invitrogen™

SILAC™ Protein Identification (ID) and Quantitation Kits

For identifying and quantifying phosphoproteins and membrane proteins

Catalog no. SP10001, SM10002, SP10005, SM10006

MS10030, MS10031, MS10032, MS10033

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User Manual

Table of Contents

Kit Contents and Storage

Shipping

Each product contains the following components.

Product	Catalog no.
SILAC™ **Phosphoprotein** Identification (ID) and Quantitation Kit	
*with [U-$^{13}C_6$]-L- Lysine (*Lys) and D-MEM*	SP10001
*with [U-$^{13}C_6$]-L-Lysine (*Lys) and RPMI 1640*	SP10005
SILAC™ **Membrane** Protein Identification and Quantitation Kit	
*with [U-$^{13}C_6$]-L-Lysine (*Lys) and D-MEM*	SM10002
*with [U-$^{13}C_6$]-L-Lysine (*Lys) and RPMI 1640*	SM10006
SILAC™ Protein Identification and Quantitation **Media** Kit	
*with [U-$^{13}C_6$]-L-Lysine (*Lys) and D-MEM-Flex*	MS10030
*with [U-$^{13}C_6$]-L-Lysine (*Lys) and RPMI-Flex*	MS10031
*with [U-$^{13}C_6$]-L-Lysine (*Lys) and IMDM-Flex*	MS10032
*with [U-$^{13}C_6$]-L-Lysine (*Lys) and Advanced D-MEM/F-12-Flex*	MS10033

SILAC™ Phosphoprotein and Membrane Kit Contents

The kit contents, shipping, and storage for SILAC™ Phosphoprotein and Membrane Protein ID and Quantitation Kits are listed below. For a detailed description of kit contents, see page 4.

These kits include appropriate media components, amino acids, and Lysis Buffer. Store all media protected from light.

Component	SP10001	SP10005	SM10002	SM10006	Shipping	Storage
SILAC™ D-MEM	√		√		Blue ice	4°C
SILAC™ RPMI 1640		√		√	Blue ice	4°C
Fetal Bovine Serum (FBS), Dialyzed	√	√	√	√	Dry ice	–20°C
L-Glutamine (100X), Liquid	√	√	√	√	Dry ice	–20°C
SILAC™ Phosphoprotein Lysis Buffer	√	√			Blue ice	4°C
PiMAC™ Resin	√	√			Blue ice	4°C
SILAC™ Membrane Protein Lysis Buffer			√	√	Blue ice	4°C
Benzonase® Nuclease			√	√	Blue ice	–20°C
SILAC™ L-Lysine HCl and L-Arginine	√	√	√	√	Blue ice	4°C
SILAC™ [U-$^{13}C_6$]-L-Lysine HCl (*Lys)	√	√	√	√	Blue ice	4°C

Continued on next page

Kit Contents and Storage, Continued

SILAC™ Media Kit Contents

The kit contents, shipping, and storage for SILAC™ Protein ID and Quantitation Media Kits are listed below. For a detailed description of kit components, see page 4.

These kits include appropriate media components and amino acids. Store all media protected from light.

Box	Component	MS10030	MS10031	MS10032	MS10033	Shipping	Storage
1	SILAC™ D-MEM-Flex Media	√				Room temperature	4°C
	SILAC™ RPMI 1640-Flex Media		√			Room temperature	4°C
	SILAC™ IMDM-Flex Media			√		Room temperature	4°C
	SILAC™ Advanced D-MEM/F-12-Flex Media				√	Room temperature	4°C
2	Fetal Bovine Serum (FBS), Dialyzed	√	√	√	√	Dry ice	–20°C
	L-Glutamine (100X), Liquid	√	√	√	√	Dry ice	–20°C
3	SILAC™ Glucose Solution (200 g/L)	√	√	√	√	Room temperature	4°C
	SILAC™ Phenol Red Solution (10 g/L)	√	√	√	√	Room temperature	4°C
	SILAC™ L-Lysine HCl and L-Arginine	√	√	√	√	Room temperature	4°C
	SILAC™ [U-$^{13}C_6$]-L-Lysine HCl (*Lys)	√	√	√	√	Room temperature	4°C

Continued on next page

Kit Contents and Storage, Continued

Phosphoprotein and Membrane Kit Components

The kit components for each SILAC™ Phosphoprotein and Membrane Identification and Quantitation Kits are listed below.

Store all components at 4°C except FBS, Benzonase® Nuclease, and L-Glutamine, which are stored at –20°C.

Component	SP10001	SM10002	SP10005	SM10006
SILAC™ D-MEM	2 × 1000 mL	2 × 1000 mL	—	—
SILAC™ RPMI 1640	—	—	2 × 1000 mL	2 × 1000 mL
Fetal Bovine Serum, Dialyzed	2 × 100 mL	2 × 100 mL	2 × 100 mL	2 × 100 mL
L-Glutamine (100X), Liquid	20 mL	20 mL	20 mL	20 mL
SILAC™ Phosphoprotein Lysis Buffer and PiMAC™ Resin (see next page for details)	1 kit	—	1 kit	—
SILAC™ Membrane Protein Lysis Buffer (see next page for details)	—	50 mL	—	50 mL
SILAC™ L-Lysine HCl	100 mg	100 mg	100 mg	100 mg
SILAC™ L-Arginine	2 × 100 mg	2 × 100 mg	2 × 100 mg	2 × 100 mg
SILAC™ [U-$^{13}C_6$]-L-Lysine HCl (*Lys)	100 mg	100 mg	100 mg	100 mg

Media Kit Components

The kit components for each SILAC™ Protein Identification and Quantitation Media Kits are listed below.

Store all components at 4°C except FBS and L-Glutamine, which are stored at –20°C.

Component	MS10030	MS10031	MS10032	MS10033
SILAC™ D-MEM-Flex Media	2 × 1000 mL	—	—	—
SILAC™ RPMI 1640-Flex Media	—	2 × 1000 mL	—	—
SILAC™ IMDM-Flex Media	—	—	2 × 1000 mL	—
SILAC™ Advanced D-MEM/F-12-Flex Media	—	—	—	2 × 1000 mL
Fetal Bovine Serum, Dialyzed	2 × 100 mL	2 × 100 mL	2 × 100 mL	2 × 100 mL
L-Glutamine (100X), Liquid	20 mL	20 mL	20 mL	20 mL
SILAC™ Glucose Solution (200 g/L)	50 mL	50 mL	50 mL	50 mL
SILAC™ Phenol Red Solution (10 g/L)	5 mL	5 mL	5 mL	5 mL
SILAC™ L-Lysine HCl	100 mg	100 mg	100 mg	100 mg
SILAC™ L-Arginine	2 × 100 mg	2 × 100 mg	2 × 100 mg	2 × 100 mg
SILAC™ [U-$^{13}C_6$]-L-Lysine HCl (*Lys)	100 mg	100 mg	100 mg	100 mg

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Kit Contents and Storage, Continued

SILAC™ Phosphoprotein Lysis Buffer

The kit components for SILAC™ Phosphoprotein Lysis Buffer Kit (supplied with Cat. nos. SP10001 and SP10005) are listed below.

Store SILAC™ Phosphoprotein Lysis Buffer Kit at 4ºC.

Component	Composition	Amount
SILAC™ Phosphoprotein Lysis Buffer A	Tris-HCl, pH 8.0 NP-40 NaCl Sodium vanadate Sodium fluoride Protease inhibitors (AEBSF, aprotonin, and leupeptin)	100 mL
SILAC™ Phosphoprotein Lysis Buffer B	Tris-HCl, pH 8.0 Triton X-100 Sodium dodecyl sulfate (SDS) Sodium deoxycholate NaCl Sodium vanadate Sodium fluoride Protease inhibitors (AEBSF, aprotonin, and leupeptin)	100 mL

SILAC™ Membrane Protein Lysis Buffer

The components for SILAC™ Membrane Protein Lysis Buffer (supplied with Cat. nos. SM10002 and SM10006) are listed below.

Store SILAC™ Membrane Protein Lysis Buffer at 4ºC and store Benzonase® Nuclease at –20ºC.

Component	Composition	Amount
SILAC™ Membrane Protein Lysis Buffer	Tris-HCl, pH 8.0 Magnesium chloride Protease inhibitors (AEBSF, aprotonin, and leupeptin)	50 mL
Benzonase® Nuclease	25 units/µL Benzonase® Nuclease in 50% glycerol	40 µL

PiMAC™ Resin

The kit components for PiMAC™ Resin (supplied with Cat. nos.SP10001 and SP10005) are listed below.

Component	Composition	Amount
PiMAC™ Resin	50% slurry in 20% ethanol (v/v)	500 µL
PiMAC™ Filter	Polyethylene sheet (1 cm × 2 cm)	1 Filter

Intended Use

For research use only. Not intended for any animal or human therapeutic or diagnostic use.

Introduction

Product Overview

Description of the System

The SILAC™ (Stable Isotopic Labeling by Amino Acids in Cell Culture) Protein Identification (ID) and Quantitation Kits provide a simple, efficient, and reproducible method for quantitative analysis of differential phosphoprotein or membrane protein expression. The kits are designed to allow efficient metabolic labeling of cells followed by sample preparation and analysis using mass spectrometry (MS).

SILAC™ Technology

The SILAC™ Technology is a powerful tool for quantitative analysis of post-translational modifications, low abundance proteins, phosphoproteins, and membrane proteins using mammalian cells. The SILAC™ Protein ID and Quantitation Kits are based on the metabolic labeling technology developed by Brian Chait (Oda *et al.*, 1999) using isotopic nutrients (N^{15}) in cell culture media and performing comparative MS analysis. Chen and coworkers modified this method and used stable isotope of amino acids instead of simple salts (Chen *et al.*, 2000). Because isotopic amino acids are incorporated into proteins in a sequence specific manner, you can employ Amino Acid Coded mass Tags (AACT) to confirm the identity of a protein with higher confidence by comparing the sequence dependent mass shifts of an entire protein digest to the peptide mass fingerprint of the unlabeled protein. Residue specific mass alterations to efficiently detect protein modifications such as phosphorylation and oxidation were also demonstrated using isotopically labeled amino acids (Bae & Chen, 2004; Zhu *et al.*, 2002).

The SILAC™ Technology is a result of further developments to his method by Mathias Mann (Ong *et al.*, 2002) using stable isotopic labeled amino acids in cell culture, which when combined with global, differential MS analysis provides a tool to identify and quantitate complex protein samples.

In SILAC™ experiments, two mammalian cell populations are grown in identical cell culture media deficient in some essential amino acids. One cell population is grown in medium with heavy (isotopic) amino acid while the other cell population is grown in medium with light (normal) amino acids. The natural metabolic machinery of the cells is utilized to label all cellular proteins with the heavy amino acid (Amanchy *et al.*, 2005). After trypsin digestion, the peptides containing the light or heavy amino acids are chemically identical and can be processed together using any protein separation method eliminating quantification errors due to unequal sampling. Since the peptides are isotopically distinct, they can be easily distinguished by mass using MS analysis. Based on the relative peak intensity of the isotopic peptide pairs, you can quantitate differential protein expression and identify differential post-translational modifications between different samples.

For a system overview, see page 3. For details on light and heavy amino acids, see page 20.

Continued on next page

Product Overview, Continued

SILAC™ Kits

Three types of SILAC™ Kits are available. For detailed description on each kit component, see page 4.

SILAC™ Phosphoprotein Identification and Quantitation Kit

Protein phosphorylation is an important regulatory pathway in mammalian cells. SILAC™ Phosphoprotein Identification and Quantitation Kits allow you to study and quantify regulated phosphorylation pathways. The kits include high quality GIBCO® cell culture media and dialyzed FBS, normal and isotope labeled amino acids, pre-made lysis buffers compatible with downstream applications and phosphopeptide enrichment resin.

SILAC™ Membrane Protein Identification and Quantitation Kit

Membrane proteins play an important role in mammalian cells but are usually difficult to isolate and analyze due to their high hydrophobicity. SILAC™ Membrane Protein Identification and Quantitation Kits provide a complete solution for studying membrane proteomics. The kits include high quality GIBCO® cell culture media and dialyzed FBS, normal and isotope labeled amino acids, pre-made hypotonic membrane lysis buffers, and an optimized protocol to isolate crude membrane fraction.

SILAC™ Protein Identification and Quantitation Media Kit

Mammalian cells are cultured in a variety of defined media based on the cell line and application. The SILAC™ Flex Media Kits allow you to customize your media to suit your application and cell line. Each SILAC™ Flex Media is depleted in glucose, phenol red, glutamine, L-Lysine, and L-Arginine. The depleted media components are supplied separately with each kit to allow you to prepare your defined culture medium for specific cell line or application.

Advantages

Using SILAC™ Technology for quantitative proteomics offers the following advantages:

- Simple, easy-to use labeling protocol designed for cell biologists and protein biochemists, and performed using standard laboratory equipment
- Produces >98% labeling efficiency as compared to other labeling methods currently available
- Allows specific sequence labeling of peptides since isotope labeled amino acid medium is used instead of isotopic nuclei labeled medium
- Generates uniformly labeled proteins to analyze several peptides for accurate results and increased sequence coverage
- Eliminates quantification error due to unequal sample preparation and increases reproducibility as the two cell populations are mixed after treatment and treated as a single sample in all subsequent steps
- Provides flexibility in the choice of amino acids used for labeling, cell culture media for culturing your specific cell line, and the types of treatment that can be applied to the cells
- Kits offer reagents for labeling and sample preparation to produce samples compatible with downstream MS analysis

Continued on next page

Applications

SILAC™ Technology can be used to:

- Quantitatively analyze differential protein expression in the presence of a stimulus or in response to stress
- Perform proteomic profiling of normal and diseased cells
- Identify inducible protein complex components

System Overview

To perform quantitative analysis of protein expression using SILAC™ Technology, you will:

- Grow your mammalian cells as two different populations.
- Metabolically label one cell population using non-radioactive isotopic labeled essential amino acids (heavy amino acid) while labeling the second cell population using normal essential amino acids (light amino acid) during cell culture.
- Harvest cells from each population after the isotopic labeled amino acids are incorporated into the cellular proteins (usually complete incorporation is achieved within six doublings).
- Mix the cells from each population using a 1:1 ratio based on cell number.
- Lyse the cells using appropriate lysis buffers supplied with the SILAC™ Kits.
- Process the lysates using SDS-PAGE and perform in-gel trypsin digestion. Purify phosphopeptides for phosphoprotein analysis.
- Analyze tryptic peptides or phosphopeptides by MS analysis.
- Perform protein identification and quantification.

