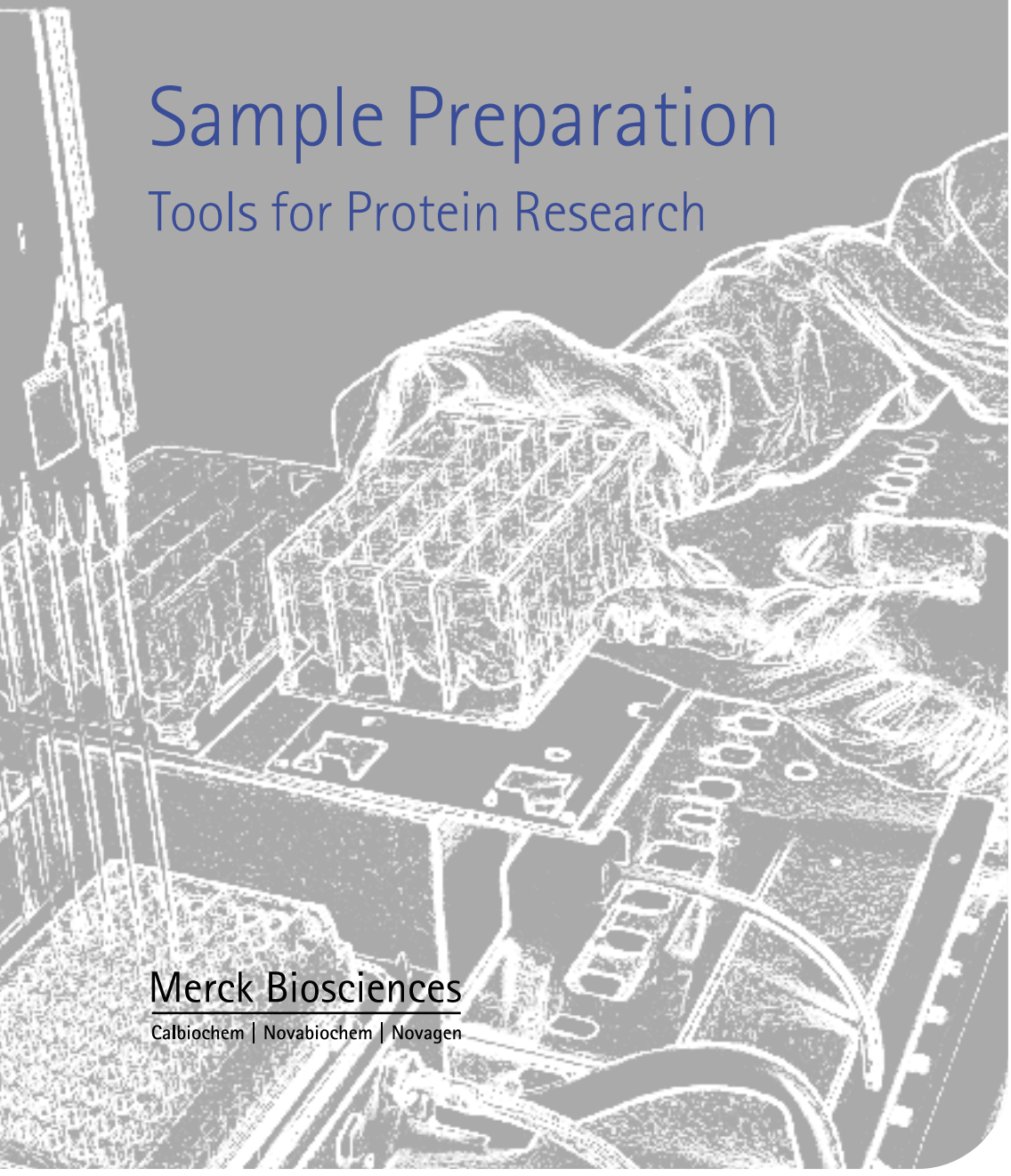
Novagen® & Calbiochem®

Sample Preparation

Tools for Protein Research



Merck Biosciences

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What's Inside!

MERCK

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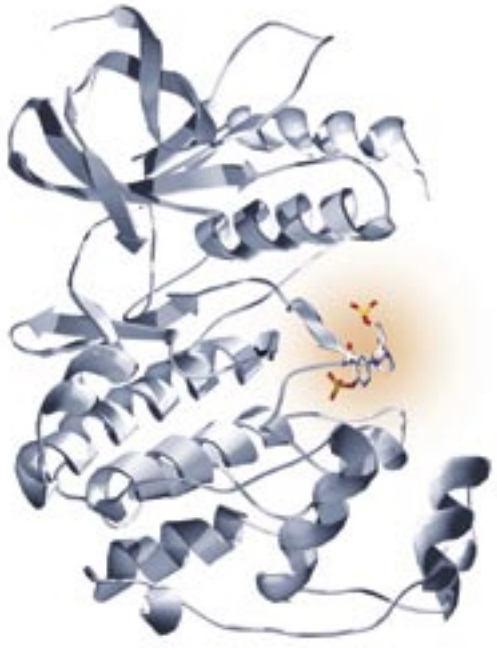


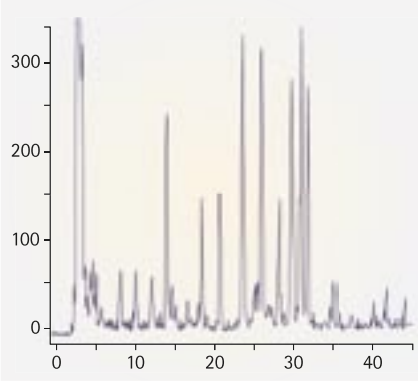
ProteoEnrich™ ATP-Binders™ Kit allows group separation of protein kinases and other ATP-binding proteins, yielding partially purified cell extracts enriched in active protein kinases. Based on an affinity resin that contains immobilized ATP, the method is compatible with 2D electrophoresis, SDS-PAGE/tandem mass spectrometry, Western blot analysis, and activity assays. The unique affinity resin contains ATP covalently linked through the γ-phosphate, presenting an ideal configuration for recognition by the conserved ATP-binding pocket of kinases.

ProteoExtract® Native Membrane Protein Extraction Kit is designed for the isolation of membrane proteins from mammalian cells and tissues. The extremely mild procedure yields a solution of integral membrane and membrane-associated proteins in their non-denatured state. The straightforward, two-step procedure does not require ultracentrifugation or incubation at elevated temperatures.

ProteoExtract Phosphopeptide Capture Kit enables isolation of phosphorylated peptides derived from cleaved or digested protein samples or kinase reactions designed for phosphorylation site identification. Utilizing the specific interaction of phosphate groups with zirconium ions immobilized on the surface of magnetic particles, phosphopeptides are captured and purified for LC-MS or MALDI-MS analysis.

ProteoExtract Protein Precipitation Kit provides efficient concentration of proteins and removal of interfering substances from dilute protein samples in a single step. The kit delivers protein solutions with very low conductivity, making it ideally suited for a wide range of proteomics applications including isoelectric focusing, 2D electrophoresis, and mass spectrometry.

ProteoExtract® All-in-One Trypsin Digestion Kit contains a set of optimized reagents for tryptic digestion of various protein samples: spots or bands excised from polyacrylamide gels, protein solutions, or cell and tissue extracts.



BugBuster® Master Mix extracts active, soluble protein with maximum yield. It combines BugBuster Protein Extraction Reagent with Benzonase® Nuclease and rLysozyme™ Solution in one convenient reagent. The all-in-one protein extraction reagent efficiently lyses bacteria and digests nucleic acids. Following centrifugation to remove debris, the extract is ready for protein purification or functional protein analysis.

D-Tube™ Dialyzers are easy-to-handle dialyzers for buffer exchange and removal of urea, detergents, and ethidium bromide while providing > 97% sample volume recovery. Available with molecular weight cutoffs from 3.5 to 14 kDa, the D-Tube Dialyzers are designed in three volume capacities: mini (10–250 µl), midi (50–800 µl), and maxi (100–3000 µl). In addition, D-Tubes can be used for dialysis and electroelution of proteins, RNA, DNA, and oligonucleotides from polyacrylamide or agarose gels.

D-Tube Electroelution Accessory Kit provides optimized reagents for protein and nucleic acid precipitation following electroelution.

Sample Preparation Overview

Successful proteome analysis depends on standardized, reliable, and convenient sample preparation. Merck Biosciences, through its Calbiochem and Novagen brands, offers a comprehensive selection of sample preparation tools for three main areas of protein research: expression proteomics, functional proteomics, and structural proteomics. Most often, expression proteomics includes the comparative analysis of proteins expressed in cells under varying conditions, whether in response to a disease, chemical compound, or therapeutic drug. These differentially expressed proteins can serve as potential drug targets or as biological markers for disease detection. The ProteoExtract® and ProteoEnrich™ kits cover the different steps in sample preparation, from protein extraction and abundant protein removal to concentration of protein mixtures, removal of interfering substances, digestion of proteins, selective capturing of phosphorylated peptides, and selective enrichment for specific protein classes. All kits are compatible with each other to provide samples that can be used directly in analytical applications such as SDS-PAGE, immunoblotting, two-dimensional gel electrophoresis (2DGE), and mass spectrometry (MS). Many kits are compatible with ELISA, activity assays, and protein microarrays.

Structural protein analysis requires a considerable amount of purified protein (20–30 mg on average) and, therefore, these analyses often begin with recombinant protein expression. When purifying proteins for functional or structural proteomics studies, the first step is to disrupt the cells or tissue sample and extract the relevant protein fraction. This step is critical because processing methods that require harsh mechanical, chemical, or enzymatic treatments can directly affect target protein integrity and activity, or otherwise expose proteins to degradative conditions. The “Buster” and “PopCulture®” families of protein extraction reagents are innovative combinations of detergents and other ingredients that enable gentle, efficient, nonmechanical extraction of soluble proteins from bacteria, yeast, plant, mammalian, and insect cells. The reagents are compatible with high-throughput recombinant protein purification and solubility screening.

ProteoExtract & ProteoEnrich Kits for Sample Preparation

ProteoExtract Kits are unique tools for differential display proteomics studies that allow analysis of complete or partial proteomes of bacterial, yeast, or mammalian cells, and of mammalian cells in normal versus diseased or disease-induced states. The Complete Proteome Extraction Kits (C-PEK) are designed for total protein extraction from bacterial, yeast, and mammalian cells. The Partial Proteome Extraction Kits (P-PEK) are designed for sequential extraction of protein fractions from bacterial, yeast, and mammalian cells according to differences in protein solubility. Both types of kits produce proteome samples ready for 2D gel electrophoresis and mass spectrometry. The Subcellular Proteome Extraction Kit (S-PEK) is designed for sequential extraction of protein fractions from mammalian cells according to their subcellular localization. The S-PEK produces native, functional proteins that can be used in activity assays, microarrays, and 1D and 2D gel electrophoresis. ProteoExtract Native Membrane Protein Extraction Kit (M-PEK) is designed for the isolation of non-denatured integral membrane or membrane-associated proteins from mammalian cells and tissues.

ProteoExtract Abundant Protein Removal Kits

These kits facilitate highly specific depletion of either albumin or albumin/IgG from serum, plasma, or cerebrospinal fluid. Selective removal of high-abundance protein improves the detection of low-abundance proteins of interest.

ProteoEnrich ATP-Binders™ Kit

This kit allows group separation of protein kinases and other ATP-binding proteins, yielding partially purified cell extracts enriched in active protein kinases. Based on an affinity resin that contains immobilized ATP, the method is compatible with 2D electrophoresis, SDS-PAGE/tandem mass spectrometry, Western blot analysis, and activity assays.

ProteoExtract® Kits for Peptide Analysis

The ProteoExtract Phosphopeptide Capture Kit is designed for the analysis of phosphoproteome. Combined with the ProteoExtract All-in-One Trypsin Digestion Kit, this kit has a unique magnetic affinity resin to isolate phosphorylated peptides with a high degree of specificity.

BugBuster® Protein Extraction Reagents

Fast, Efficient, Gentle Protein Extraction from Bacteria

BugBuster Protein Extraction Reagents are innovative combinations of detergents and other ingredients that enable gentle, efficient, nonmechanical extraction of soluble proteins from bacterial cells. rLysozyme™ Solution increases the efficiency of bacterial lysis with BugBuster Reagent. Addition of Benzonase® Nuclease degrades contaminating DNA and RNA for the preparation of nonviscous, nucleic acid-free extracts ready for target protein purification. Lysonase™ Bioprocessing Reagent combines the functional activities of rLysozyme and Benzonase Nuclease in an optimized, ready-to-use reagent that significantly increases protein extraction efficiency and facilitates processing of protein extracts. In addition, Lysonase can be used to enhance the effectiveness of non-detergent–based cell lysis procedures.

YeastBuster™ and CytoBuster™ Protein Extraction Reagents for Yeast, Plant, Mammalian, and Insect Cell Lysis

YeastBuster and CytoBuster Protein Extraction Reagents are formulated for a fast, efficient, and gentle extraction of soluble proteins from yeast, plant, mammalian, and insect cells. The YeastBuster reagent avoids harsh conditions of vigorous mechanical or chemical treatment that often result in degradation of target proteins from yeast and plant cells. The CytoBuster reagent is a proprietary formulation of detergents optimized for efficient extraction of proteins from mammalian and insect cells. Both reagents are compatible with downstream protein purification and functional assays.

NucBuster™ Protein Extraction Kit for Nuclear Extract Preparation

The NucBuster Protein Extraction Kit is designed for rapid isolation of the nuclear protein fraction from mammalian cells and avoids tedious traditional nuclear extract preparation methods. Nuclear extracts obtained by the NucBuster method are ideal for transcription factor assays.

Reportasol™ and PhosphoSafe™ Extraction Reagents for Functional Activity Assays

Reportasol and PhosphoSafe Extraction Reagents are specifically optimized for efficient extraction of reporter enzymes and phosphoproteins, respectively, from mammalian cells. Both formulations allow for maximal retention of the relevant enzymatic activity of extracted proteins, and do not require shaking or mixing to obtain efficient extraction.

High-throughput Systems: Recombinant Protein Extraction/Purification

PopCulture® Reagent for *E. coli* Lysis in Medium

PopCulture Reagent is used for extraction of proteins from liquid cultures of *E. coli* without harvesting cells. Addition of 0.1 culture volume of PopCulture Reagent directly to cells in medium, grown at any scale, efficiently extracts proteins while retaining their biological activity. The reagent is used in combination with rLysozyme Solution for efficient cell lysis and is compatible with Benzonase Nuclease and protease inhibitors to reduce viscosity and minimize protein degradation, respectively. This extraction method, combined with the Solubility Screening Plate or with the magnetic- or filtration-based affinity purification provided by the RoboPop™ Kits, enables truly high-throughput protein solubility screening and purification in automated formats.

Insect PopCulture Reagent for Insect Cell Lysis in Medium

Insect PopCulture Reagent allows centrifugation-free protein extraction from total cultures of insect cells in suspension or on tissue culture plates. The method increases processing efficiency and target protein yields and is amenable to automated expression screening and affinity purification methods.

Protein Extraction Reagents Application Guide

Cell Type	Product	Starting Material		Applications								Comments
					Analysis							
		Total Culture	Cell Pellet	HT Compatible	1D PAGE	2D PAGE	IEF	MS	Western Blot	Activity Assay	Purification	
E. coli												
	BugBuster® Protein Extraction Reagent		✓		✓	✓	✓		✓	✓	✓	Efficient protein extraction from *E. coli* under non-denaturing conditions. Extraction enhanced by the addition of rLysozyme™ Solution and Benzonase® Nuclease. Can be used on cell pellets from any size culture.
	BugBuster HT Protein Extraction Reagent		✓	✓	✓	✓	✓		✓	✓	✓	Rapid protein extraction and nucleic acid degradation. Ideal for processing many samples of any volume. Benzonase Nuclease is premixed in the lysis reagent. Extraction enhanced by the addition of rLysozyme Solution.
	BugBuster Master Mix		✓	✓	✓	✓	✓		✓	✓	✓	BugBuster Master Mix combines BugBuster Protein Extraction Reagent with Benzonase Nuclease and rLysozyme Solution. Convenient, all-in-one protein extraction reagent efficiently lyses bacteria and digests nucleic acids.
	BugBuster (primary amine-free) Extraction Reagent		✓		✓	✓	✓		✓	✓	✓	Ideal as an extraction method for purifying metal-dependent proteins or proteins to be used for immobilization or crosslinking. Extraction enhanced by the addition of rLysozyme Solution and Benzonase Nuclease.
	BugBuster 10X Protein Extraction Reagent		✓		✓	✓	✓		✓	✓	✓	A concentrated form of BugBuster Protein Extraction Reagent. Ideal for extraction when a specific buffer is required for protein stability. Extraction enhanced by the addition of rLysozyme Solution and Benzonase Nuclease.
	PopCulture® Reagent	✓		✓	✓				✓	✓	✓	Protein extraction from cells directly in the culture medium; no centrifugation required. Designed for small volumes. Extraction enhanced by the addition of rLysozyme Solution and Benzonase Nuclease.
	RoboPop™ Purification Kits (magnetic- or filtration-based)	✓		✓	✓			✓	✓	✓	✓	Protein extraction and purification in 96-well format. Ideal for robotic or manual processing; GST•Tag™- or His•Tag®-based purification by magnetic or filtration methods. rLysozyme Solution and Benzonase Nuclease are included.
	ProteoExtract® Bacterial Complete Proteome Extraction Kit		✓		✓	✓	✓	✓	✓			Total proteome extracted into one fraction.
	ProteoExtract Bacterial Partial Proteome Extraction Kit		✓		✓	✓	✓	✓	✓			Produces four protein fractions based on solubility.
Yeast												
	YeastBuster™ Protein Extraction Reagent		✓		✓				✓	✓	✓	Efficient protein extraction from yeast under non-denaturing conditions from any volume of culture. Add 0.5 M THP Solution (included) and Benzonase Nuclease for enhanced efficiency.
	ProteoExtract Yeast Complete Proteome Extraction Kit		✓		✓	✓	✓	✓	✓			Total proteome extracted into one fraction.
	ProteoExtract Yeast Partial Proteome Extraction Kit		✓		✓	✓	✓	✓	✓			Produces three protein fractions based on solubility.

Key: 1D PAGE = One-dimensional Polyacrylamide Gel Electrophoresis; 2D PAGE = Two-dimensional Polyacrylamide Gel Electrophoresis; IEF = Isoelectric Focusing; MS = Mass Spectrometry

Cell Type	Product	Starting Material		Applications								Comments
					Analysis							
		Total Culture	Cell Pellet	HT Compatible	1D PAGE	2D PAGE	IEF	MS	Western Blot	Activity Assay	Purification	
Insect												
	CytoBuster™ Protein Extraction Reagent		✓+		✓				✓	✓	✓	Gentle lysis of mammalian cells with retention of protein activity for assays and purification. Can use with monolayers or pellets derived from suspension cultures.
	Reportasol™ Extraction Buffer		✓+		✓	✓	✓		✓	✓R		Optimized for maximal activity of reporter enzymes (β-gal, firefly, and *Renilla* luciferases). Passive lysis of monolayers.
	Insect PopCulture® Reagent	✓		✓	✓				✓	✓	✓	Lysis of insect cells directly in serum-free medium. Ideal for expression screening of many small samples. Compatible with affinity purification.
Mammalian												
	CytoBuster Protein Extraction Reagent		✓+		✓				✓	✓	✓	Gentle lysis of mammalian cells with retention of protein activity for assays and purification. Can use with monolayers or pellets derived from suspension cultures.
	Reportasol Extraction Reagent		✓+	✓	✓		✓		✓	✓R		Optimized for maximal activity of reporter enzymes (β-gal, firefly, and *Renilla* luciferases). Passive lysis of adherent cells.
	PhosphoSafe™ Extraction Reagent		✓	✓	✓		✓	✓	✓	✓	✓	Ideal for extraction of phosphorylated proteins.
	NucBuster™ Protein Extraction Kit		✓		✓G				✓	✓	✓	Rapid isolation of nuclear protein fraction from mammalian cells. Ideal for electrophoretic mobility shift
	ProteoExtract® Mammalian Complete Proteome Extraction Kit		✓		✓	✓	✓	✓	✓			Total proteome extracted into one fraction.
	ProteoExtract Mammalian Partial Proteome Extraction Kit		✓		✓	✓	✓	✓	✓			Produces four protein fractions based on solubility
	ProteoExtract Subcellular Proteome Extraction Kit		✓		✓	✓*	✓*	✓	✓	✓		Produces four native protein fractions based on subcellular localization.
	ProteoExtract Native Membrane Proteome Extraction Kit		✓		✓	✓	✓	✓	✓	✓		Produces two native protein fractions, membrane and non-membrane.
Lysis and Extraction Enhancement												
Gram-negative bacteria (*E. coli*)	rLysozyme™ Solution	✓	✓	✓	✓				✓	✓	✓	Cleaves bond in peptidoglycan layer of *E. coli* cell wall. Use alone or combined with BugBuster® or PopCulture reagents for improved protein extraction. Use with Benzonase Nuclease to reduce sample viscosity and degrade nucleic acids.
	Lysonase™ Bioprocessing Reagent	✓	✓	✓	✓				✓	✓	✓	Convenient mixture of rLysozyme and Benzonase Nuclease minimizes pipetting steps
Gram-positive bacteria	Chicken Egg White Lysozyme Solution	✓	✓	✓	✓				✓	✓	✓	Cleaves bond in peptidoglycan layer of bacterial cell wall.
All cells	Benzonase® Nuclease	✓	✓	✓	✓				✓	✓	✓	Degrades all types of nucleic acids for more efficient protein extraction, faster chromotagraphy, and reduced interference in assays.

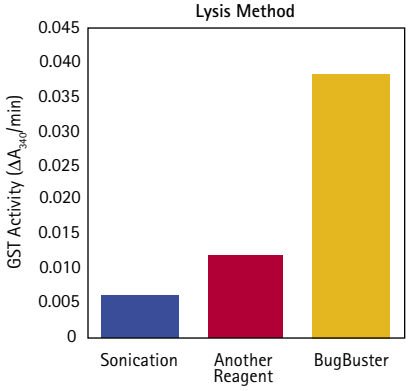
Key: 1D PAGE = One-dimensional Polyacrylamide Gel Electrophoresis
2D PAGE = Two-dimensional Polyacrylamide Gel Electrophoresis
IEF = Isoelectric Focusing
MS = Mass Spectrometry
+ = Monolayer
G = Gel Shift
R = Reporter Assay
* = SDS must be removed before IEF

BugBuster® Protein Extraction Reagents

Extraction of active protein from *E. coli* without sonication

Product	Size	Cat. No.
BugBuster® Protein Extraction Reagent	100 ml	70584-3
	500 ml	70584-4
BugBuster Plus Benzonase® Nuclease	1 kit	70750-3
Components: • 500 ml BugBuster Protein Extraction Reagent • 10 KU Benzonase Nuclease, Purity > 90%		
BugBuster HT Protein Extraction Reagent	100 ml	70922-3
	500 ml	70922-4
	2 × 500 ml	70922-5
Benzonase Nuclease, Purity > 90%	10 KU	70746-3
	2.5 KU	70746-4
rLysozyme™ Solution (30 KU/µl)	300 KU	71110-3
	1200 KU	71110-4
	6000 KU	71110-5

Note: 1 KU = 1000 units



Comparison of *E. coli* lysis methods
Samples (50 ml) of an induced 500-ml culture of BL21(DE3) containing pET-41a(+) encoding GST were harvested by centrifugation and resuspended in 2 ml 1X PBS, another commercially available protein extraction reagent, or BugBuster Reagent. The sample in PBS was sonicated with 10 pulses at 50% duty for 30 s total. Samples in lysis reagent were treated according to their respective protocols. Extracts were clarified by centrifugation and assayed for GST enzymatic activity using the Novagen GST•Tag™ Assay Kit. (Cat. No. 70532-3).

BugBuster® Protein Extraction Reagent

BugBuster Protein Extraction Reagent gently disrupts the cell wall of *E. coli* to liberate active proteins. It provides a simple, rapid, low-cost alternative to mechanical methods, such as French Press or sonication, for releasing expressed target protein in preparation for purification or other applications. The proprietary formulation utilizes a detergent mix that is capable of cell wall perforation without denaturing proteins.

In practice, cells are harvested by centrifugation and suspended in BugBuster. At this point, Benzonase® Nuclease can be added to reduce the viscosity of the extract due to liberation of chromosomal DNA. The addition of highly specific rLysozyme™ Solution, which hydrolyzes *N*-acetylmuramide linkages in the cell wall, enhances the extraction efficiency, especially for larger proteins. Following a brief incubation, insoluble cell debris is removed by centrifugation. The clarified extract is ready to use and fully compatible with Novagen affinity supports, including GST•Bind™, GST•Mag™, His•Bind®, His•Mag™, and S•Tag™ Resins, or other chromatography matrices. Following binding to affinity resin, excess BugBuster is easily removed by washing the column with the appropriate buffer. BugBuster is also useful for the preparation of high-purity inclusion bodies in instances where expressed proteins are insoluble. The reagent is available in a variety of configurations.

The standard BugBuster reagent is supplied as a ready-to-use "1X" Tris-buffered liquid that is stable at room temperature. The 500-ml size is also available bundled with 10,000 U Benzonase Nuclease (provided in a separate vial) for the preparation of low viscosity extracts and/or removal of nucleic acids from protein preparations. BugBuster and Benzonase are compatible with common protease inhibitors.

BugBuster HT Protein Extraction Reagent

BugBuster HT combines BugBuster Protein Extraction Reagent and Benzonase Nuclease in one convenient reagent. BugBuster HT eliminates common bioprocessing problems resulting from traditional cell lysis procedures. Soluble proteins are gently extracted from *E. coli* without exposure to heat or oxidative damage and viscosity is eliminated by nucleic acid digestion in a single step. The resulting protein extract can easily be fractionated by conventional purification techniques. BugBuster HT is ideally suited for application in high-throughput protein purifications.

BugBuster® Protein Extraction Reagents

Additional configurations increase convenience and versatility

BugBuster® 10X Protein Extraction Reagent

BugBuster 10X is a concentrated formulation of the proprietary detergents employed in BugBuster without the addition of buffer components. Concentrated BugBuster provides a flexible alternative to the ready-to-use standard 1X BugBuster, allowing user-defined dilution and addition of optimal buffer components. BugBuster 10X has all of the bioprocessing benefits of standard BugBuster plus the freedom to control pH, reagent concentration, and buffer additives necessary for maximum extraction and activity of target proteins.

BugBuster (primary amine–free) Protein Extraction Reagent

BugBuster (primary amine-free) is a special formulation of BugBuster designed for applications where primary amines would interfere if present in the protein extract, such as protein immobilization or cross-linking. The PIPPS buffer used in BugBuster (primary amine-free) has a similar buffering capacity and pH range as the original Tris-buffered BugBuster, but will not complex metal ions, making it also ideally suited for extraction of metal-dependent proteins.

BugBuster Master Mix

BugBuster Master Mix combines BugBuster Protein Extraction Reagent with Benzonase® Nuclease and rLysozyme™ Solution in one convenient reagent. BugBuster Master Mix allows for maximum recovery of active, soluble protein from both Gram-negative and Gram-positive bacteria. With the Master Mix, there is no need for dilution or separate addition steps. The two available package sizes provide sufficient reagents for protein extraction from 20 g and 100 g cell paste.

BugBuster Plus Lysonase™ Kit

The BugBuster Plus Lysonase Kit combines the activities of both reagents to significantly increase protein extraction efficiency and facilitate downstream processing of protein extracts, thereby enabling maximum recovery of active soluble protein from both Gram-negative and Gram-positive bacteria. Lysonase Bioprocessing reagent is an optimized, ready-to-use blend of rLysozyme Solution and Benzonase Nuclease. Use 5 ml BugBuster and 10 µl Lysonase per gram of cell paste. Two kit sizes provide sufficient reagents for protein extraction from either 20 or 100 g cell paste.

Product	Size	Cat. No.
BugBuster® 10X Protein Extraction Reagent	10 ml	70921-3
	50 ml	70921-4
	2 × 50 ml	70921-5
BugBuster (primary amine-free) Extraction Reagent	100 ml	70923-3
	500 ml	70923-4
BugBuster Master Mix Extraction Reagent	100 ml	71456-3
	500 ml	71456-4
BugBuster Plus Lysonase™ Kit	1 kit*	71370-3
	1 kit†	71370-4
rLysozyme™ Solution (30 KU/µl)	300 KU	71110-3
	1200 KU	71110-4
	6000 KU	71110-5
Benzonase® Nuclease, Purity > 90%	10 KU	70746-3
	2.5 KU	70746-4
Note: 1 KU = 1000 units		
Lysonase Bioprocessing Reagent	0.2 ml	71230-3
	1 ml	71230-4
	5 × 1 ml	71230-5

* Includes 100 ml BugBuster Protein Extraction Reagent and 0.2 ml Lysonase Bioprocessing Reagent, sufficient for protein extraction from 20 g cell paste.

† Includes 500 ml BugBuster Protein Extraction Reagent and 1 ml Lysonase Bioprocessing Reagent, sufficient for protein extraction from 100 g cell paste.

Lysonase™ Bioprocessing Reagent

Convenient blend of rLysozyme™ Solution and Benzonase® Nuclease

Product	Size	Cat. No.
Lysonase™ Bioprocessing Reagent	0.2 ml	71230-3
	1 ml	71230-4
	5 × 1 ml	71230-5

Lysonase™ Bioprocessing Reagent is an optimized, ready-to-use blend of rLysozyme Solution and Benzonase Nuclease. rLysozyme Solution contains a highly purified and stabilized recombinant lysozyme with specific activity 250 times greater than that of chicken egg white lysozyme. Benzonase Nuclease is a genetically engineered nonspecific endonuclease that degrades all forms of DNA and RNA (single stranded, double stranded, circular, linear), reducing extract viscosity, and increasing protein yield. The combined activities of rLysozyme and Benzonase Nuclease significantly increase protein extraction efficiency and facilitate downstream processing of protein extracts.

For efficient protein extraction with BugBuster® Protein Extraction Reagent, use 10 µl Lysonase per 1 g cell paste. For efficient protein extraction with PopCulture® Reagent, add 2 µl Lysonase per 1 ml culture. Store at –20°C. In addition, Lysonase can be used to enhance the effectiveness of non-detergent based cell lysis procedures.

rLysozyme™ Solution

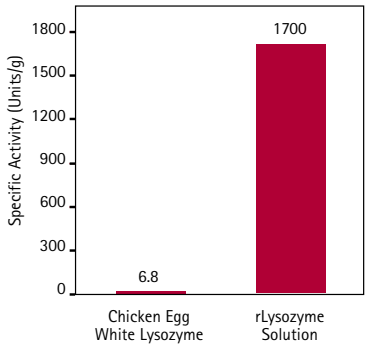
Stabilized recombinant lysozyme

Product	Size	Cat. No.
rLysozyme™ Solution (30 KU/µl)	300 KU	71110-3
	1200 KU	71110-4
	6000 KU	71110-5

Components:

- 300 KU *or* 1200 KU *or* 6000 KU rLysozyme Solution
- 1 ml rLysozyme Dilution Buffer (71110-3 only)

Note: 1 KU = 1000 units



rLysozyme™ Solution contains a highly purified and stabilized recombinant lysozyme that can be used for lysis of *E. coli*. The enzyme catalyzes the hydrolysis of *N*-acetylmuramide linkages in bacterial cell walls. The specific activity of rLysozyme (1,700 KU/mg) for *E. coli* lysis is 250 times greater than that of chicken egg white lysozyme. rLysozyme is optimally active at physiological pH. Very small amounts of rLysozyme (3–5 KU/g cell paste) enhance the efficiency of protein extraction with BugBuster, BugBuster HT, and PopCulture reagents. In the absence of protein extraction reagents, direct lysis of *E. coli* can be achieved by treatment of 1 g cell paste with 45–60 KU rLysozyme. The product is supplied as a ready-to-use solution at a concentration of 30 KU/µl in 50% glycerol containing 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 0.1 mM EDTA, 1 mM DTT, and 0.1% Triton® X-100. rLysozyme Solution is stable at –20°C.

