).

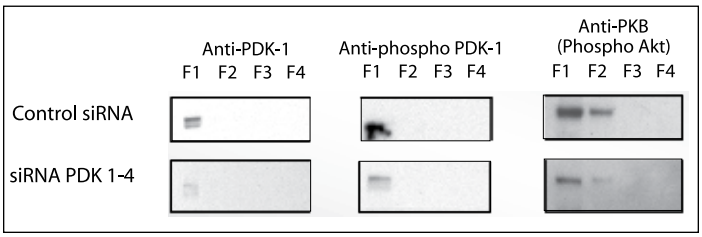
Stimulation or repression of signal transduction events can lead to changes in expression levels of protein isoforms, which are involved in the signal cascade. Such a signal transduction pathway can be silenced through the use of targeted siRNAs. Using the "modified S-PEK procedure", it should be possible to specifically analyse the silencing effect not only on the target RNA and protein level, but also on further down stream effector molecules. This assumption was proven to be true by investigation of the PDK1-AKT signal transduction pathway, whereby PDK1 expression should be decreased through RNA interference. For this purpose MDA MB 468 cells grown to a density of 6x10^5 cells per 10 cm dish were transfected with 20 µM PDK1-4 siRNA or 20 µM control siRNA for 6 h at 37°C and subsequently grown in serum-containing medium. Using the modified S-PEK procedure, RNA and protein was extracted and fractionated from the siRNA-treated cells. RNA was precipitated and purified with silica membranes. The level of down regulation of PDK1 mRNA was quantified using qRT-PCR in relation to the level of two house keeping genes HPRT and RNA polymerase II (RPII). As depicted in table below, the siRNA PDK1 knock down led to a ~5 fold down regulation of the PDK1 mRNA.

Down regulation of PDK1 mRNA by specific siRNA

	Ratio:	
siRNA treatment	PDK-1 / HPRT	PDK-1 / RPII
Control	0.44	0.43
PDK-1	0.1	0.08
Down regulation measured:	4.4 fold	5.4 fold

The effect of the PDK1 mRNA down regulation was investigated on the protein level through immunoblot analysis of the subcellular protein extracts with antibodies against total PDK1: Ser^{214} Phospho-PDK1 (phosphorylation of which is required for activation of PDK1) and Thr^{308} Phospho-AKT/PKB. The reduced PDK1 mRNA level resulted in a diminished level of PDK1 protein and phosphorylated activated PDK1 to about 40% and 22%, respectively. Although the level of AKT/PKB is not directly influenced by the PDK1 mRNA knock-down, the level of activated AKT/PKB, the immediate downstream effector of PDK1, was diminished to 25%, both in the cytosolic (F1) and the membrane-enriched fraction (F2). In conclusion, the subcellular proteome extraction procedure can be modified to enable quantitative extraction of both RNA and protein from a single sample, thus facilitating both transcriptomic and proteomic investigations in RNAi experiments.

Western blot analysis of siRNA-mediated protein levels



Down regulation of PDK1 mRNA by RNAi leads to down regulation of PDK1 protein and subsequently to less activated Akt/PKB, the down stream effector in the related signal transduction cascade.

siRNA treated MDA MB 468 cells were extracted using the described modified S-PEK procedure and proteins of designated cytosolic (F1), membrane (F2), nuclear (F3) and cytoskeletal (F4) fractions, separated by SDS-PAGE, transferred to nitrocellulose, and blotted using specific antibodies against PDK1, Ser^{214} Phospho-PDK1 and Thr^{308} Phospho-AKT/PKB.

Application Notes

High throughput screening by subcellular fractionation: Measuring small drug uptake

When searching for drug candidates potential substances might inhibit most of the current tested drug targets *in vitro*. One major problem is that inhibitors with very low IC50 values lose their inhibitory potential in cell culture and animal models. In order to get more insight into the action of various candidates, the subcellular proteome extraction procedure was used for measuring drug uptake/and drug penetration into living cells.