Important

The SILAC™ Kits are designed for cell labeling experiments performed by cell biologists and protein biochemists while working with a protein core facility for sample processing and MS analysis. You need to identify a protein core facility capable of identifying proteins from Coomassie or silver stained gel bands for MS analysis. Review the information on page 12 before starting the labeling experiments.

Purpose of the Manual

This manual provides the following information:

- Basic information for preparing cell culture media and growing cells
- Performing isotopic labeling of cells
- Preparing cell lysates using lysis buffers included with the kit
- Processing the lysates for analysis
- Guidelines for MS analysis, protein identification and quantititation
- Troubleshooting

Description of Kit Contents

Contents of the SILAC™ Kits

The SILAC™ Protein ID and Quantitation Kits include the following major components:

- GIBCO® Cell Culture Basal Media for growth of mammalian cell line of choice
- GIBCO® Dialyzed FBS (dFBS) for efficient and reproducible cell growth without any interfering amino acids for SILAC™
- SILAC™ Normal (light) amino acids for supplementing the basal medium for cell culture
- SILAC™ Isotope labeled (heavy) amino acids for performing isotope labeling in cell culture
- Pre-made, qualified SILAC™ Phosphoprotein (supplied with Cat. nos. SP10001 and SP10005) and Membrane Protein Lysis Buffers (supplied with Cat. nos. SM10002 and SM10006) containing protease inhibitors for efficient cell lysis and high fidelity
- PiMAC™ (Pi Metal Ion Affinity Chromatography) Resin for purification of phosphopeptides after trypsin digestion (supplied with Cat. nos. SP10001 and SP10005)

D-MEM and RPMI 1640

D-MEM and RPMI 1640 are high-quality basal media from GIBCO® that provide consistent and reproducible growth of mammalian cells. See next page for details on SILAC™ Flex Media.

D-MEM (Dulbecco's Modified Eagle Media)

D-MEM is suited for growth of a wide variety of mammalian cells (suspension or adherent). The D-MEM is a basal medium that requires supplementation with amino acids and dialyzed FBS for cell culture (see page 23 for preparing media).

The D-MEM medium in SILAC™ Kits supplied with Cat. nos. SP10001 and SM10002 has the following basic composition.

D-MEM with high glucose (4,500 mg/L) is formulated without L-Arginine, L-Glutamine, L-Lysine, sodium pyruvate, and HEPES Buffer, and contains phenol red, methionine, and $CaCl_2$.

RPMI 1640

RPMI 1640 Media are enriched formulations that support the growth of a variety of mammalian cells (suspension or adherent) including primary cells (with the addition of growth factors). The RPMI 1640 media is a basal media that requires supplementation with amino acids and dialyzed FBS for cell culture (see page 23 for preparing the media).

The RPMI 1640 medium supplied in SILAC™ Kits with Cat. nos. SP10005 and SM10006 has the following basic composition:

RPMI 1640 is formulated without L-Arginine, L-Glutamine, and L-Lysine, and contains glucose, phenol red, and folate.

Detailed formulation for each medium is available on www.invitrogen.com.

Description of Kit Contents, Continued

SILAC™ Flex Media

The SILAC™ Flex Media Kits allow you to customize your media to suit your application and cell line. Each SILAC™ Flex Media is depleted in glucose, phenol red, glutamine, L-Lysine, and L-Arginine. Each of the depleted media components is supplied separately with each kit to allow you to prepare your defined culture medium for specific cell line or application.

Four types of SILAC™ Flex Media Kits are available. Detailed formulation for each medium is available on www.invitrogen.com.

D-MEM (Dulbecco's Modified Eagle Media) Flex Media

D-MEM is suited for growth of a wide variety of mammalian cells (suspension or adherent). The D-MEM is a basal medium that requires supplementation with amino acids and dialyzed FBS for cell culture (see page 23 for preparing media).

D-MEM-Flex Medium supplied with Cat. no. MS10030 is formulated without glucose, phenol red, L-Arginine, L-Glutamine, L-Lysine, sodium pyruvate, and HEPES Buffer and contains methionine and $CaCl_2$.

RPMI 1640 Flex Media

RPMI 1640-Flex Medium is enriched formulations that support the growth of a variety of mammalian cells (suspension or adherent) including primary cells (with the addition of growth factors). The RPMI 1640-Flex Media is a basal media that requires supplementation with amino acids and dialyzed FBS for cell culture (see page 24 for preparing the media).

RPMI 1640-Flex Medium supplied with Cat. no. MS10031 is formulated without glucose, phenol-red, L-Arginine, L-Glutamine, and L-Lysine, and contains HEPES Buffer.

IMDM (Iscove's Modified Dulbecco's Media) Flex Media

IMDM Medium is highly enriched synthetic media that is suited for rapidly proliferating, high-density cell cultures. formulations that support the growth of a variety of mammalian cells (suspension or adherent) including primary cells (with the addition of growth factors). The IMDM-Flex Media is a basal media that requires supplementation with amino acids and dialyzed FBS for cell culture (see page 24 for preparing the media).

IMDM-Flex Medium supplied with Cat. no. MS10032 is formulated without glucose, phenol-red, L-Arginine, L-Glutamine, and L-Lysine, and α-thioglycerol or 2-mercaptoethanol and contains HEPES Buffer and sodium bicarbonate.

Advanced D-MEM/F-12 Flex Media

Advanced D-MEM/F-12-Flex Media is a standard basal medium formulation enriched in ingredients that are normal constituents of normal serum. The use of this medium reduces the FBS requirements by 50–90% without any loss in performance. When supplemented with 1–2% FBS, the Advanced D-MEM/F-12-Flex Media is capable of supporting cellular proliferation and maximum cell densities which are comparable to the conventional basal formulation supplemented with 5–10% FBS.

The Advanced D-MEM/F-12-Flex Medium supplied with Cat. no. MS10033 is formulated without glucose, phenol-red, L-Arginine, L-Glutamine, and L-Lysine, and contains sodium pyruvate.

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Dialyzed FBS

Dialyzed FBS (dFBS) is high-quality serum from GIBCO® that supports growth, proliferation, and differentiation of cells. The FBS is dialyzed against 0.15 M NaCl using 10,000 molecular weight cut-off filters using a Tangential flow filtration process. The Dialyzed FBS has low endotoxin (≤50 EU/mL) level and a hemoglobin level of ≤25 mg/mL.

The Dialyzed FBS is ideal for labeling experiments as the dialysis process removes any low molecular weight species such as free amino acids and peptides that may interfere with SILAC™ labeling.

Do not use regular FBS to perform SILAC™ labeling experiments. Trace amounts of amino acids present in regular FBS will interfere with the incorporation of labeled amino acid and produce erroneous results.

SILAC™ Amino Acids

SILAC™ Amino Acids are used for supplementing the basal media to prepare complete media. The SILAC™ Amino Acids include the normal (light) and isotope labeled (heavy) amino acids.

SILAC™ Light Amino Acids

The SILAC™ Kits include L-Lysine HCl and L-Arginine as light amino acids. These amino acids are normal, essential amino acids and do not contain any isotopic label. Use the light amino acids to prepare the light (unlabeled) medium as directed in the protocol (page 24).

SILAC™ Heavy Amino Acid

The SILAC™ Heavy Amino Acid includes the isotope labeled (heavy) amino acid, [U-$^{13}C_6$]-L-Lysine HCl(MW = 152.1259). The labeled *Lys is a stable isotope of [$^{12}C_6$]-L-Lysine (MW = 146.1055). The *Lys is 6 daltons heavier than the light L-Lysine. Use the heavy amino acid to prepare the heavy (labeled) medium as directed in the protocol (page 24).

Note

If you need maximal sequence coverage or need to monitor all possible phosphorylation sites, we recommend performing a double-labeling experiment wherein the proteins are labeled with [U-$^{13}C_6$]-L-Lysine and [U-$^{13}C_6$, $^{15}N_4$]-L-Arginine. See page 20 for details.

[U-$^{13}C_6$, $^{15}N_4$]-L-Arginine and [U-$^{13}C_6$]-L-Arginine (available separately from Invitrogen, page 58) are stable isotopes of [$^{12}C_6$, $^{14}N_4$]-L-Arginine and [$^{12}C_6$]-L-Arginine, respectively. After trypsin digestion and MS analysis, you will observe peak pairs that are separated by 10 Da (for Arg and [U-$^{13}C_6$, $^{15}N_4$]-L-Arg pairs) or 6 Da (for Arg and [U-$^{13}C_6$]-L-Arg pairs).

The Arg-containing peptides ionize better than Lys-containing peptides resulting in better sensitivity and sequence coverage. Using double labeling increases the number of informative peptides making the method more sensitive.

Use [U-$^{13}C_6$]-L-Arginine and [U-$^{13}C_6$]-L-Lysine for routine quantitative protein analysis.

Use [U-$^{13}C_6$, $^{15}N_4$]-L-Arginine and [U-$^{13}C_6$]-L-Lysine for quantitative protein analysis when a higher level of confidence is required in the identification.

Continued on next page

Description of Kit Contents, Continued

Lysis Buffers

The SILAC™ Phosphoprotein Kits are supplied with SILAC™ Phosphoprotein Lysis Buffer A and B while the SILAC™ Membrane Protein Kits are supplied with the SILAC™ Membrane Protein Lysis Buffer. The composition of each lysis buffer is included on page vii. To obtain the best results, always use the lysis buffers supplied with each kit for cell lysis. Avoid using your own buffers.

The lysis buffers are pre-made, qualified buffers used for lysis of mammalian cells after labeling and harvesting. The use of pre-made buffers provides consistent results, minimizes quantitation errors among replicate experiments, and eliminates the time required to prepare reagents.

Each lysis buffer includes protease inhibitor cocktails (AEBSF, leupeptin, and aprotinin which inhibit cysteine and serine proteases) to prevent protein degradation.

The SILAC™ Phosphoprotein Lysis Buffers A and B also include sodium vanadate which is an inhibitor of tyrosine phosphatase increasing the fidelity of the analysis and interpretation of phosphorylation changes induced by a stimulus.

The SILAC™ Membrane Lysis Buffer includes Benzonase® Nuclease to reduce the viscosity of the lysate and increase the protein yield. Benzonase® Nuclease is a genetically engineered nuclease capable of cleaving all forms of DNA and RNA.

PiMAC™ Resin

PiMAC™ (Pi Metal Ion Affinity Chromatography) Resin is a metal chelate resin used for the enrichment of phosphopeptides after trypsin digestion. Since phosphoproteins are low abundant proteins (usually 1–10% of the total protein) and higher sequence coverage is usually required to identify individual phosphorylation sites, it is important to use a method to enrich the phosphoproteins prior to MS analysis.

The PiMAC™ Resin is derivatized with iminodiacetic acid (IDA) at the specified concentration. The PiMAC™ Resin is charged with the $FeCl_3$ and the IDA binds Fe^{3+} ions by three coordination sites. The resulting metal chelating resin is used to process trypsin digested samples. The phosphorylated peptides from the trypsin digested samples bind to the Fe^{3+} ions on the PiMAC™ resin under acidic conditions while the non-phosphorylated peptides are not bound and collected in the flow through. Impurities are washed away and the phosphopeptides are eluted using ammonium hydroxide solution.

PiMAC™ Resin Specifications

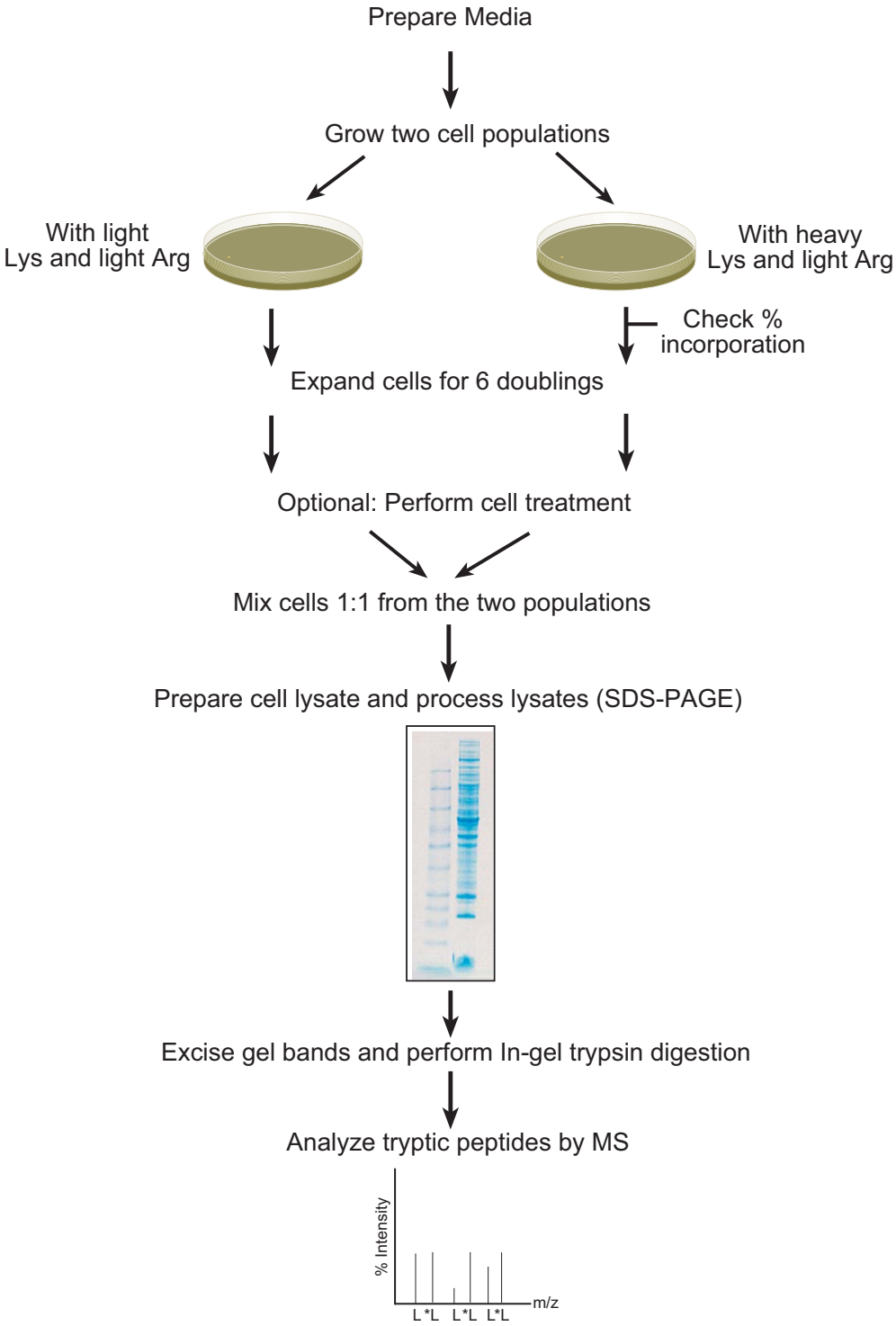
General specifications of the PiMAC™ Resin are listed below:

Particle Size:	40–90 µm
Ligand Density:	25–45 µeq/mL
Adsorption Capacity:	≥60 mg/mL
PiMAC™ Resin in Storage Buffer:	50% slurry in 20% ethanol

Experimental Overview

Flow Chart

The flow chart for the experimental outline using the SILAC™ kits is shown below. See next page for the experimental outline.