Unit definition: one unit of rLysozyme is defined as the amount of enzyme necessary to cause a decrease in absorbance of 0.025 units per minute at 450 nm at 25°C in a 1-ml suspension (1 mg/ml) of Tuner™(DE3) cells in 0.5X BugBuster diluted with 50 mM Tris-HCl, pH 7.5.

rLysozyme™ Solution, Veggie™ Grade

Certified animal-free recombinant lysozyme

rLysozyme™ Solution, Veggie™ Grade is a special grade of rLysozyme prepared using certified animal-free or disease-free reagents. All of the steps in the preparation of the recombinant enzyme are carried out using reagents of nonanimal origin, with the exception of the IPTG used to induce protein expression. IPTG is chemically synthesized by a stringent process from D-galactose isolated from lactose, a milk sugar. The lactose is derived from certified disease-free cows. rLysozyme Solution, Veggie Grade has the same stability and specific activity as the rLysozyme Solution and requires no change to the protocol. With its high specific activity (1700 KU/mg; Note: 1 KU = 1000 units), only a small amount of rLysozyme Solution, Veggie Grade is required to break the cell wall of *E. coli*. Use the enzyme at 3–5 KU/g cells to enhance protein recovery from cells treated with BugBuster® or BugBuster HT protein extraction reagents or with PopCulture® Reagent. Or, treat thawed cell pellets with only the enzyme using 45–60 KU/g cells. Optimal protein purification is achieved by treating the cell lysate with Benzonase® Nuclease, which efficiently reduces extract viscosity by degrading the nucleic acids liberated as cells lyse. rLysozyme Solution, Veggie Grade is supplied as a ready-to-use solution at a concentration of 30 KU/µl in 50% glycerol containing 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 0.1 mM EDTA, 1 mM DTT, and 0.1% Triton X-100. The solution is stable at –20°C.

Product	Size	Cat. No.
rLysozyme™ Solution, Veggie™ Grade (30 KU/µl)	1200 KU	71297-4
	6000 KU	71297-5

Note: 1 KU = 1000 units

Chicken Egg White Lysozyme Solution

A ready-to-use, stabilized lysozyme solution

Chicken Egg White Lysozyme Solution is a ready-to-use, stabilized solution of lysozyme. The enzyme catalyses the hydrolysis of *N*-acetylmuramide linkages in bacterial cell walls. The solution can be used for the purification of both DNA and protein. We recommend Chicken Egg White Lysozyme Solution for Gram-positive bacterial lysis. The solution is supplied as 10 mg/ml lysozyme in 25 mM sodium acetate with 50% glycerol, pH 4.5 (200 KU/ml, Note: 1 KU = 1000 units). The product is stable at –20°C.

Product	Size	Cat. No.
Chicken Egg White Lysozyme Solution	10 × 1 ml	71412-3

Unit definition: One unit of Chicken Egg White Lysozyme is defined as the amount of enzyme that causes a decrease in absorbance of 0.001 unit per minute at 450 nm, 25°C, pH 6.2, using a suspension of *Micrococcus lysodeikticus* as the substrate.

ProteoExtract® Complete Bacterial Proteome Extraction Kit

Fast and easy extraction of total proteins from bacteria

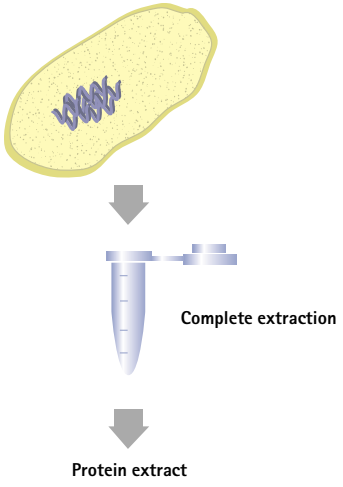
Product	Size	Cat. No.
ProteoExtract® Complete Bacterial Proteome Extraction Kit (Includes Wash Buffer, Resuspension Buffer, Extraction Reagent, Reducing Agent, Benzonase® Nuclease, and Glass Beads. Components are sufficient for approximately 20 extractions based on 2 × 10^{11} cells per sample.)	20 rxn	539770
Benzonase Nuclease, Purity > 99%	10 KU	70664-3
Benzonase Nuclease HC, Purity > 99%	25 KU	71206-3
Benzonase Nuclease, Purity > 90%	10 KU 2.5 KU	70746-3 70746-4
Benzonase Nuclease HC, Purity > 90%	25 KU	71205-3

Note: 1 KU = 1000 units

ProteoExtract® Complete Bacterial Proteome Extraction Kit (C-PEK) is designed for fast and easy extraction of total proteins from bacteria without the need for sonication or precipitation. C-PEK provides a straightforward, two-step isolation of complete proteomes in a single microcentrifuge tube. C-PEK uses optimized extraction reagents to provide improved solubilization of proteins resulting in an increased total number of spots on 2D gels. The kit includes proprietary Benzonase® Nuclease, a nonspecific nuclease leading to clear, nonviscous protein solutions and improving resolution on 2D gels. Using the C-PEK procedure, additional concentration of proteins is not necessary—extracted proteins are ready for immediate use in standard downstream proteomics applications.

Features

- **Improved solubilization of total cellular protein**
- **Ready-to-use proteins for 2D gel electrophoresis—no concentration needed**
- **Excellent spot resolution due to Benzonase Nuclease addition**



ProteoExtract® Partial Bacterial Proteome Extraction Kit

Efficient and standardized extraction of cellular proteins into four distinct partial proteomes

ProteoExtract® Partial Bacterial Proteome Extraction Kit (P-PEK) provides efficient and standardized extraction of cellular proteins into four distinct partial proteomes. Analysis of the partial proteome fractions enriched in subsets of cellular proteins allows for the detection of low-abundance proteins by 2D gel electrophoresis and Western blot analysis.

P-PEK contains uniquely balanced combinations of chaotropes and detergents to solubilize and enrich a different subset of cellular proteins in each of four fractions. The most soluble proteins are released by mechanical disruption in extraction reagent 1, while extraction reagent 2 releases proteins of intermediate solubility. Reagent 3 provides efficient extraction of membrane proteins. SDS buffer solubilizes proteins still insoluble in reagent 3.

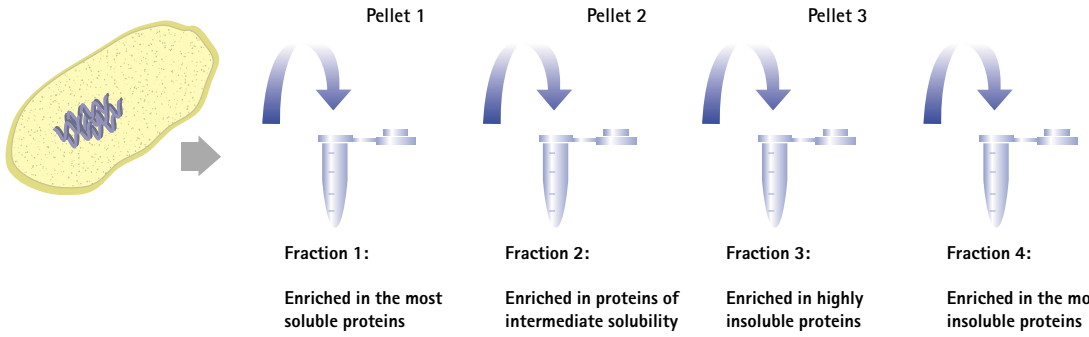
Effective removal of nucleic acids with Benzonase® Nuclease eases sample handling and highly improves resolution on 2D gels. P-PEK protocols generally work without a sonication step, thereby avoiding protein modifications due to temperature effects. P-PEK–extracted proteins are ready for immediate use in 2D gel electrophoresis applications.

Product	Size	Cat. No.
ProteoExtract® Partial Bacterial Proteome Extraction Kit (Includes Wash Buffer, Extraction Reagents, SDS-Buffers, Protease Inhibitor Cocktail, Benzonase® Nuclease, and Glass Beads. Components are sufficient for approximately 20 extractions based on 2 × 10^{11} cells per sample.)	20 rxn	539780
Benzonase Nuclease, Purity > 99%	10 KU	70664-3
Benzonase Nuclease HC, Purity > 99%	25 KU	71206-3
Benzonase Nuclease, Purity > 90%	10 KU 2.5 KU	70746-3 70746-4
Benzonase Nuclease HC, Purity > 90%	25 KU	71205-3

Note: 1 KU = 1000 units

Features

- **Protocols developed and tested specifically for different sample types**
- **Protein fractions enriched in subsets of cellular proteins to visualize low-abundance proteins**
- **Ready-to-use proteins for 1D and 2D gel electrophoresis**
- **Excellent spot resolution due to Benzonase Nuclease addition**
- **Includes protease inhibitors to preserve protein profiles**



Sequential partial proteome extraction: the P-PEK method
Samples are sequentially extracted using four buffers with increasing solubilization strengths. The proteome of the organism of interest is partitioned into four partial proteomes enriched in proteins of increasing hydrophobicity.

YeastBuster™ Protein Extraction Reagent

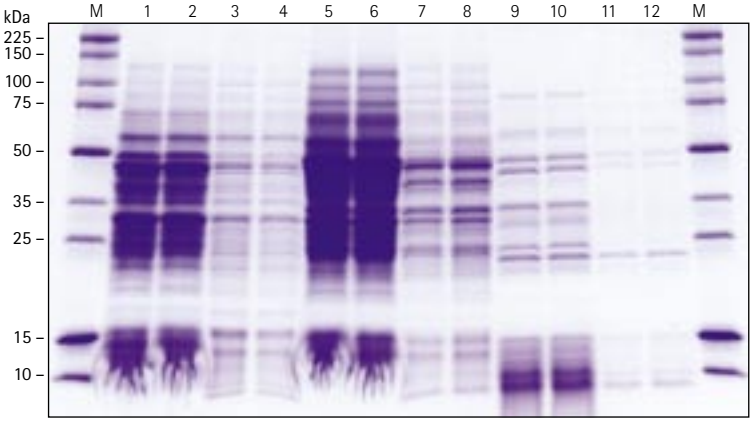
Efficient extraction of protein from yeast and plants without mechanical disruption and enzymatic lysis

Product	Size	Cat. No.
YeastBuster™ Protein Extraction Reagent (Includes 100X THP Solution)	100 ml 500 ml	71186-3 71186-4
0.5 M THP Solution	1 ml 5 × 1 ml	71194-3 71194-4

Related application note on page 60

YeastBuster™ Protein Extraction Reagent is formulated for fast, efficient, and gentle extraction of active proteins from yeast cells. The reagent avoids harsh conditions of vigorous mechanical or chemical treatment that often result in heat degradation of target proteins. The proprietary formulation utilizes a mix of mild detergents, protein stabilization buffer, and tris(hydroxypropyl)phosphine (THP) reducing agent (THP concentrate provided separately). This powerful combination eliminates the inconsistencies associated with tedious mechanical disruption of yeast cells with glass bead abrasives, ultrasonication and pressure disruption, or enzymatic digestion with β-1,3-glucanase lytic enzymes. The reagent has been tested with *Saccharomyces cerevisiae*, *Pichia pastoris*, *P. stipidis*, and *Schizosaccharomyces pombe* strains and with plant cells.

In practice, cells are harvested by centrifugation and suspended in YeastBuster. Following a brief incubation, insoluble cell debris is removed by centrifugation, and the clarified extract is ready to use. In addition to greater total protein yields in crude extracts and recovery of enzymatically active protein, the extracts are fully compatible with GST•Bind™ and Ni-NTA His•Bind® immobilized metal affinity chromatography (IMAC) purification methods. The reagent is available in 100- and 500-ml sizes.



Lane	Sample
M	Perfect Protein™ Markers, 10–225 kDa
1	*S. cerevisiae* YB
2	*S. cerevisiae* YB
3	*S. cerevisiae* GB
4	*S. cerevisiae* GB
5	*P. stipidis* YB
6	*P. stipidis* YB
7	*P. stipidis* GB
8	*P. stipidis* GB
9	*S. pombe* YB
10	*S. pombe* YB
11	*S. pombe* GB
12	*S. pombe* GB

YB = YeastBuster
GB = Glass bead lysis buffer

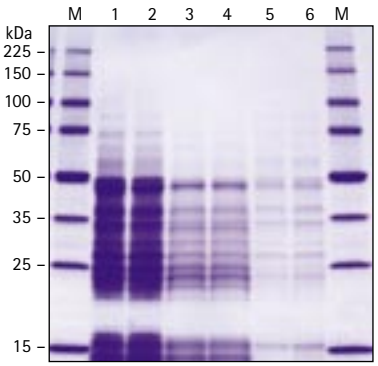
Protein yield (mg)	*S. cerevisiae*	*P. stipidis*	*S. pombe*
YeastBuster	2.7	3.3	0.9
Glass Bead Lysis Buffer	0.8	1.0	0.2

SDS-PAGE analysis and Coomassie™ blue staining of extracted soluble proteins from *S. cerevisiae*, *P. stipidis*, and *S. pombe*
Duplicate samples of pelleted yeast cells (100 mg wet weight) in 1.5-ml microcentrifuge tubes were resuspended in 500 µl YeastBuster Reagent plus 5 µl 100X THP Solution, or 500 µl of glass bead lysis buffer containing 50 mM Tris-HCl, 250 mM LiCl, 100 mM $(NH)_2SO_4$, 1 mM DTT, and 2% glycerol, plus 100 µl acid-washed glass beads (100–150 µm diameter). All samples contained protease inhibitors: 1 mM EDTA, 0.5 µM AEBSF, and 15 µg/ml benzamidine. After resuspension, the pellets were processed according to the standard YeastBuster and glass bead protocols. All samples were centrifuged at 16,000 × g for 5 min prior to SDS-PAGE analysis. Equal-volume samples (7.5 µl) of the extracts were loaded on the gel. Protein yields based on Non-Interfering Protein Assay™ (Calbiochem Cat.No. 488250) are also shown.

Features

- Gentle, rapid, efficient extraction of proteins from yeast cells
- Eliminates the inconsistencies associated with abrasive grinding, ultrasonication, and pressure disruption of yeast cells
- Higher yield of total and enzymatically active proteins
- Fully compatible with Ni–NTA His•Bind® and GST•Bind™ affinity purification methods

A. SDS–PAGE



Lane	Sample
M	Perfect Protein™ Markers, 10–225 kDa
1	5 µl YeastBuster extract
2	5 µl YeastBuster extract
3	5 µl competitor reagent extract
4	5 µl competitor reagent extract
5	5 µl glass bead extract
6	5 µl glass bead extract

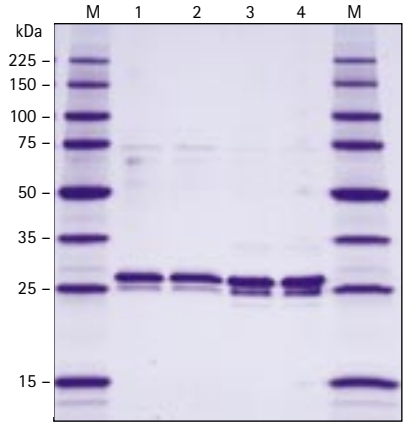
B. Total protein and reporter activities

	YeastBuster	Competitor	Glass Beads
Protein (mg/ml)	6.1	3.2	0.65
GST (ΔA_{340}/min)	0.071	0.023	0.007
β-gal (ΔA_{570}/min)	0.113	0.003	0.187

Performance comparison of YeastBuster Protein Extraction Reagent, another commercial reagent, and the glass bead method

Panel A. SDS–PAGE analysis (4–20% gradient gel) and Coomassie™ blue staining of extracted proteins. *S. cerevisiae* cells containing a recombinant plasmid expressing a 35.6-kDa GST•Tag™/His•Tag® fusion protein were incubated at 30°C, induced for expression, and harvested at OD_{600} of 1.2. Cells were collected by centrifugation at 3000 × g and resuspended in ice cold sterile water. Equal volumes of cells were dispensed into microcentrifuge tubes and pelleted at 3000 × g. Cell pellets (65 mg wet weight) were resuspended in 330 µl of the respective extraction reagents supplemented with 0.5 mM AEBSF and 15 µg/ml benzamidine. The YeastBuster Reagent also included 0.01 volume 100X THP Solution as directed in the protocol. Glass bead extraction was performed according to the standard protocol. All samples were centrifuged at 16,000 × g for 5 min prior to SDS-PAGE analysis.

Panel B. Analysis of total protein and reporter activities. Total protein extracted by the three methods was determined using Non-Interfering Protein Assay™ Kit. (Calbiochem Cat. No. 488250) GST activity was determined using GST•Tag Assay Kit. (Novagen Cat. No. 70532-3) β-gal activity was determined using the host expressing *lacZ*. Cells were grown and processed as described for panel A. Samples of the extracts were assayed using the Novagen BetaRed™ β-Gal Assay Kit. (Cat. No. 70978-3). Data reflect the average of duplicate assays.



Lane	Sample
M	Perfect Protein Markers, 10–225 kDa
1	2 µg GST•Bind eluate
2	2 µg GST•Bind eluate
3	2 µg Ni–NTA His•Bind eluate
4	2 µg Ni–NTA His•Bind eluate

SDS–PAGE analysis of GST•Bind and Ni–NTA His•Bind purified samples
S. cerevisiae cells containing a recombinant plasmid expressing a 30.5-kDa GST•Tag/His•Tag fusion protein were incubated and processed as described for panel A in the figure above. The sample was centrifuged at 16,000 × g for 5 min and 4.5-ml aliquots of the supernatant were purified using GST•Bind or Ni–NTA His•Bind Resins. The protein content of the eluates was determined by BCA and Coomassie blue binding assays and duplicate samples were analyzed by SDS-PAGE (4–20% gradient) and Coomassie blue staining.

NucBuster™ Protein Extraction Kit

Rapid and convenient extraction of nuclear proteins

Product	Size	Cat. No.
NucBuster™ Protein Extraction Kit	100 rxn	71183-3

Components:
- 2 × 7.5 ml NucBuster Extraction Reagent 1
- 7.5 ml NucBuster Extraction Reagent 2
- 100 µl 100 mM DTT
- 100 µl Protease Inhibitor Cocktail Set I

Product	Size	Cat. No.
Protease Inhibitor Cocktail Set I	1 vial 10 vials	539131
EMSA Accessory Kit		71282-3

Components:
- 1 ml 4X EMSA Buffer
- 125 µl Poly(dI-dC)•Poly(dI-dC) Solution
- 150 µl Salmon Sperm DNA
- 100 µl 100 mM DTT

Product	Size	Cat. No.
NoShift™ Transcription Factor Assay Kit	100 rxn	71377-3

Components:
- 4 × 1 ml 4X NoShift Bind Buffer
- 150 µl Salmon Sperm DNA
- 125 µl Poly(dI-dC)•Poly(dI-dC)
- 30 ml 10X NoShift Wash Buffer
- 20 ml NoShift Antibody Dilution Buffer
- 1 Streptavidin Plate
- 12 ml TMB Substrate
- 3 Aluminum Plate Sealer

The NucBuster™ Protein Extraction Kit provides an alternative to the time-intensive and cumbersome traditional methods for preparing nuclear extracts from mammalian cells. Time-consuming traditional methods (requiring up to 7 hours), based on a procedure originally described by Dignam et al. (1), include suspending cells in hypotonic solution, Dounce homogenization, centrifugation, and dialysis. The NucBuster Kit protocol is rapid and allows for the easy processing of multiple samples. The entire procedure yields ready-to-use nuclear extract within 30 minutes from start to finish. The composition of gentle detergents and salt in the final NucBuster extract is directly compatible with electrophoretic mobility shift assays (EMSA).

The NucBuster protocol is based on two proprietary detergent-based solutions, NucBuster Extraction Reagents 1 and 2, optimized for cell lysis and removal of cytoplasmic components and for extraction of nuclear proteins, respectively. In addition, NucBuster extract is free of the "stickiness" associated with release of genomic DNA, a problem associated with some traditional methods. No Dounce homogenization is required and there is no need for dialysis. The entire procedure is performed in a single microcentrifuge tube and requires only a vortex mixer and microcentrifuge. The kit provides enough reagents for 100 preparations of nuclear extract from 1–5 × 10^7 cells and the protocol is scalable.

Features

- No homogenization or dialysis required
- Nuclear extract preparation obtained within 30 minutes
- Entire procedure performed in a single microcentrifuge tube
- Extracts suitable for activity assays, electrophoretic mobility shift assays (EMSA), and NoShift™ Transcription Factor Assays

1. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489.

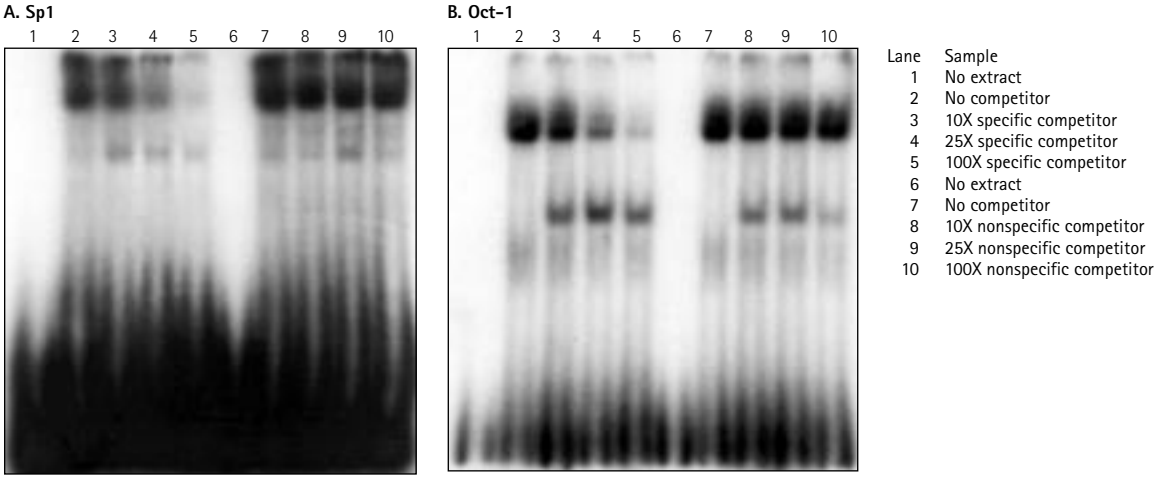
Lane	Sample
1	No extract
2	Traditional extract
3	No extract
4	NucBuster extract

NucBuster Protein Extraction Kit versus traditional methodology
The table illustrates the protocol steps and processing times of the NucBuster Protein Extraction Kit and the traditional method. The gel photo shows an electrophoretic mobility shift assay (EMSA) performed with CHO-K1 nuclear extracts prepared using the NucBuster Kit and traditional methods. Extracts were run on a 6% DNA retardation gel, dried on DEAE paper, and exposed to film.

Comparison of procedure times by method

Procedure	NucBuster Kit	Traditional method*
Cell preparation	10 min	10 min
Cytoplasmic extraction	10 min	25 min
Nuclear extraction	10 min	65 min
Dialysis	–	3 h
Final centrifugation	–	20 min
Total	30 min	5 h

*Traditional method is based on a modification of the procedure of Dignam et al. (1). NP-40 is used in the extraction and the dialysis is shortened to 3 h.



Lane	Sample
1	No extract
2	No competitor
3	10X specific competitor
4	25X specific competitor
5	100X specific competitor
6	No extract
7	No competitor
8	10X nonspecific competitor
9	25X nonspecific competitor
10	100X nonspecific competitor

Binding specificity of NucBuster™ extracted nuclear proteins
Nuclear extract from 2 × 10^7 CHO-K1 cells was isolated using the NucBuster Protein Extraction Kit. Nuclear extract samples (2.5 µl) were complexed for 30 min with 0.03 pmol of ^{32}P-labeled double stranded DNA specifying an Sp1, Oct-1, or CREB binding site and no competitor, or a 10X, 25X, or 100X molar ratio of unlabeled specific or non-specific competitors. The binding reactions were performed as specified in the NucBuster protocol. Samples were analyzed by 6% DNA retardation gel, dried on DEAE paper, and exposed to film.

Reportasol™ Extraction Buffer

Extraction of maximal reporter enzyme activity from mammalian and insect cells

Reportasol™ Extraction Buffer is designed to efficiently extract soluble reporter enzymes from mammalian and insect cells while maintaining maximal activity. The formulation has been optimized for high-throughput assays of firefly luciferase, *Renilla* luciferase, and β-galactosidase. Efficient extraction is achieved in a passive mode (i.e., no shaking or mixing is required) while maintaining extremely high reporter enzyme activity. Reportasol is also compatible with standard protein assay methods. This buffer has a different formulation than CytoBuster™ Protein Extraction Reagent and should be stored at –20°C.

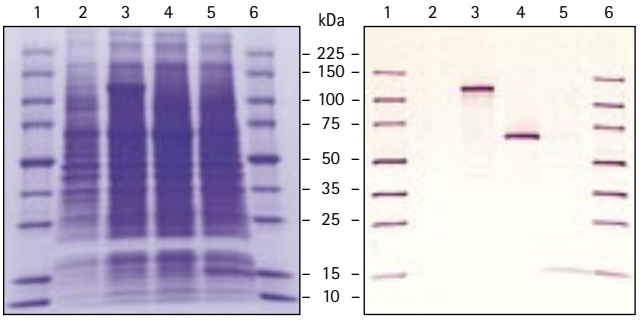
Product	Size	Cat. No.
Reportasol™ Extraction Buffer	25 ml	70909-3
	5 × 25 ml	70909-4

CytoBuster™ Protein Extraction Reagent

Extraction of soluble protein from mammalian and insect cells

Product	Size	Cat. No.
CytoBuster™ Protein Extraction Reagent	50 ml	71009-3
	5 × 50 ml	71009-4

The CytoBuster™ Protein Extraction Reagent is a proprietary formulation of detergents optimized for efficient extraction of soluble proteins from mammalian and insect cells. The gentle, non-ionic composition of CytoBuster enables isolation of functionally active endogenous or expressed proteins without a need for secondary treatment such as sonication or freeze/thaw. CytoBuster has been specifically formulated for utilization in Western blot analysis, immunoprecipitation, and kinase/phosphatase assays. The reagent is compatible with protease inhibitors, kinase inhibitors and phosphatase inhibitors. Store at room temperature.



Lane	Sample
1	Perfect Protein™ Markers, 10–225 kDa (stained gel), Perfect Protein Western Markers (blot)
2	Mock transfection
3	S•Tag β-galactosidase
4	S•Tag firefly luciferase
5	S•Tag 11-kDa fusion protein
6	Perfect Protein Markers, 10–225 kDa (stained gel), Perfect Protein Western Markers (blot)

Analysis of S•Tag™ fusion proteins extracted with CytoBuster Reagent
COS-1 cells were transfected with a pTriEx™ vector encoding the indicated S•Tag fusion proteins using GeneJuice® Transfection Reagent. After 48 h the cells were treated with CytoBuster Protein Extraction Reagent and equal sample volumes were analyzed by Coomassie blue–stained SDS-PAGE and Western blot. The S•Tag fusion proteins were detected on the Western blot using the S-protein AP Conjugate and NBT/BCIP AP substrates. The Perfect Protein Western Markers were detected simultaneously with the S-protein AP Conjugate.

PhosphoSafe™ Extraction Reagent

When phosphorylation state matters

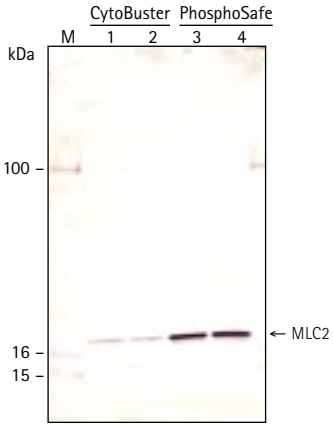
PhosphoSafe™ Extraction Reagent efficiently extracts cytosolic proteins from mammalian and insect cells while preserving their phosphorylation state. This reagent contains the same formula as CytoBuster™ Protein Extraction Reagent (1), but also includes four phosphatase inhibitors: sodium fluoride, sodium vanadate, β-glycerophosphate, and sodium pyrophosphate. PhosphoSafe is compatible with kinase assays, protein interaction analysis, and other applications.

Features

- **Phosphorylation state preserved during extraction**
- **Compatible with kinase assays and other applications**

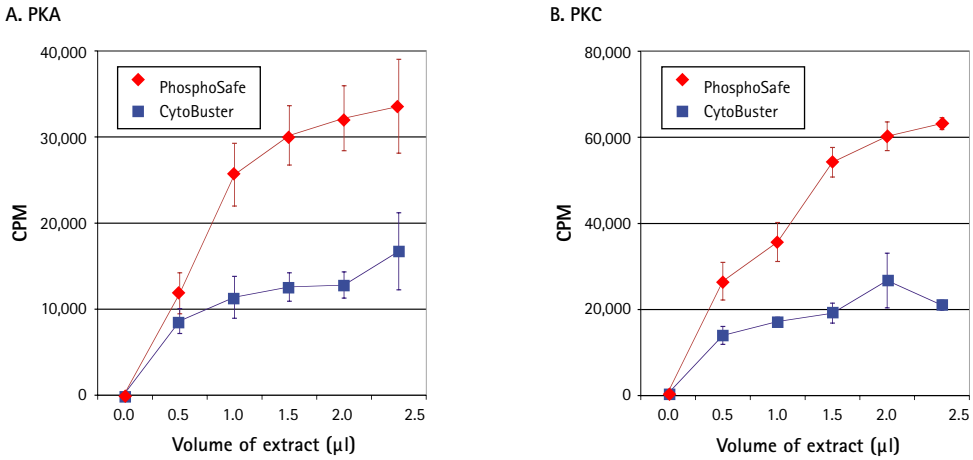
1. Trim, J. E. and Sawyer, D. L. (2001) *inNovations* 12, 5–6.

Product	Size	Cat. No.
PhosphoSafe™ Extraction Reagent	25 ml 5 × 25 ml	71296-3 71296-4
Protein Kinase Assay Kit Universal	1 kit	539551
AP Detection Reagent Kit (NBT, BCIP, 20X AP Buffer)	1 ea 5 ea	69264-3 69264-4
Trail Mix™ Western Markers	25 lanes	70982-3
BCA Protein Assay Kit	500 assays (2500 microplate assays)	71285-3
Components: • 500 ml BCA Solution • 15 ml 4% Cupric Sulfate • 3 × ml BSA Standard, 2 mg/ml		
Goat Anti-Rabbit IgG AP Conjugate (Fc specific)	40 µl	69265-3



Detection of phosphorylated MLC2
Monolayers of subconfluent L6 myoblasts were extracted with CytoBuster reagent or PhosphoSafe buffer for 10 min at room temperature. Extracts were centrifuged and assayed for protein concentration using the BCA Protein Assay Kit (Novagen Cat. No. 71285-3). Duplicates of each extract (10 µg) were analyzed by SDS-PAGE (4–20% gradient gel). Proteins were transferred to a nitrocellulose membrane and protein phosphorylation state was assessed with anti-phospho-MLC2 as the primary antibody. Goat Anti-Rabbit IgG AP Conjugate (Novagen Cat. No. 69265-3) was added and detected by staining with the AP Detection Reagent Kit (Novagen Cat. No. 69263-3).

Related application note on page 75



Kinase assays for PKA and PKC
Monolayers of subconfluent CHO-K1 cells were extracted with CytoBuster reagent or PhosphoSafe reagent for 10 min at room temperature. Biotinylated peptides corresponding to the PKA phosphorylation site of HNF-6 and the pseudosubstrate region of PKC were incubated with increasing amounts of extract (0.6 mg/ml) in the presence of γ-^{32}P ATP using the Protein Kinase Assay Kit, Universal (Calbiochem Cat. No. 539551). Phosphate transfer mediated by PKA (panel A) and PKC (panel B) was detected by scintillation counting. Protein concentration was determined using the BCA Protein Assay Kit (Novagen Cat. No. 71285-3).

ProteoExtract® Complete Proteome Extraction Kits

Fast and easy extraction of total proteins from yeast and mammalian cells and tissues

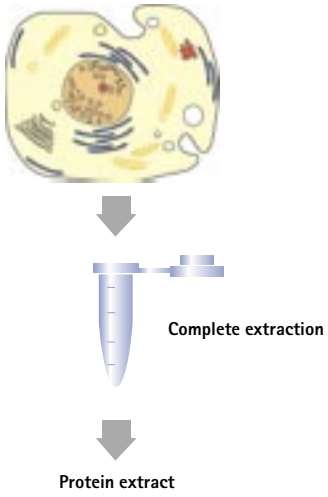
Product	Size	Cat. No.
ProteoExtract® Complete Yeast Proteome Extraction Kit (Includes Wash Buffer, Yeast Extraction Reagent, Benzonase Nuclease®, and Glass Beads. Components are sufficient for approximately 20 extractions based on 2 × 10^7 cells per sample.)	20 rxn	539775
ProteoExtract Complete Mammalian Proteome Extraction Kit (Includes Wash Buffer, Resuspension Buffer, Extraction Reagent, Reducing Agent, Benzonase Nuclease, and Glass Beads. Components are sufficient for approximately 20 extractions based on 1–2 × 10^8 cells per sample.)	20 rxn	539779
Benzonase Nuclease, Purity > 99%	10 KU	70664-3
Benzonase Nuclease HC, Purity > 99%	25 KU	71206-3
Benzonase Nuclease, Purity > 90%	10 KU 2.5 KU	70746-3 70746-4
Benzonase Nuclease HC, Purity > 90%	25 KU	71205-3

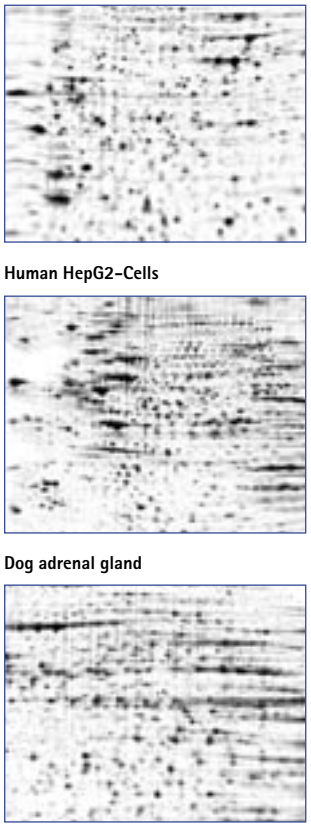
Note: 1 KU = 1000 units

ProteoExtract® Complete Proteome Extraction Kits (C-PEKs) are designed for fast and easy extraction of total proteins from yeast and mammalian cells and tissues, without the need for sonication or precipitation. C-PEKs provide a straightforward two-step isolation of complete proteomes in a single microcentrifuge tube. C-PEKs use optimized extraction reagents to provide improved solubilization of proteins resulting in an increased total number of spots on 2D gels. The procedure includes proprietary Benzonase® Nuclease, a nonspecific nuclease leading to clear, nonviscous protein solutions and improving resolution on 2D gels. Using the C-PEK procedure, protein concentration is not necessary—extracted proteins are ready for immediate use in standard downstream proteomics applications.