In the following experiment, the cellular uptake of small molecules was followed. As described in the attached protocol, cells were incubated with the desired small molecule. MultiScreen® Assay Plates (Millipore) were used to facilitate the collection of fractions after each extraction step. The concentration depends on the detection efficiency in mass-spectrometry analysis.

After treating epidermal human cancer cells with each drug over night the subcellular protein extraction protocol was performed. To test the integrity of the fractionation, assays for lactase dehydrogenase (marker protein for the cytoplasmic fraction), and alkaline phosphatase (marker protein for the membrane fraction) were employed. Propidium iodine (PI) staining was used to confirm the nuclear fraction.

The isolated protein populations were analyzsed using Quattropol ESI LC MS analysis. Calibration curves with pure substance in acetonitrile- water solution were recorded in the range from 0.8 to 1000 ng/ml. The sample (fraction) was prepared by adding 20 µl to 15µl internal standard [(IS)/250 ng/ml] plus 150 µl acetonitile/H2O (ratio 60:40). A volume of 10 µl sample was injected using a Chromolith® Speedrod 55-3 mm column (Merck KGaA). The subcellular protein fractions were analyzed to determine the lower limit of quantification (LLOQ) of the particular drug in each fraction. Moreover for each measurement the coefficient of correlation (r) was determined and shows the robustness of the method.

The results show that drug A, which exhibits a low IC50 value *in vitro*, is not able to pass the membrane barrier *in vivo*. Thus it is not a useful drug candidate, because in all four tested fractions the concentration of drug A is below the LLOQ. By contrast, drug B is detectable in various concentrations in all four fractions. High concentrations are found in the cytosolic fraction. The drug is also detectable passing the membrane and accumulating in the cytoskeleton, with the signal intensity higher than in the nuclear fraction (F3). Another important point regarding the function of the drug is that after passing the membrane barrier, drug A is not degraded or metabolized. The ESI LC-MS analysis can be used for the detection of changes in mass to determine the metabolic degradation of a drug, and thus its function *in vivo*.

Protocol:

Cell-culture and treatment

- Seed 5 x 10^4 cells per 96-well (A431 cells) in 180 µl RPMI 1640 and incubate in an atmosphere of 5 % CO_2 at 37°C for 4 h.
- Add test substance in 20 µl volume (final 10µM).
- Incubate the cells for 18 h in 5 % CO_2 at 37°C.
- Proceed with the S-PEK procedure as follows.:

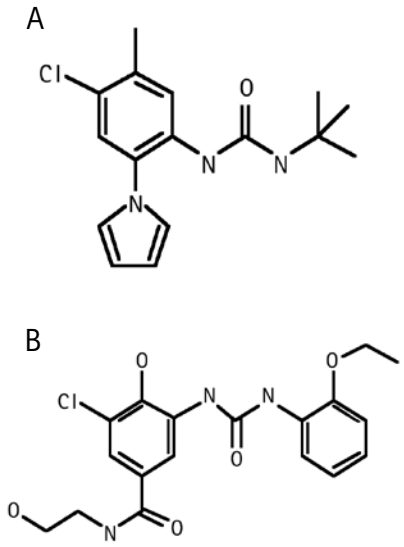


Fig. 1
Chemicals tested for drug uptake in A431 cells. A: 1-tert-butyl-3-(4-chloro-5-methyl-2 pyrrol-1-yl-phenyl)-urea (MW 305,81) B:3-chloro-5-[3-(2-ethoxy-phenyl)-ureido]-4-hydroxy-N-(2-hydroxy-ethyl)-benzamide (MW 393.83).