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Experimental Overview, Continued

Experimental Outline

The experimental outline for using the SILAC™ kits is shown below. See next page for the experimental workflow.

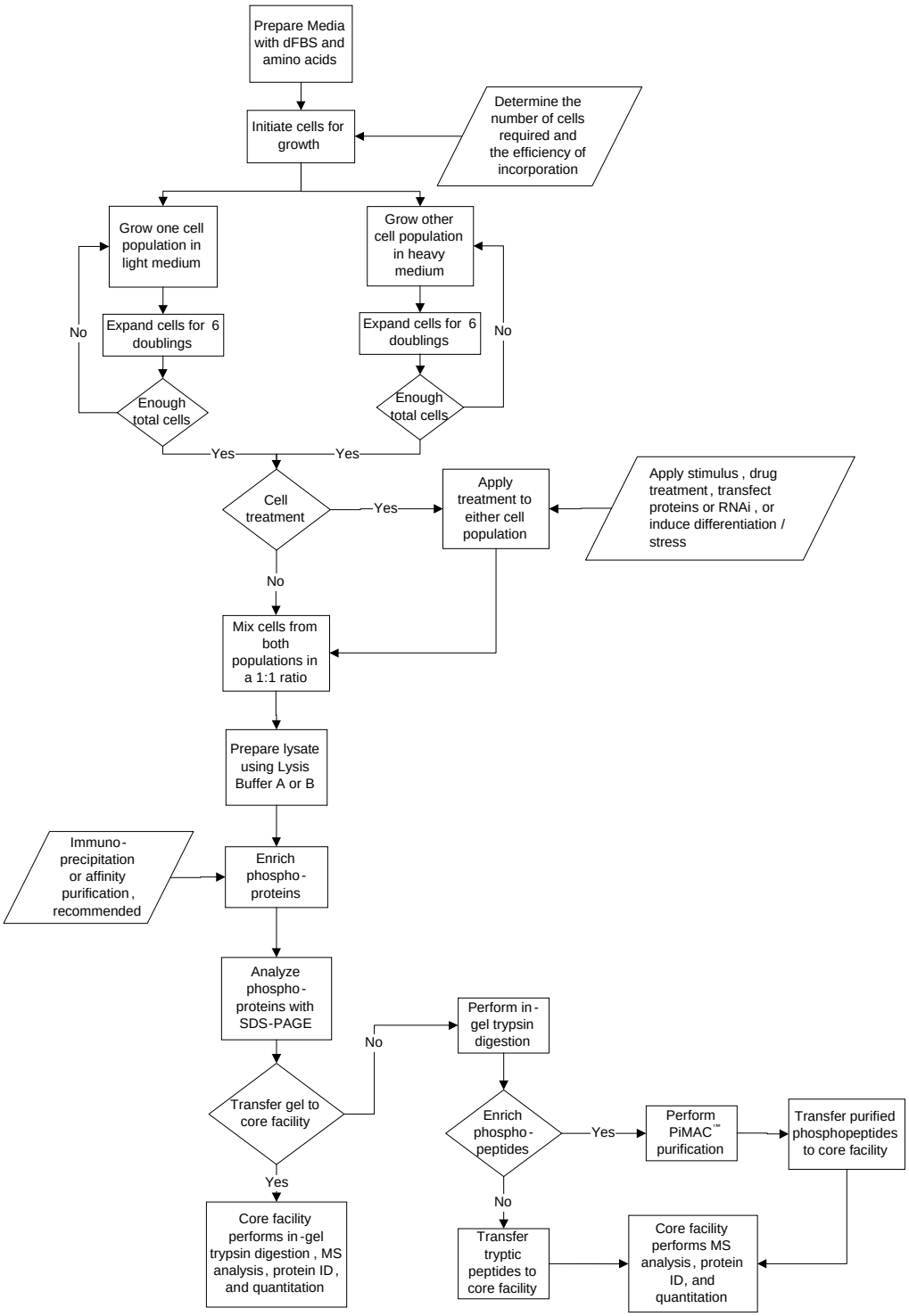
Step	Action	Page no.
1	Initiate your cell line of interest for growth.	16
2	Perform experiments to determine the cell number required for MS analysis.	17
3	Prepare SILAC™ medium with supplements, and normal lysine or isotope labeled lysine.	23
4	Grow your cells as two different populations; grow one cell population in medium containing light (normal) lysine and grow the other cell population in medium containing heavy (isotope labeled) lysine.	23
5	Expand the two cell populations for six doubling times to achieve complete incorporation of the labeled amino acid.	23
6	Perform cell treatment, if needed.	27
7	Harvest cells from each population and mix the cells using a 1:1 ratio based on cell number.	31
8	Prepare cell lysates using appropriate lysis buffers.	32
9	Process the cell lysates using a suitable method (immunoprecipitation or SDS-PAGE).	34
10	Perform in-gel trypsin digestion to generate tryptic peptides.	39
11	For phosphoprotein analysis, purify phosphopeptides using the PiMAC™ resin.	40
12	Analyze tryptic peptides and purified phosphopeptides using MALDI-TOF MS or LC-MS.	44
13	Perform protein identification using MS instrument software or Mascot software suite.	47
14	Perform protein quantitation using instrument software such as GPS Explorer™ or manual calculations.	50

Continued on next page

Experimental Overview, Continued

Phosphoprotein Workflow

Below is the experimental workflow for using the SILAC™ Phosphoprotein ID and Quantitation Kits.



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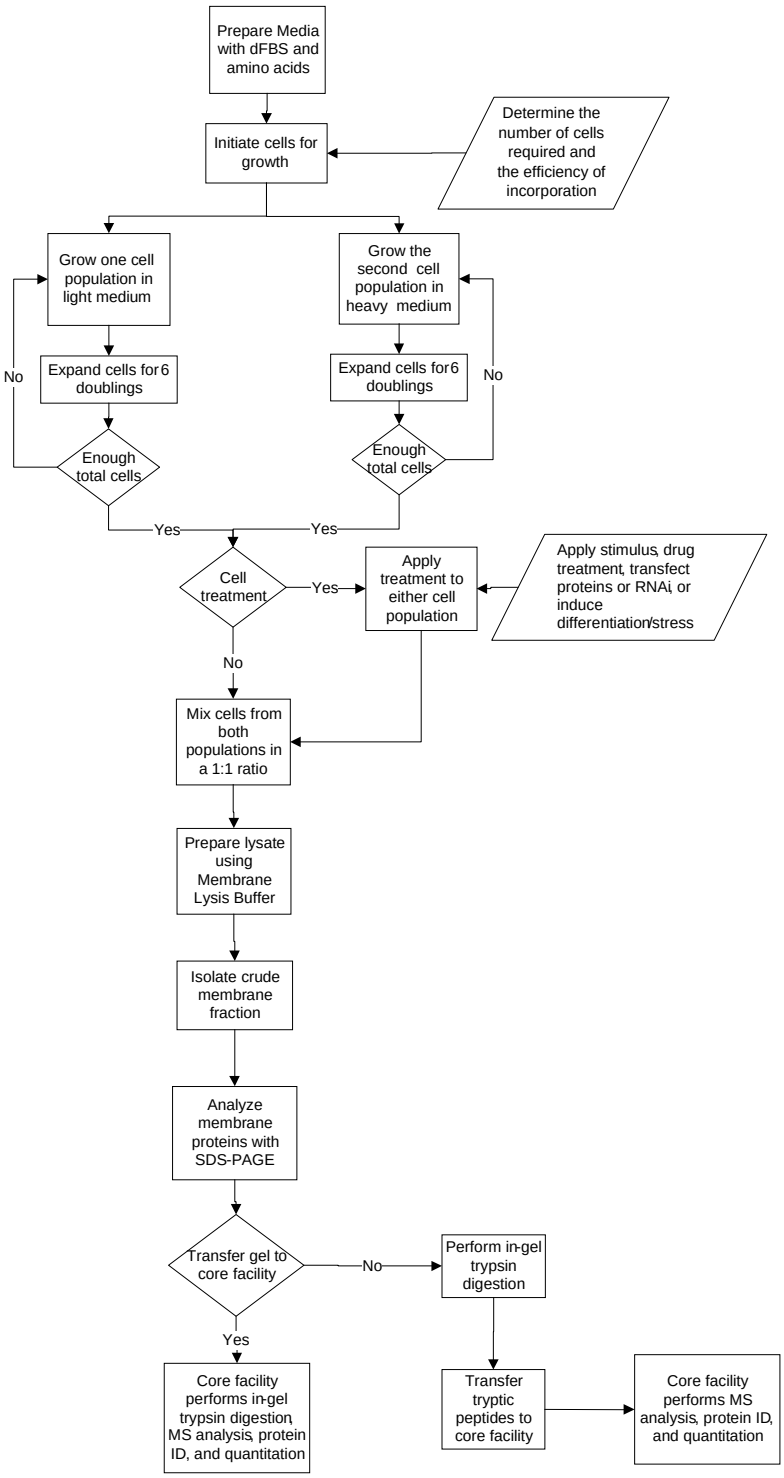
Experimental Overview, Continued

Membrane Protein Workflow

Below is the experimental workflow for using the SILAC™ Membrane Protein Kits.



Methods

Before Starting

Important

Review the information in this section prior to starting your SILAC™ experiments. You need to perform certain experiments and need to purchase some reagents before proceeding with the isotope labeling experiments.

Cell number

It is important to pre-determine the number of cells required to detect significant signal of the peptides of interest using MALDI-TOF MS analysis.

To perform the experiment for determining the number of cells, use standard cell culture medium (see page 58 for ordering information). **Do not** use the medium prepared with isotope labeled amino acid as described on page 23.

See page 17 for more details on determining the cell number.

Efficiency of Incorporation

To obtain easily interpretable results, it is important to obtain >95% incorporation of the isotope-labeled lysine into proteins. You need to determine the efficiency of incorporation as described on page 27.

Based on the doubling time of your cell line, you can determine the efficiency of incorporation before starting the actual labeling experiment (if the doubling time of your cells is 16–18 hours) or along with your labeling experiment (if the doubling time of your cells is 24–48 hours).

Greater than 98% incorporation of the isotope labeled lysine into proteins is recommended for SILAC™ labeling experiments.

MS Core Facility

The SILAC™ Kits are designed for use by cell biologists and protein biochemists to perform the labeling experiments and then coordinate and work with the protein core facility for sample processing and MS analysis.

Based on your expertise with certain protocols and the options provided by the core facility, you can transfer the samples to the core facility for MS analysis at various points as indicated in the protocols.

As each core facility has specific requirements for sample preparation and handling, it is important that you consult with your core facility about the sample requirements prior to preparing the samples. You also need to work closely with the core facility to schedule time for the MS analysis when your samples are ready.

Recommended Core Facilities for SILAC™

If you do not have access to a core facility or the core facility is not equipped to perform MS analysis for SILAC™, contact Technical Support (page 60) for a list of recommended core facilities. We have identified and qualified some core facilities for performing MS analysis, protein identification, and quantitation for SILAC™ Technology.

Continued on next page

Note

If you are an experienced user of MS, have access to various MS instruments, and are able to perform MALDI-MS or LC-MS analysis, you may chose to perform the MS analysis yourself without working with a core facility.

MS Instruments

SILAC™ experimental data can be analyzed using MALDI-TOF MS analysis for simple samples or using MS/MS analysis for complex samples.

SILAC™ Kits were developed using the 4700 Proteomics Analyzer MALDI TOF/TOF® equipped with GPS Explorer™ software that allowed protein identification and quantitation after labeling.

If you have access to the AB/MDS Sciex Family of MALDI TOF/TOF® Analyzers (includes 4700 Proteomics Analyzer MALDI TOF/TOF®) equipped with GPS Explorer™ software, you can perform fully automated analysis of SILAC™ raw data including protein identification with Mascot and quantitation.

If you have other MS instrument, you can perform semi-automated analysis of SILAC™ raw data using the MS instrument for protein identification, but you will need to perform protein quantitation using manual calculations as described on page 57 or contact the instrument vendor.