Features

- Improved solubilization of total cellular protein
- Ready-to-use proteins for 2D gel electrophoresis—no concentration needed
- Excellent spot resolution due to Benzonase Nuclease addition





Different samples show high resolution in 2D gel electrophoresis

ProteoExtract® Partial Proteome Extraction Kits

Efficient and standardized extraction of cellular proteins into four distinct partial proteomes

ProteoExtract® Partial Proteome Extraction Kits (P-PEKs) provide efficient and standardized extraction of yeast and mammalian cellular proteins into four distinct partial proteomes. Analysis of the partial proteome fractions enriched in subsets of cellular proteins allows for the detection of low abundance proteins and screening by means of 2D gel electrophoresis and Western blot analysis.

P-PEKs contain uniquely balanced combinations of chaotropes and detergents to solubilize and enrich a different subset of cellular proteins in each of four fractions. The most soluble proteins are released by mechanical disruption in extraction reagent 1, while extraction reagent 2 releases proteins of intermediate solubility. Reagent 3 provides efficient extraction of membrane proteins. SDS buffer solubilizes proteins still insoluble in reagent 3.

Effective removal of nucleic acids with Benzonase® Nuclease eases sample handling and highly improves resolution on 2D gels. P-PEK protocols generally work without a sonication step, thereby avoiding protein modifications by temperature effects. P-PEK–extracted proteins are ready for immediate use in 2D gel electrophoresis applications.

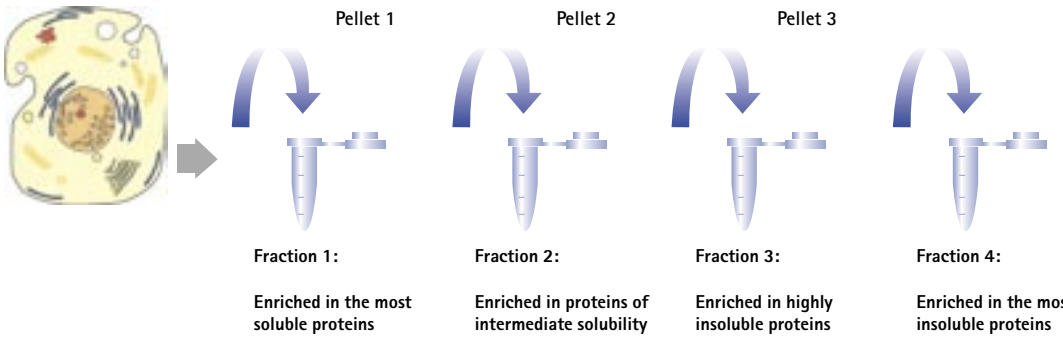
Features

- **Specifically developed and tested protocols for different sample types**
- **Protein fractions enriched in subsets of cellular proteins to visualize low-abundance proteins**
- **Ready-to-use proteins for 1D and 2D gel electrophoresis**
- **Excellent spot resolution through Benzonase Nuclease addition**
- **Includes protease inhibitors to preserve protein profiles**

Product	Size	Cat. No.
ProteoExtract® Partial Yeast Proteome Extraction Kit (Includes Wash Buffer, Extraction Reagents, Rehydration Reagent, SDS Buffers, Protease Inhibitor Cocktail, Benzonase® Nuclease, and Glass Beads. Components are sufficient for approximately 20 extractions based on 2 × 10^7 cells per sample.)	20 rxn	539785
ProteoExtract Partial Mammalian Proteome Extraction Kit (Includes Wash Buffer, Extraction Reagents, SDS buffers, Protease Inhibitor Cocktail, Benzonase Nuclease, and Glass Beads. Components are sufficient for approximately 20 extractions based on 1–2 × 10^8 cells per sample.)	20 rxn	539789
Benzonase Nuclease, Purity > 99%	10 KU	70664-3
Benzonase Nuclease HC, Purity > 99%	25 KU	71206-3
Benzonase Nuclease, Purity > 90%	10 KU 2.5 KU	70746-3 70746-4
Benzonase Nuclease HC, Purity > 90%	25 KU	71205-3

Note: 1 KU = 1000 units

Related application note on page 62



Sequential partial proteome extraction: the P-PEK method
Samples are sequentially extracted using four buffers with increasing solubilization strengths. The proteome of the organism of interest is partitioned into four partial proteomes enriched in proteins of increasing hydrophobicity.

ProteoExtract® Subcellular Proteome Extraction Kit

Fast and reproducible extraction of subcellular proteomes from mammalian cells

Product	Size	Cat. No.
ProteoExtract® Subcellular Proteome Extraction Kit (Includes Wash Buffer, Extraction Buffers, Protease Inhibitor Cocktail, and Benzonase® Nuclease. Components are sufficient for approximately 20 extractions based on T-25 flask of adherent cells or 1 × 10^6 suspended cells per sample.)	20 rxn	539790
Benzonase Nuclease, Purity > 99%	10 KU	70664-3
Benzonase Nuclease HC, Purity > 99%	25 KU	71206-3

Note: 1 KU = 1000 units

Related application note on page 66

ProteoExtract® Subcellular Proteome Extraction Kit (S-PEK) is designed for fast and reproducible extraction of subcellular proteomes from mammalian tissue and adherent and suspension-grown cells. S-PEK takes advantage of the different solubilities of certain subcellular compartments in the four selected reagents. In the case of adherent cells, the procedure is performed directly in the tissue culture dish without the need for cell removal. Cells or the parts of the cells remain attached to the plate during sequential extraction of subcellular compartments until the appropriate extraction reagent is used. Thus, the early destruction of the cellular structure by enzymatic or mechanical detachment of cells from the tissue culture plate and any mixing of different subcellular compartments is prevented. For suspension-grown cells, extraction starts with gentle sedimentation and washing of the cells. For tissues, fragmentation is required before proceeding with the extraction protocol.

The stepwise extraction delivers four distinct protein fractions from one sample:

- Cytosolic protein fraction
- Membrane/organelle protein fraction
- Nuclear protein fraction
- Cytoskeletal protein fraction

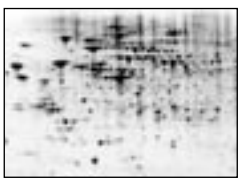
Proteins are obtained in the native state making the S-PEK suitable for many downstream applications such as 1D and 2D gel electrophoresis, immunoblotting, enzyme activity assays, and protein microarrays.

Features

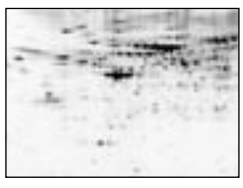
- Stepwise extraction resulting in four distinct subcellular proteomes from one sample
- Highly reproducible
- No ultracentrifugation steps
- Fast— just 2 hours, only 45 minutes hands-on time
- Produces proteins suitable for functional studies

Fraction 1: Cytosol

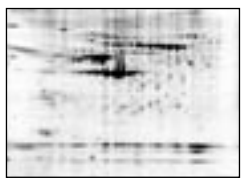
Fraction 2: Membrane/Organelle



Fraction 3: Nucleus

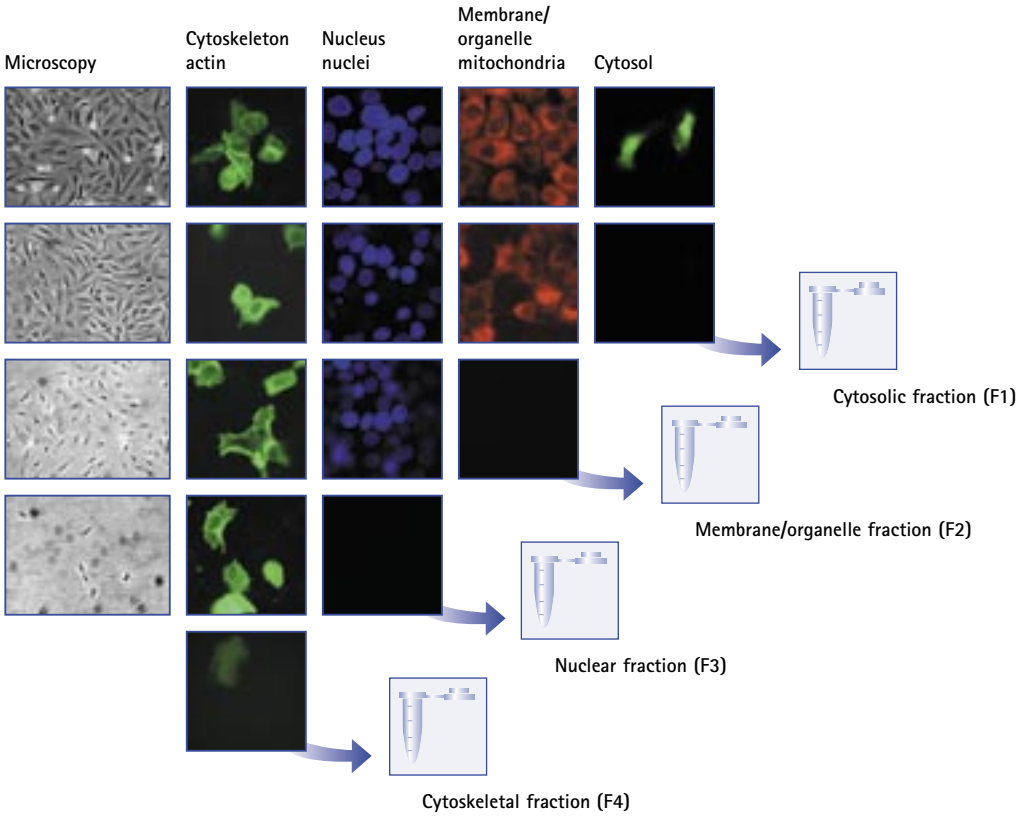


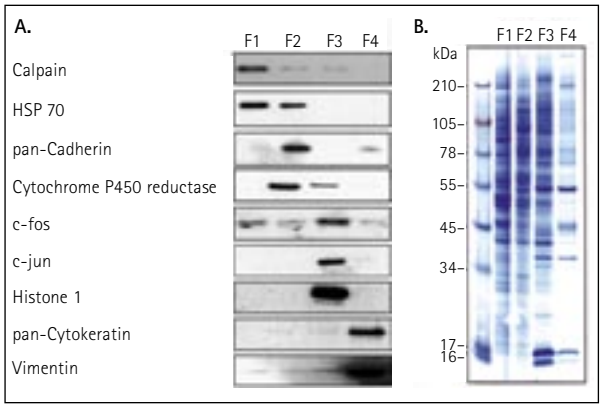
Fraction 4: Cytoskeleton



Protein profiling by 2D gel electrophoresis

The comparison of fractions 1 through 4 by two-dimensional SDS-PAGE indicates a large number of protein spots that are specific for the respective subcellular fraction.





Stepwise extraction with S-PEK demonstrates distinct protein patterns for each isolated subcellular fraction
Panel A: Immunoblots of selected marker proteins demonstrate separation efficiency greater than 80% for subcellular compartments of mammalian tissue culture cells. For detection of c-fos, the protein was immunoprecipitated before electrophoresis.
Panel B: Polyacrylamide gel analysis of subcellular fractions after S-PEK extraction of adherent tissue culture cells demonstrates clearly distinct protein patterns for each fraction. Fractions are as follows: F1, cytosolic fraction; F2, membrane/organelle fraction; F3, soluble nuclear fraction; F4, cytoskeletal/nuclear matrix fraction.

ProteoExtract® Native Membrane Protein Extraction Kit

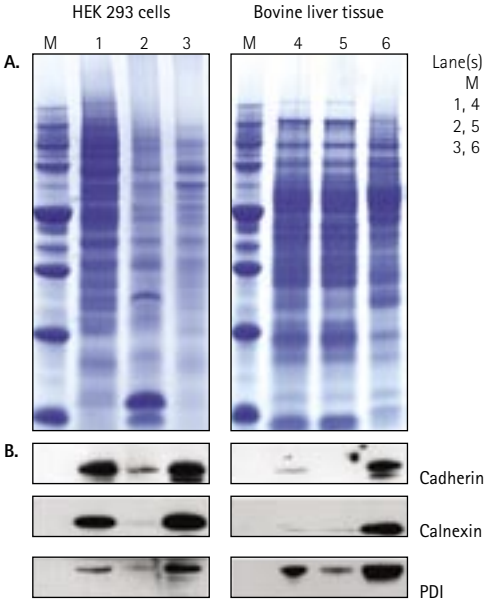
For the selective extraction of native membrane proteins

Product	Size	Cat. No.
ProteoExtract® Membrane Native Protein Extraction Kit (Includes Wash Buffer, Extraction Buffers, and Protease Inhibitor Cocktail)	20 rxn	444810

The ProteoExtract® Native Membrane Protein Extraction Kit (M-PEK) is designed for the isolation of native membrane proteins from mammalian cells and tissue. Rather than separating proteins solely by their intrinsic hydrophobicity, M-PEK extracts proteins from mammalian samples based on an actual association of proteins with cellular membranes. Samples from adherent and suspension tissue culture cells (2–5 × 10^6 cells) or mammalian tissue (25–50 mg) can be separated as the membrane protein fraction in less than two hours without the need for ultracentrifugation or incubation of samples at elevated temperatures. The extremely mild conditions yield a solution of integral membrane and membrane-associated proteins in their native state. These proteins are suitable for enzyme activity assays, non-denaturing gel electrophoresis, Western blot analysis, ELISAs, assays to examine post-translational modifications of membrane proteins, SELDI-profiling of integral and membrane-associated proteins, and NHS ester labeling of membrane proteins for array detection with dyes or biotin.

Features

- Offers three- to five-fold enrichment of native membrane proteins
- Yields proteins in their native, functional state
- Allows parallel processing of multiple samples in just two steps

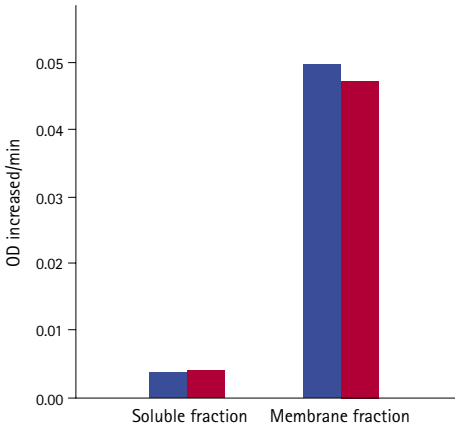


Lane(s)	Sample
M	Markers
1, 4	Total lysates
2, 5	Nonmembrane fraction
3, 6	Membrane fraction

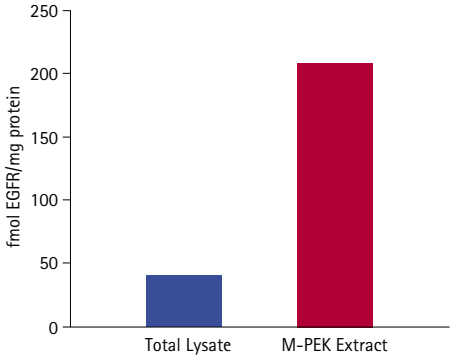
Selective extraction of membrane proteins from cell and tissue samples

Panel A. HEK 293 suspension cells and frozen bovine liver tissue were extracted either with SDS to yield a total lysate or with M-PEK to yield a membrane fraction and remaining "nonmembranous" proteins. Protein equivalents of extracted fractions were separated on SDS-PAGE and visualized by Coomassie™ blue staining. The membrane protein pattern (lanes 3 and 6) is clearly distinct from the patterns of both total and nonmembraneous fractions (lanes 1, 2, 4, and 5), indicating the selectivity of the M-PEK extraction.

Panel B. Immunoblotting of an equivalent gel using membrane-associated and integral membrane protein markers demonstrates the selectivity of the M-PEK extraction procedure.



Mild extraction conditions are compatible with enzyme assays
HEK 293 cells were extracted with M-PEK using the two-step procedure to yield a soluble protein and a membrane protein extract. Volume equivalents of the two fractions obtained from two independent experiments were assayed for endogenous alkaline phosphatase activity. The activity profile not only reveals selective separation of this GPI-anchored, membrane-associated enzyme, but also demonstrates that the assayed component is extracted in an active state.



Enrichment for EGF-receptor (EGFR)
HEK 293 cells were extracted with buffered 1% Triton® X-100 to generate a total lysate or extracted with M-PEK to yield a membrane fraction. The membrane extract obtained with the M-PEK Kit demonstrated a 4.5-fold enrichment of EGFR.

ProteoExtract® Abundant Protein Removal Kits

Enhancing resolution of low-abundance proteins

A major challenge for disease marker identification in human serum or plasma is the extraordinary range of protein abundance present in the sample. Proteins in plasma differ in concentration by a factor of one billion. Serum albumin can constitute 55% of total serum proteins and IgG can be as much as 25%. The presence of these proteins makes the resolution of lower-abundance proteins difficult. The new ProteoExtract® Abundant Protein Removal Kits provide highly specific and efficient depletion of either albumin alone or albumin and IgG from serum, plasma, or cerebrospinal fluid (CSF). Removal of serum albumin and IgG eliminates approximately 75% of the total protein present in serum. A sample enriched three to four times can be loaded on 2D gels or liquid chromatography columns. Sample complexity is significantly reduced, thereby improving the detection and analysis of low-abundance proteins and peptides in biomarker discovery, toxicological studies for new drugs, protein profiling using SELDI analysis, and other applications.

ProteoExtract Albumin Removal Kit

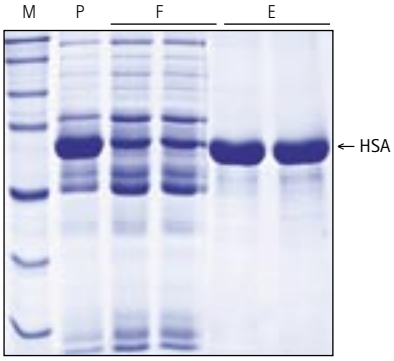
Highly specific albumin removal

Product	Size	Cat. No.
ProteoExtract® Albumin Removal Kit (Includes 12 Albumin Removal Columns and Albumin Binding Buffer)	20 rxn	122640

The ProteoExtract Albumin Removal Kit is based on a new affinity resin which is highly specific for albumin. Depletion of albumin from a typical human serum sample is consistently higher than 80% without binding significant amounts of other serum proteins. The remarkable selectivity provided by the resin and the optimized design of the columns result in less than 10% background binding of other serum proteins. Each kit contains 12 disposable columns pre-packed with 300 µl albumin-binding resin and a binding buffer for optimal resin performance. Resin binding capacity is 2 mg albumin per column and sample volumes of up to 60 µl can be processed without any loss of selectivity. The gravity-flow column format minimizes hands-on time making it highly suitable for processing multiple samples in parallel. The ProteoExtract Albumin Removal Kit depletion procedure is convenient and straightforward: equilibrate, load the diluted sample, allow to pass by gravity-flow, wash the column, combine collected flow-through and wash, and concentrate the proteins. Depleted samples are compatible with 1DGE, 2DGE, LC/MS, and MALDI-TOF MS. Although ProteoExtract Albumin Removal Kit has been optimized to bind human serum albumin, it is also compatible with rabbit, rat, and mouse samples.

Features

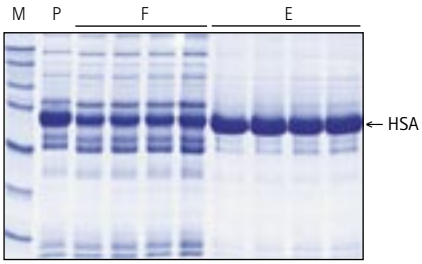
- **Efficient removal of albumin with albumin-specific resin (not Cibacron based)**
- **Highly specific, exhibiting little non-specific binding**
- **Increased loading of enriched sample on 2DGE or LC**
- **Fast, 20–30 minute protocols**
- **Easy, column-based procedure to process multiple samples in parallel**



Lane	Sample
M	Markers
P	Human plasma
F	Flow-through
E	Eluate fraction

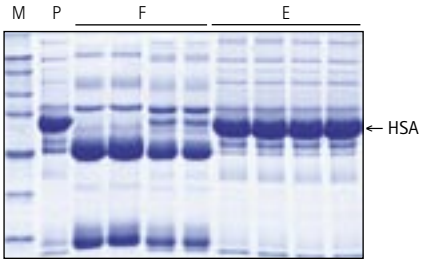
Efficient removal of albumin from human serum samples
Human plasma (35 µl) was processed with ProteoExtract® Albumin Removal Kit. From each fraction, 15 µg protein was separated on SDS-PAGE and visualized by Coomassie™ blue staining. Densitometric analysis of stained bands demonstrated that more than 80% of albumin was removed from the serum using the ProteoExtract Albumin Removal Kit.

A. Supplier I

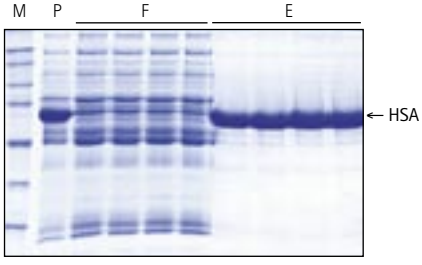


Lane	Sample
M	Markers
P	Human plasma
F	Flow-through
E	Eluate fraction

B. Supplier II



C. Calbiochem



Lower background binding
Depletion of albumin from human plasma using the ProteoExtract Albumin Removal Kit is more specific and more efficient than with products from other suppliers. The same experiment was performed in parallel using the Calbiochem ProteoExtract Albumin Removal Kit and albumin depletion products from Supplier I and Supplier II. From each fraction, 15 µg of protein was separated on SDS-PAGE and visualized by Coomassie blue staining. The absence of proteins other than albumin in the eluate fraction demonstrates the low background binding using the ProteoExtract Albumin Removal Kit (panel C). High background binding of proteins and less efficient removal of albumin is seen using products from Supplier I (panel A) and Supplier II (panel B).

ProteoExtract® Albumin/IgG Removal Kit

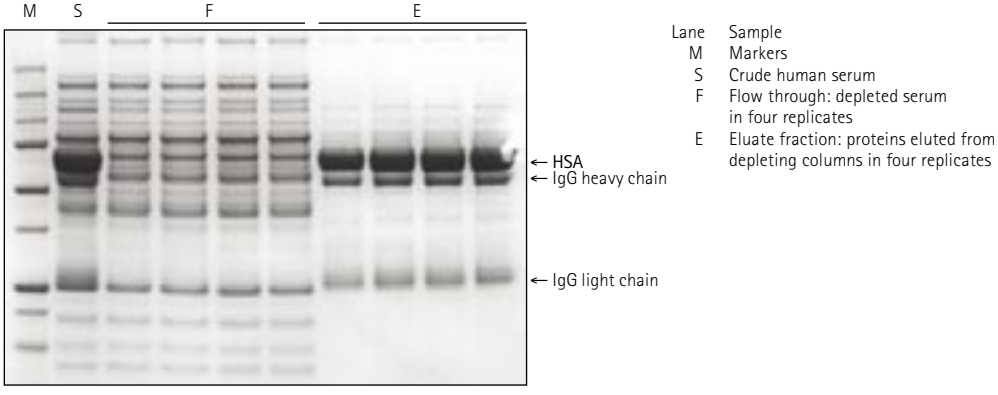
Enhancing resolution of low abundance proteins

Product	Size	Cat. No.
ProteoExtract® Albumin/IgG Removal Kit	20 rxn	122642
(Includes 12 Albumin Removal Columns and Albumin Binding Buffer. One kit is sufficient to process 12 plasma samples.)		

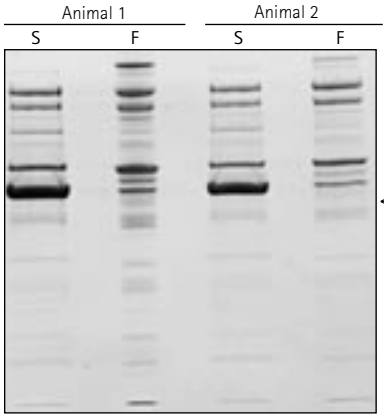
The ProteoExtract® Albumin/ IgG Removal Kit uses a combination of the albumin-specific resin and a unique immobilized protein A polymeric resin. Depletion of albumin and IgG from typical human serum samples is consistently higher than 80% without binding significant amounts of other serum proteins. The excellent selectivity provided by the resins and the optimized design of the columns result in less than 10% background binding of other serum proteins. Sample volumes up to 60 µl can be processed without any loss of selectivity. The ProteoExtract Albumin/IgG Protein Removal Kit has been optimized to bind human serum albumin and IgG, but will also deplete rabbit, rat, mouse, or pig samples effectively. Each kit contains 12 disposable columns pre-packed with 450 µl of the resin mix and an optimized binding buffer that promotes selective binding of albumin and IgG. Binding capacity of each column is 0.7 mg IgG and 2 mg albumin. The gravity-flow column format minimizes hands-on time, allowing the parallel processing of multiple samples. The kit procedure is similar to the ProteoExtract Albumin Removal Kit procedure. Depleted samples are compatible with downstream proteomics methods such as 1D and 2D gel electrophoresis, LC/MS, and MALDI-TOF MS.

Features

- **Efficient removal of albumin and IgG from serum samples**
- **Highly specific, exhibiting very little non-specific binding**
- **Compatible with human, rabbit, rat, mouse, and pig serum samples**
- **Increased loading of enriched sample on 2D gel electrophoresis or LC**
- **Fast, 20–30 minute protocols**
- **Easy, column-based procedure to process multiple samples in parallel**

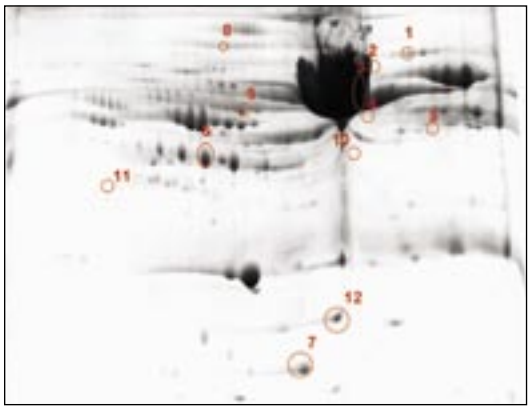


Efficient and reproducible removal of albumin and IgG from serum samples
Using the ProteoExtract Albumin/IgG Removal Kit, 35 µl human serum was processed, analyzed on 10% SDS-PAGE, and visualized by Coomassie™ blue staining. Densitometric analysis of stained bands of the eluate (E) fractions demonstrates that 88% ±5% albumin and 90% ±4% immunoglobulin are removed from the crude serum (S) using the ProteoExtract Albumin/IgG Removal Kit.



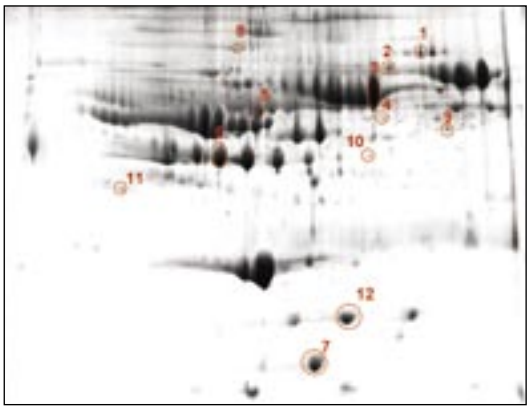
Lane	Sample
S	Crude rat serum
F	Flow through

Removal of albumin and IgG from rat serum
Using the ProteoExtract® Albumin/IgG Removal Kit, 35 µl rat serum from two different animals was processed. Equal protein amounts were separated by 10% SDS-PAGE gels and visualized by Coomassie™ blue staining. Densitometric analysis of stained flow-through demonstrates that the ProteoExtract Albumin/IgG Removal Kit removes more than 78% of rat albumin.



Unprocessed human serum

Spot	Identity
1	Complement Factor B precursor
2	Human serum albumin fragment
3	Human serum albumin
4	Human serum albumin
5	Antithrombin III, chain L
6	Haptoglobin chain β
7	Transthyretin
8	Inter-α trypsin inhibitor—related protein precursor
9	Human serum albumin precursor
10	Non-identified
11	Non-identified
12	Haptoglobin chain α 2



Albumin- and IgG-depleted serum

Spot	Identity
1	Complement Factor B precursor
2	Gelsolin precursor
3	Human serum albumin
4	Transferrin n-terminal lobe
5	Antithrombin III, chain L
6	Haptoglobin chain β
7	Transthyretin
8	Inter-α trypsin inhibitor – related protein precursor
9	Transferrin fragment HUMTF12 NID
10	Apolipoprotein L1 precursor
11	α-1-antitrypsin chain A
12	Haptoglobin chain α 2

Improving gel resolution
Human serum (35 µl) was either used directly (panel A) or subjected to albumin/IgG depletion (panel B) using the ProteoExtract Albumin/IgG Removal Kit. From each fraction, 1.5 mg was precipitated, resolubilized in IEF buffer, subjected to 2DGE, and visualized by Coomassie™ blue staining. Selected spots were excised from the gel and proteins were digested with trypsin and identified by peptide mapping using nano-LC/MS and the Mascot® search algorithm. The identification of five additional proteins (spots 2, 4, 9, 10, and 11) in the depleted serum sample (panel B) demonstrates that removal of albumin and IgG allows visualization and identification of proteins otherwise obscured by albumin and IgG.

ProteoEnrich™ ATP-Binders™ Kit

Affinity enrichment of ATP-binding proteins

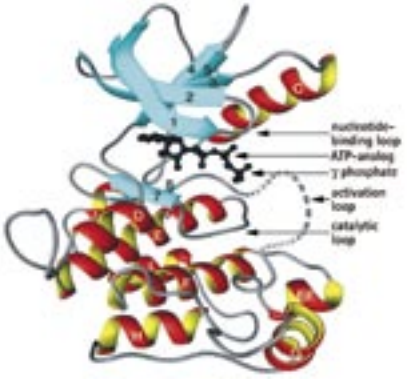
Product	Size	Cat. No
ProteoEnrich™ ATP-Binders™ Kit	1 kit	71438-3

Components:

- 100 mg ATP-Binders Resin
- 1 PBS Tablet
- 2 × 5 ml 100 mM EDTA
- 1.6 ml 1M DTT
- 2 ml ATP-Binders 6X Resin Conditioning Buffer
- 7 ml ATP-Binders 5X Bind Buffer
- 5 ml ATP-Binders Wash Buffer Concentrate
- 1 ml ATP-Binders Elution Buffer Concentrate
- 5 ml ATP-Binders 10X Nucleotide Mix (10 mM ADP, 10 mM AMP, 10 mM NADH, pH 7.0)
- 1 ml Protease Inhibitor Cocktail Set V, EDTA-Free, Lyophilized
- 2 × 250 µl 100 mM DTT
- 100 µl Activated Sodium Vanadate (200 mM Na_3VO_4, pH 10.0)
- pkg/10 Spin Filter, 2-ml capacity

Product	Size	Cat. No.
ATP-Binders Resin	100 mg	71445-3

Related application note on page 75



Ribbon diagram of the protein tyrosine kinase domain of the insulin receptor bound to an ATP analog
β-strands (numbered) are shown in cyan (•) and α-helices (lettered) in red (•) and yellow (•). An ATP analog is shown in ball and stick representation (black). The dashed gray line indicates the portion of the A-loop that is disordered (residues 1155–1171). Adapted from Till, J. H., Ablooglu, A. J., Frankel, M., Bishop, S. M., Kohanski, R. A., and Hubbard, S. R. (2001) *J. Biol. Chem.* **276**, 10049–10055, with permission from the American Society for Biochemistry and Molecular Biology.