Fractionation

- Set the 96-well plates into an adaptor plate and centrifuge 5 minutes at 200 × g at 4°C to remove the cell culture media.
- Wash the cells four times by adding 200 µl ice cold PBS and centrifuge 5 minutes at 200 × g at 4°C.
- Add 50 µL S-PEK Extraction Buffer I and incubate at 4°C under gentle agitation.
- Centrifuge the sample for 5 minutes at 200 × g at 4°C, collecting the flow through in a fresh 96-well plate. This is cytosolic fraction (F1).
- Wash the cells by adding 200 µl ice cold PBS and centrifuge 5 minutes at 200 × g at 4°C.
- Add 50 µL S-PEK Extraction Buffer II and incubate at 4°C under gentle agitation.
- Centrifuge the sample for 5 min at 200 × g at 4°C, collecting the flow through in a fresh 96-well plate. This is membrane fraction (F2).
- Wash the cells by adding 200 µl ice cold PBS and centrifuge 5 min at 200 × g at 4°C.
- Add 50 µL S-PEK Extraction Buffer III and incubate at 4°C under gentle agitation.
- Centrifuge the sample for 5 min at 200 × g at 4°C, collecting the flow through in a fresh 96-well plate. This is nuclear fraction (F3).
- Wash the cells by adding 200 µl ice cold PBS and centrifuge 5 min at 200 × g at 4°C.
- Add 50 µL S-PEK Extraction Buffer IV and incubate at 4°C under gentle agitation.
- Centrifuge the sample for 5 min at 200 × g at 4°C, collecting the flow through in a fresh 96-well plate. This is cytoskeletal fraction (F4).

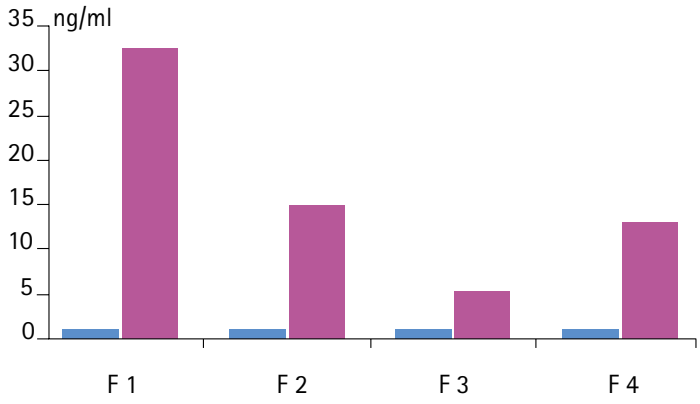


Fig. 2. Concentration of drug A and B in subcellular fractions
Quantification of molecule A (blue bar) and B (purple bar) in subcellular fractions (cytosolic: F1, membrane: F2, nuclear: F3 and cytoskeletal: F4) by Quattropol ESI LC-MS analysis. The lower limit of quantification (LLOQ) of drug A and B was determined to be 0.8 ng/ml. Coefficient of correlation (r) were in all fractions between 0.94 and 0.99.

Measuring drug response of receptor tyrosine kinase inhibitors on EGFR signaling

Over the past 30 years, a relatively simple growth factor and its membrane receptor have provided seminal insights into the understanding of the development of cancer, as well as modes of growth factor signalling. The epidermal growth factor (EGF), its cognate receptor (EGFR) and related family members have been shown to be important in normal, and malignant growth of many cell types. The following experiment shows that after cellular stimulation of epidermal cancer cells with EGF followed by the EGF receptor inhibition the signalling events in the receptor mediated membrane fraction are efficiently silenced. For inhibition different therapeutically relevant receptor tyrosine kinase inhibitors were used. To analyse phosphorylation events the described procedure combines the subcellular protein fractionation procedure with capture of tryptic phosphopeptides and mass spectrometry analysis.

The MS data depicted in Fig. 2 show the effect of three different RTK inhibitors after cellular stimulation of epidermal cancer cells with EGF. The signalling events in the membrane fraction by phosphorylation are efficiently silenced. As expected, the inhibitors repress the signalling from the receptor (membrane fraction) to the nucleus. More importantly nuclear signalling is repressed in different manners depending on the individual RTK inhibitor mode of action.