Continued on next page

Enriching Phosphorylated Proteins/Peptides

Phosphoproteins are low-abundant proteins and account for only 1–10% of the total proteins in a cell. To obtain a complete profile of the phosphoproteins in the cell in the presence of other high-abundant proteins, it is important to enrich or purify the phosphoproteins.

Prior to cell labeling experiments, you should have an optimized method for enriching phosphoproteins or the protein of interest involved in the phosphorylation cascade from the cell lysate.

Various methods are available such as:

Immunoprecipitation

Phosphoproteins can be immunoprecipitated using anti-phosphotyrosine antibodies that bind to phosphorylated tyrosine residues in the protein (Amanchy *et al.*, 2005; Ibarrola *et al.*, 2003). A large variety of anti-phosphotyrosine antibodies are commercially available (see next page for details on antibodies).

If you have a polyclonal or monoclonal antibody against your phosphorylated protein (against the protein backbone or an epitope on the protein), you can use the protein specific antibody for immunoprecipitation.

Precipitating Protein Complexes

Protein phosphorylation is a highly-regulated event occurring in response to a specific stimulus via a signal mediated pathway and involves the formation of multiple protein complexes. Complexes of phosphoproteins can be precipitated as follows:

- Allow specific proteins to bind the complex and precipitate the resulting protein complex using protein specific antibodies coupled to Protein A or G resin **or**
- Use specific expressed epitope tagged beads such as GST-agarose (Blagoev *et al.*, 2003) or Streptavidin agarose for precipitating phosphoprotein complexes.

Affinity Purification

You may purify the phosphoprotein of interest using affinity purification.

PiMAC™ Resin

The SILAC™ Phosphoprotein ID and Quantitation Kits include a PiMAC™ Resin for purifying phosphopeptides after in-gel trypsin digestion. **Do not** use the PiMAC™ Resin for purification of intact phosphoproteins as the PiMAC™ Resin is designed to bind peptides under acidic conditions. Using the phosphopeptide purification protocol for phosphoprotein purification can cause aggregation or precipitation of intact proteins.

Continued on next page

Antibodies

Phosphotyrosine Antibodies

Various anti-phosphotyrosine antibodies are commercially available. The SILAC™ Phosphoprotein ID and Quantitation Kits were developed using the anti-phosphotyrosine antibodies from Santa Cruz Antibodies (sc-7020 AC).

Note: Since different monoclonal and polyclonal anti-phosphotyrosine antibodies can bind to a variety of tyrosine phosphorylated proteins, using a mixture of two anti-phosphotyrosine antibodies may product better results (Amanchy *et al.*, 2005).

Antibodies against specific proteins

A large variety of antibodies against various proteins are available from Invitrogen (page 59). Antibodies against specific epitope-tags such as 6X His- V5-, *Myc*- are also available from Invitrogen. Visit www.invitrogen.com for more information.

Preparing the Cells

Introduction

To perform SILAC™ experiments, you will need a mammalian cell line of choice. You may use any mammalian adherent, suspension, or primary cell line.

General guidelines are included below for handling cells. If you are performing cell culture for the first time, refer to published protocols for more information (Ausubel *et al.*, 1994).

Mammalian Cells

SILAC™ Technology has been tested on various cell lines (Amanchy *et al.*, 2005) including adherent cells (NIH 3T3, HEK, 293T, HeLA, HepG2, and 3T3L1) and suspension cells (HeLaS3, Jurkat, BaF3, PC-12), prostrate cancer cell lines; PC3M and PC3M-LN4 (Everley *et al.*, 2004). The SILAC™ labeling does not affect the growth, morphology of the cells, or enzymatic activity of proteins (Ong *et al.*, 2002).

The cell line of choice must be able to grow in supplemented D-MEM, RPMI 1640, IMDM, or Advanced D-MEM/F-12 medium under the conditions used for labeling (see page 20 for details). If your specific cells require certain growth factors for growth, you may add the growth factors to the medium but **do not** add any additional amino acids to the growth medium.

Optimize the growth conditions for primary cell lines prior to performing the labeling experiment.

General Guidelines

Follow the general guidelines below to grow and maintain your mammalian cells.

- **All solutions and equipment that come in contact with the cells must be sterile.** Always use proper sterile technique and work in a laminar flow hood.
- Before starting the labeling experiments, be sure to have your cell line of interest established and have some frozen stocks on hand.
- Always use log phase cultures with >90% cell viability. Determine cell viability using the trypan blue dye exclusion method.
- Optimize the growth conditions for primary cells isolated from animals or patients using growth factors.
- Handle mammalian cells as potentially biohazardous material under the appropriate Biosafety Level as required by your institution.

Cells for Labeling

You will need log-phase cells with >90% viability to perform successful labeling. Perform a control experiment to determine how many cells you need for labeling (see next page for details).

Determining the Cell Number Required for Isotopic Labeling

Introduction

Pre-determine the number of cells required to detect a significant signal of the peptides for a protein of interest using MALDI-TOF MS analysis as described in this section.

Important

Prior to cell labeling, perform this experiment to determine the number of cells required if you are performing phosphoprotein analysis or using specific methods to enrich your proteins of interest (for example, immunoprecipitation or affinity purification).

If you are analyzing the entire proteome, consult with the protein core facility to determine the level of detection available.

General Guidelines

- Use equivalent, standard cell culture medium available from Invitrogen for determining the number of cells required.
- **Do not** use the medium prepared with isotope labeled amino acid as described on page 23.
- Do not use isotopic labeled amino acids supplied in the kit. You can use standard cell culture medium supplemented with normal amino acids.
- You can use normal FBS, as you are not performing any quantitation at this point. If desired, you can use dFBS.

  Note: If you are using primary cell lines or cell lines that require specific growth factors, ensure the cells are able to grow at similar growth rates in medium supplemented with dFBS before performing the experiment. This will allow you to ensure the cell number determined using FBS is still applicable when cells are grown in dFBS.
- A starting cell number for phosphoprotein or membrane protein analysis is recommended in the protocol. Based on your initial MS results, you can optimize the number of cells required for detection by MS.

Experimental Outline

1. Prepare medium with FBS, amino acids, and supplements.
2. Grow cells in the complete medium to obtain the cell density.
3. Harvest cells and prepare cell lysates using the lysis buffers supplied with the kit.
4. Analyze the lysates by SDS-PAGE.
5. Excise the desired band and perform in-gel trypsin digestion.
6. Analyze the tryptic peptides by MS.

Continued on next page

Determining the Cell Number Required for Isotopic Labeling, Continued

Materials Needed

- Mammalian cells of choice
- Cell culture basal medium (see page 58 for ordering information)
- FBS (page 58)
- Antibiotics (Penicillin, Streptomycin, see page 58)
- *Optional:* growth factors if needed for your cells
- Appropriate tissue culture dishes and flasks
- 37°C incubator with a humidified atmosphere of 8% CO_2
- Sterile centrifuge tubes
- Reagents to determine viable and total cell counts (see 58)
- 0.22 µm filtration unit to filter sterilize the medium
- Appropriate lysis buffer included with the kit
- NuPAGE® Novex® Bis-Tris Gel
- NuPAGE® MES/MOPS SDS Running Buffer
- XCell *SureLock*™ Mini-Cell for electrophoresis of the gel
- Sequencing grade trypsin
- 25 mM ammonium bicarbonate buffer, pH 8.0 for trypsin digestion
- 100% and 70% (v/v) acetonitrile

Prepare Medium

Prepare 1000 mL complete medium as follows:

1. Replace 100 mL of basal medium with 100 mL FBS or dFBS.

   Note: Since Advanced D-MEM/F-12 requires only 5–20% FBS, remove the appropriate amount of medium.
2. Add 10 mL 100X L-Glutamine, if basal medium does not contain glutamine.
3. Add 10 mL 100X Penicillin-Streptomycin, if needed.
4. Add any additional growth factors required for your cell line.
5. Mix well and filter sterilize the medium using 0.22 µm filtration device.
6. Store the complete medium at 2 to 8°C protected from light until use.

Note

L-Glutamine concentrations can vary from 2–4 mM depending on cell line requirements and media formulation. We recommend adding 10 mL L-Glutamine to obtain a final glutamine concentration of 2 mM, but if desired, higher concentrations of L-Glutamine (available separately, see page 58) can be used.

Higher concentrations of L-Glutamine are recommended if the media is used over an extended period of time (3–6 months), as L-Glutamine degrades over time.

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Determining the Cell Number Required for Isotopic Labeling, Continued

Procedure

Determine the number of cells required for detection by MS as below.

1. Grow the mammalian cells of choice in the complete medium prepared as described on the previous page.
2. Split the cells every 3–4 days (depending on the cell line) using the prepared medium (previous page)
3. Expand the cells to obtain the following cell numbers:
   - For phosphoprotein analysis, use a starting cell number of ~ 2×10^8 cells
   - For membrane protein analysis, use a starting cell number of ~ 2×10^6 cells

   Note: Based on your initial MS analysis results, you may need to optimize the number of cells.
4. Harvest and lyse cells using the appropriate lysis buffer supplied in the kit (see page 32).
5. Enrich for the proteins of interest using immunoprecipitation (page 36) or affinity purification.
6. Analyze the purified or enriched protein fraction using SDS-PAGE (page 37).
7. Stain the gel with Coomassie R-250 Stain.

   Note: Depending on your protein core facility, you may transfer the gel to the core facility to perform trypsin digestion and MS analysis. For more information on protein core facilities that offer MS analysis for SILAC™, see page 12.
8. Excise 3–4 protein bands of interest or cut the gel into 20 pieces (if you are analyzing uncharacterized proteins).
9. Perform in-gel trypsin digestion (page 39).
10. Perform MS analysis (page 44)

What You Should Expect

You should be able to detect peaks and identify the protein of interest after MS analysis, if you had enough cells.

If you are unable to identify the protein, review the following solutions:

- Fractionate the sample using nano HPLC and MS.
- Make sure the stained protein band is your protein of interest. Perform a western detection, if needed to confirm the presence of the protein. If you transferred a protein band that was validated using western detection and still failed to obtain a positive identification, this suggests that the protein of interest is a low abundant protein and you may need to enrich for the specific protein. After enriching for the protein, you are still unable to obtain a positive identification, you may need to use more starting material. Increase the number of cells used for analysis by 5-fold.
- Enrich the protein of interest using a suitable technique.
- Increase the number of cells used for analysis by 5-fold. Be sure you are not increasing the background by using more cells. Make sure you have used a method to enrich for the protein of interest.

Isotopic Labeling in Cell Culture

Introduction

Instructions for performing cell labeling are described in this section. Be sure you have determined the number of cells required for analysis as described on page 17 prior to labeling. At this point, you should have initiated your cell line of interest for growth and prepared any frozen stocks, if needed.

Isotopic Labeling

Metabolic labeling with stable isotope is performed using the SILAC™ Technology. To obtain complete incorporation of the isotope labeled amino acid into the proteins, you need to adapt the cells to the medium containing the labeled medium. Complete incorporation is usually achieved within 6 passages of the cells in the medium containing the isotope labeled amino acid.

Labeling with Isotopically Labeled Amino Acid

The SILAC™ Phosphoprotein and Membrane Protein Kits are supplied with [U-$^{13}C_6$]-L-Lysine HCl (MW = 152.1259) which is a stable isotope of [$^{12}C_6$]-L-Lysine (MW = 146.1055). The heavy *Lys is 6 daltons heavier than normal Lys.

For most of your experiments, performing single labeling with *Lys is sufficient to determine the relative expression of proteins.

Trypsin is the most widely used enzyme to generate peptides for MS analysis. Trypsin cleaves the proteins at the C-terminus of arginine and lysine residues. Labeling the cells with heavy labeled *Lys and performing trypsin digestion yields peptides isotopically labeled with Lys. When these isotopically labeled peptides with C-terminal *Lys are mixed with non-labeled peptides with C-terminal Lys and MS analysis is performed, the peptides are detected as "peak pairs" that are precisely 6.0204 Da apart. Using labeling with *Lys only, you will detect peak pairs only for the subset of peptides with C-terminal Lys residues, while not detecting the peptides with C-terminal Arg residues.

If you need maximal sequence coverage or need to monitor all possible phosphorylation sites, we recommend performing a double-labeling experiment wherein the proteins are labeled with [U-$^{13}C_6$]-L-Lysine HCl and [U-$^{13}C_6$, $^{15}N_4$]-L-Arginine (MW=184.1241). The Arg-containing peptides ionize better than Lys-containing peptides resulting in better sensitivity and sequence coverage. Using double labeling increases the number of informative peptides making the method more sensitive.

[U-$^{13}C_6$, $^{15}N_4$]-L-Arginine (*Arg) is available separately from Invitrogen (see page 58) and is a stable isotope of [$^{12}C_6$, $^{14}N_4$]-L-Arginine (MW=174.1117). After trypsin digestion and MS analysis, you will observe peak pairs that are separated by 6.0204 Da (for Lys and *Lys pairs) and 10.0124 Da (Arg and *Arg pairs).

Continued on next page

Isotopic Labeling in Cell Culture, Continued

Experimental Outline

1. Prepare light (normal) and heavy (isotope labeled) supplemented medium with dialyzed FBS.
2. Harvest cells and initiate two cultures. Grow one culture in the light (normal) supplemented medium and the other culture in heavy (isotope labeled) supplemented medium.
3. Grow the two cell populations for at least six doublings to allow complete incorporation of the labeled amino acid.
4. Perform the cell treatment (see below), if appropriate.

General Experimental Timelines

General experimental timelines for cell culture and labeling for a typical mammalian epithelial cell with a doubling time of ~18 hours are **~5–6 days**. If you are applying a stimulus or performing a cell treatment, the timeline is **~7–10 days**. See below for detailed timelines.

You can use these timelines as a guideline and adjust the timelines accordingly for your specific cell line.

Day 1

Initiate the growth of cells in light and heavy supplemented medium. Start with 1×10^5 cells for each cell population.

Days 3–4

Change the medium or split the cells every 3–4 days using the appropriate medium.

Days 5–6

Each cell population has achieved six doublings resulting in 6.4×10^6 cells for each population.