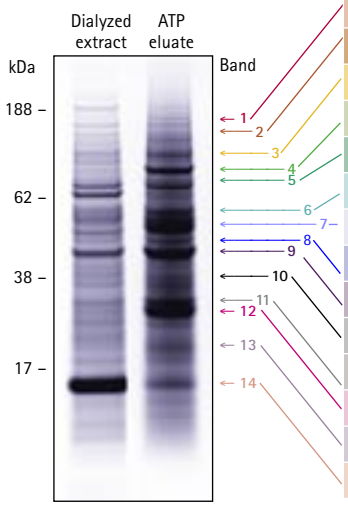
The ProteoEnrich™ ATP-Binders™ Kit allows group separation of protein kinases and other ATP binding proteins, yielding partially purified cell extracts enriched in active protein kinases. Based on an affinity resin that contains immobilized ATP, the method is compatible with 2D gel electrophoresis, SDS-PAGE/tandem mass spectrometry, Western blot analysis, and activity assays. The unique affinity resin contains ATP covalently linked through its γ-phosphate and a unique, flexible 13-atom linker to a polyacrylamide-based resin. The structure of the ATP-Binders resin presents an ideal configuration for recognition by the conserved ATP-binding pocket of kinases. This resin packs more tightly than agarose beads and so facilitates binding and washing steps. The resin is compatible with mild detergents and suitable for isolation of cytosolic and solubilized membrane proteins. The gentle elution conditions (20 mM ATP) enable recovery of active proteins, including interacting partners such as scaffolding proteins, from crude cell or tissue lysates. The resin-based, batchwise purification scheme is well suited for multiple separations and can be used to selectively enrich for functional protein groups in proteomic studies or in individual kinase purification schemes. Further, this kit can remove interfering non-ATP–binding proteins and can be used as a "polishing" step in an ATP-binding protein purification strategy.

The kit contains the ATP-polyacrylamide–based resin, spin filters, and optimized buffers required to process extracts prepared from crude mammalian cell or tissue lysates by a standard protocol. The kit provides sufficient reagents to perform enrichments from up to 17.5 mg crude protein.

Features

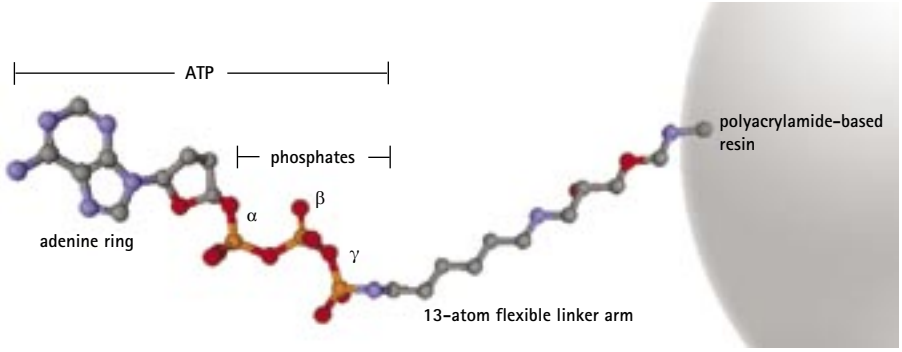
- **Based on novel resin for sample enrichment with functionally active protein kinases and other ATP-binding proteins**
- **Contains optimized buffers for maximum active protein recovery**
- **Compatible with 1D and 2D gel electrophoresis, LC/MS, and MALDI-TOF MS.**
- **Compatible with ProteoExtract® All-in-One Trypsin Digestion Kit (see page 36)**
- **Compatible with protein tyrosine kinase activity assays**

1. Bartnicki, D., Batenjany, M., Loomis, K., Wong, S., Menezes, R., Suleman, A., and Andrecht, S. (2004) *inNovations* **19**, 6–9.

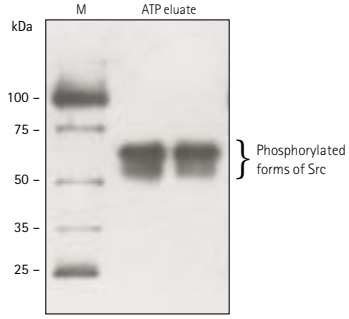


Band	Assignment	Function
1	Ca-ATPase, guanylate cyclase precursor	ATPase, nucleotide binding
2	Protein kinase splicing protein, guanylate cyclase	Protein kinase, nucleotide binding
3	Actinin, NOSIII, xylosyl transferase	Scaffolding protein, ATPase, nucleotide binding
4	ErbB kinase, DNA topoisomerase, HSP 86	Protein kinase, nucleotide binding, ATPase
5	HSP 70	ATPase
6	Chaperonin s3, Ser/Thr kinase 10	Nucleotide binding, protein kinase
7	Calreticulin	Activator ATPase
8	Protein disulfide isomerase	ATP binder
9	Actin-β	ATP binder
10	Actin-γ fragment	ATP binder
11	60S ribosomal protein L6	Related to histone H1
12	14-3-3 Protein (θ)	Scaffolding protein
13	14-3-3 Protein (ζ)	Scaffolding protein
14	Hemoglobin	Respiratory protein (very abundant)

Identification of enriched proteins from rat spleen
A rat spleen homogenate was processed with the ProteoEnrich™ ATP-Binders™ Kit. The crude dialyzed extract and the ATP eluate were subjected to SDS-PAGE and stained with colloidal Coomassie™ blue. The indicated bands from the ATP eluate were excised from the gel and washed several times with 100–250 µl of 50 mM ammonium hydrogen carbonate in 30% acetonitrile until completely colorless. These samples were digested with trypsin using the ProteoExtract® All-in-One Trypsin Digestion Kit according to the standard protocol and analyzed by RPLC combined with tandem mass spectrometry. Assignments of numbered bands are shown in the table.



Structure of ATP-Binders Resin



Detection of phosphorylated Src
Cell extract from the A-431 cell line was prepared by the standard PhosphoSafe™ Extraction Reagent protocol and dialyzed to remove endogenous ATP. Dialyzed extract was processed according to the standard ProteoEnrich ATP-Binders Kit protocol (1). Duplicate extract samples (5 µg) were separated on SDS-PAGE (4–20% gradient gel). After transfer to nitrocellulose, phosphorylated Src was detected with a 1:500 dilution of primary antibody for rabbit anti-human Src, followed by an Anti-Rabbit IgG, H & L Chain Specific (Goat) Peroxidase Conjugate. Detection was by chemiluminescence using SuperSignal® HRP Substrate.

ProteoExtract® Phosphopeptide Capture Kit

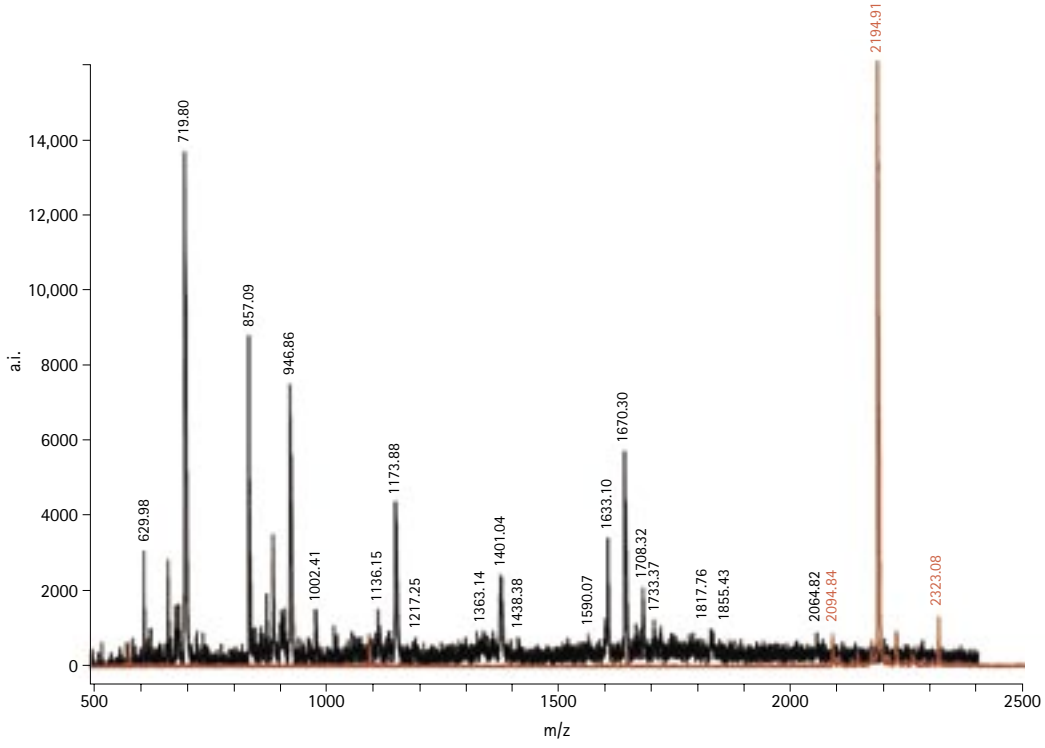
Specific capture of phosphorylated peptides combined with high yields

Product	Size	Cat. No.
ProteoExtract® Phosphopeptide Capture Kit (Includes Phospho Binding Buffer, Wash Buffers, Elution Buffer, and MagPrep® PhosphoBind)	100 rxn	525250

The ProteoExtract® Phosphopeptide Capture Kit is a dedicated tool for the analysis of the phosphoproteome. The kit enables the user to isolate both specifically and quantitatively phosphorylated peptides derived from cleaved or digested protein samples or kinase reactions designed for phosphorylation site identification. Utilizing the specific interaction of phosphate groups with immobilized zirconium ions on the surface of magnetic particles, phosphopeptides are captured and highly purified for identification by LC-MS or MALDI-MS.

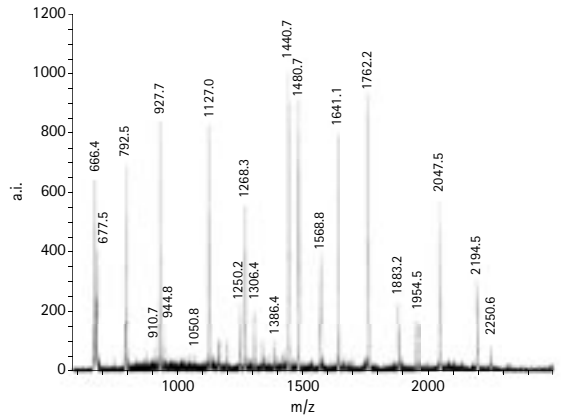
Features

- **Fast—just 30 minutes to isolate phosphorylated species from a complex mixture of tryptic peptides**
- **Specific, high-affinity capture**
- **Scalable, adaptable to various sample quantities**
- **Solid phase extraction (SPE) for desalting not required**
- **Validated, compatible protocols for LC/ESI- and MALDI-MS**

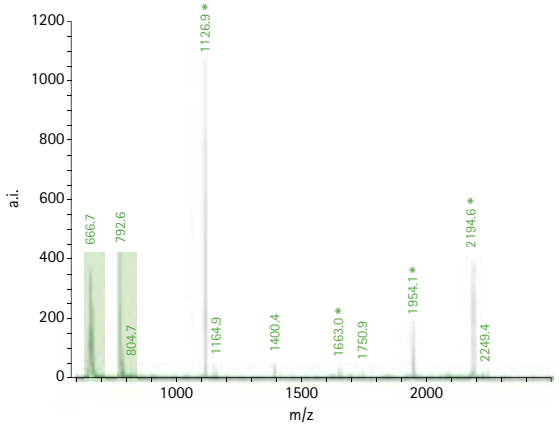


Specific isolation of phosphopeptides for MALDI-MS
A mixture of various peptides spiked with low amounts of one monophosphate peptide (M.W. 2194 Da) was processed according to the protocol of the ProteoExtract Phosphopeptide Capture Kit. The unprocessed sample (black) as well as the elution fraction (red) were directly applied on the MALDI target. The resulting peak pattern shows a single signal corresponding to the 2194-Da peptide in the elution fraction.

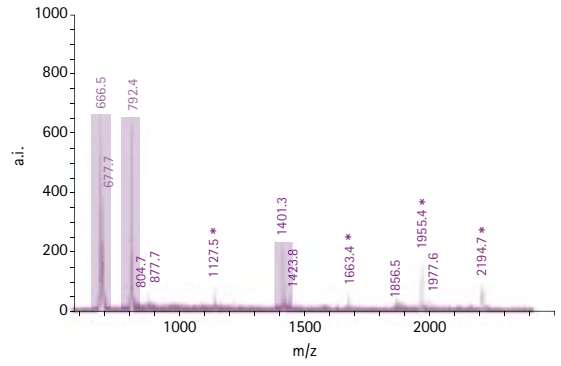
A. Unprocessed sample



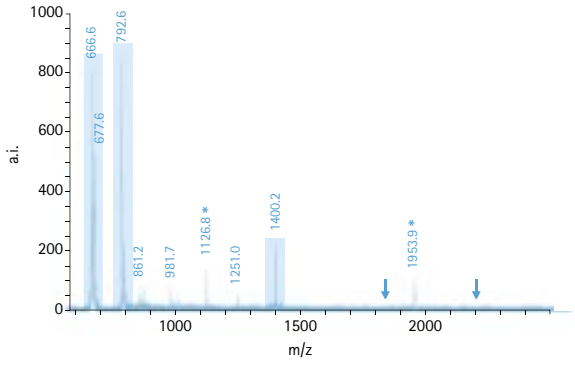
B. ProteoExtract Phosphopeptide Capture Kit



C. Kit from Supplier I



D. Kit from Supplier II



MALDI-MS spectra from various phosphopeptide isolation methods
BSA, casein alpha, and histone type IIB1 were mixed, digested with trypsin, and supplemented with a serine-phosphopeptide and a tyrosine-phosphopeptide. Analysis was performed on a MALDI-TOF instrument in linear mode, positive ion selection, with 4-hydroxy-α-cyanocinnamic acid as the matrix. Resulting spectra show phosphopeptide ions, identified by asterisks (*), and contaminating peptides, identified by shading. Panel A shows an unprocessed sample. Panel B shows a sample processed using the ProteoExtract® Phosphopeptide Capture Kit, which detected four phosphopeptides. Panel C shows a sample processed using a kit from Supplier I, which also detected four phosphopeptides, but at reduced signal intensity and with contaminating non-phosphorylated peptides. Panel D shows a sample processed using a kit from Supplier II that detected only two of the phosphopeptides (positions of the missing phosphopeptide ions are marked by arrows); the most intense peaks represent contaminating non-phosphorylated peptides.

ProteoExtract® All-in-One Trypsin Digestion Kit

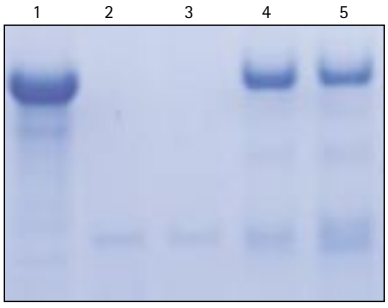
High yield of tryptic peptides from many sample types

Product	Size	Cat. No.
ProteoExtract® All-in-One Trypsin Digestion Kit (Includes Extraction Buffers, Extraction Buffer Solvent, Digest Buffer Reagent, Digest Buffer, Reducing Agent, Blocking Agent, Trypsin, and Wash Buffer)	100 rxn	650212

The ProteoExtract® All-in-One Trypsin Digestion Kit contains a set of optimized reagents that allows tryptic digests of various protein sample types: spots or bands excised from polyacrylamide gels, protein solutions, or cell and tissue extracts. Each protocol is optimized using affinity-purified trypsin, which guarantees efficient digestion of protein samples regardless of their origin. High digestion efficiency results in a high yield of tryptic peptides—even with hard-to-digest proteins—leading to improved LC-MS peak patterns and sequence coverage after MS analysis. This allows a comprehensive analysis of post-translational modifications such as phosphorylation site identification.

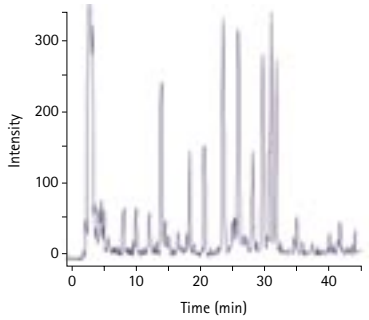
Features

- **Compatible with a variety of protein sample types**
- **Fast procedure, complete digestion in 3 hours**
- **20–30% increase in sequence coverage**

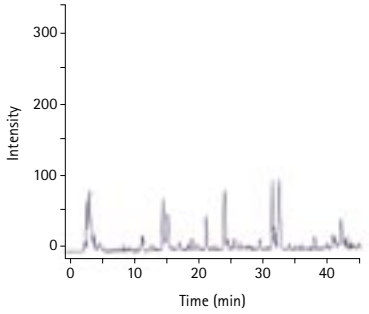


Improved digestion of hard-to-digest proteins
Ovalbumin and ovalbumin digests were analyzed as follows: lane 1: ovalbumin, chicken egg, 43 kDa; lane 2: digest incubated 3 h using ProteoExtract All-in-One Trypsin Digestion Kit; lane 3: digest incubated 4 h using the same kit; lane 4: digest incubated 3 h using reagent from Supplier I; lane 5: digest incubated 3 h using kit from Supplier II.

A. ProteoExtract All-in-One Trypsin Digestion Kit



B. Kit from Supplier III



Enhanced detection allows comprehensive analysis
Ovalbumin was digested using ProteoExtract All-in-One Trypsin Digestion Kit (panel A) or according to Supplier III kit protocol (panel B). Base peak chromatograms of nano-LC/MS analyses are shown.

PopCulture® Reagent

Protein extraction from *E. coli* cultures without harvesting cells

Product	Size	Cat. No.
PopCulture® Reagent	15 ml	71092-3
	75 ml	71092-4
	250 ml	71092-5
PopCulture GST•Mag™ Purification Kit		71113-3

Components:
- 15 ml PopCulture Reagent
- 3 × 1 ml GST•Mag Agarose Beads
- 2 × 100 ml 10X GST Bind/Wash Buffer
- 40 ml 10X Glutathione Reconstitution Buffer
- 1 g Glutathione, Reduced
- 300 KU rLysozyme™ Solution
- 1 ml rLysozyme Dilution Buffer

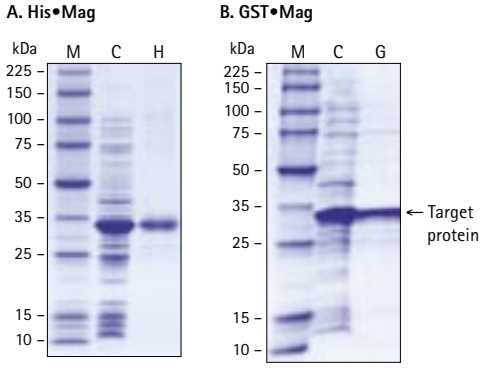
PopCulture His•Mag™ Purification Kit		71114-3

Components:
- 15 ml PopCulture Reagent
- 3 × 1 ml His•Mag Agarose Beads
- 80 ml 8X Binding Buffer
- 2 × 25 ml 8X Wash Buffer
- 2 × 25 ml 4X Elute Buffer
- 300 KU rLysozyme Solution
- 1 ml rLysozyme Dilution Buffer

Note: 1 KU = 1000 units

PopCulture® Reagent* is a detergent-based concentrate that can be added directly to cultures of *E. coli* to effectively extract proteins without the need for cell harvest. Recombinant proteins can be directly screened in the crude extract or purified by adding an affinity matrix, washing the matrix-target protein complex to remove spent culture medium and cellular contaminants, and eluting the purified protein from the matrix. The entire culturing, extraction, and purification process can be performed in the original culture tube or multiwell plate. This "in-media" protein screening or purification procedure can be adapted to high-throughput robotic processing of samples for proteomics research and any application that would benefit from the increased speed and convenience it provides. Successful purification of intact fusion proteins from total culture extracts has been demonstrated using His•Bind® and GST•Bind™ Resins (1). The use of His•Mag™ or GST•Mag™ Agarose Beads allows the entire procedure to be carried out in a single tube without the need for columns or centrifugation. Addition of rLysozyme™ Solution or the use of pLysS hosts increases the efficiency of protein extraction with the procedure. Benzonase® Nuclease may also be added to reduce the viscosity of the extract.

PopCulture Reagent is supplied as a ready-to-use Tris-buffered liquid concentrate that is stable at room temperature.



Lane	Sample
M	Perfect Protein™ Markers (10–225 kDa)
C	Crude extract (standard protocol)
H	PopCulture + His•Mag Agarose
G	PopCulture + GST•Mag Agarose

PopCulture His•Mag and GST•Mag purification
Induced cultures of *E. coli* strain BL21(DE3) containing pET-41b(+), which encodes a 35.6-kDa GST•Tag/His•Tag fusion protein, were processed using PopCulture Reagent and either His•Mag (panel A) or GST•Mag (panel B) Agarose Beads. Samples of a crude extract prepared with BugBuster and the purified proteins were analyzed by SDS-PAGE and Coomassie™ blue staining.

Features

- No separation of cells from culture media
- No mechanical disruption of cells
- No clarification of cell extracts prior to purification
- Direct affinity adsorption of target proteins to resin from the total culture extract
- Ability to rapidly perform the entire cell growth and purification process in a single tube or well

PopCulture Purification Kits

PopCulture Reagent is available bundled with His•Mag and GST•Mag Agarose Beads and corresponding buffers, plus rLysozyme Solution, for convenient extraction and affinity purification using magnetic separation. These kits enable processing of 40 × 3-ml cultures with yields up to 375 µg His•Tag® or up to 150 µg GST•Tag™ fusion protein per 3 ml culture, based on bead binding capacity. For 96-well processing using PopCulture, please refer to RoboPop™ Purification Kits later in this section.

1. Grabski, A. C., Drott, D., Handley, M., Mehler, M., and Novy, R. (2001) *inNovations* 13, 1–4.

* Patent pending

RoboPop™ Solubility Screening Kit

Expression and solubility screening in a 96-well format

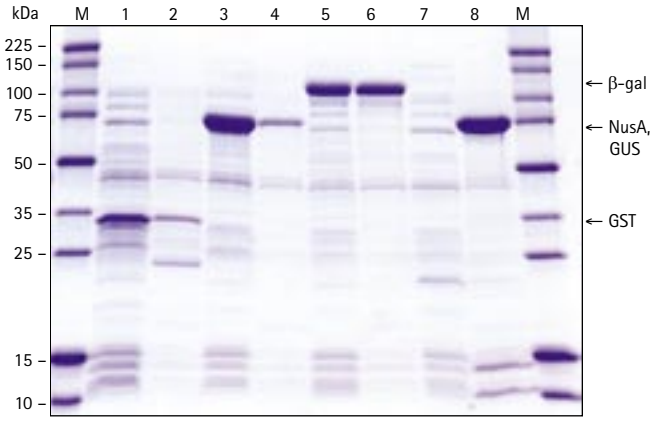
The RoboPop™ Solubility Screening Kit is designed for protein expression–level and solubility screening in a 96-well format and includes all reagents and plates necessary to perform parallel processing of 96 bacterial cultures. The kit contains PopCulture® Reagent and Lysonase™ Bioprocessing Reagent for efficient extraction of recombinant proteins from *E. coli* directly from the culture medium without cell harvest, mechanical disruption, or extract clarification. Lysonase significantly increases protein extraction efficiency and reduces sample viscosity, facilitating downstream processing and robotic pipetting.

The RoboPop Solubility Screening Kit also incorporates an innovative filtration plate capable of retaining insoluble inclusion bodies while allowing soluble proteins to be collected for rapid quantitation and analysis. Insoluble proteins retained by the filtration plate are solubilized with 4% SDS, collected, and quantified separately. The Filter Plate is compatible with standard vacuum manifolds for manual processing and the entire protocol has been validated for robotic sample processing with the Genesis® Freedom™ Workstation from Tecan and the Packard-brand MultiPROBE® II liquid handling work station from PerkinElmer Life Sciences. Two collection plates with sealers are provided for convenient storage of separated soluble and insoluble fractions.

Product	Size	Cat. No.
RoboPop™ Solubility Screening Kit	1 plate	71255-3
Components:		
• 15 ml PopCulture® Reagent		
• 0.2 ml Lysonase™ Bioprocessing Reagent		
• 1 Solubility Screening 96-well Filter Plate		
• 20 ml 4% SDS		
• 2 Collection Plate and Sealer		
Product	**Size**	**Cat. No.**
Solubility Screening 96-well Filter Plate	1 plate	71258-3
FRETWorks™ S•Tag™ Assay Kit	100 assays	70724-3
	1000 assays	70724-4
PopCulture Reagent	15 ml	71092-3
	75 ml	71092-4
	250 ml	71092-5
Lysonase Bioprocessing Reagent	0.2 ml	71230-3
	1 ml	71230-4
	5 × 1 ml	71230-5
Overnight Express™ Instant TB Medium	1 EasyPak	71491-3
	5 EasyPaks	71491-4
	1 kg	71491-5

Related application note on page 77

A. SDS-PAGE analysis of soluble and insoluble fractions



Lane	Sample
M	Perfect Protein™ Markers, 10–225 kDa
1	GST, soluble
2	GST, insoluble
3	NusA, soluble
4	NusA, insoluble
5	β-gal, soluble
6	β-gal, insoluble
7	GUS, soluble
8	GUS, insoluble

B. FRETWorks S•Tag Assay of soluble and insoluble fractions

Construct	Fraction	pmol/ml	µg/ml	% in fraction
GST	soluble	116	209	65.1
GST	insoluble	62.2	112	34.9
NusA	soluble	33.2	121	74.7
NusA	insoluble	11.2	41	25.3
β-gal	soluble	33.7	202	44.7
β-gal	insoluble	41.7	250	55.3
GUS	soluble	1.0	3	0.7
GUS	insoluble	140	477	99.3

Solubility screening using four different vector constructs

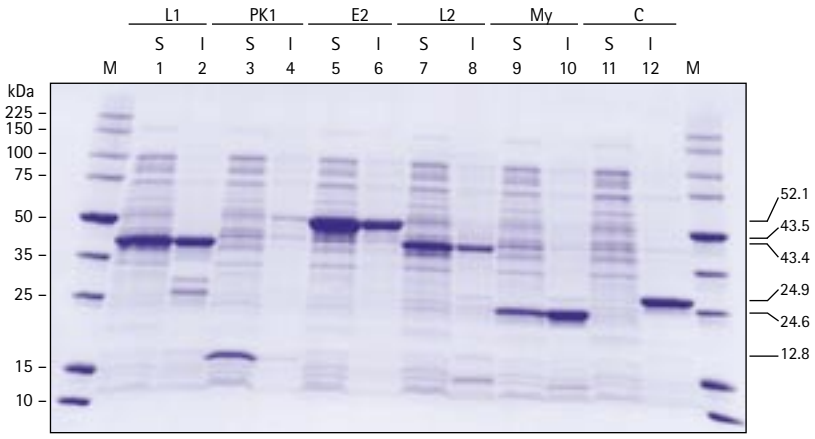
Cultures of *E. coli* strain BL21(DE3) containing four separate pET vector constructs were incubated at 37°C to an OD_{600} = 1.5 and target protein expression was induced by adding IPTG to a final concentration of 1 mM. Following further incubation for approximately 3 h at 30°C (final OD_{600} = 5–6), the cultures were dispensed (1 ml/well) into sequential rows of 2-ml 96-well plates, and 100 µl PopCulture Reagent containing 2 µl Lysonase Reagent was added to each well. Soluble and insoluble protein fractions were generated according to the RoboPop Solubility Screening Protocol. Panel A shows SDS-PAGE analysis (Coomassie™ blue staining) of the indicated samples (15 µl extract). Panel B shows the results of the FRETWorks™ S•Tag™ Assay performed with the same fractions. For the assay, samples were serially diluted 1:25 to 1:2500, and the dilutions analyzed according to the standard protocol (20 µl diluted sample was used per assay). The S•Tag fusion proteins were quantified with a standard curve based on known amounts of S•Tag Standard.

RoboPop™ Solubility Screening Kit *continued*

A. FRETWorks™ S•Tag™ assays of soluble and insoluble fractions

pET-30 Ek/LIC Construct	% in Fraction	
	Soluble	Insoluble
Lipocortin I	51	49
PK1 alpha	74	26
Enolase 2	58	42
Lipocortin II	54	46
Myosin regulatory light chain 2	45	55
Casein alpha	34	66

B. SDS-PAGE analysis of soluble and insoluble fractions



Lane	Sample
L1	Lipocortin 1
PK1	Protein kinase inhibitor alpha
E2	Enolase 2
L2	Lipocortin II
My	Myosin regulatory light chain 2
C	Casein alpha
S	Soluble
I	Insoluble
M	Perfect Protein™ Markers, 10–225 kDa

Automated solubility screening using the RoboPop™ Kit and Tecan Genesis® Freedom™ Workstation
Cultures of *E. coli* strain BL21(DE3) containing the vector constructs listed in the table above were incubated at 30°C and target protein expression was induced by Overnight Express™ Instant TB Medium (Novagen Cat. No. 71491). Following further incubation for approximately 16 h at 30°C, the cultures were processed robotically according to the RoboPop Solubility Screening protocol. Panel A shows the results of the FRETWorks™ S•Tag™ Assay performed with the same fractions. Panel B shows SDS-PAGE analysis (Coomassie™ blue staining) of the indicated samples (15 µl extract).

RoboPop™ Magnetic Purification Kits

PopCulture extraction and His•Mag or GST•Mag purification in a 96-well format

The RoboPop™ Purification Kits are designed for 96-well format purification of His•Tag® and GST•Tag™ fusion proteins directly from *E. coli* cultures without harvesting cells. The kits feature PopCulture® Reagent for extraction of proteins from total cultures without the need for centrifugation, and His•Mag™ or GST•Mag™ Agarose Beads for high-capacity magnetic affinity purification. The combination of PopCulture and magnetic agarose beads enables the entire procedure to be carried out in a single culture plate.

The kits contain one 96-well Deep Well Culture Plate (2 ml wells) with air-permeable sealing membranes for bacterial cell growth and protein purification, and one 96-well Collection Plate (450 µl wells) with an air-tight aluminum foil sealer for storage of the purified proteins. rLysozyme™ Solution, Benzonase® Nuclease, and purification buffers are also included.

The Culture Plate is compatible with the Novagen Magnetight™ HT96™ Separation Stand, which is recommended for efficient processing of magnetic affinity supports in deep-well plates. The RoboPop His•Mag and GST•Mag Purification Kits will purify up to 12 mg of His•Tag fusion proteins per plate (up to 125 µg/well) or up to 4.8 mg of GST•Tag fusion proteins per plate (up to 50 µg/well), respectively. Stated yields are based on 1 ml cultures and binding capacities of the beads, and will vary with the folding properties, expression levels, and solubility of individual fusion proteins.

Product	Cat. No.
RoboPop™ His•Mag™ Purification Kit	71103-3

Components:

- 15 ml PopCulture® Reagent
- 1 Sterile 96-well Deep Well Culture Plate with Sealers (3)
- 1 Collection Plate with Aluminum Plate Sealer
- 3 × 1 ml His•Mag Agarose Beads
- 80 ml 8X Binding Buffer
- 2 × 25 ml 8X Wash Buffer
- 2 × 25 ml 4X Elute Buffer
- 300 KU rLysozyme™ Solution
- 1 ml rLysozyme Dilution Buffer
- 2.5 KU Benzonase® Nuclease, Purity > 90%

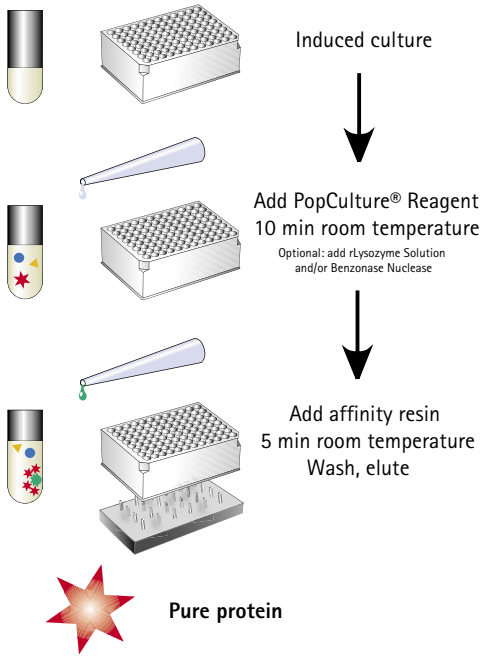
Product	Cat. No.
RoboPop GST•Mag™ Purification Kit	71102-3

Components:

- 15 ml PopCulture Reagent
- 1 Sterile 96-well deep well Culture Plate with Sealers (3)
- 1 Collection Plate with Aluminum Plate Sealer
- 3 × 1 ml GST•Mag Agarose Beads
- 2 × 100 ml 10X GST Bind/Wash Buffer
- 40 ml 10X Glutathione Reconstitution Buffer
- 1 g Glutathione, Reduced
- 300 KU rLysozyme Solution
- 1 ml rLysozyme Dilution Buffer
- 2.5 KU Benzonase Nuclease, Purity > 90%

Product	Culture scale	Processing method	Capacity*
RoboPop His•Mag Purification Kit	96 × 1 ml	Magnetic	125 µg/1 ml culture
RoboPop GST•Mag Purification Kit	96 × 1 ml	Magnetic	50 µg/1 ml culture
RoboPop Ni-NTA His•Bind® Purification Kit	96 × 5 ml	Filtration	1 mg/5 ml culture
RoboPop GST•Bind™ Purification Kit	96 × 5 ml	Filtration	0.8 mg/5 ml culture

* Capacities are based on 1- or 5-ml cultures and binding capacities of the resins. Yields will vary with the expression levels, folding properties, and solubility of individual fusion proteins.