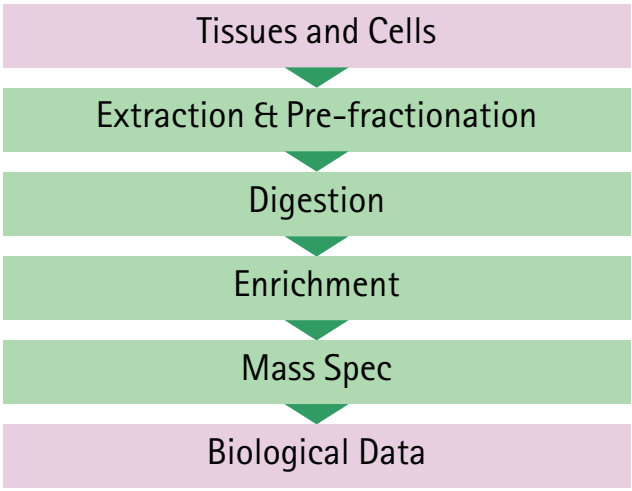


Fig. 1 Work flow for phosphoproteome profiling
After treatment of cell lines the proteomes are fractionated using the ProteoExtract® Subcellular Proteome Extraction Kit. The subcellular fractions were digested with trypsin followed by phosphopeptide enrichment using zirconium based IMAC prior to MS analysis.

Phosphopeptide profiles in subcellular protein fractions

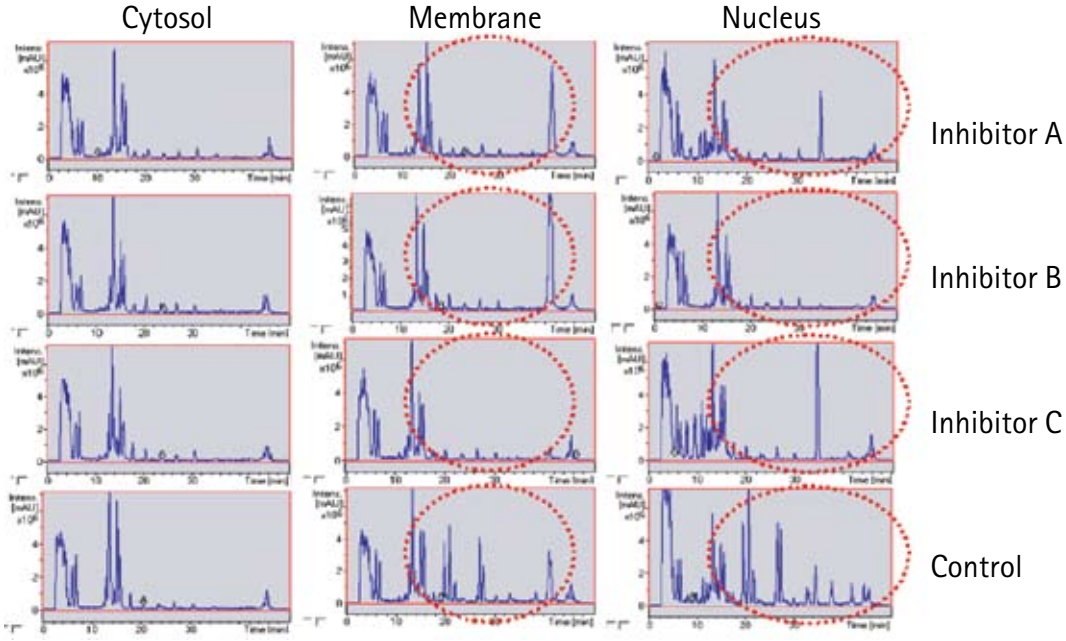


Fig. 2 Pathway analysis of therapeutically relevant RTK-inhibitors. The A431 cancer cells were seeded at a density of 3×10^6 cells per 75 cm^2 flask in RPMI 1640 with 10% FCS and 2 mM L-Glutamine. The cells were stimulated with EGF prior to RTK-inhibitor treatment. After subcellular protein fractionation, 100 µg of each protein fraction were precipitated followed by trypsin digestion. The phosphopeptides of digested S-PEK fractions were captured and analysed by ESI LC/MS.