Days 7–10

Apply the appropriate cell treatment or stimulus if needed (see below for details).

Treatment of Cells

Since the SILAC™ labeling experiments are performed in cell culture, various types of cell treatments can be performed to compare the effect of the treatment on protein expression.

Examples of various cell treatments are listed below. The time for the treatment is highly variable from 5 minutes to several days depending on the treatment.

- Growth factor stimulation
- Drug treatment
- Induction of cell differentiation (stem cells)
- Response to stress (withdrawal of serum)
- Transfecting proteins (for expression of specific proteins) or RNAi (to study knockdown effects)

While analyzing results after performing the treatment, always compare the results with cells grown in heavy medium and cells grown in light medium, both media containing the same concentration of the light (normal) amino acid or the heavy (isotope labeled) amino acid.

Continued on next page

Isotopic Labeling in Cell Culture, Continued

Materials Needed

- Mammalian cells of choice (see page 17 to determine the number of cells needed for labeling)
- Antibiotics (Penicillin, Streptomycin, see page 58)
- *Optional:* growth factors if needed for your cells
- Appropriate tissue culture dishes and flasks
- 37°C incubator with a humidified atmosphere of 8% CO_2
- Sterile centrifuge tubes
- Reagents to determine viable and total cell counts (see page 58)
- 0.22 µm filtration unit to filter sterilize the medium
- *Optional:* [U-$^{13}C_6$, $^{15}N_4$]-L-Arginine or [U-$^{13}C_6$]-L-Arginine for double labeling experiments (page 58)
- Appropriate reagents for cell treatment, if applicable

For determining the efficiency of incorporation, you will also need:

- NuPAGE® LDS Sample Buffer (4X)
- NuPAGE® Sample Reducing Agent (10X)
- NuPAGE® Novex® Bis-Tris Gel
- NuPAGE® MES/MOPS SDS Running Buffer (20X)

Components Supplied in the Kit

You will need the following items (supplied with the kit):

- SILAC™ D-MEM or RPMI 1640 (deficient in lysine, arginine, and glutamine) or SILAC™ Flex Media (D-MEM-Flex, RPMI-1640-Flex, IMDM-Flex, Advanced D-MEM/F-12-Flex—deficient in lysine, arginine, glutamine, and glucose, phenol red)
- Dialyzed Fetal Bovine Serum, thaw and store on ice until use
- L-Lysine HCl
- L-Arginine
- L-Glutamine, thaw and store on ice until use
- SILAC™ Glucose Solution and SILAC™ Phenol Red Solution to prepare SILAC™ Flex Media
- [U-$^{13}C_6$]-L-Lysine HCl (*Lys)

Note

Before performing the isotopic labeling experiments, be sure:

- To determine the number of cells required for labeling (page 17).
- You have the required number of cells actively growing with >90% viability.
- To keep some cells aside to measure the percentage of incorporation as directed in the protocol.

Continued on next page

Preparing D-MEM and RPMI Medium

Prepare the D-MEM or RPMI 1640 labeling medium containing 10% dialyzed FBS and supplemented with 100 mg/mL L-Lysine, 100 mg/mL L-Arginine, and 100X L-Glutamine using the basal medium (supplied with Cat. nos. SP10001, SP10005, SM10002, and SM10006) as described below. Perform all steps in a tissue culture hood under sterile conditions and filter sterilize complete medium (see Step 8).

To prepare SILAC™ Flex Media, see next page.

Note: D-MEM Medium does not contain sodium pyruvate. Purchase sodium pyruvate separately from Invitrogen (page 58), if sodium pyruvate is required for cell growth.

D-MEM Labeling Medium

1. Resuspend 100 mg L-Lysine HCl and 100 mg [U-$^{13}C_6$]-L-Lysine (*Lys) each in 1 mL basal, unsupplemented D-MEM medium supplied with the kit. Mix well until completely dissolved.
2. Resuspend 100 mg L-Arginine from each vial (2 vials are supplied in the kit) in 1 mL basal, unsupplemented D-MEM medium each supplied with the kit. Mix well until completely dissolved.

   Note: If you are using double labeled arginine (available separately from Invitrogen, see page 58), resuspend 100 mg [U-$^{13}C_6$, $^{15}N_4$]-L-Arginine (*Arg) or 100 mg [U-$^{13}C_6$]-L-Arginine (*Arg) in 1 mL basal, unsupplemented D-MEM supplied with the kit. Mix well until completely dissolved.
3. Remove 100 mL D-MEM from each 1 L D-MEM bottle supplied with the kit and replace with 100 mL dialyzed FBS supplied with the kit.
4. To one 1 L bottle of D-MEM from Step 3, add L-Lysine HCl (100 mg/mL) from Step 1 and L-Arginine (100 mg/mL) from Step 2 to prepare **light D-MEM medium** supplemented with Light (normal) lysine and arginine. Mix well and mark the bottle appropriately
5. To the second 1 L bottle of D-MEM from Step 3, add *Lys (100 mg/mL) from Step 1 and L-Arginine (100 mg/mL) from Step 2 to prepare **D-MEM single labeling medium** supplemented with light arginine and heavy (isotope labeled) lysine. Mix well and mark the bottle appropriately.

   Optional: If you are preparing **double** labeled medium, add *Lys (100 mg/mL) from Step 1 and *Arg (100 mg/mL) from Step 2 to prepare D-MEM double labeling medium supplemented with heavy (isotope labeled) arginine and lysine. Mix well and mark the bottle appropriately.
6. To each 1 L medium bottle, add 10 mL 100X L-Glutamine supplied with the kit.
7. *Optional:* Add 10 mL 100X Penicillin-Streptomycin (page 58), if needed (highly recommended). You may supplement the medium with additional growth factors or cytokines, if needed for your specific cell line.
8. **Filter sterilize each medium using 0.22 µm filtration device.**
9. Store the medium at 2 to 8°C, protected from light until use. The medium is stable for 6 months when properly stored (avoid introducing any contamination into the medium).

RPMI 1640 Labeling Medium

Prepare the RPMI 1640 heavy labeling medium and light medium as described above for the D-MEM medium except, you will use RPMI 1640 basal medium supplied with the kit instead of D-MEM medium.

Continued on next page

Preparing SILAC™ Flex Medium

Prepare the SILAC™ Flex labeling medium containing dialyzed FBS and supplemented with 100 mg/mL L-Lysine, 100 mg/mL L-Arginine, 100X L-Glutamine, glucose, and phenol red using the basal SILAC™ Flex Medium (supplied with Cat. nos MS10030, MS10031, MS10032, and MS10033) as below.

Notes: Review the following notes prior to preparing the media.

- D-MEM-Flex Medium does not contain sodium pyruvate. Purchase sodium pyruvate separately from Invitrogen (page 58), if sodium pyruvate is required for cell growth.
- Do not add Phenol Red Solution to the medium if you are studying secreted proteins. If Phenol Red Solution is not added to the medium, monitor the pH of the medium or cell density.

  Caution: When handling Phenol Red Solution, avoid contact with skin and eyes.
- Supplemented SILAC™ Flex Medium contains 10% dialyzed FBS, except Advanced D-MEM/F-12-Flex Media which contains 0.5–2% dFBS. If higher concentration of dialyzed FBS is required for cell growth, purchase dialyzed FBS separately from Invitrogen (page 58).

  Caution: For SILAC™ Advanced D-MEM/F-12-Flex Media, human origin materials are non-reactive (donor level) for Anti-HIV 1 and 2, Anti-HCV, and HBs Ag. Handle in accordance with established biosafety practices.

Perform all steps in a tissue culture hood under sterile conditions and filter sterilize complete medium (see Step 8).

1. Resuspend 100 mg L-Lysine HCl and 100 mg [U-$^{13}C_6$]-L-Lysine (*Lys) each in 1 mL basal, unsupplemented medium supplied with the kit. Mix well until completely dissolved.
2. Resuspend 100 mg L-Arginine from each vial (2 vials are supplied in the kit) in 1 mL basal, unsupplemented medium each supplied with the kit. Mix well.

   Note: If you are using double labeled arginine (available separately from Invitrogen), resuspend 100 mg [U-$^{13}C_6$, $^{15}N_4$]-L-Arginine (*Arg) or 100 mg [U-$^{13}C_6$]-L-Arginine (*Arg) in 1 mL basal, unsupplemented medium supplied with the kit. Mix well.
3. Remove the appropriate amount of medium from each 1 L SILAC™ Flex Media bottle and add the components listed on the following page to prepare the supplemented medium in a final volume of 1 L.

Continued on next page

Preparing SILAC™ Flex Medium, Continued

Reagent	D-MEM-Flex	RPMI-Flex	IMDM-Flex	Advanced D-MEM-F/12-Flex
SILAC™ Glucose Solution (200 g/L)	High Glucose 22.5 mL Low Glucose 5 mL	10 mL	22.5 mL	15.8 mL
L-Glutamine 200 mM (100X)	20 mL	10 mL	20 mL	20 mL
SILAC™ Phenol Red Solution (10 g/L)	1.5 mL	0.5 mL	1.5 mL	0.8 mL
FBS, Dialyzed	100 mL	100 mL	100 mL	5–20 mL
Penicillin-Streptomycin (100X)	10 mL	10 mL	10 mL	10 mL

*Optional: Add 20 mL L-Glutamine for each 1L of medium (see **Note** on page 18)

4. Add L-Lysine HCl (100 mg/mL) from Step 1 and L-Arginine (100 mg/mL) from Step 2 to one 1 L bottle of medium from Step 3 to prepare light medium supplemented with Light (normal) lysine and arginine. Mix well and mark the bottle appropriately.
5. To the second 1 L bottle of medium from Step 3, add *Lys (100 mg/mL) from Step 1 and L-Arginine (100 mg/mL) from Step 2 to prepare D-MEM **single** labeling medium supplemented with light arginine and heavy (isotope labeled) lysine. Mix well and mark the bottle appropriately.

   Optional: If you are preparing **double** labeled medium, add *Lys (100 mg/mL) from Step 1 and *Arg (100 mg/mL) from Step 2 to prepare D-MEM double labeling medium supplemented with heavy (isotope labeled) arginine and lysine. Mix well and mark the bottle appropriately.
6. *Optional:* You may supplement the medium with additional growth factors or cytokines, if needed for your specific cell line.
7. After addition of the supplements (glucose, glutamine, and phenol red) to the basal Flex medium, the pH and osmolality is usually in the range below.

Target Range	D-MEM-Flex	RPMI-Flex	IMDM-Flex	Advanced D-MEM-F/12-Flex
pH Range	7.0–7.4	7.0–7.4	6.9–7.3	7.0–7.4
Osmolality Range (mOsm/kg)	High Glucose 320–350 Low Glucose 310–340	265–300	270–310	290–330

8. **Filter sterilize each medium using 0.22 µm filtration device.**
9. Store the medium at 2 to 8°C, protected from light until use. The medium is stable for 6 months when properly stored (avoid introducing any contamination into the medium).

Continued on next page

Isotopic Labeling in Cell Culture, Continued

Labeling and Cell Culture

Instructions for performing labeling with *Lys are described below.

1. Determine the viable and total cell count on an aliquot of cells using the trypan blue exclusion method.
2. Using the cell density determined in Step 1, transfer the appropriate volume of cell suspension in two separate sterile 15 mL conical tubes to obtain 1×10^5 cells per tube.
3. Centrifuge the cells at 1000 × *g* for 5 minutes at room temperature.
4. Aspirate the medium and resuspend the cells as follows:
   - **Tube 1:** Resuspend the cells in 3 mL medium containing **light lysine** (prepared as described on pages 23–24)
   - **Tube 2:** Resuspend the cells in 3 mL medium containing **heavy lysine** (prepared as described on pages 23–24)
5. Grow the cells separately as follows:
   - **Suspension Cells:** Transfer the cells into two separate T-25 tissue culture flasks containing 5–10 mL appropriate heavy and light medium with dFBS
   - **Adherent Cells:** Split the cells into two tissue culture dishes (60 mm × 15 mm) containing 3–5 mL appropriate heavy and light medium with dFBS
6. Incubate the flasks or dishes in a 37°C incubator containing a humidified atmosphere of 8% CO_2.
7. Change the medium or split the cells every 3–4 days (depending on the cell line) using the appropriate light or heavy medium.

   Note: Cells will grow at a similar rate in each media.
8. Expand each cell population for at least six doubling times to achieve >95% incorporation of labeled amino acid into the proteins.
9. After six doublings, harvest a small aliquot of cells (~1×10^6 cells) from each cell population to determine the efficiency of incorporation. Store the cell pellet at –80°C until use. See next page for details on sample processing.
10. At the end of six doublings, you will have 6.4×10^6 cells for each cell population. Based on the kit that you purchased and the number of cells needed for analysis (determined as described on page 17), you need:
    - ~2×10^6 cells for **membrane** protein analysis (Membrane Kit)
    - ~2×10^8 cells for **phosphoprotein** analysis (Phosphoprotein Kits)

    Note: You may freeze the remaining cells or continue to maintain or expand the two cell populations in the light or heavy medium if you wish to repeat the experiment.
11. Proceed to **Performing Cell Treatment** (next page, if needed) or **Harvesting Cells** (page 31).

Continued on next page

Performing Cell Treatment

Perform the cell treatment as described below. You may label the cells in light or heavy medium.

1. Determine the viable and total cell count using the trypan blue exclusion method.
2. Save an aliquot of cells as control prior to starting the treatment.
3. To either cell population, apply the desired treatment such as stimulation by growth factor, drug treatment, RNAi transfection, or induce cell differentiation.
4. Perform the treatment for the desired time (usually 5 minutes to several days depending on the treatment).
5. At the end of the treatment, proceed to **Harvesting Cells**, page 31.

Determining the Efficiency of Incorporation

To ensure >95% incorporation of the heavy amino acid into proteins, analyze small aliquots of cells (10^6) labeled with light or heavy amino acids and determine the efficiency of incorporation.