RoboPop™ Filtration-based Purification Kits

High-throughput, milligram-scale purification of His•Tag and GST•Tag fusion proteins

Product	Cat. No.
RoboPop™ Ni-NTA His•Bind® Purification Kit	71188-3

Components:

- 75 ml PopCulture® Reagent
- 25 ml Ni-NTA His•Bind Resin
- 125 ml 4X Ni-NTA Bind Buffer
- 2 × 125 ml 4X Ni-NTA Wash Buffer
- 50 ml 4X Ni-NTA Elute Buffer
- 1 2-ml 96-well Filter Plate
- 1 1-ml 96-well Collection Plate with Aluminum Plate Sealer
- 300 KU rLysozyme™ Solution
- 1 ml rLysozyme Dilution Buffer
- 10 KU Benzonase® Nuclease, Purity > 90%

Product	Cat. No.
RoboPop GST•Bind™ Purification Kit	71189-3

Components:

- 75 ml PopCulture Reagent
- 25 ml GST•Bind Resin
- 100 ml 10X GST•Bind/Wash Buffer
- 1 g Glutathione, Reduced
- 40 ml 10X Glutathione Reconstitution Buffer
- 1 2-ml 96-well Filter Plate
- 1 1-ml 96-well Collection Plate with Aluminum Plate Sealer
- 300 KU rLysozyme Solution
- 1 ml rLysozyme Dilution Buffer
- 10 KU Benzonase Nuclease, Purity > 90%

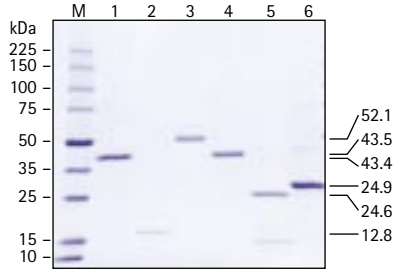
Note: 1 KU = 1000 units

The RoboPop™ Ni-NTA His•Bind® and GST•Bind™ Purification Kits are designed for high-throughput (HT) purification of soluble His•Tag® and GST•Tag™ fusion proteins directly from *E. coli* cultures. Like the corresponding RoboPop His•Mag™ and GST•Mag™ Kits (1), the new RoboPop Kits feature PopCulture® Reagent, rLysozyme™ Solution, and Benzonase® Nuclease for centrifugation-free cell lysis and extract preparation in one step. However, these kits are designed for larger-scale cultures (up to 5 ml) and include Ni-NTA His•Bind or GST•Bind Resin plus a filtration-based processing protocol (see details at right). Magnetic-based GST•Mag and His•Mag Kits purify up to 50–125 µg target protein per 1 ml culture; filtration-based kits purify up to 1 mg His•Tag fusion protein or 0.8 mg GST•Tag fusion protein per well (see table on page 41).

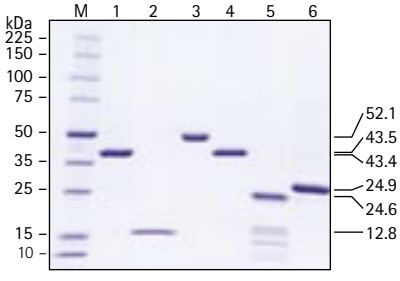
Bacterial culture, cell lysis, and resin binding steps are carried out in standard 24-well plates (not supplied), which accommodate a maximum volume of 5 ml per well. The reaction slurry is then transferred to a 96-well Filter Plate (included) and the washing and elution steps are carried out on a vacuum manifold. The Filter Plate is compatible with standard filter manifolds for manual sample processing, and the entire purification has been validated for robotic sample processing with the Packard-brand MultiPROBE® II liquid handling workstation from PerkinElmer Life Sciences and with the Genesis® Freedom™ workstation from Tecan. A 96-well Collection Plate (1 ml wells) with an air-tight aluminum foil sealer is provided for storage of the purified proteins.

1. Grabski, A., Mehler, M., Drott, D., and Van Dinther, J. (2002) *inNovations* **14**, 2–5.

A. Magnetic-based affinity purification



B. Filtration-based affinity purification



Lane	Target protein	Magnetic (His•Mag Agarose Beads) Expected size (kDa)	Magnetic (His•Mag Agarose Beads) Yield (µg/ml culture)	Magnetic (His•Mag Agarose Beads) % Purity	Filtration (Ni-NTA His•Bind Resin) Yield (µg/ml culture)	Filtration (Ni-NTA His•Bind Resin) % Purity
1	Lipocortin I	43.5	61	> 98	61	> 98
2	Protein kinase inhibitor alpha	12.8	26	> 98	20	> 98
3	Enolase	52.1	40	> 98	146	> 98
4	Lipocortin II	43.4	47	> 98	36	> 98
5	Myosin regulatory light chain 2	24.6	56	90	35	72
6	Casein alpha	24.9	122	> 98	54	> 98

Automated purification of His•Tag fusion proteins using Novagen magnetic- and filtration-based affinity purification kits and Tecan® workstation
Cultures of *E. coli* strain BL21(DE3) containing various pET vector constructs were incubated at 30°C and target protein expression was induced by Overnight Express™ Autoinduction System 1 (Novagen Cat. No. 71300-3). Following further incubation for approximately 16 h at 30°C, the cultures were processed robotically according to the RoboPop purification protocols. Panel A (approximately 1 µg protein load) and panel B (approximately 2 µg protein load) show purified protein samples analyzed by SDS-PAGE (10–20% gradient gel with Coomassie™ blue staining). The entire purification process after cell culture and induction was performed automatically by the Tecan Genesis 200. Protein assays were performed by the Bradford method and purity was determined by densitometry of the scanned gel. Lane M: Perfect Protein™ Markers, 10–225 kDa.

Insect PopCulture® Reagent

Protein extraction directly from insect cell cultures

Insect PopCulture® Reagent is a detergent-based lysis reagent specifically formulated for total insect cell culture extraction without the need for centrifugation. The improved method recovers both protein released into the medium and intracellular protein, increasing processing efficiency and target protein yields (1). It is amenable for automated expression-level screening and is fully compatible with the Ni-NTA His•Bind® affinity purification method. Insect PopCulture reagent can be used for protein extraction from insect cells grown in suspension and adherent cells grown on tissue culture plates.

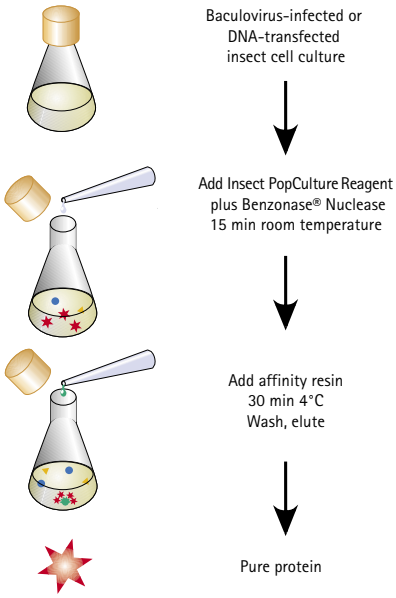
Product	Size	Cat. No.
Insect PopCulture® Reagent	50 ml 250 ml	71187-3 71187-4
Benzonase® Nuclease, Purity > 99%	10 KU	70664-3
Benzonase Nuclease, Purity > 90%	10 KU 2.5 KU	70746-3 70746-4

Note: 1 KU = 1000 units

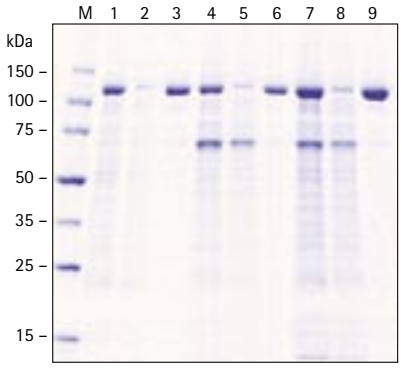
Features

- No need to separate cells from culture media
- No need to clarify cell extracts prior to purification
- Higher protein yield due to target protein recovery from both medium and cells
- Direct affinity adsorption of target proteins to IMAC affinity resin from the total culture extract
- Compatible with protease inhibitor cocktails
- Ideal for high-throughput, expression-level screening and protein purification
- Compatible with transfected and baculovirus-infected insect cell cultures

1. Loomis, K., Grabski, A., and Wong, S. C. (2002) *inNovations* **15**, 16–17.



Insect PopCulture method



Lane	Sample
M	Perfect Protein™ Markers, 15–15- kDa
1	Cell pellet, crude
2	Cell pellet, flow-through
3	Cell pellet, eluate
4	Medium, crude
5	Medium, flow-through
6	Medium, eluate
7	Insect PopCulture, crude
8	Insect PopCulture, flow-through
9	Insect PopCulture, eluate

Sample	Purified protein
Cell pellet	56 µg/ml culture
Medium	64 µg/ml culture
Insect PopCulture	131 µg/ml culture

Purification of His•Tag β-galactosidase from baculovirus-infected insect cell cultures
The bacterial β-galactosidase coding sequence, *lacZ*, was amplified by PCR and cloned into the pTriEx™-4 Ek/LIC Vector. Recombinant baculoviruses were generated by cotransfection using BacVector®-3000 Triple Cut Virus DNA (Novagen Cat. No. 70078-3) according to the recommended Novagen protocol. For protein expression, TriEx Sf9 cells (Novagen Cat. No. 71023-3) in a shaker culture in TriEx Insect Cell Medium (Novagen Cat. No. 71022-3) were infected with baculoviruses at MOI of 5. At 72 h post infection, 1 ml of the culture was used for direct in-media cell lysis with Insect PopCulture Reagent, and 1 ml was processed by standard extraction with CytoBuster™ Extraction Reagent. The His•Tag® β-galactosidase fusion protein was purified with the Ni-NTA His•Bind affinity purification method. Protein concentration of the pooled eluates was determined by the BCA method. The crude extract, flow-through, and pooled eluates were analyzed by SDS-PAGE and Coomassie™ blue staining.

Insect RoboPop™ Ni-NTA His•Bind® Purification Kit

Filtration-based, 96-well format purification directly from transfected cultures of insect cells

Product	Cat. No.
Insect RoboPop™ Ni-NTA His•Bind® Purification Kit	71257-3

Components:

- 50 ml Insect PopCulture® Reagent
- 10 KU Benzonase® Nuclease, Purity > 90%
- 10 ml Ni-NTA His•Bind Resin
- 125 ml 4X Ni-NTA Bind Buffer
- 2 × 125 ml 4X Ni-NTA Wash Buffer
- 50 ml 4X Ni-NTA Elute Buffer
- 1 2-ml 96-well Filter Plate
- 1 Collection Plate with Aluminum Plate Sealer

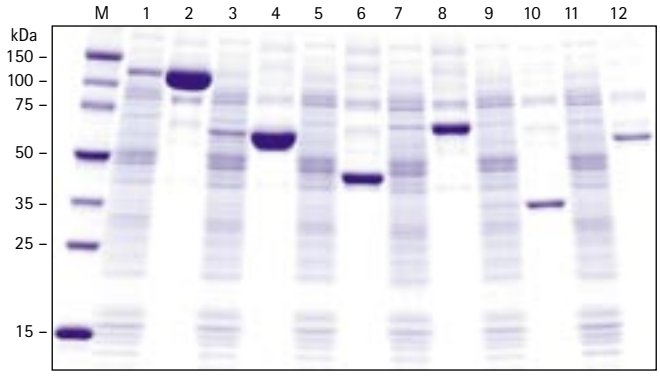
Note: 1 KU = 1000 units

Rapid purification of recombinant proteins from 96-insect cell cultures is possible with the Insect RoboPop™ Ni-NTA His•Bind® Purification Kit. This kit includes Insect PopCulture® Reagent for protein extraction from total cultures, Benzonase® Nuclease for viscosity reduction, Ni-NTA His•Bind Resin and buffers, 2 ml 96-well Filter Plate, and Collection Plate with Sealer. The kit is configured for robotic processing of 10 ml suspension cultures and purifies up to 400 µg His•Tag® fusion protein per culture based on binding capacity of the resin. Protein yields ranging from 60 to 140 µg per 10 ml culture were obtained with β-gal, Fluc, MAP kinase, and cdc2 kinase expressed in the pIEx™ transient protein expression system (1).

In practice, Insect PopCulture Reagent is added directly to the cell culture, followed by the addition of Benzonase Nuclease. The lysate is transferred to a tube of appropriate size or deep-well plate and Ni-NTA His•Bind resin is added. After mixing, the lysate-resin slurry is transferred to the Filter Plate, and washing and elution steps are carried out on a vacuum manifold. The Filter Plate is compatible with standard vacuum manifolds for either manual or automated processing, and the entire purification has been validated for robotic sample processing with the Packard-brand MultiPROBE® II liquid handling workstation from PerkinElmer Life Sciences and with the Genesis® Freedom™ workstation from Tecan.

Also use the Insect RoboPop Ni-NTA His•Bind Purification Kit with centrifugation when a vacuum manifold is not available.

1. Loomis, K., Grabski, A., and Wong, S. C. (2002) *inNovations* 15, 16–17.



Lane	Sample	Protein yield (µg)
M	Perfect Protein™ Markers, 15–150 kDa	
1	pIEx-1/β-gal, total cell protein	
2	pIEx-1/β-gal, purified	137
3	pIEx-1/Fluc, total cell protein	
4	pIEx-1/Fluc, purified	123
5	pIEx-1/MAP kinase, total cell protein	
6	pIEx-1/MAP kinase, purified	91
7	pIEx-2/MAP kinase, total cell protein	
8	pIEx-2/MAP kinase, purified	89
9	pIEx-1/cdc2 kinase, total cell protein	
10	pIEx-1/cdc2 kinase, purified	63
11	pIEx-2/cdc2 kinase, total cell protein	
12	pIEx-2/cdc2 kinase, purified	63

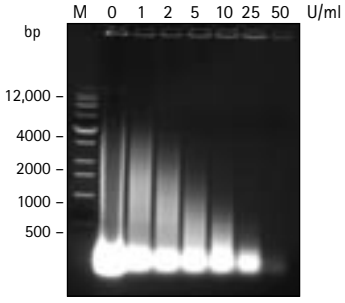
Target protein expression levels and purification from transfected Sf9 cells
Sf9 cells in 10-ml suspension cultures (1 × 10^6 cells/ml) were transfected with 20 µg of the indicated pIEx™ recombinants using Insect GeneJuice® Transfection Reagent (Novagen Cat. No. 71259-3). Total culture extracts were prepared 48 h later by the addition of Insect PopCulture Reagent (500 µl) followed by the addition of Benzonase Nuclease (5 µl). Samples were taken at this point to represent the total cell protein. Ni-NTA His•Bind Resin (50 µl per culture) was then added to the extracts. Samples were processed robotically using a Genesis Freedom workstation from Tecan. Target protein was eluted in a volume of 150 µl. Crude and purified fractions (10 µl) were loaded in adjacent lanes of a 10–20% SDS polyacrylamide gel, which was stained with Coomassie™ blue. Purified protein yields were determined by BCA assay.

Benzonase® Nuclease

Effective viscosity reduction and removal of nucleic acids from protein solutions

Product	Size	Cat. No.
Benzonase® Nuclease, Purity > 99%	10 KU	70664-3
Benzonase Nuclease HC, Purity > 99%	25 KU	71206-3
Benzonase Nuclease, Purity > 90%	10 KU 2.5 KU	70746-3 70746-4
Benzonase Nuclease HC, Purity > 90%	25 KU	71205-3

Note: 1 KU = 1000 units



Nucleic acid digestion by Benzonase
E. coli BL21(DE3) cells containing a pET construct were suspended in BugBuster Reagent (5 ml/g wet weight). Aliquots of the suspension were treated with the indicated amounts of Benzonase for 30 min at room temperature. Samples were clarified by centrifugation and analyzed by agarose gel electrophoresis and ethidium bromide staining.

Benzonase® Nuclease is a genetically engineered endonuclease from *Serratia marcescens*. It degrades all forms of DNA and RNA (single stranded, double stranded, linear, and circular) while having no proteolytic activity. It is effective over a wide range of conditions and possesses an exceptionally high specific activity. The enzyme completely digests nucleic acids to 5′-monophosphate terminated oligonucleotides 2 to 5 bases in length (below the hybridization limit), which is ideal for removal of nucleic acids from recombinant proteins, enabling compliance with FDA guidelines for nucleic acid contamination. The ability of Benzonase to rapidly hydrolyze nucleic acids makes the enzyme an excellent choice for viscosity reduction, to reduce processing time, and to increase yields of protein. For example, the enzyme is compatible with BugBuster® and PopCulture® Reagents and can be added along with these reagents to eliminate viscosity and remove nucleic acids from *E. coli* extracts.

The enzyme consists of two subunits of 30 kDa each. It is functional between pH 6 and 10 and from 0°C to 42°C and requires 1–2 mM Mg^{2+} for activation. The enzyme is also active in the presence of ionic and non-ionic detergents, reducing agents, PMSF (1 mM), EDTA (1 mM) and urea (relative activity depends on specific conditions). Activity is inhibited by > 150 mM monovalent cations, > 100 mM phosphate, > 100 mM ammonium sulfate, or > 100 mM guanidine hydrochloride. Benzonase Nuclease is available in ultrapure (> 99% by SDS-PAGE) and pure (> 90%) grades at a standard concentration of 25 U/µl and at a high concentration (HC) of 250 U/µl. Both preparations are free of detectable protease and have specific activity > 1×10^6 U/mg protein. The > 99% purity grade is tested for endotoxins and contains < 0.25 EU/1000 units. The product is supplied as a 0.2-µm filtered solution in 50% glycerol. Store at –20°C.

Unit definition: one unit is defined as the amount of enzyme that causes a ΔA_{260} of 1.0 in 30 minutes, which corresponds to complete digestion of 37 µg DNA.

Viscosity reduction by Benzonase
E. coli BL21(DE3) cells containing a pET construct were suspended in BugBuster Reagent (5 ml/g wet weight). Aliquots of the suspension were treated with the indicated amounts of Benzonase for 10 min at room temperature, centrifuged at 350 × g for 3 min and photographed.

Protease Inhibitors

Protection against proteolysis during purification

Protease Inhibitor Cocktail Set I (with EDTA)

Protease Inhibitor Cocktail Set I is a specially formulated mixture of five protease inhibitors with broad specificity for the inhibition of various proteases and esterases. It is provided as a lyophilized solid, ready for reconstitution, and is available in either of two configurations: as a single vial or as a set of 10 vials. Each vial, when reconstituted with 1 ml of water, makes 1 ml of 100X stock solution.

When reconstituted, each vial of Protease Inhibitor Cocktail Set I contains 50 mM AEBSF-HCl, 15 µM Aprotinin, 0.1 mM E-64, 50 mM EDTA, and 0.1 mM Leupeptin Hemisulfate. Note that the presence of EDTA may interfere with purification of His•Tag® fusion proteins if a cell extract is applied directly to IDA or Ni-NTA resins. For these applications we recommend Protease Inhibitor Cocktail Set III.

Protease Inhibitor Cocktail Set II (with EDTA)

This lyophilized cocktail includes five protease inhibitors with broad specificity for the inhibition of aspartic, cysteine, serine, and metalloproteases, as well as aminopeptidases. It is recommended for use with bacterial cell extracts (except those being used for metal chelation chromatography). Reconstitute each vial with 1 ml DMSO and 4 ml water to obtain a 5-ml stock solution. Slight turbidity in the reconstituted solution is normal. When reconstituted, each vial contains 20 mM AEBSF-HCl, 1.7 mM Bestatin, 200 µM E-64, 85 mM EDTA, and 2 mM Pepstatin A. One set contains 1 vial of lyophilized inhibitors plus 1 vial DMSO. Five sets contain 5 vials of lyophilized inhibitors plus 5 vials DMSO, enough for 25 ml total after addition of water. Use of 5 ml is recommended for the inhibition of proteases extracted from 20 g *E. coli*.

Protease Inhibitor Cocktail Set III (without EDTA)

This liquid cocktail includes six protease inhibitors with broad specificity for the inhibition of aspartic, cysteine, and serine proteases, as well as aminopeptidases. It is recommended for use with bacterial cell extracts being used for metal chelation chromatography, mammalian cell and tissue extracts. Each 1 ml vial contains 100 mM AEBSF-HCl, 80 µM Aprotinin, 5 mM Bestatin, 1.5 mM E-64, 2 mM Leupeptin Hemisulfate, and 1 mM Pepstatin A as a solution in DMSO. Contains no metal chelators. Use of 1 ml is recommended for the inhibition of proteases extracted from 20 g of bovine liver or 20 g *E. coli*.

Product	Size	Cat. No.
Protease Inhibitor Cocktail Set I (with EDTA)	1 vial 10 vials	539131
Protease Inhibitor Cocktail Cocktail Set II (with EDTA)	1 set 5 sets	539132
Protease Inhibitor Cocktail Set III (without EDTA)	1 ml 5 × 1 ml	539134

Protease Inhibitor Cocktail Set I (with EDTA) contents and properties

Inhibitor	M.W.	Concentration after reconstitution	Target protease
AEBSF, Hydrochloride	239.5	50 mM	Serine proteases
Aprotinin	6512	15 µM	Broad spectrum, serine proteases
E-64	357.4	0.1 mM	Cysteine proteases
EDTA, Disodium	372.2	50 mM	Metalloproteases
Leupeptin Hemisulfate	475.6	0.1 mM	Cysteine proteases and trypsin-like proteases

Protease Inhibitor Cocktail Set II (with EDTA) contents and properties

Inhibitor	M.W.	Concentration after reconstitution	Target protease
AEBSF, Hydrochloride	239.5	20 mM	Serine Proteases
Bestatin	308.4	1.7 mM	Aminopeptidase B and Leucine Aminopeptidase
E-64	357.4	0.2 mM	Cysteine proteases
EDTA, Disodium	372.2	85 mM	Metalloproteases
Pepstatin A	685.9	2 mM	Aspartic proteases

Protease Inhibitor Cocktail Set III contents and properties

Inhibitor	M.W.	Concentration after reconstitution	Target protease
AEBSF, Hydrochloride	239.5	100 mM	Serine proteases
Aprotinin	6512	80 µM	Broad spectrum, serine proteases
Bestatin	308.4	5 mM	Aminopeptidase B and leucine aminopeptidase
E-64	357.4	1.5 mM	Cysteine proteases
Leupeptin Hemisulfate	475.6	2 mM	Cysteine proteases and trypsin-like proteases
Pepstatin A	685.9	1 mM	Aspartic proteases

Product	Size	Cat. No.
Protease Inhibitor Cocktail Set IV (without EDTA)	1 set	539136
Protease Inhibitor Cocktail Set V, EDTA-Free	10 vials	539137

Protease Inhibitor Cocktail Set IV (without EDTA)

This liquid cocktail includes four protease inhibitors with broad specificity for the inhibition of aspartic, cysteine, metallo-, and serine proteases. It is recommended for fungal and yeast cell extracts. One set includes five 1-ml vials. Each 1-ml vial contains 100 mM AEBSF-HCl, 1.5 mM E-64, 2 mM Pepstatin A, and 500 mM 1,10-Phenanthroline as a solution in DMSO.

Protease Inhibitor Cocktail Set IV (without EDTA) contents and properties

Inhibitor	M.W.	Concentration after reconstitution	Target protease
AEBSF, Hydrochloride	239.5	100 mM	Serine proteases
E-64	357.4	1.5 mM	Cysteine proteases
Pepstatin A	685.9	2 mM	Aspartic proteases
1,10-Phenanthroline	198.2	500 mM	Metalloproteases

Protease Inhibitor Cocktail Set V, EDTA-Free

This cocktail includes four protease inhibitors for the inhibition of serine and cysteine proteases, but not metalloproteases. Reconstitute each vial with 1 ml H_2O to obtain 1 ml of 100X concentrated stock solution. A 1X stock solution contains 500 µM AEBSF-HCl, 150 nM Aprotinin, 1 µM E-64, and 1 µM Leupeptin Hemisulfate. Note: this product is hygroscopic.

Protease Inhibitor Cocktail Set V, EDTA-Free contents and properties

Inhibitor	M.W.	Concentration after reconstitution	Target protease
AEBSF, Hydrochloride	239.5	50 mM	Serine proteases
Aprotinin	6512	15 µM	Broad spectrum, serine proteases
E-64	357.4	0.1 mM	Cysteine proteases
Leupeptin Hemisulfate	475.6	0.1 mM	Cysteine proteases and trypsin-like proteases

Protease inhibitors: mechanism of action				
Inhibitor	**M.W.**	**Target protease class and mechanism of action**	**Solubility**	**Suggested concentration**
AEBSF, Hydrochloride	239.5	Water-soluble, non-toxic alternative to PMSF. Irreversible inhibitor of serine proteases. Reacts covalently with a component of the active site. Inhibits chymotrypsin, kallikrein, plasmin, trypsin, and related thrombolytic enzymes.	H_2O	0.1–1 mM
Aprotinin	6512.0	A competitive and reversible inhibitor of proteolytic and esterolytic activity. A serine protease inhibitor. In cell culture, it extends the life of cells.	H_2O	0.6–2.0 µg/ml
Benzamidine, Hydrochloride	156.6	Inhibitor or trypsin and trypsin-like enzymes.	H_2O, ethanol	0.5–4.0 mM
Bestatin	308.4	Binds to cell surfaces and reversibly inhibits aminopeptidase B and leucine aminopeptidase.	DMSO, methanol	1–10 µM
E-64	357.4	An irreversible cysteine protease inhibitor that has no effect on cysteine residues in other proteins. Specific active site titrant.	H_2O, DMSO	1–10 µM
EDTA, Disodium	372.2	A reversible metalloprotease inhibitor. A chelator that may interfere with other metal ion–dependent processes.	H_2O	1–10 mM
Leupeptin	475.6	An inhibitor of aspartic proteases. Reversibly inhibits cathepsin D, cathepsin G, pepsin, and renin.	H_2O	10–100 µM
Pepstatin A	685.9	An inhibitor of aspartic proteases. Reversibly inhibits cathepsin D, cathepsin G, pepsin, and renin.	DMSO, methanol	1 µM

Phosphatase Inhibitors

Convenient phosphatase inhibitor cocktails

Product	Size	Cat. No.
Phosphatase Inhibitor Cocktail Set I	1 set	524624
Phosphatase Inhibitor Cocktail Set II	1 set	524625

Phosphatase Inhibitor Cocktail Set I

Phosphatase Inhibitor Cocktail Set I is a mixture of three inhibitors that will inhibit alkaline phosphatase as well as serine/threonine protein phosphatases such as PP1 and PP2. The inhibitors are provided at the following concentrations: 2.5 mM (–)-*p*-Bromotetramisole Oxalate, 500 µM Cantharidin, and 500 nM Microcystin-LR in DMSO. Dilute 1:100 immediately before use.

Phosphatase Inhibitor Cocktail Set I

Inhibitor	M.W.	Inhibits	Concentration in vial
(–)-*p*-Bromotetramisole oxalate	373.2	Tyrosine Phosphatase	2.5 mM
Cantharidin	196.2	Serine/Threonine Phosphatase	500 µM
Microcystin-LR	995.2	Protein Phosphatases 1 and 2A	500 nM

Phosphatase Inhibitor Cocktail Set II

Phosphatase Inhibitor Cocktail Set II is an aqueous solution of five phosphatase inhibitors for the inhibition of acid and alkaline phosphatases as well as protein tyrosine phosphatases (PTPs). It is suitable for use with tissue and cell extracts, including extracts containing detergents. The inhibitors are provided at the following concentrations: 200 mM Imidazole, 100 mM Sodium Fluoride, 115 mM Sodium Molybdate, 100 mM Sodium Orthovanadate, and 400 mM Sodium Tartrate Dihydrate. Dilute 1:100 immediately before use.

Phosphatase Inhibitor Cocktail Set II

Inhibitor	M.W.	Inhibits	Concentration in vial
Imidazole	68.1	Alkaline Phosphatase	200 mM
Sodium Fluoride	42.0	Acid Phosphatase	100 mM
Sodium Molybdate	205.9	Acid Phosphatase	115 mM
Sodium Orthovanadate	183.9	Protein Tyrosine Phosphatase, Alkaline Phosphatase	100 mM
Sodium Tartrate Dihydrate	230.1	Acid Phosphatase	400 mM

ProteoExtract® Protein Precipitation Kit

Efficient concentration and sample clean-up

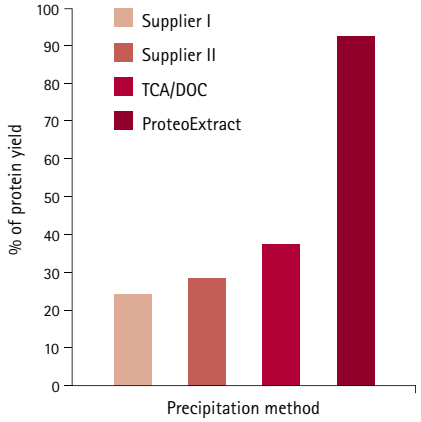
The new ProteoExtract® Protein Precipitation Kit provides efficient concentration of proteins and removal of interfering substances from dilute protein samples in a single step. The ProteoExtract Protein Precipitation Kit uses a unique combination of precipitation agents to quantitatively precipitate proteins and remove interfering substances, for one-step concentration and clean-up of proteins.

The ProteoExtract Protein Precipitation Kit delivers protein solutions with very low conductivity, making it ideally suited for a wide range of proteomics applications including IEF, 1DGE, 2DGE, and MS.

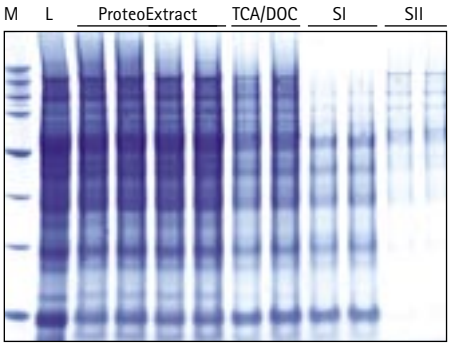
Product	Size	Cat. No.
ProteoExtract® Protein Precipitation Kit	200 × 200 µl	539180

Features

- **Highest yields and quantitative protein recovery including low–molecular weight proteins**
- **High solubility of protein pellets**



Protein methods by various methods
Protein at a concentration of 2 mg/ml was precipitated using the ProteoExtract® Kit; protein pellets were redissolved in IEF buffer prior to determination of protein concentration. The same experiment was performed in parallel using TCA/deoxycholate (DOC) and products from Supplier I and Supplier II. All experiments were performed in duplicate. The ProteoExtract Protein Precipitation Kit delivers up to 3 times greater protein yields as compared to other precipitation methods.



Quantitative recovery of proteins
Equal volume equivalents from samples precipitated with different methods were separated by 1D SDS-PAGE and visualized by Coomassie™ blue staining. Protein patterns from the ProteoExtract-precipitated proteins (P) are identical to the pattern from the liver extract (L), clearly demonstrating the quantitative recovery of proteins. Weaker staining is achieved by TCA/DOC indicating protein loss. Selective protein loss is observed using products from Supplier I (SI) and Supplier II (SII). M: molecular weight markers.

D-Tube™ Dialyzers

Dialysis and electroelution from polyacrylamide or agarose gels

Product	Size	Cat. No.
D-Tube™ Dialyzer Mini, MWCO 6–8 kDa	1 kit	71504-3
D-Tube Dialyzer Mini, MWCO 12–14 kDa	1 kit	71505-4
D-Tube Dialyzer Midi, MWCO 3.5 kDa	1 kit	71506-3
D-Tube Dialyzer Midi, MWCO 6–8 kDa	1 kit	71507-3
D-Tube Dialyzer Maxi, MWCO 3.5 KDa	1 kit	71508-3
D-Tube Dialyzer Maxi, MWCO 6–8 KDa	1 kit	71509-3
D-Tube Dialyzer Maxi, MWCO 12–14 KDa	1 kit	71510-3

Components:
- 10 D-Tubes
- 1 Floating Rack

The D-Tube™ Dialyzers can be used for dialysis and electroelution of proteins, RNA, DNA, and oligonucleotides from polyacrylamide or agarose gels. The disposable, single-use tubes require no syringes, microcentrifuge, or laborious steps to manipulate small sample volumes. The sample is added and removed using a standard laboratory pipette. Available with molecular weight cutoffs (MWCO) from 3.5 to 14 kDa, the D-Tube Dialyzers are designed in three volume capacities: mini (10–250 µl), midi (50–800 µl), and maxi (100–3000 µl). The membrane is ultra-clean, EDTA-treated regenerated cellulose, sulfur- and heavy metal-free. Each kit contains one floating rack that can hold up to four D-Tubes in the exchange buffer.