Selected ProteoExtract® Subcelluar Proteome Extraction Kit Citations

Apoptosis Research

Bivik, C., et al. 2007. Hsp70 protects against UVB induced apoptosis by preventing release of cathepsins and cytochrome c in human melanocytes. *Carcinogenesis* **28**, 537.

de Kozak, Y., et al. 2007. Protein kinase Cς(PKCς) regulates ocular inflammation and apoptosis in endotoxin-induced uveitis (EIU): signaling molecules involved in EIU resolution by PKCςinhibitor and interleukin-13. *Am. J. Pathol.* **170**, 1241.

Luciano, F., et al. 2007. Nur77 converts phenotype of Bcl-B, an antiapoptotic protein expressed in plasma cells and myeloma. *Blood* **109**, 3849.

Masud, A., et al. 2007. Endoplasmic reticulum stress-induced death of mouse embryonic fibroblasts requires the intrinsic pathway of apoptosis. *J. Biol. Chem.* **282**, 14132.

Borradaile, N.M., et al. 2006. Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. *J. Lipid Res.* **47**, 2726.

Nakata, S., et al. 2006.15-Deoxy-Delta12,14-prostaglandin J(2) induces death receptor 5 expression through mRNA stabilization independently of PPARgamma and potentiates TRAIL-induced apoptosis. *Mol. Cancer Ther.* **5**, 1827.

Sakurai, N. and Utsumi, T. 2006. Posttranslational N-myristoylation is required for the anti-apoptotic activity of human tGelsolin, the C-terminal caspase cleavage product of human gelsolin. *J. Biol. Chem.* **281**,14288.

Yu, F., et al. 2006. Overexpression of SOD1 in transgenic rats attenuates nuclear translocation of endonuclease G and apoptosis after spinal cord injury. *J. Neurotrauma* **23**, 595.

Brecht, K., et al. 2005. Hematopoietic transcription factor GATA-2 promotes upregulation of alpha globin and cell death in FL5.12 cells. *Apoptosis 10*, **1063**.

Bunin, A., et al. 2005. Regulation of Bim by TCR signals in CD4/CD8 double-positive thymocytes. *J. Immunol.* **175**,1532.

Feng, Y., et al. 2005. Death-signal-induced relocalization of cyclin-dependent kinase 11 to mitochondria. *Biochem. J.* **392**, 65.

Mourtada-Maarabouni, M., et al. 2005. Functional expression cloning reveals a central role for the receptor for activated protein kinase C 1 (RACK1) in T cell apoptosis. *J. Leukoc. Biol.* **78**, 503.

Rios-Doria, J. and Day, M.L. 2005. Truncated E-cadherin potentiates cell death in prostate epithelial cells. *Prostate* **63**, 259.

Chang, T.S., et al. 2004. Peroxiredoxin III, a mitochondrion-specific peroxidase, regulates apoptotic signaling by mitochondria. *J. Biol. Chem.* **279**, 41975.

Zhang, L. and Insel, P.A. 2004. The pro-apoptotic protein Bim is a convergence point for cAMP/protein kinase A- and glucocorticoid-promoted apoptosis of lymphoid cells. *J. Biol. Chem.* **279**, 20858.

Cancer Research

Chiu, C.-M., et al. 2007. Inaugural article: hepatitis B virus X protein enhances androgen receptor-responsive gene expression depending on androgen level. *Proc. Natl. Acad. Sci. USA* **104**, 2571.

Fang, D., et al. 2007. Phosphorylation of β-catenin by AKT promotes β-catenin transcriptional activity. *J. Biol. Chem.* **282**, 11221.

Kinoshita, K., et al. 2007. Adenovirus-mediated expression of BMP-7 suppresses the development of liver fibrosis in rats. *Gut* **56**, 706.

Uchiyama, S., et al. 2007. Enhanced expression of hepatocyte growth factor activator inhibitor type 2-related small peptide at the invasive front of colon cancers. *Gut* **56**, 215.

Alao, J.P., 2006. The cyclin D1 proto-oncogene is sequestered in the cytoplasm of mammalian cancer cell lines. *Mol. Cancer online* **5:7**.

Chen, H.J., et al. 2006. The role of microtubule actin cross-linking factor 1 (MACF1) in the Wnt signaling pathway. *Genes Dev.* **20**,1933.