1. After six doublings, harvest a small aliquot of cells (~1×10^6 cells) from each cell population as described in Step 9, previous page.
2. Lyse each cell pellet separately in 500 µL 1X NuPAGE® LDS Sample Buffer and 50 µL NuPAGE® Reducing Agent (10X).
3. Heat the samples at 70°C for 8–10 minutes.
4. Load the samples from light and heavy medium side by side on a NuPAGE® Novex® 4–12% Bis-Tris Gel and perform electrophoresis using NuPAGE® Novex® MES or MOPS SDS Running Buffer. Be sure to load appropriate protein standards on the gel.
5. Stain the gel with Coomassie R-250 Stain.

   Note: Depending on your protein core facility, you may transfer the gel to the core facility to perform trypsin digestion and MS analysis. For more information on protein core facilities that offer MS analysis for SILAC™, see page 12.
6. Excise 3–4 side by side protein bands from each lane.
7. Perform in-gel trypsin digestion (page 39).
8. Perform MS analysis (page 44).

See next page for **Example of Results**.

Continued on next page

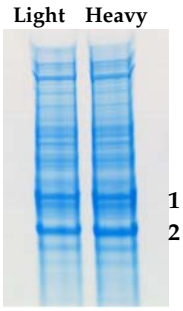
Example of Results

An example of results obtained after determining the efficiency of incorporation is shown below.

The MS analysis should show an increase in mass by 6 daltons for peptides labeled with *Lys when compared to peptides labeled with normal Lys (see figure below).

Note: If you have used double labeling with *Arg and *Lys, then the MS analysis should show an increase in mass by 6 and 10 daltons for peptides labeled with heavy *Lys and *[U-$^{13}C_6$, $^{15}N_4$]-Arg, respectively or 6 daltons for peptides labeled with heavy *Lys and *[U-$^{13}C_6$]-Arg, when compared to peptides labeled with normal (light) Lys and Arg.

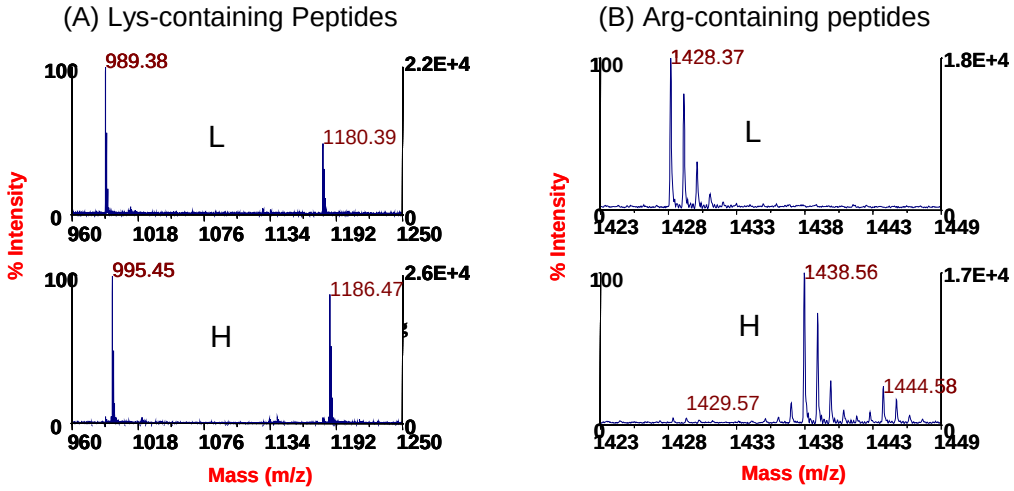
SDS-PAGE Analysis



Samples were lysed and analyzed by SDS-PAGE using NuPAGE® Novex® 4–12% Bis-Tris Gel as described on the previous page and stained with a Coomassie stain. Protein bands (1 and 2) were excised from each side by side lane and subjected to in-gel trypsin digestion and MS analysis (see below).

MS Analysis

MALDI-TOF MS analysis was performed on samples using the Voyager DE™-STR MALDI-TOF MS instrument.



L: light Lys or Arg
H: heavy [U-$^{13}C_6$] Lys or [U-$^{13}C_6$, $^{15}N_4$]Arg

Preparing Cell Lysates

Introduction

After performing cell labeling, harvest the cells and prepare cell lysates as described in this section. Choose the appropriate buffer for cell lysis as described below. To obtain the best results, use the lysis buffers supplied with each kit. Avoid using your own buffers.

Choosing the Lysis Buffer

The SILAC™ Phosphoprotein and Membrane Protein Kits are supplied with qualified lysis buffers to perform cell lysis. The pre-made buffers provide consistent results, optimal protein recovery, and eliminate the time required to prepare reagents. The buffers are compatible with downstream applications such as SDS-PAGE, immunoprecipitation, and affinity purification.

Based on the type of kit that you have purchased and the application that you wish to perform, choose the appropriate lysis buffer as described below.

SILAC™ Phosphoprotein Lysis Buffer A

This buffer is supplied with SILAC™ Phosphoprotein ID and Quantitation Kits. The Lysis Buffer A contains NP-40 detergent for cell lysis and is mainly used for analysis of cytosolic proteins. This buffer is compatible with downstream applications such as SDS-PAGE, immunoprecipitation, precipitating protein complexes, and affinity purification.

SILAC™ Phosphoprotein Lysis Buffer B

This buffer is supplied with SILAC™ Phosphoprotein ID and Quantitation Kits. The Lysis Buffer B contains stronger detergents such as SDS for cell lysis and is mainly used for analysis of cytosolic and membrane-associated proteins. This buffer is compatible with downstream applications such as SDS-PAGE and immunoprecipitation. **Do not** use this buffer if you wish to precipitate protein complexes as Lysis Buffer B includes SDS.

SILAC™ Membrane Protein Lysis Buffer

This buffer is supplied with SILAC™ Membrane Protein ID and Quantitation Kits. The Membrane Protein Lysis Buffer is a hypotonic lysis buffer and is used with 1.25 M sucrose solution for cell lysis. The buffer is used for analysis of membrane proteins. This buffer is compatible with downstream applications such as SDS-PAGE and immunoprecipitation.

Experimental Outline

1. Count the cells from each cell population after six doublings.
2. Harvest cells from each cell population using a method of choice.
3. Mix the cells from each cell population at 1:1 ratio based on the cell number.
4. Lyse cells using the buffers supplied in the kit.

Continued on next page

Preparing Cell Lysates, Continued

Materials Needed

- Appropriate Lysis Buffer stored on ice until use (see previous page for details on choosing the buffer)
  - SILAC™ Phosphoprotein Lysis Buffer A (supplied with the SILAC™ Phosphoprotein Kits) **or**
  - SILAC™ Phosphoprotein Lysis Buffer B (supplied with the SILAC™ Phosphoprotein Kits **or**
  - SILAC™ Membrane Protein Lysis Buffer (supplied with the SILAC™ Membrane Protein Kits) with Benzonase® Nuclease
- PBS, keep on ice until use (page 58)
- Reagents to determine viable and total cell counts (page 58)
- Centrifuge capable of centrifuging at 10,000 × *g*
- Ultracentrifuge capable of centrifuging at 100,000 × *g* and ultracentrifuge tubes (if using Lysis Buffer B and Membrane Lysis Buffer)

Additional materials needed with Membrane Protein Lysis Buffer

- 1.25 M sucrose solution in ultra pure water, stored on ice until use

  Note: Use high quality sucrose and water to prepare 1.25 M sucrose solution to prevent any keratin contamination.
- 4X NuPAGE® LDS Sample Buffer (58)
- NuPAGE® Sample Reducing Agent (10X, page 58)
- Dounce homogenizer or equivalent

Continued on next page

Harvesting Cells

After performing the labeling for six doubling times and performing the cell treatment, if appropriate, harvest cells from each cell population as below.

Based on the kit that you purchased and the number of cells needed for analysis (determined as described on page 17), you will need:

- ~2×10^6 cells for **membrane** protein analysis (Membrane Kit)
- ~2×10^8 cells for **phosphoprotein** analysis (Phosphoprotein Kits)

1. Determine the viable and total cell count on an aliquot of cells using the trypan blue method.
2. Harvest the required number of cells from each population using a suitable method for the cell line.
   - **For adherent cells:** Aspirate the growth medium from the culture plates. Wash the cells once with PBS. Remove the cells from the plate using trypsin or a rubber policeman. Wash the cells twice in PBS.
   - **For suspension cells**: Harvest the cells and centrifuge cells at 1000 × *g* for 5 minutes to pellet cells. Remove the growth medium. Wash the cells twice with PBS.
3. Resuspend the cell pellets in 1 mL chilled PBS.
4. Mix the cells grown in light (normal) medium and heavy (isotope labeled) medium in a 1:1 ratio based on the cell number.
5. Centrifuge the cells at 1000 × *g* for 5 minutes at 4°C to remove PBS.
6. Proceed immediately to cell lysis using the appropriate lysis buffers (see next page).

Note

If you have performed any type of cell treatment, be sure to lyse the control cells (from Step 2, page 27) using the same lysis method used for treated cells.

Using Phosphoprotein Lysis Buffers

Use the SILAC™ Phosphoprotein Lysis Buffer A and B for Phosphoprotein analysis. Each buffer is supplied in the SILAC™ Phosphoprotein ID and Quantitation Kits (Cat. nos. SP10001 and SP10005).

1. Resuspend the cell pellet from Step 5, previous page, in 8–10 mL SILAC™ Phosphoprotein Lysis Buffer A or B.
2. Mix well by pipetting up and down. After using the Lysis Buffer A or B, immediately return the remaining buffers to 4°C.
3. Centrifuge the lysate as follows:
   - If using Lysis Buffer A, centrifuge at 10,000 × *g* for 20 minutes at 4°C
   - If using Lysis Buffer B, centrifuge at 100,000 × *g* for 20 minutes at 4°C
4. The supernatant (lysate) contains the cytosolic proteins (if Lysis Buffer A was used), and cytosolic and membrane-associated proteins (if Lysis Buffer B was used). Save the pellet at –80°C, if you are interested in analysis of membrane proteins.
5. Proceed immediately to **Processing the Cell Lysate**, page 34.

Using Membrane Protein Lysis Buffer

Use the SILAC™ Membrane Protein Lysis Buffer for membrane protein analysis. The buffer is supplied in the SILAC™ Membrane Protein ID and Quantitation Kits (Cat. nos. SM10002 and SM10006).

1. To 50 mL of Membrane Protein Lysis Buffer, add 40 µL Benzonase® Nuclease (supplied in the kit). Mix well. Store the buffer on ice until use.
2. Resuspend the cell pellet from Step 5, previous page in 1.6 mL SILAC™ Membrane Protein Lysis Buffer.
3. Mix well by pipetting up and down. After using the Lysis Buffer, immediately return the remaining buffer to 4°C.
4. Incubate on ice for 30 minutes.
5. Homogenize the lysate on ice using a Dounce homogenizer or equivalent for 30 strokes.
6. Add 0.4 mL 1.25 M sucrose solution to the lysate and mix well by pipetting up and down 5 times.
7. Centrifuge the lysate at 500 × *g* for 10 minutes at 4°C to remove nuclear fraction. Remove the supernatant and discard the nuclear pellet.
8. Centrifuge the supernatant at 100,000 × *g* for 1 hour at 4°C to obtain the membrane pellet.
9. Carefully remove the supernatant and save the supernatant, if you are interested in analysis of cytosolic proteins.
10. Resuspend the membrane pellet in 30–60 µL 1X NuPAGE® LDS Sample Buffer. Add 3–6 µL NuPAGE® Sample Reducing Agent (10X).
11. Proceed to **Processing the Cell Lysate** (page 34) or store the pellet at –80°C for up to 2 months.

Continued on next page

Note

After preparing the lysates and depending on your protein core facility, you may transfer the lysates to the core facility to process the lysates, perform in-gel trypsin digestion, and MS analysis as described in this manual.

For more information on protein core facilities that offer MS analysis for SILAC™, see page 12.

Processing the Cell Lysate

Enriching Phosphoproteins

Phosphoproteins are low-abundant proteins and account for only 1–10% of the total cell protein in a cell. To obtain a complete profile of the phosphoproteins in the cell in the presence of other high-abundant proteins and for proper identification of the phosphorylated peptide, it is important to enrich or purify the phosphoproteins prior to analysis. Review the information on page 14 to choose the best option for your sample.

Recommended Methods for Protein Analysis

After preparing the lysates, process the lysates using the following recommended methods for membrane protein analysis and phosphoprotein enrichment for best results.

Quantification of proteins present in spots focused on two-dimensional gels can be subject to unusual migration influenced by ampholytes and salt in first dimension gel and may also be isoform-specific. To avoid these problems, do not use two-dimensional gel electrophoresis for SILAC™ sample analysis.

For SILAC™ Membrane Kits:

Analyze the membrane pellet from Step 10, page 32 using SDS-PAGE (page 37) followed by in-gel trypsin digestion (page 39). Avoid using two-dimensional gel electrophoresis for analysis of membrane proteins.

For SILAC™ Phosphoprotein Kits:

Enrich for phosphoproteins from the lysate using immunoprecipitation, affinity purification, or precipitating protein complexes of interest. See page 14 for more details.

Experimental Outline

1. Process the lysate using SDS-PAGE (see above for recommended methods).
2. Stain the SDS-PAGE gel using Coomassie or silver staining.
3. Excise the bands of interest from the gel or cut the gel into 40 equal pieces.
4. Perform in-gel trypsin digestion.

Continued on next page

Materials Needed

You will need the following items. Ordering information is on page 58.

- NuPAGE® Novex® Bis-Tris Gel (see **Note** below)
- NuPAGE® MES/MOPS SDS Running Buffer
- NuPAGE® Sample Reducing Agent (10X)
- NuPAGE® LDS Sample Buffer (4X)
- NuPAGE® Antioxidant
- XCell *SureLock*™ Mini-Cell for electrophoresis of the gel
- Sterile tubes
- Antibody for immunoprecipitation (see previous page)
- Protein A or Protein G Agarose (for immunoprecipitation)
- Sequencing grade trypsin
- 25 mM ammonium bicarbonate buffer, pH 8.0 for trypsin digestion
- 5% formic acid (FA)
- 100% and 70% (v/v) acetonitrile

Note

To obtain the best results, we recommend using NuPAGE® Novex® Bis-Tris Gels. You may use Novex® 4–20% Tris-Glycine Gel or any other SDS/PAGE gel of choice for performing SDS/PAGE. Use an appropriate percentage of acrylamide gel that best resolves your proteins of interest.