Features:

- **Easy-to-handle dialyzers for buffer exchange and removal of urea, detergents, and ethidium bromide**
- **One-step procedure that does not require syringes or any special equipment**
- **Sample volume recovery > 97%**
- **Protease, RNAse, DNAse, and PCR product free**
- **Ideal for electroelution of proteins, protein-DNA complexes, oligonucleotides, DNA, and RNA from polyacrylamide and agarose gels**

D-Tube Dialyzer Size	Volume	MW Cutoff
Mini	10 to 250µl 10 to 250µl	6-8 kDa 12-14 kDa
Midi	50 to 800µl 50 to 800µl	3.5 kDa 6-8 kDa
Maxi	1000 to 3000 1000 to 3000 1000 to 3000	3.5 kDa 6-8 kDa 12-14 KDa

D-Tube™ Electroelution Accessory Kit Midi

Optimized reagents for protein and nucleic acid precipitation following electroelution

The combination of D-Tube™ Dialyzers and the D-Tube Electroelution Accessory Kit Midi provides a unique tool for extraction of any protein, protein-protein complex, or protein-DNA complex from non-denaturing and denaturing (SDS) polyacrylamide gels with 60% recovery yield in less than 2 hours. Extracted proteins are compatible with most downstream applications such as MALDI-MS, animal immunization for antibody production, HPLC, peptide mapping, and functional assays. In addition, D-Tube Dialyzers can be used for oligonucleotides, RNA, and DNA extraction from both polyacrylamide and agarose gels. Efficient extraction (> 90%) is achieved for 15-nt oligos and for DNA fragments of up to 80 kbp. The D-Tube Electroelution Accessory Kit provides one D-Tube support tray which is compatible with most commercially available horizontal electrophoresis units and optimized reagents for protein and nucleic acid precipitation following electroelution.

Product	Size	Cat. No.
D-Tube™ Electroelution Accessory Kit Midi	1 kit	71511-3

Components:

- 1 ml MS Precipitation Buffer
- 10 ml TCA, 20%
- 2 × 1 ml 3 M NaAc, pH 5.2
- 1 Supporting Tray, Midi

Features:

- Efficient extraction of protein, protein–DNA complexes, oligonucleotides, DNA, and RNA from 1D and 2D polyacrylamide and agarose gels
- More than 60% protein recovery
- More than 90% recovery of oligonucleotides , RNA, and DNA from 15 nt to 80 kbp in size
- Procedure compatible with variety of downstream applications including MALDI-MS, functional assays, and HPLC
- High-throughput electroelution from multiple samples simultaneously

BCA Protein Assay Kit

Simple and reliable protein quantification

Product	Size	Cat. No.
BCA Protein Assay Kit	500 assays (2500 microplate assays)	71285-3

Components:
- 500 ml BCA Solution
- 15 ml 4% Cupric Sulfate
- 3 × 1 ml BSA Standard, 2 mg/ml

Product	Size	Cat. No.
BugBuster® Protein Extraction Reagent	100 ml	70584-3
	500 ml	70584-4
PopCulture® Reagent	15 ml	71092-3
	75 ml	71092-4
	250 ml	71092-5
CytoBuster™ Protein Extraction Reagent	50 ml	71009-3
	5 × 50 ml	71009-4
Reportasol™ Extraction Buffer	25 ml	70909-3
	5 × 25 ml	70909-4
Insect PopCulture Reagent	50 ml	71187-3
	250 ml	71187-4

The BCA protein assay is based on a biuret reaction, which is the reduction of Cu^{+2} to Cu^{+1} by proteins in an alkaline solution with concentration-dependent detection of the monovalent copper ions. Bicinchoninic acid is a chromogenic reagent that chelates the reduced copper, producing a purple complex with strong absorbance at 562 nm. This assay can be used to quantify protein concentration with a wide variety of samples and can be performed in minutes.

The Novagen BCA Protein Assay Kit can be used to determine protein concentration in the range of 20 to 2000 µg/ml in either a standard assay or microassay configuration. Kit components are sufficient to complete 500 standard-size reactions (50 µl protein sample plus 1 ml reagent) or 2500 micro-scale reactions (25 µl protein sample plus 200 µl reagent). A BSA standard (3 × 1 ml at 2 mg/ml) is provided for convenient preparation of standard curves.

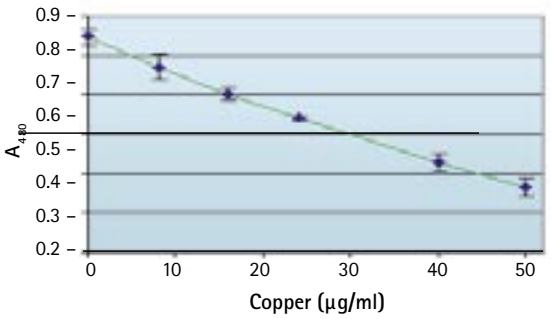
This assay is robust and can be performed in the presence of many chemical compounds. Some reagents, including chelating agents, strong acids or bases, and reducing agents, interfere with the reduction and chelating reactions on which this assay depends. The BCA assay is compatible with the following Novagen protein extraction and lysis reagents: BugBuster® Protein Extraction Reagent, PopCulture® Reagent, CytoBuster™ Protein Extraction Reagent, Reportasol™ Extraction Buffer, and Insect PopCulture Reagent. Options for the removal or dilution of interfering substances are described in the kit literature.

Non-Interfering Protein Assay™ Kit

Overcomes interference of agents found in protein solutions

The easy-to-use Non-Interfering Protein Assay™ Kit overcomes interference of agents found in protein solutions including detergents, chelating agents, reducing agents, amines, sugars, urea, and others. The Universal Protein Precipitating Agent (UPPA™ reagent) is used to precipitate and immobilize the protein in the tube while the interfering reagents are removed. Protein concentration is based on the specific binding of copper to the peptide backbone. As the protein concentration increases, the concentration of unbound copper ions decreases, and the color density is inversely related to the amount of protein present in solution. The kit includes UPPA reagents I and II, Copper Solution I, Color Agents A and B, and BSA standard.
Note: one kit is sufficient for 500 individual protein determinations.

Product	Size	Cat. No.
Non-Interfering Protein Assay™ Kit	1 kit	488250



Standard curve generated with the BSA standard and the Non-Interfering Protein Assay Kit
Assay measures copper ions in solution. A predetermined concentration of copper reagent binds to the peptide backbone of proteins. Higher concentrations of protein in the standards or sample bind more copper, leaving less unbound copper in solution to react with the colorimetric reagent and resulting in lower absorbance readings at 480 nm.

Perfect Protein™ Markers

Precisely sized, conveniently spaced for accurate protein size determination

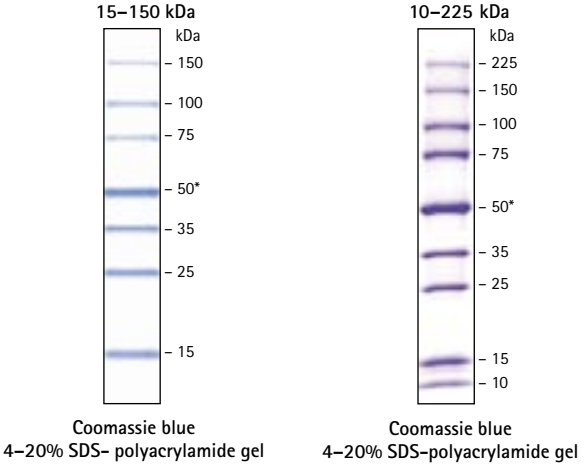
Product	Size	Cat. No.
Perfect Protein™ Markers, 15–150 kDa	100 lanes	69149-3
Perfect Protein Markers, 10–225 kDa	100 lanes	69079-3
4X SDS Sample Buffer	2 ml	70607-3

The Perfect Protein™ Markers are a novel set of recombinant proteins with defined sizes at convenient intervals. Designed for routine use in SDS-polyacrylamide gel electrophoresis, the Perfect Protein Markers enable highly accurate size determination of unknown samples. Unlike many conventional markers (e.g., ovalbumin, serum albumin, etc.), the Perfect Protein Markers contain no oligosaccharides that cause anomalous migration, heterogeneous "fuzzy" bands, or inaccurate size estimation. The known mass of each Perfect Protein Marker band also enables estimation of concentration of sample proteins. The markers are optimized for use with Coomassie™ blue staining, but adjusted amounts can also be used with other gel staining methods (e.g., silver staining, fluorescent dyes, etc.).

The Perfect Protein Markers, 15–150 kDa, include protein sizes of 15, 25, 35, 50, 75, 100, and 150 kDa. Each vial contains 400 µg protein (50 µg per band except for 100 µg of the 50-kDa high-intensity reference band).

The Perfect Protein Markers, 10–225 kDa, include the protein sizes listed above and two additional proteins, 10 kDa and 225 kDa, for applications requiring a broader size range. Each vial contains 500 µg protein (50 µg per band except for 100 µg of the 50-kDa high-intensity reference band).

The 4X SDS Sample Buffer is a standard formulation commonly used for SDS-PAGE analysis of proteins. The solution includes DTT for complete denaturation of disulfide bonds. The buffer can be used at a final concentration of 2X for most applications.



Coomassie blue 4–20% SDS- polyacrylamide gel

Coomassie blue 4–20% SDS-polyacrylamide gel

* High-intensity reference band

Trail Mix™ Protein Markers

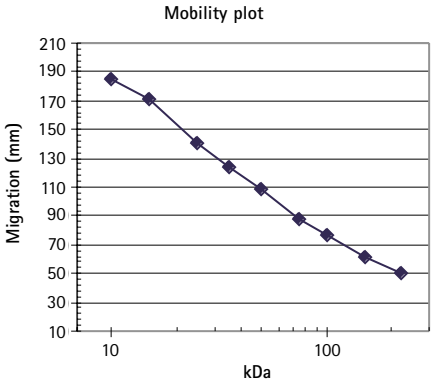
Novel protein markers for visible tracking and accurate sizing in stained gels

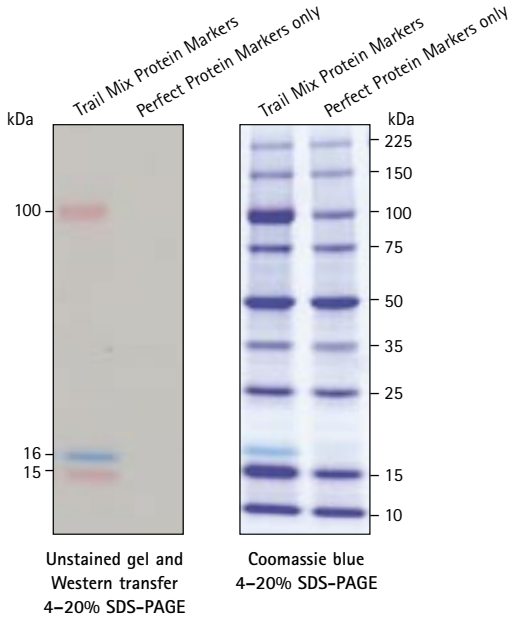
Trail Mix™ Protein Markers are a mixture of the Novagen Perfect Protein™ Markers supplemented with a group of three prestained indicator proteins to allow direct visualization of protein migration during electrophoresis. Unlike other marker sets in which the entire ladder is prestained, Trail Mix uses only three reference bands (at 100, 16, and 15 kDa) to confirm separation and indicate gel orientation. Prestaining can cause band broadening or affect mobility, reducing the precision with which mobility and molecular weight determinations can be made. The migration or band sharpness of the Perfect Protein Markers is unaffected by the presence of the prestained bands in Trail Mix.

When stained with Coomassie™ blue, 10 bands appear, ranging from 10 to 225 kDa. In addition to the prestained bands, the 50 kDa marker serves as a landmark on stained gels because it is present at a higher concentration in the mixture relative to the adjacent bands.

The 4X SDS Sample Buffer is a standard formulation commonly used for SDS-PAGE analysis of proteins. The solution includes DTT for complete denaturation of disulfide bonds. The buffer can be used at a final concentration of 2X for most applications.

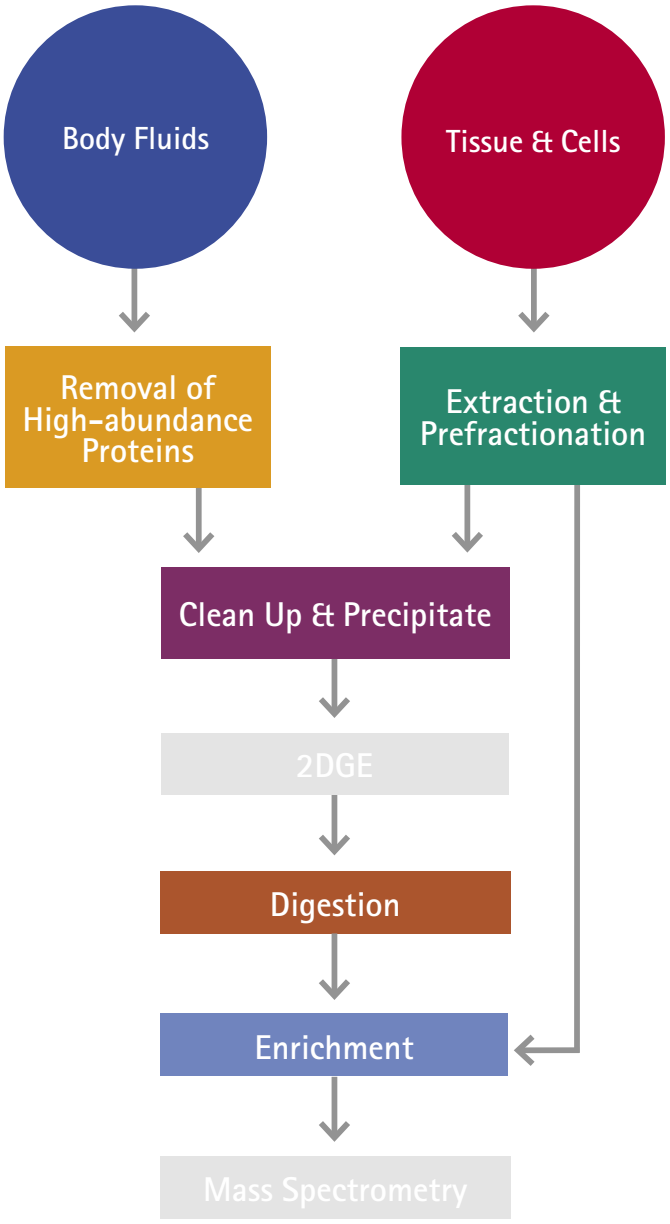
Product	Size	Cat. No.
Trail Mix™ Protein Markers	100 lanes	70980-3
4X SDS Sample Buffer	2 ml	70607-3





Sample Preparation Processes

Example of Protein Sample Preparation Workflow



A rapid procedure for protein extraction from filamentous fungi and plants using YeastBuster™ Protein Extraction Reagent*

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Department of Molecular Biology and Biotechnology, Krebs Institute for Biomolecular Research, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK

Efficient release of proteins is demonstrated using YeastBuster™ Protein Extraction Reagent with the mycelium of a fungus (*Aspergillus nidulans*) and the seeds of bambara groundnut (*Voandzeia subterrannea*).

The main methods for preparing protein extracts from filamentous fungi and plants involve the homogenization of mortar-pulverized material in liquid nitrogen and extraction under either non-denaturing or denaturing conditions. The simple non-denaturing method used in our laboratory with *Aspergillus nidulans* involves extraction with a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM PMSF, 2 mM EDTA, and 2 mM DTT under agitation at room temperature; proteins are recovered as a soluble fraction after centrifugation. The protein recovery is variable and, furthermore, most of the proteins remain in the pellet. Denaturing methods provide proteins in a single extract fraction; however the protein extracts are not fully compatible with a variety of downstream applications, such as enzyme assays.

Recently, YeastBuster Protein Extraction Reagent was developed for gentle release of soluble proteins from yeast cells (1). The success in our laboratory with *in vitro* enzyme assays using *Saccharomyces cerevisiae* protein extracts obtained with YeastBuster reagent prompted us to investigate whether a similar approach could be used for filamentous fungi.

Here we present an optimized protocol for *A. nidulans* that also enables the processing of seeds of *Voandzeia subterrannea* (L) Thouars (bambara groundnut), widely used as a main source of dietary protein by rural communities in many African countries.

A. nidulans strain G191 (2) was cultured at 37°C in rich liquid medium (YEPD broth with supplements as required) and mycelia were collected by filtration through Miracloth. Both *Aspergillus* mycelial biomass and seeds of bambara groundnut (accession number 2, as described in reference 3) were ground to a fine powder with a mortar and pestle under liquid nitrogen. The pulverized materials were used immediately or after storage at –80°C.

YeastBuster Protein Extraction Reagent extracted proteins with a similar efficiency over a wide molecular mass range. Starting with 50 mg tissue, the YeastBuster method extracted approximately 50% more protein (9.4 mg) than the denaturing method (6.3 mg).

Pulverized material (50 mg) was suspended in a 1.5 ml microcentrifuge tube by pipetting in 1 ml of extraction buffer [978 µl YeastBuster Protein Extraction Reagent, 10 µl THP, 1 µl Benzonase® Nuclease (25 U), and 11.4 µl 100 mM PMSF] and then agitated in a MiniMix microcentrifuge shaker (Thermo Life Sciences) at top speed for 20 minutes. Insoluble debris was removed by centrifugation at 16,000 × g at 4°C for 10 minutes. The supernatant containing the soluble fraction was transferred to another tube and retained. The pellet was resuspended in 1 ml denaturing buffer (see Figure 1).

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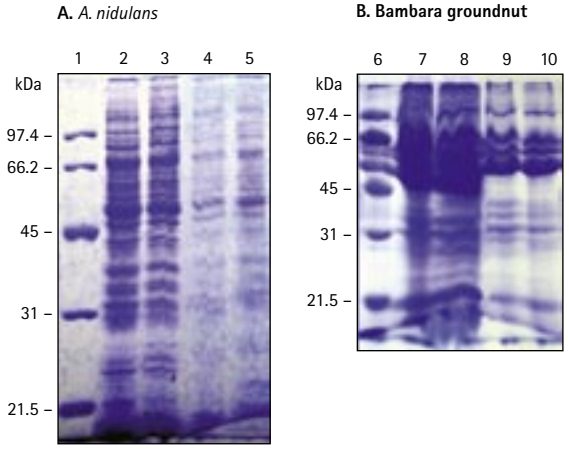
*Adapted from *inNovations* (2004) **19**, 15–16.

For comparison, protein extracts were also prepared according to a described method (4) with modifications. In a microcentrifuge tube, 50 mg ground material was suspended in 1 ml 10% (w/v) TCA. Next, 10 µl cold acetone containing 0.07% (v/v) 2-mercaptoethanol was added, mixed, and left overnight at –20°C. After centrifugation at 16,000 × g at 4°C for 20 minutes, the supernatant was discarded and the pellet was washed with 900 µl cold acetone/mercaptoethanol mixture and dried under vacuum for 10 minutes. Proteins were resuspended in 1 ml denaturing buffer [40 mM Tris, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 100 mM DTT], sonicated (six 10-second bursts with a 10-second cooling period between each burst) on ice, and then spun at 16,000 × g at 4°C for 20 minutes. The supernatant (soluble fraction) was transferred to a new tube and the remaining pellet resuspended in 500 µl denaturing buffer. A Bradford assay was used to determine the concentration of recovered protein.



Lane	Sample
1	Protein markers
2	5 µl denaturing extract
3	5 µl YeastBuster extract
4	10 µl pellet obtained with the denaturing method and resuspended in denaturing buffer
5	10 µl pellet obtained with YeastBuster Protein Extraction Reagent and resuspended in denaturing buffer
6	Protein markers
7	10 µl denaturing extract
8	10 µl YeastBuster extract
9	10 µl pellet obtained with the denaturing method and resuspended in denaturing buffer
10	10 µl pellet obtained with YeastBuster Protein Extraction Reagent and resuspended in denaturing buffer

Figure 1. Comparison of YeastBuster and denaturing extraction methods
Proteins extracted using the methods described in the text were analyzed by SDS-PAGE (10% gel) and Coomassie™ blue staining. Panel A: Protein extracts from *A. nidulans*. Panel B: Protein extracts from bambara groundnut.

Figure 1, panel A shows that YeastBuster™ Protein Extraction Reagent extracted proteins with a similar efficiency over a wide molecular mass range. Starting with 50 mg tissue, the YeastBuster method extracted approximately 50% more protein (9.4 mg) than the denaturing method (6.3 mg). Figure 1, panel B shows that the YeastBuster method was equally effective for release of proteins from seeds of bambara groundnut: both YeastBuster and denaturing extracts contained similar amounts of protein (6.7 mg and 7.1 mg, respectively). Most of the extractable protein was released (Figure 1, lanes 4, 5, 9, and 10). It should be noted that the same YeastBuster method described here yielded up to 15 mg protein from *S. cerevisiae* extract (data not shown).

YeastBuster Protein Extraction Reagent is capable of extracting proteins from *A. nidulans* and seeds of bambara groundnut with similar or even higher efficiencies than the denaturing method. Protein extracts can be used immediately or frozen as portions in liquid nitrogen and stored at –80°C for future use.

Summary

The YeastBuster non-denaturing method is rapid, simple, efficient, relatively inexpensive, flexible enough to be scaled up or down, and compatible with downstream applications. The results shown here suggest that the YeastBuster extraction method also could be used with a variety of filamentous fungi and plant tissues.

References

1. Drott, D., Bahairi, S., and Grabski, A. (2002) *inNovations* **15**, 14–17.
2. Keszenman-Pereyra, D., Lawrence, S., Twfieg, M.-E., Price, J., and Turner, G. (2003) *Curr. Genet.* **43**, 186–190.
3. Odeigah, P. G. C. and Osanyinpeju, A. O. (1998) *Genet. Resour. Crop Evol.* **45**, 451–458.
4. Protocol for protein extraction, at: http://www.biotech.unl.edu/proteomics/mercaptoethanol.html

See the following page for YeastBuster Protein Extraction Reagent product details and ordering information:

Product	Cat. No.	Page
YeastBuster™ Protein Extraction Reagent	71186	16

Double your chance to discover less-abundant, low-solubility proteins on 2D electrophoresis gels*

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Introduction

Proteome analysis requires the handling of highly complex samples consisting of both a large number of analytes as well as large variances in the amount of the analytes. A typical eukaryotic cell contains approximately 70 pg of total protein, representing roughly 4000 unique proteins. In this mixture, the 250 most abundant protein species (mainly cellular housekeeping proteins) account for roughly 90% of the protein mass. These proteins are most easily visualized by standard methods and, without prefractionation, result in redundancy of collected MS-spectra during protein identification as often more than 30 MS spectra are collected per housekeeping protein.

Currently, two-dimensional electrophoresis (2DE) is the highest resolution technique for protein separation (1), capable of routinely separating 1000 proteins on a single gel. Despite representing approximately 50% of cellular proteins, low-abundance proteins are less frequently identified by 2DE of total proteomes (2) due to sample loading considerations. Furthermore, despite the high resolving power of 2DE, co-migration of proteins is a common problem (2), making differential protein analysis of complete proteomes by image analysis software unreliable, if at all possible. Hence, identification of less-abundant proteins and accurate protein expression profiling would profit significantly from robust and easy-to-use prefractionation schemes to reduce the protein complexity prior to analysis. This article describes the use of the recently developed ProteoExtract® Mammalian Partial Proteome Extraction Kit (P-PEK). The kit is designed for a serial sample preparation of complex protein mixtures using reagents with increasing solubilization power. Up to four sub-proteomes can be obtained with the provided reagents. Due to the reduction of protein complexity within each of the partial proteomes combined with better solubilization of proteins, the use of the ProteoExtract kit enables the production of 2D displays in which the full proteome is better characterized. Standardization of both protocol and reagents results in optimal resolution and reproducibility of 2DGE-protein patterns.

Methods and Results

Sample preparation, analytical 2DGE, and image analysis

Human HepG2 and A-431 skin carcinoma tissue culture cells were grown in the appropriate media until 1–2 × 10^8 cells at 80% confluency were reached. To extract complete proteomes in one fraction, cell pellets were treated with the ProteoExtract Complete Mammalian Proteome Extraction Kit (C-PEK) (Calbiochem Cat. No. 539779), according to the manufacturer's instructions. For selective extraction of proteins based on differential solubility, cell pellets were prepared and proteins sequentially extracted with the ProteoExtract Partial Mammalian Proteome Extraction Kit (Calbiochem Cat. No. 539789) according to the manufacturer's instructions.

For analytical gels, 200 µg of protein was used for each 17-cm pH 4–7 immobilized pH gradient (IPG) strip (BioRad). SERVA ampholytes were added to 0.5% and the appropriate extraction reagent for dilution and rehydration (two for fraction 2 and three for fraction 3). In the case of partial proteome fraction 4, which contains SDS, proteins were precipitated using TCA/acetone (3) and resuspended in extraction buffer 3. IEF was performed in parallel for each extract in one tray for 50,000 Vh according to standard focusing conditions (4). Following SDS-PAGE using 13% gels, proteins were visualized by silver staining as described by Heukeshoven and Dernick (5). With the ProteoExtract Complete Mammalian Proteome Extraction Kit, the proteins of a given sample are in one extract (Figure 1, panel A). Sequential extraction with the ProteoExtract Partial Mammalian Proteome Extraction Kit expands the proteome into four partial proteome fractions, each successive fraction containing proteins of increasing hydrophobicity (Figure 1, panel B). Computer-assisted image analysis using PDQuest™ software v6.2.1 (Bio-Rad) indicated 891 protein spots in the complete extract and a total of 2545 spots in the four gels of the sequential extraction. By deducing the number of protein spots detected in more than one fraction, 2004 spots are found to be unique to their, respective fraction (see also Table 1). This means that after

*Adapted from Calbiochem ProteoExtract Partial Mammalian Proteome Extraction Kit Application Note PE 0302.

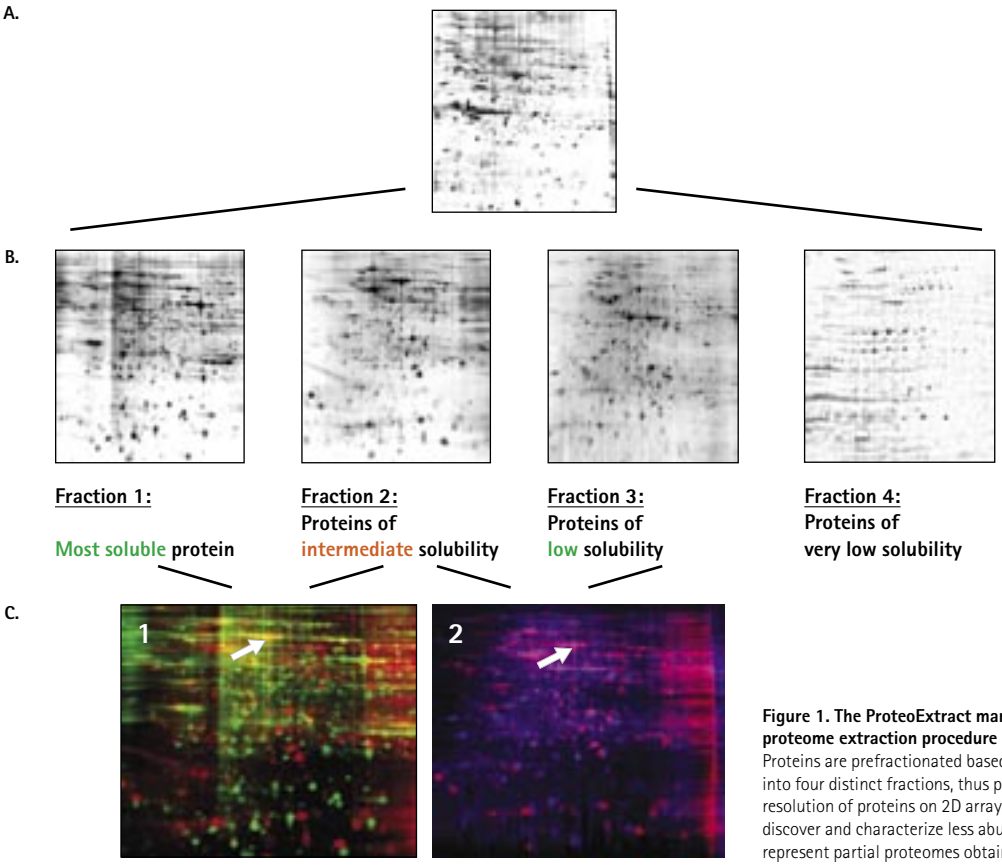

Figure 1. The ProteoExtract mammalian partial proteome extraction procedure
Proteins are prefractionated based on differential solubility into four distinct fractions, thus providing increased resolution of proteins on 2D arrays and a better chance to discover and characterize less abundant proteins. Images represent partial proteomes obtained from HepG2 cells.

sequential extraction of human tissue culture cells with the ProteoExtract® Partial Mammalian Proteome Extraction Kit 1113 *additional* protein spots could be detected as compared to the complete extract. Since gels with partial proteomes of fractions 2–4 contain less complex 2D patterns, the corresponding image analysis is easier.

To readily visualize the differences in 2D patterns of the obtained partial proteomes, the gel images of fractions 1–3 were matched using different colormodes. Image 1 in Figure 1, panel C shows the comparison of protein pattern of fraction 1 containing the most soluble proteins from HepG2 cells (green) with fraction 2 containing proteins of intermediate solubility (red). The comparison of the protein pattern of fraction 2 containing proteins of intermediate solubility from HepG2 cells (red) with fraction 3 containing less soluble proteins (green) is depicted in image 2 in Figure 1, panel C. Note that the majority of protein spots appear to be unique for the respective fraction (also documented in Table 1). Despite successful prefractionation, some of the higher-abundance proteins appear in more than one fraction (yellow), thereby facilitating the matching during the image analysis process. For example, the white arrow points to a highly abundant protein spot detected in all three fractions, which was used as a landmark for matching the gel images. Protein quantitation reveals that approximately 60% of the total amount of extracted protein is obtained in fraction 1. This fraction contains the most soluble proteins, many of which are highly abundant. Detection of additional protein spots in the subsequent fractions 2 and 3 (Table 1) significantly increases the chance to discover and characterize less-abundant proteins. The supplementary SDS-soluble proteins in fraction 4, which can optionally be analyzed by 1DE rather than 2DE, would even enhance this but have not been considered in this analysis.

Table 1. Sequential extraction of human tissue culture cells enables detection of additional protein spots

ProteoExtract kit used	Protein spots	Unique protein spots
Complete	891	**891**
Partial fraction 1 Partial fraction 2 Partial fraction 3	1024 860 661	91 684 407
Combined partial fraction 1–3	2545	**2004**

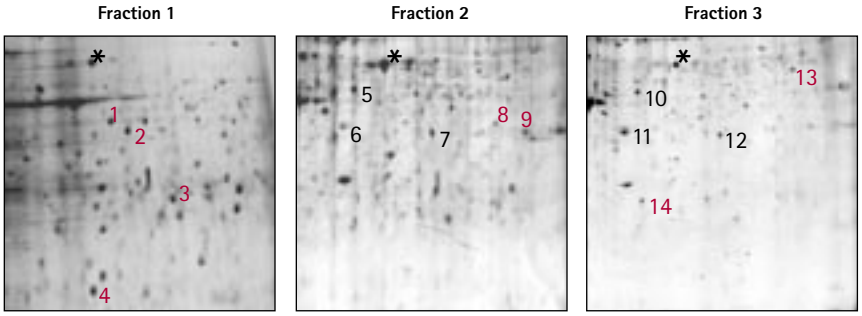

Figure 2. Two-dimentional electrophoresis protein arrays of fractions from A-431 cells following the ProteoExtract® mammalian partial proteome extraction procedure
Spots used as landmark (*) and for identification by nLC-MS/MS peptide mapping (numbers 1–14) are indicated and correspond to the proteins listed in Table 2.