Feng, Y.H., et al. 2006. A truncated P2X7 receptor variant (P2X7-j) endogenously expressed in cervical cancer cells antagonizes the full-length P2X7 receptor through hetero-oligomerization. *J. Biol. Chem.* **281**,17228.

Horinaka, M., et al. 2006. The dietary flavonoid apigenin sensitizes malignant tumor cells to tumor necrosis factor-related apoptosis-inducing ligand. *Mol. Cancer Ther.* **5**, 945.

Ito, S., et al. 2006. Differential expression of the human α-enolase gene in oral epithelium and squamous cell carcinoma. *Cancer Sci.* **98**, 499.

Janigro, D., et al. 2006. Alternating current electrical stimulation enhanced chemotherapy: a novel strategy to bypass multidrug resistance in tumor cells. *BMC Cancer online* **6:72**.

Katuri, V., et al. 2006. Critical interactions between TGF-beta signaling/ELF, and E-cadherin/beta-catenin mediated tumor suppression. *Oncogene* **25**,1871.

Kuphal, S. and Bosserhoff, A.K. 2006. Influence of the cytoplasmic domain of E-cadherin on endogenous N-cadherin expression in malignant melanoma. *Oncogene* **25**, 248.

Benetti, R., et al. 2005. The calpain system is involved in the constitutive regulation of beta-catenin signaling functions. *J. Biol Chem.* **280**, 22070.

Liebig, B., et al. 2005. Forced expression of deltaN-TCF-1B in colon cancer derived cell lines is accompanied by the induction of CEACAM5/6 and mesothelin. *Cancer Lett.* **223**,159.

Polette, M., et al. 2005. Membrane-type 1 matrix metalloproteinase expression is regulated by zonula occludens-1 in human breast cancer cells. *Cancer Res.* **65**, 7691.

Rios-Doria, J., et al. 2004. Cleavage of β-Catenin by Calpain in Prostate and Mammary Tumor Cells. *Cancer Res.* **64**, 7237.

Cardiovascular Research

Boateng, S.Y., et al. 2007. Cardiac dysfunction and heart failure are associated with abnormalities in the subcellular distribution and amounts of oligomeric muscle LIM protein. *Am. J. Physiol. Heart Circ. Physiol.* **292**, H259.

Li, H., et al. 2007. FoxO4 regulates tumor necrosis factor alpha-directed smooth muscle cell migration by activating matrix metalloproteinase 9 gene transcription. *Mol. Cell. Biol.* **27**, 2676.

Sayed, D., et al. 2007. MicroRNAs play an essential role in the development of cardiac hypertrophy. *Circ. Res.* **100**, 416.

Cai, J., et al. 2006. Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of vascular endothelial growth factor receptor 1. *J. Biol. Chem.* **281**, 3604.

Chen, I.Y., et al. 2006. Histone H2A.Z is essential for cardiac myocyte hypertrophy but opposed by silent information regulator 2_. *J. Biol. Chem.* **281**,19369.

Lin, T., 2006. Cardiac histones are substrates of histone deacetylase activity in hemorrhagic shock and resuscitation. *Surgery* **139**, 365.

Nagata, D., et al. 2006. Molecular mechanism of the inhibitory effect of aldosterone on endothelial NO synthase activity. *Hypertension* **48**,165.

Takeda, N., et al. 2006. Role of RhoA Inactivation in Reduced Cell Proliferation of Human Airway Smooth Muscle by Simvastatin. *Am. J. Respir. Cell Mol. Biol.* **35**, 722.

Zhao, H., et al. 2006. RECS1 deficiency in mice induces susceptibility to cystic medial degeneration. *Genes Genet. Syst.* **81**, 41.

Crespo, J., et al. 2005. Simvastatin inhibits NOR-1 expression induced by hyperlipemia by interfering with CREB activation. *Cardiovasc. Res.* **67**, 333.

Lypowy, J., et al. 2005. An alliance between Ras GTPase-activating protein, filamin C, and Ras GTPase-activating protein SH3 domain-binding protein regulates myocyte growth. *J. Biol. Chem.* **280**, 25717.