Important

Due to the large variety of antibodies that can be used for immunoprecipitation, it is not possible to have a single immunoprecipitation protocol that is suitable for all antibodies. Use the immunoprecipitation procedure from this section as a starting protocol and based on your initial results, empirically determine the immunoprecipitation protocol by optimizing the antibody concentration, buffer formulation, wash stringency, and incubation time.

If you have an optimized immunoprecipitation protocol for a specific antibody, use the optimized protocol.

Continue don next page

Immuno-precipitation

Immunoprecipitation protocol to enrich for phosphoproteins using Protein G Agarose is described below. You may use Protein A beads, if desired.

1. To 10 mL lysate from Step 4, page 32, add 15 µL Protein-G Agarose slurry (50% slurry in lysis buffer) per 1 mL lysate to pre-clear the lysate.
2. Rock the lysate at 4°C for 1 hour.
3. Centrifuge at 10,000 × *g* for 1 minute at 4°C.
4. Transfer the supernatant to a sterile tube and place on ice.
5. Add 50–100 µg of the anti-phosphotyrosine antibody or antibody against the phosphoprotein of interest.

   Note: You may optimize the amount of antibody used based on the initial results.
6. If the antibody is already coupled to Protein A or Protein G agarose, proceed to Step 8 directly.
7. Add 100 µL of the Protein-G Agarose slurry to the supernatant.
8. Rock for 8–16 hours at 4°C.
9. Centrifuge at 10,000 × *g* for 5 minutes at 4°C. Remove the supernatant.
10. Wash the agarose pellet twice with 1 mL SILAC™ Phosphoprotein Lysis Buffer A or B.
11. Resuspend the pellet in 50 µL 1X NuPAGE® LDS Sample Buffer. Add 5 µL NuPAGE® Sample Reducing Agent (10X).
12. Heat the sample at 70°C for 8–10 minutes.
13. Centrifuge the sample for 1 minute at 10,000 × *g* and load supernatant onto a NuPAGE® Novex® Bis-Tris Gel and analyze the protein immune complexes using SDS-PAGE, next page.

Continue d on next page

Analyzing Protein Complexes

Instructions for analyzing protein complexes in solution using protein specific antibodies and Protein G Agarose are described below. You may use Protein A beads, if desired.

1. To 10 mL lysate from Step 4, page 32, add 30–50 µg of the bait protein that allows binding to the protein complex.

   Note: You may optimize the amount of protein used based on the initial results.
2. Add 20–50 µL epitope-tagged resin such as GST agarose or Streptavidin agarose to precipitate the protein complex, if your protein of interest contains an expressed GST tag or a biotin tag.
3. Rock for 2–24 hours at 4°C.
4. Centrifuge at 10,000 × *g* for 5 minutes at 4°C. Remove supernatant.
5. Wash the pellet twice with 1 mL SILAC™ Phosphoprotein Lysis Buffer A.
6. Resuspend the pellet in 16–20 µL 1X NuPAGE® LDS Sample Buffer and add 2 µL of NuPAGE® Sample Reducing Agent (10X).
7. Heat the sample at 70°C for 8–10 minutes.
8. Centrifuge the sample for 1 minute at 10,000 × *g* and load supernatant onto a NuPAGE® Novex® Bis-Tris Gel and analyze the protein immune complexes using SDS-PAGE, below.

SDS-PAGE Analysis

The following procedure uses NuPAGE® Novex® Bis-Tris Gels with the XCell *SureLock*™ Mini-Cell. If you are using any other electrophoresis system, refer to the manufacturer's recommendations.

1. Assemble the gel cassette/Buffer Core sandwich as described in the XCell *SureLock*™ Mini-Cell manual (download the manual from www.invitrogen.com). If you are using only one gel, use the Buffer Dam to replace the second gel cassette.
2. Fill the Lower Buffer Chamber and Upper Buffer Chamber with the recommended volume of 1X NuPAGE® MES or MOPS SDS Running Buffer. Add 0.5 mL of NuPAGE® Antioxidant to the Upper Buffer Chamber.
3. Load the processed samples and load protein molecular weight standards in a different well.
4. Place the XCell *SureLock*™ Mini-Cell lid on the Buffer Core. With the power on the power supply turned off, connect the electrode cords to the power supply.
5. Perform SDS-PAGE at 200 V for 40–50 minutes for NuPAGE® Novex® Bis-Tris Gel.
6. At the end of electrophoresis, turn off the power and disassemble the gel cassette/Buffer Core sandwich assembly as described in the XCell *SureLock*™ Mini-Cell manual.
7. Proceed to gel staining, next page.

Continued on next page

Staining the Gel

After SDS-PAGE, stain the gel with a protein stain to visualize the protein bands. Use a Coomassie stain such as SimplyBlue™ SafeStain for staining or silver stain such as SilverQuest™ Silver Staining Kit for staining low abundant proteins

SimplyBlue™ SafeStain is a ready-to-use, proprietary Coomassie G-250 stain that is specially formulated for fast, sensitive detection and safe, non-hazardous disposal. Proteins stained using the SimplyBlue™ SafeStain are compatible with mass spectrometry analysis. Refer to the manual supplied with stain for protocol details. See page 58 for ordering information.

SilverQuest™ Silver Staining Kit provides a rapid and easy method to silver stain proteins in polyacrylamide gels. This kit is specifically designed to provide sensitive silver staining compatible with mass spectrometry analysis. The SilverQuest™ Silver Staining Kit includes destaining solutions that effectively remove silver ions from protein bands in polyacrylamide gels. This improves trypsin digestion and subsequent mass spectrometry coverage of the protein, as silver ions are known to inhibit trypsin digestion of proteins (Chambers *et al.*, 1974). Refer to the manual supplied with stain for protocol details. See page 58 for ordering information.

Note: If you are destaining the gel using the destaining solutions included in the SilverQuest™ Kit, wash the gel piece thoroughly with ultrapure water until the gel piece is completely destained, no yellow color is visible before trypsin digestion.

Note

After staining the gel, you may transfer the stained gel to the core facility to perform in-gel trypsin digestion and MS analysis as described in this manual.

If you wish to stain the gel and perform in-gel trypsin digestion, follow the protocol described on the next page.

For more information on protein core facilities that offer MS analysis for SILAC™, see page 12.

Follow the guidelines below for trypsin digestion to obtain the best results:

- Always use sequencing/proteomics grade trypsin for MS analysis (page 58)
- Always prepare the trypsin digestion buffer (25 mM ammonium bicarbonate buffer, pH 8.0) using ultra pure reagents and water
- Avoid touching the gel with bare hands to prevent contamination from keratin
- Be sure to use polypropylene microcentrifuge tubes and HPLC grade solvents to avoid any contamination from polymers

Continued on next page

In-gel Trypsin Digestion

A general protocol for in-gel trypsin digestion is provided below. You may use any method of choice or a method recommended by your protein core facility. For more information, refer to published reference sources (Coligan *et al.*, 1998; Helmann *et al.*, 1995).

Note: The digestion protocol given below is generally used for protein identification. If you need more protein coverage, you may need to perform reduction and alkylation of peptides (Shevchenko *et al.*, 1996).

1. Rinse the stained gel in water for 10 minutes to remove any particulate material.
2. Excise the desired gel band from the stained gel. Mince the excised gel piece into smaller pieces (1 mm × 1 mm). Transfer the gel pieces to a clean microcentrifuge tube.
3. Add 500 µL 50% acetonitrile/25 mM ammonium bicarbonate, pH 8.0. Incubate at room temperature for 15 minutes for destaining the gel pieces, discard the supernatant carefully without removing the gel pieces.
4. Repeat Step 3 until the gel pieces are sufficiently destained.
5. Add 200 µL 100% acetonitrile to dehydrate the gel pieces.
6. Incubate for 5–10 minutes at room temperature, discard the supernatant carefully without removing the gel pieces.
7. Dry the gel pieces in a centrifugal vacuum concentrator (e.g., Thermo Savant SpeedVac® centrifuge).
8. Add enough trypsin solution (10 ng/µL dissolved in 25 mM ammonium bicarbonate, pH 8.0,) to cover the gel pieces.
9. Incubate on ice for at least one hour to allow the trypsin solution to penetrate the gel pieces. The cold temperature helps to prevent autolysis of the trypsin.
10. Incubate overnight at 37°C.
11. Add 25 µL 5% formic acid (FA), and incubate for 30 minutes at room temperature.
12. Vortex for 30 seconds, centrifuge at 14,000 × *g* for 1 minute, and collect the supernatant.
13. Add 25 µL 5% FA, 50% acetonitrile, and incubate for 30 minutes at room temperature.
14. Vortex for 30 seconds, centrifuge at 14,000 × *g* for 1 minute, and collect the supernatant, pooling it with the supernatant from Step 12.
15. Concentrate the supernatant using a centrifugal vacuum concentrator to ~5 µL. **Do not allow the samples to dry out.**

The Next Step

For phosphoprotein analysis, you may further enrich for phosphopeptides after in-gel trypsin digestion by purification of tryptic peptides using the PiMAC™ Resin included in the phosphoprotein kits.

For membrane protein analysis, proceed directly to MS analysis after trypsin digestion. Submit your tryptic peptides to the protein core facility for analysis.

Purifying Phosphopeptides

Introduction

Instructions for purification of phosphorylated peptides from the tryptic peptide mix are described below. If you are interested in identifying the phosphorylation sites on the protein of interest, we recommend that you enrich the phosphopeptides prior to MS analysis.

Note

- Use the following protocol for purification of phosphopeptides using PiMAC™ resin included with the SILAC™ Phosphoprotein ID and Quantitation Kits after in-gel trypsin digestion. This method results in packing the resin in a very narrow column that allows the elution of purified phosphopeptides in a very small volume of 4–6 µL that can be directly used for MS analysis.
- Some protein core facilities may offer to purify the phosphopeptides using the recommended protocol below. Check with the core facility prior to purification.
- You may use other resins to purify the phosphoproteins, if you have an optimized protocol available.

Experimental Outline

1. Insert a frit (small piece of PiMAC™ Filter) at the narrow end of the gel loading tip.
2. Pack the PiMAC™ Resin into the gel loading tip.
3. Charge the resin with $FeCl_3$ solution.
4. Wash off the excess $FeCl_3$.
5. Load tryptic peptide sample onto the column.
6. Wash the resin with acetic acid to remove unbound materials.
7. Elute phosphopeptides with ammonium hydroxide solution.

Materials Needed

- PiMAC™ Resin (included with the kit)
- Gel Loading Tips (Eppendorf, Cat. no. 0030 001.222)

  Note: The gel loading tip from Eppendorf has a thin 15 mm capillary of <0.3 mm diameter and is recommended to prepare the column. You may use any equivalent gel loading tip with narrow capillary ends of similar diameter such that after packing the column, the elution volume should not be >6 µL.
- PiMAC™ Filter (polyethylene, 15 × 45 micron, 1/15 inches thick, fine sheet, included with the kit)
- 100 mM ferric chloride ($FeCl_3$) in ultrapure water
- 0.1% acetic acid and 0.1% acetic acid containing 25% acetonitrile
- 100 mM ammonium hydroxide
- Razor blade
- Thin column tubing (360 microns) for guiding the frit into the tip

Continued on next page

Purifying Phosphopeptides, Continued

Follow the recommendations below to obtain the best results:

- Wear gloves and laboratory coat while performing the purification protocol
- Always use ultra pure reagents and water to prepare buffer
- **Do not** allow the resin to dry once packed into the tip.
- Always maintain a thin layer (~ 1 mm) of liquid over the resin
- Be sure to use polypropylene microcentrifuge tubes and HPLC grade solvents to avoid any contamination from polymers
- Avoid touching the gel loading tip with bare hands to prevent contamination from skin keratin

Preparing the Column Frit

1. Cut the PiMAC™ Filter supplied with the kit into a small 0.3 mm × 0.3 mm piece using a clean razor blade or scalpel (Fig. A).
2. Chop the PiMAC™ Filter piece into smaller pieces to use as a column frit. The frit should be able fit into the narrow end of the gel loading tip that will be used to pack the column (Fig A).
3. Hold the gel loading tip in your hand and add a few drops of water using another gel loading tip fitted onto a pipettor to wet the gel loading tip in your hand. Wetting the gel loading tip that will be used for column packing allows the PiMAC™ Filter piece to easily slide into the narrow end of the tip.
4. Using a clean, wet gel loading tip, pick up the PiMAC™ Filter frit from Step 2 and transfer the frit into the wet gel loading tip that will be used as a column (Fig. B, indicated with a circle).
5. Use a narrow column tubing to push the frit into the narrow end of the gel loading tip such that the frit is ~3 cm from the bottom to prepare a column containing a frit (Fig. C, indicated with a circle).

Figure A

Figure B

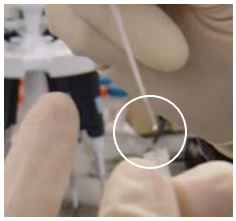
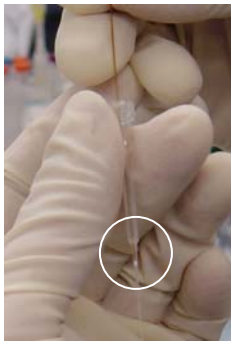


Figure C



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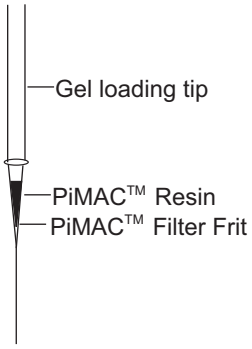
Purifying Phosphopeptides, Continued

Preparing the Column

1. Add 5–10 µL of water to the frit and place the column onto a pipette tip rack.
2. Thoroughly resuspend the PiMAC™ Resin.
3. Add 2–6 µL PiMAC™ Resin into the column under the aqueous layer using a thin gel loading tip.
4. Place a repeater pipettor fitted with 200 µL-pipette tip (cut off the end of the tip) on top of the column and slowly apply pressure to push the water out of the column and pack the resin into the column. Adjust the repeater pipettor to obtain a drop rate of 1 drop/6 seconds.