Sample preparation, preparative 2DE, and identification by MS

To validate the findings of the computer-assisted image analysis by direct protein identification using mass spectrometry, preparative 2DE was performed. For preparative loading on IPG-strips, aliquots of fractions 1–3 from A-431 cells were precipitated using TCA/acetone (3) and pellets resuspended directly in a suitable volume of extraction reagent. Extraction Reagent 2 was used for fractions 1 and 2 and Extraction Reagent 3 for fraction 3. The IEF was performed with 1 mg protein per 17-cm pH 4–7 immobilized pH gradient strips for 50,000 Vh according to standard focusing conditions. Following SDS-PAGE using 13% gels, proteins were visualized by colloidal Coomassie™ blue staining (6). The different partial proteomes of fractions 1–3 are shown in Figure 2. The predominant protein spot present in all three fractions (indicated by an asterisk) was used as a landmark during gel matching. This spot represents ER 60 protease as identified by MS (data not shown). Representative spots chosen for each fraction, were excised from the gel and the proteins were fragmented using trypsin according to standard protocols (7). Proteins were identified by peptide mapping using a capillary HPLC with 100 µm Chromolith® CapRod® monolithic silica capillary (at 3 µl/min flow rate), interfaced with a standard ESI ion source of an IonTrap Mass Spectrometer and the Mascot® search engine (8).

Protein identification by MS and MS/MS of the four spots picked from the gel array of fraction 1 (spots 1–4 in Figure 2) revealed readily soluble proteins of intermediate to high abundance (Table 2). Since the image analysis indicated that the divergence seemed to be smallest between the partial proteomes of fractions 2 and 3, three pairs of protein spots were chosen that appeared to be common between the two fractions. Spots 5 and 10, 6 and 11, and 7 and 12 (Figure 2) were either more prominent, less prominent, or equally prominent in fractions 2 and 3, respectively. MS and MS/MS analysis confirmed that the spots represent identical proteins of high abundance such as actin and ribosomal proteins (Table 2). Despite the fact that high-abundance proteins can be present in more than one fraction, protein identification of other spots, such as spots 8, 9, 13, and 14, revealed that in later fractions of the sequential extraction, membrane-associated and less-soluble proteins predominate. For example, annexin I could be identified in fraction 2 but not in fraction 3 and vice versa for NADPH reductase.

The reagents and the protocol of the extraction kit have been optimized to solubilize proteins in distinct partial proteomes, however the degree of redundancy between the different protein arrays obtained will depend on the type of cells or tissue and their condition. Therefore, in some cases it might be advantageous to perform the extraction in only three, rather than four, sequential steps, using Extraction Reagent 1, followed by either Extraction Reagent 2 or 3, and finally Extraction Reagent 4.

Conclusions

The sequential proteome extraction procedure results in distinct and reproducible partial proteomes with reduced complexity, thereby facilitating an easier computer-assisted image analysis. The discovery of low-abundance, low-solubility proteins is considerably enhanced by 2DE gel arrays that lack the most soluble and highly abundant proteins.

References

1. (1999) *2D Proteome Analysis Protocols* (Link, A. J., ed.), Humana Press, Totowa, New Jersey.
2. Gygi, S. P., Corthals, G. L., Zhang, Y., Rochon, Y., and Aebersold, R. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 9390–9395.
3. Yuan, X., Russell, T., Wood, G., and Desiderio, D. M. (2002) *Electrophoresis* **23**, 1185–1196.
4. Gorg, A. and Weiss, W. (1999) *Methods Mol. Biol.* **112**, 189–195.
5. Heukeshoven, J. and Dernick, R. (1988) *Electrophoresis* **9**, 28–32.
6. Patton, W. F. (2002) *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **771(1–2)**, 3–31.
7. Shevchenko, A., Chernushevic, I., Shevchenko, A., Wilm, M., and Mann, M. (2002) *Mol. Biotechnol.* **20(1)**, 107–118.
8. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) *Electrophoresis* **20(18)**, 3551–3567.

Table 2. MS and MS/MS analysis

Fraction	Spot	Assignment	Mascot Mowse score	Sequence coverage (in %)	
				MS	MS/MS
1	1	alpha enolase	410	66, 8	27, 6
	2	pyruvate kinase	476	43, 3	20, 5
	3	triosephosphate isomerase	578	86, 7	45,2
	4	statmin	173	59, 7	26, 8
2	5	ubiquinol-cytochrome c reductase core protein I	307	34, 8	17, 3
	6	actin	228	49, 7	19, 0
	7	ribosomal protein p0, acidic	424	56, 5	28, 7
	8	mitochondrial ribosomal protein S22	180	48, 1	13, 3
	9	annexin I	258	47, 8	20, 4
3	10	ubiquinol-cytochrome c reductase core protein I	268	34, 8	11, 7
	11	actin	209	46, 2	19, 6
	12	ribosomal protein p0, acidic	280	44, 4	19, 2
	13	RuvB like 1	234	31, 1	14, 7
	14	NADPH ubiquinone reductase	211	38, 4	19, 4

See the following pages for ProteoExtract® Kit product details and ordering information:

Product		Cat. No.	Page
Complete	Bacterial Yeast Mammalian	539770 539775 539779	14 22 22
Partial	Bacterial Yeast Mammalian	539780 539785 539789	15 23 23
Subcellular	Mammalian	539790	24

Convenient and versatile subcellular extraction procedure that facilitates classical protein expression profiling and functional protein analysis*

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Standardized sample preparation to reduce proteome complexity facilitates subsequent proteome analysis. Here we describe a robust sequential extraction method that enables simple fractionation of proteins in their native state according to their subcellular localization, yielding four subproteomes enriched in (a) cytosolic, (b) membrane and membrane organelle-localized, (c) soluble and DNA-associated nuclear, and (d) cytoskeletal proteins. Efficiency and selectivity is demonstrated by morphological, two-dimensional electrophoresis image, immunological, as well as enzymatic analysis. In pilot studies, subcellular redistribution of regulatory proteins was successfully measured.

Introduction

One of the major challenges in functional proteomics is the separation of complex protein mixtures to allow detection of low-abundance proteins and provide for reliable quantitative and qualitative analysis of proteins impacted by environmental parameters (1, 2). Prerequisites for the success of such analyses are standardized and reproducible operating procedures for sample preparation prior to protein separation by one- and two-dimensional gel electrophoresis and/or liquid chromatography (3, 4). Due to the complexity of total proteomes, especially of eukaryotic proteomes, and the divergence of protein properties, it is often beneficial to prepare standardized partial proteomes of a given organism to maximize the coverage of the proteome and to increase the chance to visualize low-abundance proteins and make them accessible for subsequent analysis (4).

Besides expression profiling, activity assays (5) or protein interaction studies (e.g., in a microarray format) (6), are extremely informative for elucidating protein function. Such analyses often require that the proteins of interest remain in a nondenatured state. Another valuable and complementary data-set for clarifying protein function is the subcellular localization of proteins (7) and the detection of redistribution events within a cell. Changes in the topology of proteins, i.e., spatial rearrangements to another subcellular compartment, are important cellular events and crucial for biological processes such as signal transduction or apoptosis.

Therefore, it is evident that there is an increasing need for standardized but versatile sample preparation methods, which yield the total proteome fractionated into nondenatured subproteomes of decreased complexity and that simultaneously provide topological information. Selective purification of cellular organelles, based on popular cell biological techniques, has been utilized previously for proteome analysis (8–10) and has meanwhile led to numerous efforts concerning organelle proteomics 11–14). Although this approach can be extremely powerful, examples of which have been described recently (15, 16), isolation of organelles with high purity is mainly realized by timely and costly techniques such as differential centrifugation and/or affinity enrichment, thereby mostly neglecting the remaining cellular components and the changes therein. Furthermore, the homogenization techniques employed require usually relatively large amounts of starting material and are generally more efficient with tissues than tissue culture cells. Hence, we have systematically investigated scalable biochemical extraction methods, applicable to both adherent as well

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Abbreviations: A-431, epidermoid carcinoma cell line; **DAPI**, 4′,6-diamidino-2-phenylindole; **GFP**, green fluorescent protein; **MAPK**, mitogen activated protein kinase; **NFκB**, nuclear factor kappa B; **Saos-2**, osteosarcoma cell line; **TNFα**, tumor necrosis factor alpha

*Adapted from (2004) *Proteomics* **4**, 1397–1405 with permission.

as suspension-grown mammalian tissue culture cells, that enable simple fractionation of the total proteome into distinct subcellular fractions. Since both membrane proteins and nuclear proteins are of particular pharmacological interest, a suitable extraction method is desirable that selectively harvests these proteins (which are frequently present in minuscule quantities) into distinct subcellular fractions, separated from high-abundance proteins present in cytosol or cytoskeleton.

Previously, an elegant sample preparation procedure based on differential detergent extraction has been described (17), however, we have found that the selectivity of extraction, especially for nuclear proteins, was insufficient for our purposes [own unpublished observation and (18)]. As a result, we have studied alternative approaches resulting in an optimized sequential extraction procedure that ensures sufficient selectivity in a convenient and rapid format. The validated procedure has recently become commercially available (Calbiochem ProteoExtract® Subcellular Proteome Extraction Kit). We assessed the selectivity of the developed procedure by several independent but complementary methods: morphological analysis, one- and two-dimensional electrophoresis (1DE, 2DE) protein analysis, immunoblot, ELISA, and enzyme activity profiling. To demonstrate the versatility of the extraction method, spatial changes of two important signaling molecules were monitored: mitogen activated protein kinase (MAPK) migrates upon phosphorylation from the cytosol to the nucleus and nuclear factor kappa B (NFκB) relocates from the cytosol to the nucleus upon stimulation with tumor necrosis factor alpha (TNFα).

Materials and methods

Materials

Laboratory chemicals were obtained in extra pure grade from Merck (Darmstadt, Germany), as well as Coomassie™ Brillant Blue R250, Benzonase® nuclease, nonfat dry milk, and BSA fraction V. ProteoExtract Subcellular Proteome Extraction Kit, Protease Inhibitor Cocktail Set III (Calbiochem Cat. No. 539134), Calpain-1 ELISA Kit (Calbiochem Cat. No. QIA118), and Calpain Activity Assay Kit, Fluorogenic (Calbiochem Cat. No. QIA120) are from EMD Biosciences. Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum was from Gibco (Grand Island, NY, USA). ER-Tracker™; MitoTracker®; 4′,6-diamidino-2-phenylindole (DAP); and phallicidin were from Molecular Probes (Eugene, OR, USA). DC protein assay, IPG strips 4–7, carrier ampholyte 3–10, Criterion precast gels, MultiProtean IEF cell, PDQuest™ software, PVDF membrane, and SYPRO® Ruby Protein Stain were from Bio-Rad (Munich, Germany). Primary antibodies that were used in this study were obtained against the following antigens listed from the supplier indicated in parentheses: calpain, actin, NFκB (Calbiochem), heat shock protein 70 (HSP 70), cadherins, cytochrome P450 reductase, c-Fos, cytokeratins, vimentin (Sigma, Steinheim, Germany), c-Jun (BD Transduction Laboratories, Lexington, KY, USA), histone-1 (Santa Cruz Biotechnology, Heidelberg, Germany), p44/ 42 MAP kinase, and Phospho-p44/42 MAP Kinase (Cell Signaling Technology, Beverly, MA, USA). Conjugated horseradish peroxidase secondary antibody, ECL Western blotting detection reagent and Hyperfilm® were from Amersham Biosciences (Freiburg, Germany). GeneTools® software was from Syngene (Cambridge, UK).

Cell culture and stimulation

An osteosarcoma cell line, Saos-2 (ATCC HTB-85), and an epidermoid carcinoma cell line, A-431 (ATCC CRL-1555) were grown to form subconfluent (80%) layers at 37°C in DMEM supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2. To study NFκB translocation, A-431 cells were stimulated with 0.2 mg/ml TNFα for 0, 5, and 15 minutes, essentially as described previously (32). Following stimulation, cells were put on ice and extracted to generate the four subcellular proteomes.

Subcellular fractionation and protein extraction

The extraction procedure was performed as described in the protocol included with the ProteoExtract® Subcellular Proteome Extraction Kit. Additives such as protease inhibitor cocktail and an unspecific nuclease, Benzonase® nuclease were included. Adherent-growing cells (such as A-431 cells or Saos-2) allowed the procedure to be performed directly in cell culture flask or coverslips without detachment of the monolayer. In the case of suspension culture cells, tissue pieces, frozen cell pellet, or when adherent cells were detached during extraction, the resulting suspension was transferred into a microcentrifuge tube and pelleted by centrifugation. The supernatants (e.g., cytosolic fraction) were stored at –70°C for later use. A flow chart describing the subcellular fractionation method is depicted in Figure 1.

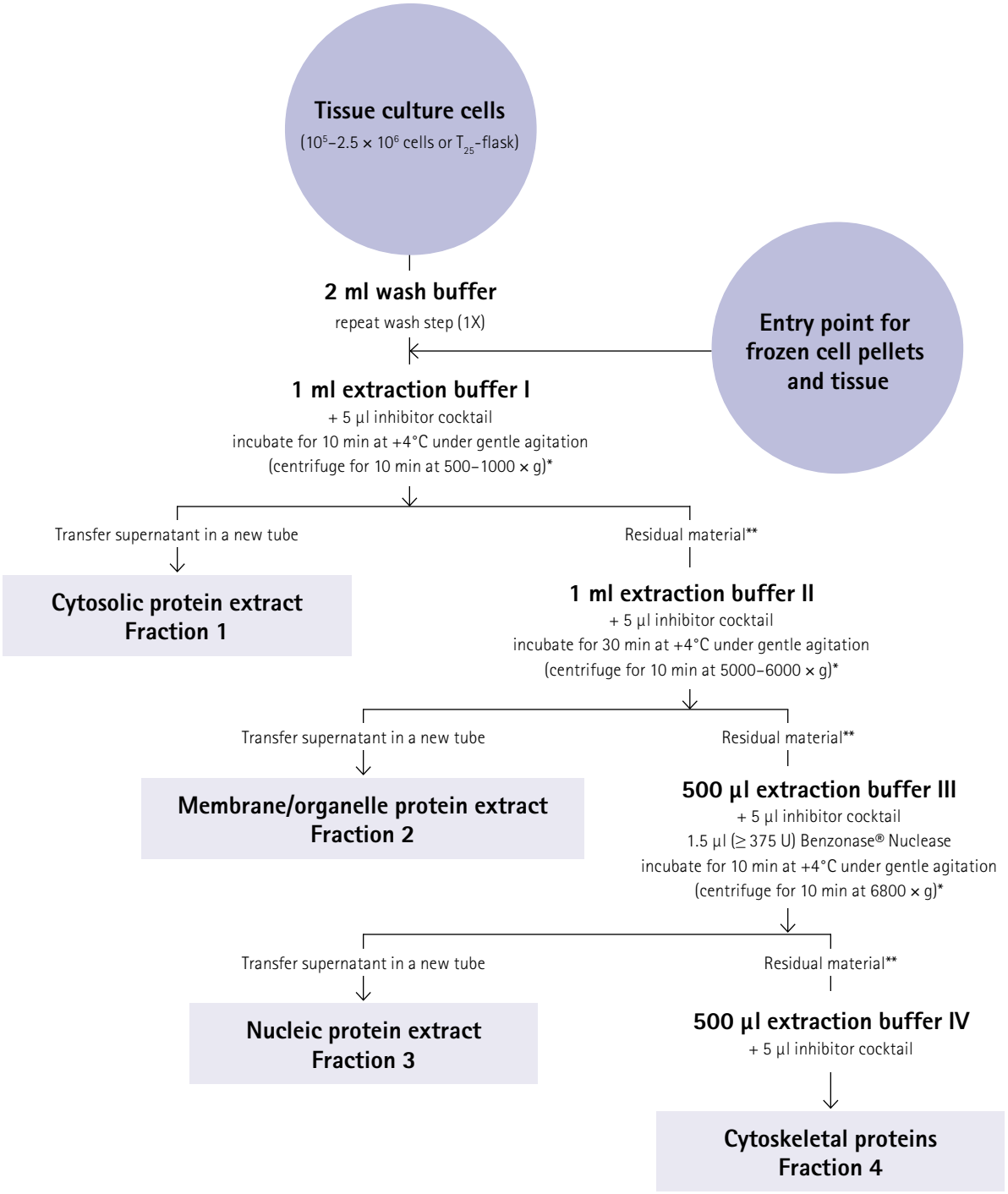

Figure 1. Schematic representation of the subcellular extraction procedure, depicting the individual steps of the sequential biochemical fractionation
The scheme provides the buffer volumes used for approximately 1 × 10⁶ cells. The asterisk (*) indicates that for adherent cells, centrifugation is not required as long as cells or cell remnants remain attached to the growth support.

Fluorescence microscopy

Prior to extraction, live cells were prestained with ER-Tracker™, MitoTracker®, DAPI, or phallicidin as fluorescent markers for endoplasmic reticulum (ER), mitochondria, nuclei, and filamentous actin cytoskeleton, respectively. For use as an indicator for cytosolic extraction, cells were transfected with a plasmid encoding green fluorescent protein (GFP). The treated cells were washed and extracted, and subcellular structures of cells or cell remnants that remained attached during extraction were documented by fluorescence microscopy prior to and after each extraction step. Exposure times for the digital imaging were kept identical throughout the extraction procedure for the individual fluorescent labels and GFP, so that subcellular disintegration, i.e., extraction, leads to a decreased signal strength. In cases where loss of signal was observed following the extraction, phase contrast images were recorded of the identical field to prove that cells or cell remnants were still present.

Gel electrophoresis and gel analysis

In the case of conventional 1DE, volume equivalents of the four subcellular fractions obtained were separated by SDS-PAGE (10% gel) (19) and stained by Coomassie™ Blue R-250 (20). For 2DE, 200 mg of each subcellular extract was precipitated using TCA/acetone, as previously described (21), and resuspended in 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, and 0.2% carrier ampholytes pH 3–10. IEF was performed in parallel on 11-cm IPG strips with linear pH gradient from 4–7 in a MultiProtean IEF cell for 38,000 Vh according to standard focusing conditions (22). Following second dimension run with Criterion precast gels (10% resolving, 4% stacking gel with a gel size of 13.3 × 8.7 cm) as previously described (23), gels were fixed, stained with SYPRO® Ruby, and scanned (24). Comparative analysis of the four subcellular proteomes by 2DE was performed with PDQuest™ software (v6.2.1) following matching using 29 landmarks.

Immunological investigations

For Western blot analysis, proteins were transferred to PVDF membranes after SDS-PAGE. Blots were blocked overnight with 5% nonfat dry milk in PBS and membranes were probed, washed, and developed according to the manufacturer's instructions. Films were scanned by densitometry analysis using GeneTools® Software, essentially as described elsewhere (25). To be able to detect c-Fos reliably, the protein was immunoprecipitated prior to SDS-PAGE. In addition, calpain was quantified using a commercial ELISA kit (Calbiochem Cat. No. QIA118) according to the manufacturer's instruction.

Protein determination and enzyme activity analysis

Protein concentrations of extracts were determined by a modified Lowry method (DC protein assay) using BSA as a standard according to the manufacturer's instruction. Calpain activity was measured with a commercial kit according to the manufacture's instruction. Alkaline phosphatase activity was determined as previously described (26). RNAse activity was measured in a proprietary versatile RNAse assay utilized in-house for quality control of molecular biology reagents. Briefly, following incubation with a subcellular sample under investigation, remaining fluorescence is measured after affinity depletion of a biotin-tagged fluorescent probe that carries consensus sequences for numerous RNAses between both the labels. All enzyme activities were measured as the change in signal strength per minute to make sure that measurements were taken within the linear range of the assay used.

Results and discussion

Morphological analysis of the subcellular extraction procedure

For the sequential extraction of cell content, the developed procedure takes advantage of the differential solubility of certain subcellular compartments in special reagent mixtures. The four extraction buffers utilized have been optimized to provide reproducible protein isolation from distinct subcellular compartments. Defined surfactants maintained in select buffers of appropriate ionic and osmotic composition enable selective extraction. A flow chart describing the method to prepare the subcellular extracts is depicted in Figure 1.

The success of such a procedure depends on the preservation of the integrity of the subcellular structures before and during the extraction, which was investigated using adherent-growing A-431 cells. This allows the procedure to be performed directly on chambered coverslips without the need for cell removal. Hence, early destruction of the cellular structure by enzymatic or mechanical detachment of cells from the growth support is prevented. In the case of strongly attached adherent cells, the cells or the parts of the cells that remain upon sequential extraction stay attached to the surface during the extraction procedure, thereby facilitating image analysis. Cells treated with fluorescent dyes that selectively label chosen subcellular compartments were washed and extracted, and subcellular structures of cells or cell remnants that remained attached during extraction were visualized by fluorescence and phase contrast microscopy prior to and after each extraction step (Figure 2). In cases where loss of signal was observed

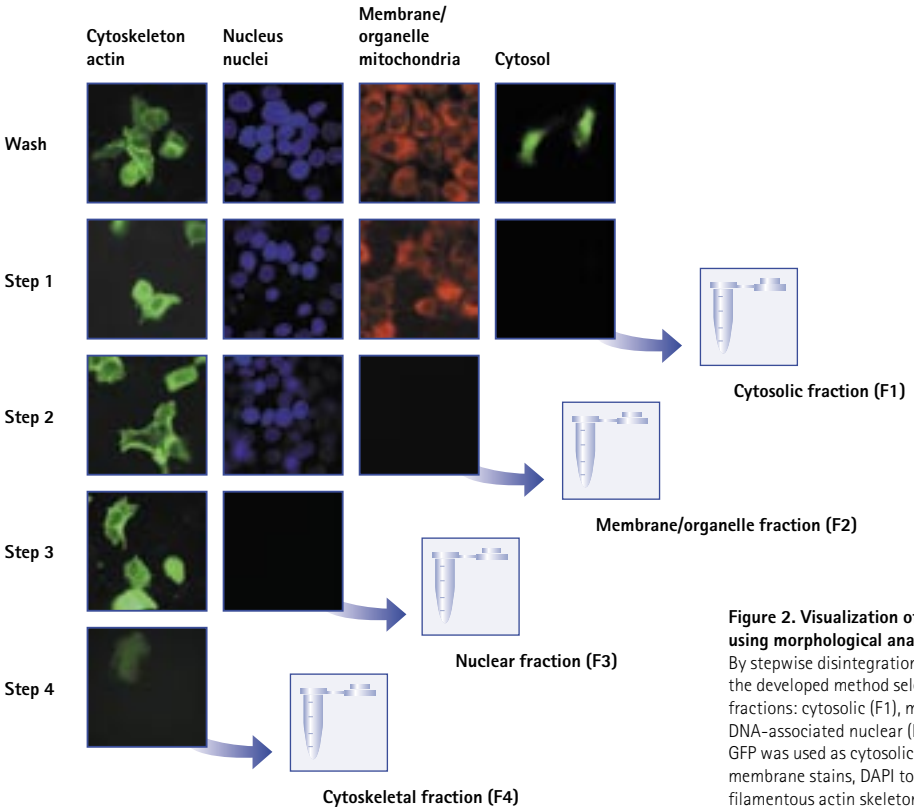

Figure 2. Visualization of subcellular extraction procedure selectivity using morphological analysis of adherently growing A-431 cells
By stepwise disintegration of the cells' structure upon sequential extraction, the developed method selectivity extracts cellular proteins into four fractions: cytosolic (F1), membrane and organelle-localized (F2), soluble and DNA-associated nuclear (F3), and cytoskeletal (F4) proteins. Recombinant GFP was used as cytosolic marker, MitoTracker® and ER-Tracker™ as membrane stains, DAPI to visualize nuclei, and phallicidin to visualize filamentous actin skeleton.

following the extraction, phase contrast images were recorded of the identical field to prove that cells or cell remnants were still present (Figure 2).

These results show that the sequential extraction results in a stepwise disintegration of the cell's structure, yielding four subcellular fractions. Upon treatment with the first extraction buffer, cells shrink as a result of the release of the cytosolic content but remain intact in their overall structure (Figure 2, step 1). After the second extraction step, due to the solubilization of membranes and membrane organelles, only isolated nuclei and the cytoskeleton remain intact (Figure 2, step 2). The treatment of the residual material with the third extraction buffer destroys the structure of the nucleus (Figure 2, step 3), thus probably solubilizing the nuclear proteins. Finally, the cytoskeleton components are liberated during the fourth and final extraction (Figure 2, step 4).

Protein pattern analysis of the subcellular extracts

The selectivity of the procedure was further investigated by 1DE and 2DE protein profiling. A typical result of the 1DE and 2DE protein profiling with subcellular extracts generated from Saos-2 cells is depicted in Figure 3, panel A, and Figure 4, respectively. Comparative analysis of the four subcellular proteomes by 2DE, which is depicted in

Table 1. Computer-assisted image analysis indicated a total of 1107 unique spots in matched gels of the sequential extracts. By deducing the number of protein spots detected in more than one subcellular fraction, 499 spots (i.e., 45% of the total of 1107 spots detected) are solely found in a single fraction. See the "Protein pattern analysis of the subcellular extracts" section and Figure 4, page 71, for further details.

Subcellular fraction	Total spots	Unique spots
Cytosolic	572	200
Membrane/organelle	484	111
Nuclear	393	99
Cytoskeleton	272	89
Combined master gel	1107	499

complementary colors for the cytosolic (red), membrane/organelle (green), nuclear (dark blue), and cytoskeletal (gray green) fractions in Figure 4, revealed that more than 35% of the total protein spots visualized (45% in the given example) are detected in only a single subcellular fraction (see also Table 1). Examples of such protein spots are SSP 6103, 7206, 6611, and 7010 in Figure 4. Spots present in all four fractions appear light blue in color. More detailed quantitative analysis revealed that for more than half (62% in the given example) of the visualized proteins, at least 80% is detected in a single subcellular fraction. The protein overlap between neighboring fractions is ≤ 25%, resulting in protein patterns of membrane/organelle and nuclear fractions that are clearly

distinct from the protein patterns of the cytosolic and the cytoskeletal fractions. Thus the subcellular prefractionation can increase the chance to visualize low-abundance proteins in membranes and organelles and nuclei since these are efficiently separated from high-abundance proteins commonly found in the cytosol and cytoskeleton that might otherwise hamper analysis. Intriguingly, although some proteins are extracted in multiple subsequent fractions (e.g., SSP 2101, Figure 4), the majority (on average 70% in example) of the proteins detected in more than one subcellular fraction do not overlap with adjacent fraction(s); see for example SSP 1503, 2603, and 1101 in Figure 4. As many of these are also located near landmarks that were set, it is unlikely that this results from misalignment during matching. Therefore, this probably reflects a functional relevance rather than resulting from inefficient extraction or mismatching.

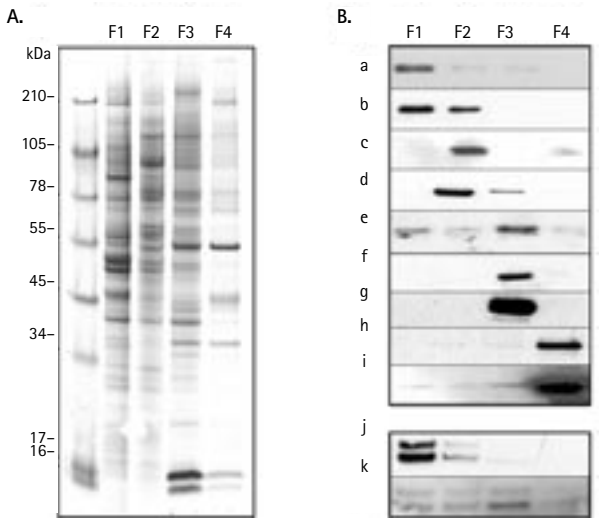

Figure 3.
Selectivity of the subcellular extraction procedure is demonstrated by 1DE protein profiling and by monitoring marker protein distribution of obtained subcellular fractions
Visualization of total protein by SDS-PAGE and Coomassie™ staining revealed clearly distinct protein profiles of the individual fractions obtained (panel A). Fractionated proteins were also subjected to immunoblot analysis, revealing a distinct topological distribution of the analyzed marker proteins to individual subcellular fractions. Marker proteins of the cytosol (a, calpain; b, HSP 70) were mainly detected in fraction 1, membrane and organelle markers (c, cadherins; d, cytochrome P450 reductase) were predominantly in fraction 2, transcription factors as well as DNA-associated proteins (e, c-Fos; f, c-Jun; g, histone 1) were in fraction 3, and cytokeratins and vimentin representing cytoskeletal markers (h and i, respectively) were found in fraction 4. To study changes in protein topology upon post-translational modification, mapping of total p44/42 MAPK (j) and phosphorylated p42/42 MAPK (k) revealed that the phosphorylated protein form is mainly present in the nucleus, i.e., fraction 3, even though p44/42 MAPK as such is found by and large in the cytosol, i.e., fraction 1.

Immunological analysis of the subcellular extracts

The efficiency and selectivity of the subcellular extraction procedure was further investigated by immunological analysis using both Western blot and ELISA. Following SDS-PAGE, proteins were electroblotted to membranes and probed with antibodies against calpain and HSP 70 as cytosolic markers (a and b in Figure 3, panel B), cadherins and cytochrome P450 reductase as membrane markers (c and d), c-Fos, c-Jun, and histone-1 as soluble and DNA-associated nuclear markers (e, f, and g), and cytokeratins and vimentin as cytoskeletal markers (h and i). Immunoblotting revealed that the majority of marker proteins were primarily (0.85%) detected in distinct subcellular fractions and that the distribution of markers expected to be localized within the same subcellular context was virtually identical. Please note that HSP 70 is known to be present in both the cytoplasm as well as associated with cell membranes (26), reflected by detection in both the cytoplasmic and membrane/organelle fraction after subcellular extraction. In the case of actin, however, immunodetection revealed that the protein was not only present in the cytoskeletal fraction, but also in the cytosolic and to some extent in the membrane/organelle and nuclear fractions as well (data not shown). The cytosolic fraction most likely corresponds to soluble actin monomers, which are not detected by the phallicidin stain during morphological analysis. Independently, calpain was quantified using an ELISA, providing highly comparable results to the data collected by immunoblotting (data not shown). Taken together, the immunological data are consistent with the morphological findings and demonstrate the efficiency and selectivity of the subcellular extraction procedure at the protein level. Thus the developed procedure readily fractionates proteins according to their subcellular localization, yielding four subproteomes enriched in (a) cytosolic, (b) membrane/organelle-localized, (c) soluble and DNA-associated nuclear, and (d) cytoskeletal proteins. In addition to the analysis for the above-mentioned marker proteins, the subcellular distribution of p44/42 mitogen-activated protein kinase (MAPK) was investigated by immunoblotting as described, using commercial antibodies detecting total (phosphorylation-state independent) p44/42 MAPK (j in Figure 3, panel B), as well as antibodies that detect endogenous levels of the kinase only when it is phosphorylated (k in Figure 3, panel B). MAPKs are known to mediate signaling from the cell membrane to the nucleus upon phosphorylation at conserved threonine and tyrosine residues within their activation loops (27, 28). Quantification by densitometric analysis revealed that although 0.80% of total p44/42 MAPK was detected in the cytosolic fraction under

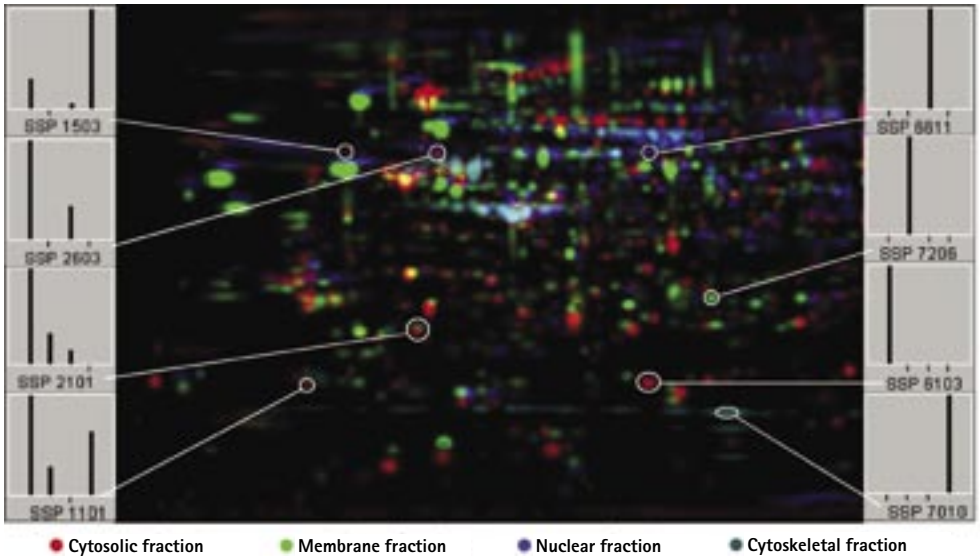

Figure 4. Separation and staining of subcellular proteomes by 2DE reveals clearly distinct protein profiles in the collected fractions. Two hundred micrograms from each sequentially extracted subcellular fraction was separated on linear 4–7 IPG strips and 10% PAGE gels and visualized by staining with SYPRO® Ruby. Gel images were matched using PDQuest™ software and artificially colored to represent individual subfractions: red for the cytosolic, green for the membrane/organellar, dark blue for the nuclear, and gray green for the cytoskeletal subcellular fraction. See Table 1, page 69, for further details.

steady-state conditions, about 50% of the phospho-p44/42 MAPK was present in the nucleus (j and k in Figure 3, panel B, respectively). These results indicate that the subcellular extraction procedure facilitates studies that monitor changes in protein topology induced by transient post-translational modification, such as after phosphorylation.