Sato, M., et al. 2005. Intrabodies against the EVH1 domain of Wiskott-Aldrich syndrome protein inhibit T cell receptor signaling in transgenic mice T cells. *FEBS J.* **272**, 6131.

Garcia, K.D., et al. 2004. Immunolocalization of type 2 inositol 1,4,5-trisphosphate receptors in cardiac myocytes from newborn mice. *Am. J. Physiol. Cell Physiol.* **287**, C1048.

Porter, K.E., et al. 2004. Simvastatin reduces human atrial myofibroblast proliferation independently of cholesterol lowering via inhibition of RhoA. Cardiovasc. *Res.* **61**, 745.

Infection and Immunology Research

Gérard, A., et al. 2007. The Par polarity complex regulates Rap1- and chemokine-induced T cell polarization. *J. Cell Biol.* **176**, 863.

Li, Y., et al. 2007. Phosphorylated ERM is responsible for increased T Cell polarization, adhesion, and migration in patients with systemic lupus erythematosus. *J. Immunol.* **178**, 1938.

Cario, E., et al. 2006. Trypsin-sensitive modulation of intestinal epithelial MD-2 as mechanism of lipopolysaccharide tolerance. *J. Immunol.* **176**, 4258.

Cunningham, N.R., et al. 2006. Immature CD4+CD8+ thymocytes and mature T cells regulate Nur77 distinctly in response to TCR stimulation. *J. Immunol.* **177**, 6660.

Dong, Y., et al. 2006. AgDscam, a hypervariable immunoglobulin domain-containing receptor of the Anopheles gambiae innate immune system. *PLoS Biol.* **4**(7) 1137.

Hu, Y. and Ivashkiv, L.B. 2006. Costimulation of chemokine receptor signaling by matrix metalloproteinase-9 mediates enhanced migration of IFN-alpha dendritic cells. *J. Immunol.* **176**, 6022.

Huang, K.J., et al. 2006. The dual-specific binding of dengue virus and target cells for the antibody-dependent enhancement of dengue virus infection. *J. Immunol.* **176**, 2825.

Lee, C.H., et al. 2006. Peroxisome proliferator-activated receptor delta promotes very low-density lipoprotein-derived fatty acid catabolism in the macrophage. *Proc. Natl. Acad. Sci. USA* **103**, 2434.

Neuroscience Research

Stamatovic, S.M., et al. 2006. Protein kinase Cα-RhoA cross-talk in CCL2-induced alterations in brain endothelial permeability. *J. Biol. Chem.* **281**, 8379.

Arndt, S., et al. 2005. Cloning and functional characterization of a new Ski homolog, Fussel-18, specifically expressed in neuronal tissues. *Lab. Invest.* **85**, 1330.

Reid, E., et al. 2005. The hereditary spastic paraplegia protein spastin interacts with the ESCRT-III complex-associated endosomal protein CHMP1B. *Hum. Mol. Genet.* **14**, 19

Williams, K.L., et al. 2005. Hsp27 and axonal growth in adult sensory neurons in vitro. *BMC Neurosci. online* **6**:24.

Metabolic Research

Battista, M.-C., et al. 2007. Seladin-1 expression in rat adrenal gland: effect of adrenocorticotropic hormone treatment. *J. Endocrinol.* **192**, 53.

Fukuhara, D., et al. 2007. Protein characterization of Na+-independent system L amino acid transporter 3 in mice: a potential role in supply of branched-chain amino acids under nutrient starvation. *Am. J. Pathol.* **170**, 888.

Pickard, B.W., et al. 2007. Type 1 parathyroid hormone receptor (PTH1R) nuclear trafficking: regulation of PTH1R nuclear-cytoplasmic shuttling by importin-α/β and chromosomal region maintenance 1/exportin 1. *Endocrinology* **148**, 2282.

Verlaeten, O., et al. 2007. Identification of Urop11, a novel leptin-modulated gene that is upregulated in the hypothalamus of mice with virus-induced obesity. *J. Mol. Endocrinol.* **38**, 3.