Enzymatic analysis of the subcellular extracts

During the optimization phase of the extraction method, we found that extracts from Chinese hamster ovary (CHO)-K1 cells expressing *Renilla* luciferase maintained luciferase activity (data not shown), despite this enzyme being sensitive to denaturation. Encouraged by these findings, we set out to test chosen endogenous enzyme activities after subcellular fractionation of Saos-2 cells. As a cytosolic marker, calpain activity was measured and alkaline phosphatase activity was used as membrane marker. Finally, as a surrogate enzyme marker for both membrane-associated (29) and nuclear fractions (30), RNAse activity was measured. Subcellular fractions were thus assayed and the measured relative enzymatic activities graphically displayed (Figure 5). The activity profiling independently confirms the selective separation of cellular components according to their subcellular localization. Moreover, it also demonstrates that the assayed components are extracted in an active and thus functional state.

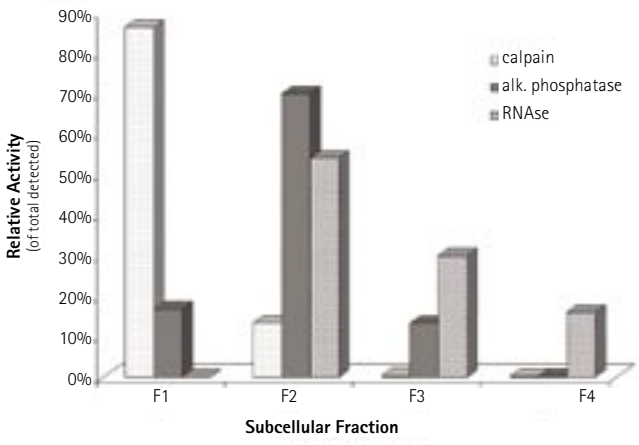

Figure 5. Documentation of enzyme activities in the obtained subcellular fractions Respective fractions were assayed for three endogenous enzymatic markers: calpain activity (localized to cytoplasm), alkaline phosphatase activity (membrane-associated), and RNAse activity (membrane- and nuclear-associated). The activity profiling independently confirms selective separation of cellular components according to their subcellular localization and demonstrates that the assayed components are extracted in an active and thus functional state.

Utilization of the subcellular extraction procedure to monitor redistribution of regulatory proteins

The developed subcellular proteome extraction procedure should be suitable to investigate changes in subcellular localization of regulatory proteins impacted by experimental or disease parameters. A strong indication for this were the encouraging results with p44/42 MAPK (Figure 3, panel B, j and k). To demonstrate this application we chose the well-described translocation of NFκB from the cytosol to the nucleus upon stimulation of cells with TNFα (31, 32). NFκB translocation, studied in A-431 cells stimulated for different times with TNFα, was detected and quantified using densitometric analysis of immunoblots. Time course analysis demonstrates a measurable translocation of NFκB from the cytoplasm to the nucleus in as little as 5 minutes with a stronger response observed at 15 minutes (Figure 6) while the control protein calpain did not translocate between fractions upon TNFα-stimulation of cells. Therefore, the subcellular extraction method allows the assessment of spatial rearrangements of various proteins.

The subcellular extraction procedure has successfully been used with a variety of adherent and nonadherent tumor cells. The procedure is scalable and has routinely been used with 4×10^5 to 2×10^7 cells. Although the relative protein amounts obtained vary slightly from cell type to cell type, the overall reproducibility is satisfying with CV < 10%. Lastly and importantly for the investigation of clinical and pharmacological relevant samples, the protocol has been applied successfully with frozen cell pellets and with dissected colon carcinoma tissue that had been fragmented into cell clusters.

Concluding remarks

Although the purity of the four subcellular fractions resulting from sequential extraction might not be as high as with dedicated purification techniques for the isolation of singular organelles, the procedure has been shown to be very selective and provides for enriched membrane/organellar or nuclear proteins in solution, separated for the most part from cytosolic and cytoskeletal proteins. The procedure is applicable to a wide variety of mammalian cellular sources, is scalable, fast, simple, and robust. The developed method has a broad application range and facilitates protein expression profiling and functional analysis of a vast array of proteins, including mode of action studies. Finally, the sequential extraction procedure can also be advantageous for protein-interaction studies (e.g., with immobilized baits), as it extracts proteins in a functional state and it decreases the risk of false positives when probing with the relevant subcellular fraction.

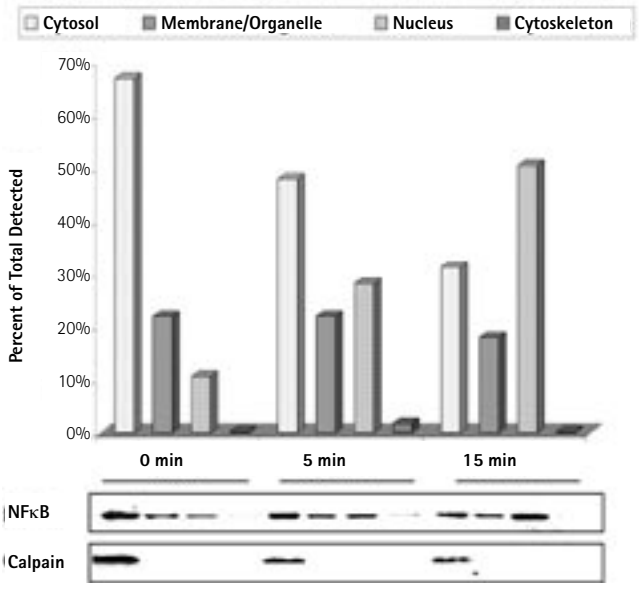

Figure 6. Feasibility demonstration of exploiting the subcellular extraction procedure to detect and measure redistribution of proteins affected by disease or experimental factors

NFκB redistribution was recorded in a time course analysis in A-431 cells that were stimulated with TNFα by quantification of immunoblots generated with the subcellular fractions and anti-NFκB antibodies. The time course analysis demonstrates a measurable translocation of NFκB from the cytoplasm to the nucleus within 5 min with a stronger response observed at 15 min upon cell stimulation, whereas the control protein calpain does not change its topology.

Parts of this work were supported by the German Federal Ministry of Education ("Proteomanalyse des Menschen"). We want to thank M. Wehsling for excellent technical assistance and C. Burger and W. Lautz for kindly providing cells.

References

1. Banks, R. E., Dunn, M. J., Hochstrasser, D. F., Sanchez, J. C., Blackstock, W., Pappin, D. J., and Selby, P. J. (2000) *Lancet* **356**, 1749–1756.
2. Westbrook, J. A., Yan, J. X., Wait, R., Welson, S. Y., and Dunn, M. J. (2001) *Electrophoresis* **22**, 2865–2871.
3. Rabilloud, T. (2000) *Proteome Research: Two-dimensional Gel Electrophoresis and Identification Methods* (Rabilloud, T. and Chevallet, M., eds) pp. 9–29, Springer-Verlag, Heidelberg.
4. Krapfenbauer, K., Berger, M., Friedlein, A., and Lubec, G. (2001) *Eur. J. Biochem.* **268**, 3532–3537.
5. Adam, G. C., Sorensen, E. J., and Cravatt, B. F. (2002) *Mol. Cell. Proteomics* **1**, 828–835.
6. Zhu, H. and Snyder, M. (2001) *Curr. Opin. Chem. Biol.* **5**, 40–45.
7. Huber, L. A., Pfaller, K., and Vietor, I. (2003) *Circ. Res.* **92**, 962–968.
8. Jung, E., Heller, M., Sanchez, J.-C., and Hochstrasser, D. F. (2000) *Electrophoresis* **21**, 3369–3377.
9. Pasquali, C., Fialka, I., and Huber, L. A. (1999) *J. Chromatogr. B Biomed. Sci. Appl.* **722**, 89–102.
10. Fountoulakis, M., Juranville, J. F., Berndt, P., Langen, H., and Suter L. (2001) *Electrophoresis* **22**, 1747–1763.
11. Scianimanico, S., Pasquali, C., Lavoie, J., Huber, L. A., Gorvel, J.P., and Desjardins, M. (1997) *Electrophoresis* **18**, 2566–2572.
12. Bell, A. W., Ward, M. A., Blackstock, W. P., Freeman, H. N., Choudhary, J. S., Lewis, A. P., Chotai, D., Fazel, A., Gushue, J. N., Paiement, J., Palcy, S., Chevet, E., Lafreniere-Roula, M., Solari, R., Thomas, D. Y., Rowley, A., and Bergeron, J. J. (2001) *J. Biol. Chem.* **276**, 5152–5165.
13. Taylor, R. S., Wu, C. C., Hays, L. G., Eng, J. K., Yates, J. R. 3rd, and Howell, K. E. (2000) *Electrophoresis* **21**, 3441–3459.
14. Fountoulakis, M. and Schlaeger, E. J. (2003) *Electrophoresis* **24**, 260–275.
15. Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P. H., Steele-Mortimer, O., Paiement, J., Bergeron, J. J., and Desjardins, M. (2002) *Cell* **110**, 119–131.
16. Desjardins, M. (2003) *Nat. Rev. Immunol.* **3**, 280–291.
17. Ramsby, M. L., Makowski, G. S., and Khairallah, E. A. (1994) *Electrophoresis* **15**, 265–277.
18. Chiang, E. T., Lim, M. J., Patton, W. F., and Shepro, D. (2000) *J. Biochem. Biophys. Methods* **46**, 53–68.
19. Laemmli, U. K. (1970) *Nature* **227**, 680–685.
20. Wong, C., Sridhara, S., Bardwell, J. C. A., and Jakob, U. (2000) *Biotechniques* **28**, 426–432.
21. Yuan, X., Russell, T., Wood, G., and Desiderio, D. M. (2002) *Electrophoresis* **23**, 1185–1196.
22. Görg, A. and Weiss, W. (1999) *Methods Mol. Biol.* **112**, 189–195.
23. Vidarte, L., Pastor, C., Mas, S., Blazquez, A. B., and Vivanco, F. (2001) *J. Biol. Chem.* **276**, 38217–38223.
24. Seliger, B., Menig, M., Lichtenfels, R., Atkins, D., Bukur, J., Halder, T. M., Kersten, M., Harder, A., Ackermann, A., Beck, J., Muehlenweg, B., Brenner, W., Melchior, S., Kellner, R., and Lottspeich, F. (2003) *Proteomics* **3**, 979–990.
25. Tanaka, A., Miyakawa, H., Luketic, V. A., Kaplan, M., Storch, W. B., and Gershwin, M. E. (2002) *Cell. Mol. Biol.* **48**, 295–299.
26. Broquet, A. H., Thomas, G., Masliah, J., Trugnan, G., and Bachelet, M. (2003) *J. Biol. Chem.* **278**, 21601–21606.
27. Marshall, C. J. (1995) *Cell* **80**, 179–185.
28. Toledano-Katchalski, H., Kraut, J., Sines, T., Granot-Attas, S., Shohat, G., Gil-Henn, H., Yung, Y., and Elson, A. (2003) *Mol. Cancer Res.* **1**, 541–550.
29. Egesten, A., Dyer, K. D., Batten, D., Domachowske, J. B., and Rosenberg, H. F. (1997) *Biochim. Biophys. Acta* **1358**, 255–260.
30. Frank, P., Braunshofer-Reiter, C., Poltl, A., and Holzmann, K. (1998) *Biol. Chem.* **379**, 1407–1412.
31. Mejdoubi, N., Henriques, C., Bui, E., and Porquet, D. (1999) *Biochem. Biophys. Res. Commun.* **254**, 93–99.
32. Butcher, B. A., Kim, L., Johnson, P. F., and Denkers, E. Y. (2001) *J. Immunol.* **167**, 2193–2201.

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Product	Cat. No.	Page
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Preserve phosphorylation state & kinase activity in cell extracts*

Scott Hayes, Yuping Ambuel, David Bartnicki, and Michael Batenjany — EMD Biosciences/Novagen

Here we highlight the advantages of combining the PhosphoSafe™ Extraction Reagent with both the ProteoEnrich™ ATP-Binders™ Kit, which extracts phosphorylated proteins, and the Calbiochem K-LISA™ PTK Screening Kit, which measures protein tyrosine kinase (PTK) activity. We prepared extracts from rat L6 myoblasts with PhosphoSafe Extraction Reagent and CytoBuster™ Protein Extraction Reagent under identical conditions (1). The PhosphoSafe buffer is based on the same formulation as CytoBuster reagent, but includes four phosphatase inhibitors (sodium fluoride, sodium vanadate, β-glycerophosphate, and sodium pyrophosphate) to help preserve phosphorylation throughout extraction and processing. Western blot analysis assessed by an antibody to phosphorylated myosin light chain 2 (anti-phospho-MLC2; Thr18/Ser19) demonstrated specific recognition of the phosphorylated MLC2 with both reagents (Figure 1). The signal intensity increased dramatically in samples prepared with the PhosphoSafe buffer, indicating that a greater number of the phosphorylated residues remained intact.

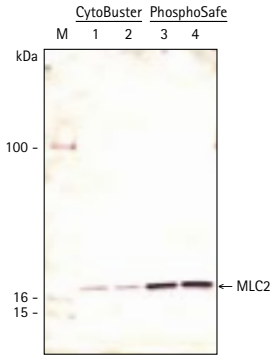

Figure 1. Detection of phosphorylated MLC2
Monolayers of subconfluent L6 myoblasts were extracted with CytoBuster reagent or PhosphoSafe buffer for 10 min at room temperature. Extracts were centrifuged and assayed for protein concentration using BCA. Duplicates of each extract (10 µg) were separated by SDS-PAGE (4–20% gradient gel). After transfer to nitrocellulose, protein phosphorylation state was probed with anti-phospho-MLC2 primary antibody. Goat Anti-Rabbit IgG AP Conjugate was added and detected by staining with the AP Detection Reagent Kit.

To test whether PhosphoSafe Extraction Reagent could maintain the phosphorylation state of target proteins through multiple processing steps, we prepared cell extracts from A-431 human cell lines using PhosphoSafe buffer and processed the extracts with the ProteoEnrich ATP-Binders Kit. The ATP-Binders protocol involves buffer exchanges, multiple wash steps, and an overnight dialysis at 4°C. We analyzed the ATP-binding protein–enriched fraction for Src, a known ATP-binding protein, by Western blotting (Figure 2). The blot revealed multiple bands, indicating that the PhosphoSafe Extraction Reagent enabled retention of multiply phosphorylated forms of Src.

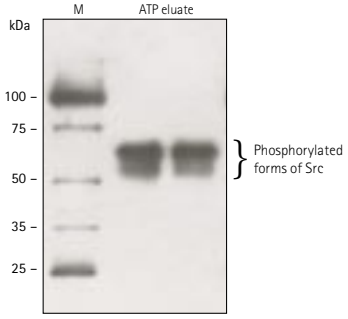

Figure 2. Detection of phosphorylated Src
Cell extract from the A-431 cell line was prepared by the standard PhosphoSafe Extraction Reagent protocol and dialyzed to remove endogenous ATP. Dialyzed extract was processed according to the standard ProteoEnrich ATP-Binders Kit protocol (1). Duplicate extract samples (5 µg) were separated by SDS-PAGE (4–20% gradient gel). After transfer to nitrocellulose, phosphorylated Src was detected with a 1:500 dilution of primary antibody for rabbit anti-human Src, followed by an Anti-Rabbit IgG, H & L Chain Specific (Goat) Peroxidase Conjugate. Detection was by chemiluminescence using SuperSignal® HRP Substrate.

*Adapted from *inNovations* (2004) **20**, 14.

To measure PTK activity of different human cell line extracts (HeLa, MCF-7, HEK 293, and A-431) prepared with PhosphoSafe Extraction Reagent, we used the K-LISA PTK Screening Kit (Calbiochem Cat. No. 539701). The kit uses an ELISA-based, colorimetric assay and plate format that includes a multiplex array of three synthetic polypeptide PTK substrates composed of glutamic acid, alanine, and tyrosine (E_4Y, EAY, and EY) to differentiate substrate specificity. Using the kit, we quantified PTK activity in less than two hours. Each of the kinase-containing extract and peptide substrate combinations produced activity levels significantly higher than the corresponding background (Figure 3).

Based on these data, we conclude that extracts prepared with PhosphoSafe™ Extraction Reagent offer significant advantages over preparations lacking phosphatase inhibitors. Specifically, the inhibitors in this formulation preserve a protein's phosphorylation state and maintain enzymatic activity. Extracts prepared with this buffer are compatible with Western blot, kinase activity, protein interaction, and other analysis methods.

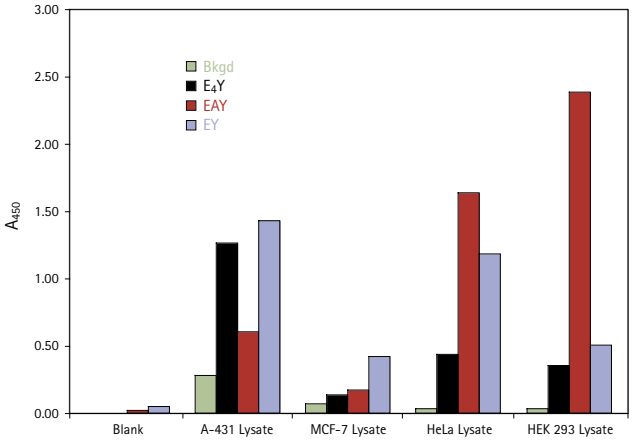

Figure 3. Detection of tyrosine kinase activity
Crude lysates from A-431, MCF-7, HeLa, and HEK 293 cells prepared with PhosphoSafe buffer were diluted in kinase reaction buffer and tested for enzymatic activity in an ELISA format using a synthetic set of tyrosine kinase substrates, E_4Y, EAY, and EY, as provided in the K-LISA™ PTK Screening Kit (Calbiochem Cat. No. 539701). Substrate phosphorylation was detected using Anti-Phosphotyrosine (PY20) (Mouse) Peroxidase Conjugate (Calbiochem Cat. No. 525320).

References

1. Trim, J. and Sawyer, D. (2001) *inNovations* **12**, 5–6.
2. Bartnicki, D., Batenjany, M., Loomis, K., Wong, S., Menezes, R., Suleman, A., and Andrecht, S. (2004) *inNovations* **19**, 6–9.

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PhosphoSafe™ Extraction Reagent	71296	21
ProteoEnrich™ ATP-Binders™ Kit	71438	32

Automated solubility screening of recombinant proteins in a 96-well format*

Anthony Grabski, Don Drott, and Mark Mehler—EMD Biosciences/Novagen

We describe here a novel HT–compatible method for protein solubility screening. The procedure incorporates a nonfouling filtration plate capable of retaining insoluble inclusion bodies while allowing soluble proteins to be collected for rapid quantification and analysis.

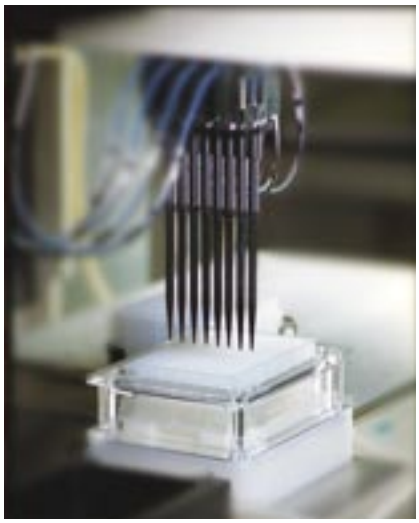


Figure 1. MultiPROBE® II HT EX workstation set up for automated solubility screening

Table 1. RoboPop solubility screening protocol

1. Culture *E. coli* cells in liquid medium under conditions for target protein production.
2. Add 0.1 culture volume premixed PopCulture® Reagent + Lysonase™ Bioprocessing Reagent to each culture, mix, and incubate 10 min at room temperature.
3. Place the 96-well Collection Plate and Filter Plate into the vacuum manifold.
4. Transfer 200 µl extract from each culture to the 96-well Filter Plate.
5. Apply vacuum*, collecting the flow-through containing soluble proteins in the Collection Plate.
6. Remove the Collection Plate containing the soluble fraction and replace it with a new Collection Plate.
7. Add 200 µl 4% SDS denaturing solution to each well of the Filter Plate and incubate 10 min at room temperature. This step solubilizes the inclusion body fraction.
8. Apply vacuum*, collecting the solubilized proteins in a Collection Plate.
9. Quantify target proteins present in soluble and insoluble fractions (step 5 and step 9, respectively). His•Tag® fusion proteins may be detected by Western blotting or ELISA using the His•Tag Monoclonal Antibody or His•Tag Antibody Plates, respectively. S•Tag™ fusion proteins may be quantified directly using the FRETWorks™ S•Tag Assay Kit (Novagen Cat. No. 70724-3). Activity of native proteins may be assayed directly from the soluble fraction. SDS-PAGE followed by staining may also be used for highly expressed proteins.

* Samples may be collected by substituting centrifugation of the Filter Plate over the Collection Plate at 2000 × g for 5 min.

Bacterial expression systems are frequently employed to produce large quantities of heterologous protein for structural and functional analysis irrespective of the source, sequence, or abundance of the protein in its natural host. Unfortunately, the ease and efficiency of bacterial expression systems for recombinant protein production do not always correlate with high yield of soluble, correctly folded, active protein. Instead, insoluble inclusion bodies are formed due to rapid, high expression of the protein, inadequate or low concentrations of chaperone helper proteins, complexities of folding, and limited solubility of folded domains (1). The yield of soluble, correctly folded protein can often be increased by optimizing the primary sequence of the target protein, the genetic background of the host strain, and growth conditions, such as temperature and induction methods (2).

Conventional methods for screening the effectiveness of these solubility optimization experiments are tedious and inefficient. These methods typically involve cell harvest by centrifugation, mechanical disruption by sonication or French press, and separation of soluble proteins from insoluble proteins, debris and residual intact cells by a second centrifugation step. The soluble supernatant fraction is subsequently analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, and/or activity assay to detect and quantify the presence and quality of the target protein.

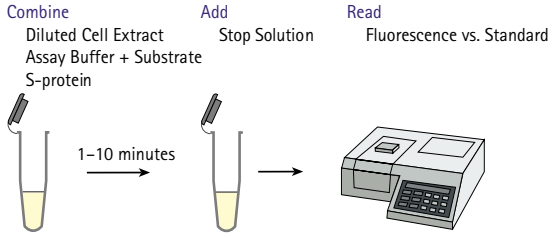
Parallel processing of hundreds of samples for high-throughput (HT) proteomics research requires biological, chemical, and engineering solutions to eliminate tasks that are difficult to automate, such as sonication and centrifugation, while minimizing multiple processing steps. We have developed specialized lysis reagents that eliminate the need for mechanical disruption of cells. These reagents include BugBuster®, YeastBuster™, and CytoBuster™ protein extraction reagents, for *E. coli*, yeast, and insect or mammalian cells, respectively. The effectiveness of BugBuster Reagent and the

*Adapted from (2003) *inNovations* **16**, 11–13.

need to simplify the purification process for automation led us to the development of PopCulture® Reagent. PopCulture is a concentrated mixture of specialized detergents that when combined with rLysozyme™ Solution and Benzonase® Nuclease enables extraction and purification of recombinant proteins from *E. coli* directly from the culture media without cell harvest, mechanical disruption, or extract clarification. The combined activities of rLysozyme and Benzonase Nuclease are now available as Lysonase™ Bioprocessing Reagent. Lysonase significantly increases protein extraction efficiency and reduces sample viscosity, thereby facilitating downstream processing and robotic pipetting. PopCulture, rLysozyme, Benzonase, affinity resins, and plasticware are conveniently formatted into the RoboPop™ Protein Purification Kits for automated protein purification from *E. coli* or insect cells.

We have now expanded the applications of PopCulture and Lysonase for use in parallel or automated expression-level solubility screening. We describe here a novel high throughput–compatible method for protein solubility screening.

The procedure incorporates a nonfouling filtration plate capable of retaining insoluble inclusion bodies while allowing soluble proteins to be collected for rapid quantification and analysis. Insoluble proteins retained by the filtration plate are solubilized with 4% SDS denaturing solution, collected, and quantified separately. The protocol has been automated on the Packard-brand MultiPROBE® II from PerkinElmer Life Sciences (Figure 1). The general protocol we used for automation is described in Table 1.



FRETWorks S•Tag Assay

Substrate	FRET ArUAA
Endpoint	fluorescence (520 nm)
Elapsed time	< 10 min
Detection limit	< 1 fmol

Figure 2. FRETWorks™ S•Tag Assay

Solubility screening of fusion proteins from *E. coli* total culture extracts

We used four different plasmid recombinants for expression-level solubility screening, as listed in Table 2 . All of these fusion proteins can be purified by immobilized metal chelation chromatography using His•Bind® affinity resins and detected using either the His•Tag® Monoclonal Antibody or the FRETWorks™ S•Tag™ Assay (Figure 2). Figure 3 shows the results of a solubility screening experiment. The data demonstrate the effectiveness of the RoboPop™ Solubility Screening Kit for fractionation and quantification of the expressed soluble and insoluble target protein from each construct. The SDS-PAGE analysis (Figure 3, panel A) shows that under the expression conditions tested, GUS was entirely insoluble, β-gal was approximately 50% soluble, NusA was approximately 90% soluble, and GST was approximately 75% soluble. Although the fractions obtained with the kit may be analyzed by conventional SDS-PAGE to estimate the degree of soluble target protein expression, sensitivity and throughput are low and quantification is difficult by this method. A highly sensitive, robot-friendly protocol has been developed to quantify S•Tag fusion proteins in crude extracts using the homogeneous FRETWorks S•Tag Assay (3). This assay is based on the affinity of the 15–amino acid S•Tag peptide in the fusion proteins for the 104–amino acid S-protein in the assay buffer. The S•Tag–S-protein interaction reconstitutes ribonuclease activity, cleaving the FRET substrate and producing a fluorescent signal as the quencher is released from the fluorescent molecule. Results of the FRETWorks assay for quantification of GUS, β-gal, NusA and GST (Figure 3, panel B) correlated well with the SDS-PAGE analysis of the same samples. The RoboPop Solubility Screening Kit may also be used without the aid of a robotic platform by employing simple multichannel pipetting for the robotic liquid handling steps, and vacuum filtration may be replaced by centrifugation in the robotic or manual protocol.

Table 2. Vector constructs used for analysis in Figure 3

Vector/construct	Fusion protein – "name"	Expected size
pET-41b(+)	GST•Tag™/His•Tag®/S•Tag™ – "GST"	35.6 kDa
pET-30b(+)/β-gal	His•Tag/S•Tag/β-gal – "β-gal"	121 kDa
pET-43.1b(+)	Nus•Tag™/His•Tag/S•Tag/HSV•Tag® – "NusA"	66.4 kDa
pTriEx™-4/GUS	His•Tag/S•Tag/GUS/HSV•Tag – "GUS"	73.5 kDa

Summary

The RoboPop Solubility Screening Kit increases the efficiency of solubility optimization by streamlining tedious cell lysis, extract fractionation, and sample analysis procedures. The screening kit provides a robot-friendly alternative to conventional labor-intensive, expression-level protein solubility screening through PopCulture® cell lysis and HT 96-well filtration-based fractionation. We have combined the chemistry of PopCulture, the biological activities of Lysonase™ Solution, and the engineering of an innovative Filtration Plate to eliminate common bioprocessing bottlenecks. Together, the RoboPop Solubility Screening Kit and RoboPop purification kits allow rapid identification and selection of ideal host-vector combinations, expression conditions, and purification parameters toward production of proteins for structural or functional analysis.

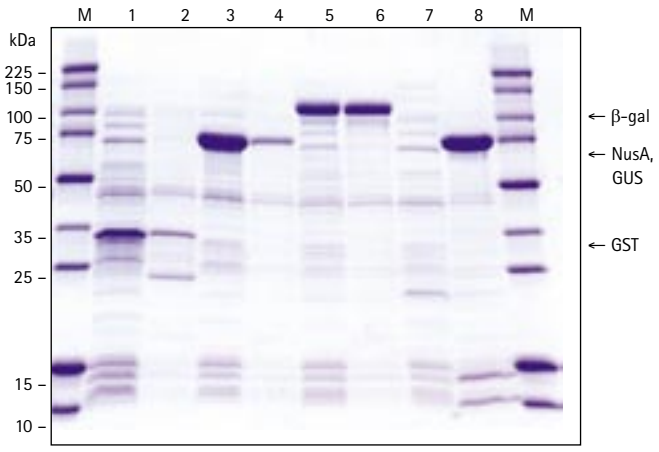
References

1. Mukhopadhyay, A. (1997) *Adv. Biochem. Eng. Biotechnol.* **56**, 61–109.
2. Wigley, W. C., Stidham, R. D., Smith, N. M., Hunt, J. F., and Thomas, P. J. (2001) *Nature Biotechnol.* **19**, 131–136.
3. Keleman, B. R., Klink, T. A., Behlke, M. A., Eubanks, S. R., Leland, P. A., and Raines, R. T. (1999) *Nucleic Acids Res.* **27**, 3696–3701.

See the following pages for related product details and ordering information:

Product	Cat. No.	Page
Lysonase™ Bioprocessing Reagent	71230	12
PopCulture® Reagent	650212	36
RoboPop™ Solubility Screening Kit	71255	39

A. SDS-PAGE analysis of soluble and insoluble fractions

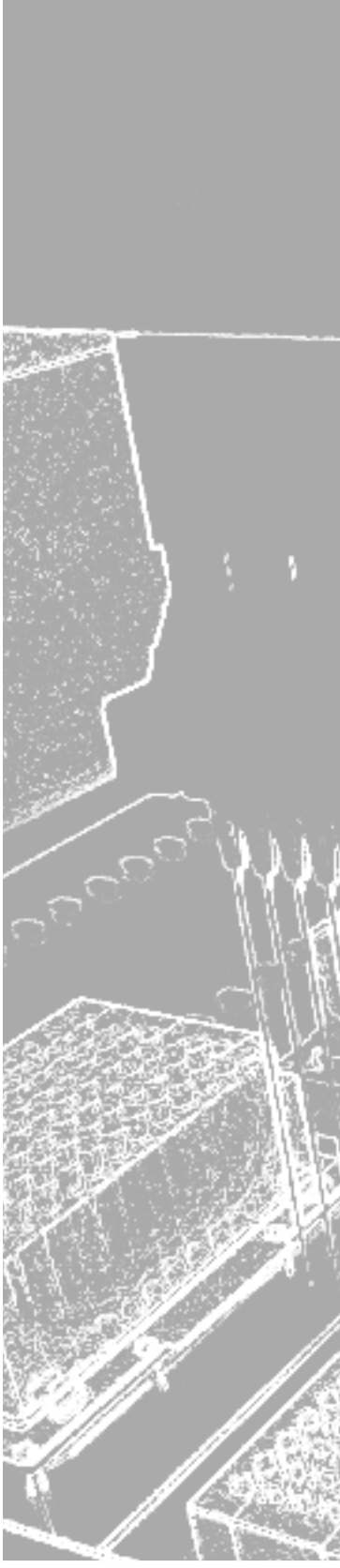


Lane	Sample
M	Perfect Protein™ Markers, 10–225 kDa
1	GST, soluble
2	GST, insoluble
3	NusA, soluble
4	NusA, insoluble
5	β-gal, soluble
6	β-gal, insoluble
7	GUS, soluble
8	GUS, insoluble

B. FRETWorks S•Tag Assay of soluble and insoluble fractions

Construct	Fraction	pmol/ml	µg/ml	% in fraction
GST	soluble	116	209	65.1
	insoluble	62.2	112	34.9
NusA	soluble	33.2	121	74.7
	insoluble	11.2	41	25.3
β-gal	soluble	33.7	202	44.7
	insoluble	41.7	250	55.3
GUS	soluble	1.0	3	0.7
	insoluble	140	477	99.3

Figure 3. Solubility screening using four different vector constructs
Cultures of *E. coli* strain BL21(DE3) containing the vector constructs described in Table 2 were grown at 37°C to an OD_{600} = 1.5 and target protein expression was induced by adding IPTG to a final concentration of 1 mM. Following incubation for approximately 3 h at 30°C (final OD_{600} = 5–6), the cultures were dispensed (1 ml/well) into sequential rows of 2-ml 96-well plates, and 100 µl PopCulture Reagent containing 2 µl Lysonase Reagent was added to each well. The procedure described in Table 1 (page 77) was used to generate soluble and insoluble protein fractions. Panel A shows SDS-PAGE analysis (Coomassie™ blue staining) of the indicated samples (15 µl extract). Panel B shows the results of the FRETWorks S•Tag Assay performed with the same fractions. For the assay, samples were serially diluted 1:25 to 1:2500, and the dilutions analyzed according to the standard protocol (20 µl diluted sample was used per assay). The S•Tag fusion proteins were quantified with a standard curve based on known amounts of S•Tag Standard.



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