Lanoix, D., et al. 2006. Expression of melatoninergic receptors in human placental choriocarcinoma cell lines. *Hum. Reprod.* **21**, 1981.

Zitzer, H., et al. 2006. Sterol regulatory element-binding protein 1 mediates liver X receptor-beta-induced increases in insulin secretion and insulin messenger ribonucleic acid levels. *Endocrinology* **147**, 3898.

Efanov, A.M., et al. 2004. Liver X receptor activation stimulates insulin secretion via modulation of glucose and lipid metabolism in pancreatic beta-cells. Diabetes 53, *Suppl* **3**:S75.

Signal Transduction

Sabio, G., et al. 2005. p38gamma regulates the localisation of SAP97 in the cytoskeleton by modulating its interaction with GKAP. *EMBO J.* **24**, 1134.

Song, Y.S., et al. 2005. Oxidative stress transiently decreases the IKK complex (IKKalpha, beta, and gamma), an upstream component of NF-kappaB signaling, after transient focal cerebral ischemia in mice. *J. Cereb. Blood Flow Metab.* **25**,1301.

Kamakura, S., et al. 2004. Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling. *Nat. Cell. Biol.* **6**, 547.

Mizutani, T., et al. 2004. Tyrosine dephosphorylation of STAT3 in SARS coronavirus-infected Vero E6 cells. *FEBS Lett.* **577**, 187.

Monte, M., et al. 2004. hGTSE-1 expression stimulates cytoplasmic localization of p53. *J. Biol. Chem.* **279**, 11744.

Singh, L.P., et al. 2004. Hexosamines and TGF-beta1 use similar signaling pathways to mediate matrix protein synthesis in mesangial cells. *Am. J. Physiol. Renal Physiol.* **286**, F409.

Other Topics

Nakano, Y., et al. 2007. Expression and localization of plasma transglutaminase factor XIIIA in bone. *J. Histochem. Cytochem.* express March 6.

Greaves, J. and Chamberlain, L.H. 2006. Dual role of the cysteine-string domain in membrane binding and palmitoylation-dependent sorting of the molecular chaperone cysteine-string protein. *Mol. Biol. Cell* **17**, 4748.

Kim, S.J., 2006. Expression of vanilloid receptor 1 in cultured fibroblast. *Exp. Dermatol.* **15,** 362.

Mitsushima, M., et al. 2006. Vinexin β regulates the phosphorylation of epidermal growth factor receptor on the cell surface. *Genes Cells online* **11**, 971.

Ruehl, M., et al. 2005. The elongated first fibronectin type III domain of collagen XIV is an inducer of quiescence and differentiation in fibroblasts and preadipocytes. *J. Biol. Chem.* **280,** 38537.

Saika, S., et al. 2005. Expression of Smad7 in mouse eyes accelerates healing of corneal tissue after exposure to alkali. *Am. J. Pathol.* **166,** 1405.

Abdolzade-Bavil, A., et al. 2004. Convenient and versatile subcellular extraction procedure that facilitates classical protein expression profiling and functional protein analysis. *Proteomics* **4,**1397.

Sun, J., et al. 2005. Cochlear gap junctions coassembled from Cx26 and 30 show faster intercellular Ca2+ signaling than homomeric counterparts. Am. J. Physiol. *Cell Physiol.* **288,** C613.

Yeretssian, G., et al. 2005. Competition on nitrocellulose-immobilized antibody arrays: from bacterial protein binding assay to protein profiling in breast cancer cells. *Mol. Cell Proteomics* **4**, 605.

Okamoto, K., et al. 2004. Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. *J. Virol.* **78,** 6370.

For more information or to order Novagen products, contact EMD

Orders
Phone 800 854 3417
Fax 800 776 0999

Technical Support
Phone 800 628 8470
E-mail novagen@emdchemicals.com

Visit our website
www.emdbiosciences.com/novagen

You can also order Novagen products through our distribution partner, VWR International
Phone 800 932 5000
Web www.vwr.com

Printed in the USA

Protein Sample Preparation Tools
10001296 USD