   Using regular pipettes to apply pressure is not effective as the resin is packed into a very thin gel loading tip.
5. Do not push out all the liquid. Always maintain the column with a thin layer of liquid. **Do not allow the column to dry anytime during the entire purification procedure.**
6. Immediately proceed to the purification procedure, next page.

An example of the packed column in the gel loading tip is shown below.



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Purifying Phosphopeptides, Continued

Purification Procedure

1. Charge the column with ferrous chloride by adding 30 µL 100 mM $FeCl_3$ to the column.
2. Allow the liquid to enter into the column using a repeater pipettor as described on the previous page.
3. Repeat Steps 1–2 with additional 30 µL 100 mM $FeCl_3$.
4. Wash excess $FeCl_3$ using 3 washes of 30 µL 0.1% acetic acid, each. Perform all washing by holding the column in your hand and pushing the liquid using a repeater pipettor. The column appears yellow after the washing step.
5. Load ~5–10 µL tryptic peptide sample (Step 14, page 39) onto the column.
6. Wash the column as follows:
   - First wash with 30 µL 0.1% acetic acid
   - Second wash with 30 µL 0.1% acetic acid containing 25% acetonitrile
7. Elute the phosphopeptides with 4–6 µL 100 mM ammonium hydroxide solution into a sterile, small PCR tube or directly onto a MALDI plate (if performing MALDI-TOF analysis).

   Note: If you are eluting into a MALDI plate, be sure the MALDI plate contains a MALDI matrix.
8. Transfer the samples to the core facility for MS analysis, next page.

Mass Spectrometric Analysis

Introduction

General guidelines for performing MALDI-TOF MS and LC-MS analysis of tryptic digested peptides (page 39) or purified phosphopeptides (page 43) are described in this section.

For details on the use of various MS instruments for analysis, refer to the manual supplied with the instruments.

Important

This section is designed for experienced users of MALDI-TOF and LC-MS analysis, especially core facility personnel that are familiar with standard techniques and instruments for MS analysis. General recommendations are included but detailed protocols for using the MS instruments are not included.

If you are a first time user of MS instruments, refer to the manuals supplied with the instrument for details or contact a protein core facility for MS analysis (see page 12).

General Guidelines

Basic guidelines for sample preparation are given below. The choice of matrix and the amount of sample needed for mass spectrometry analysis depends on the technique used for analysis and the individual protein sample.

For more details on sample preparation, contact your protein core facility. For more information, refer to published protocols (Ausubel *et al.*, 1994; Coligan *et al.*, 1998; Peter, 2000; Simpson, 2003; Speicher, 2004).

- Sample concentration of 200–500 nM in a total volume of ~5 µL
- Prepare samples preferably in ultrapure water, methanol, or acetonitrile
- Sample must contain <10 mM buffer or salts

Invitrosol™ LC/MS Protein Solubilizer

The Invitrosol™ LC/MS Protein Solubilizer is a novel surfactant blend that maintains a variety of hydrophobic proteins in solution, does not interfere with protease activity, and is compatible with reverse-phase high-pressure liquid chromatography (RP-HPLC) and LC-coupled electrospray ionization/mass spectrometry (ESI/MS) separations of the tryptic digested peptides. Use Invitrosol™ LC/MS Protein Solubilizer to remove incompatible buffer components prior to MS analysis or during in-gel trypsin digestion to improve the solubility of hydrophobic tryptic peptides.

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MS Reagents

A variety of reagents for MS analysis are available from Invitrogen (see page 58 for ordering information).

Invitrosol™ LC/MS Protein Solubilizer

The Invitrosol™ LC/MS Protein Solubilizer is a novel surfactant blend that maintains a variety of hydrophobic proteins in solution, does not interfere with protease activity, and is compatible with reverse-phase high-pressure liquid chromatography (RP-HPLC) and LC-coupled electrospray ionization/mass spectrometry (ESI/MS) separations of the tryptic digested peptides. Use Invitrosol™ LC/MS Protein Solubilizer to remove incompatible buffer components prior to MS analysis or during in-gel trypsin digestion to improve the solubility of hydrophobic tryptic peptides.

Invitrosol™ MALDI Protein Solubilizer Kit

The Invitrosol™ MALDI Protein Solubilizer Kit is specifically designed for direct MALDI-TOF MS analysis of hydrophilic or hydrophobic intact proteins and peptides eliminating the need for solid phase extraction, acid hydrolysis, and matrix crystal washing. The Invitrosol™ MALDI Protein Solubilizer A and B are ready-to-use reagents composed of unique, proprietary detergent formulations that are designed to minimize suppression effects on the ionization of peptides/intact proteins and minimize cluster formation, and effectively solubilize hydrophobic proteins and improves sequence coverage of tryptic peptides in solution without affecting the sensitivity.

Continued on next page

Recommended Methods for MS Analysis

The tryptic peptides (page 39) or purified phosphopeptides (page 40) can be analyzed using the following MS analysis methods:

Important: For identifying and quantitating proteins using SILAC™ Technology, it is important to perform MS analysis using appropriate instruments that are capable of performing MS/MS analysis.

- For samples with less complexity use MALDI-TOF MS analysis. We routinely use 4700 Proteomics Analyzer (MALDI-TOF/TOF® instrument) from Applied Biosystems. Other instruments such as Bruker Reflex III (Bruker Daltonics) or Voyager-DE™ STR MALDI TOF Workstation (Applied Biosystems) are also suitable.
- For complex samples use on-line or off-line LC-MS/MS or two-dimensional LC-MS/MS. You may use Micromass Q-Tof Premier™ Mass Spectrometer (Waters) or QSTAR® Pulsar quadrupole TOF tandem MS (Applied Biosystems) equipped with a nanoelectrospray ion source.

  Some recommended gradients for LC-MS are listed below.

Note

Depending on the type of MS instrument that you have, you may be able to:

- Perform fully automated analysis of SILAC™ raw data. This is supported through the MS instrument software for protein identification and quantitation (for example, the AB/MDS Sciex Family of MALDI TOF/TOF® Analyzers with GPS Explorer™ software)

OR

- Perform semi-automated analysis of SILAC™ raw data. This is supported through the MS instrument software for protein identification but you will need to perform protein quantitation using manual calculations as described on page 57 or consult the instrument vendor for details.

Recommended Gradients for LC-MS

If you are using LC-MS analysis, the following gradients are recommended. If you optimized the LC-MS analysis with specific gradients that are suitable for your analysis, use the optimized gradients for your analysis.

- For samples with less complexity, use a gradient of 5–45% (v/v) acetonitrile in 0.1% formic acid (or TFA) over 45 minutes and then use a gradient 45-95% acetonitrile in 0.1% formic acid (or TFA) over 5 minutes.

  Note: Use 0.1% formic acid solution on ESI based instruments and 0.1% TFA solution on off-line LC-MS/MS analysis using MALDI-TOF/TOF®.
- For a complex sample, use a gradient of 5–45% (v/v) acetonitrile, 0.1% formic acid (or TFA) over 90 minutes or up to 120 minutes, and then use a gradient of 45–95% acetonitrile, 0.1% formic acid (or TFA) over 30 minutes or up to 60 minutes.

Protein Identification and Quantitation

Important

This section is designed for experienced users of MALDI-TOF and LC-MS analysis, especially core facility personnel that are familiar with standard techniques and instruments for MS analysis.

If you are a first time user of MS instruments, refer to the manuals supplied with the instrument for details or contact a protein core facility for MS analysis (see page 12).

Note

- Be sure to always compare the results with cells grown in the light and heavy medium containing each amino acid at the same concentration.
- The screen shots included in this section are provided as guidelines and may not represent the exact screen that you may view for the software, if the software has been upgraded. These screen shots were captured using GPS Explorer™ 3.x software.

Protein Identification

Protein identification is performed by searching the peptide sequences obtained after MS analysis against non-redundant protein databases.

Most of the MS instruments are supplied with software that is capable of protein identification. You may use the instrument software to perform protein identification.

The protein identification method for SILAC™ kits was developed by processing the raw MS data files from MS with Mascot Distiller (Matrix Science, London) and then searched the NCBI database using Mascot search algorithm. Our results have shown that using Mascot to identify proteins provides ~40% better results than compared to other protein identification methods.

Certain MS instruments contain softwares that perform protein identification using the Mascot search algorithm. For example, the GPS Explorer™ 3.0 software with AB/MDS Sciex Family of MALDI TOF/TOF® Analyzers.

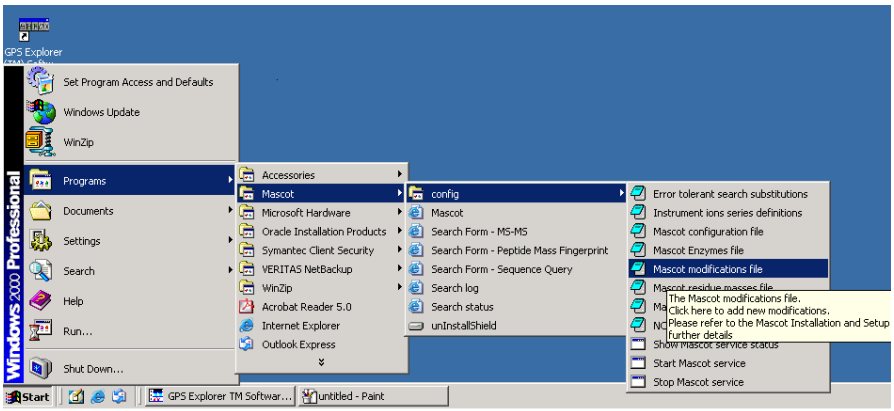
For more information on Mascot Distiller, visit www.matrixscience.com.

Continued on next page

Using Mascot for Protein Identification

Brief instructions are provided below to set up the Mascot server settings for protein identification using GPS Explorer™. For installation, set up, and detailed instructions on using Mascot, visit www.matrixscience.com.

1. Start GPS Explorer™ software on your MS instrument (AB/MDS Sciex Family of MALDI TOF/TOF® Analyzers).
2. Start Mascot server on your local computer and navigate to the Mascot Modification File screen (Mascot>Configuration>Mascot Modification Files).



3. Add the following text at the end of the Mascot Modification File to enable identification of proteins and isotopic peptide pairs for SILAC™.

   "

   Title: Lys_light
   Residues: K 128.09497 128.1741
   "

   Title: Lys_heavy
   Residues: K 134.09497 134.1741
   "

   Title: Arg_light
   Residues: R 156.10112 156.1875
   "

   Title: Arg_heavy
   Residues: R 166.10112 166.1875
   "

   This will show isotope labeled lysine (heavy lysine) 6 Da larger than normal lysine (light lysine) and isotope labeled arginine (heavy arginine) 10 Da larger than normal arginine (light arginine).
4. Set the mass tolerance of the precursor peptide ion at 200 ppm and mass tolerance for the MS/MS fragment ions at 0.5 Da.
5. Select the variables modification in the setting for data analysis as follows depending on the type of labeling experiment:
   - For a single label experiment with *Lys, select a pair of light and heavy Lys as variables
   - For a double label experiment with *Lys and *Arg, select a pair of light and heavy Lys and a pair of light and heavy Arg

An example of the Mascot search result is shown on the next page.

Continued on next page

Protein Identification and Quantitation, Continued

Example of Mascot Search Result

The Mascot search result will show identities of proteins and the output will show peptides labeled with light or heavy Lys and/or Arg as shown below.

Peptide Summary Report

Switch to Protein Summary Report

To create a bookmark for this report, right click this link: Peptide Summary Report (SampleSetID: 343, AnalysisID: 818, Path=\Xiqu Membrane\msilacexp3bqand12jrk\lysargmsms)

Select All | Select None | Search Selected | Error tolerant | Archive Report

1. gi|5453832 **Mass:** 111266 **Total score:** 729 **Peptides matched:** 37
oxygen regulated protein precursor; oxygen regulated protein (150kD) [Homo sapiens]
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Rank	Peptide
23	1005.51	1004.50	1004.54	-0.05	0	(36)	1	YFQHLLGK
47	1011.54	1010.53	1010.54	-0.01	0	48	1	YFQHLLGK + Lys_heavy
48	1011.55	1010.54	1010.54	-0.00	0	(35)	1	YFQHLLGK + Lys_heavy
49	1012.54	1011.53	1011.55	-0.01	0	(41)	1	EVQYLLNK + Lys_heavy
50	1012.56	1011.55	1011.55	0.00	0	48	1	EVQYLLNK + Lys_heavy
131	1041.49	1040.48	1040.48	0.00	1	15	7	KYPDYESK + 2 Lys_heavy
161	1047.53	1046.53	1046.55	-0.03	0	(32)	1	LAGLFNEQR
162	1047.54	1046.53	1046.55	-0.02	0	(23)	1	LAGLFNEQR
174	1053.55	1052.54	1052.55	-0.01	0	70	1	LAGLFNEQR + Arg_heavy
175	1053.56	1052.55	1052.55	-0.00	0	(19)	9	LAGLFNEQR + Arg_heavy
176	1053.56	1052.55	1052.55	-0.00	0	(39)	1	LAGLFNEQR + Arg_heavy
178	1053.56	1052.56	1052.55	0.00	0	(9)	6	LAGLFNEQR + Arg_heavy
352	1124.58	1123.58	1123.58	-0.00	0	(29)	1	TLGGLEMELR + Arg_heavy
353	1124.59	1123.58	1123.58	-0.00	0	39	1	TLGGLEMELR + Arg_heavy
656	1250.60	1249.60	1249.59	0.01	0	79	1	FFGDSAASMAIK + Lys_heavy
708	1276.66	1275.65	1275.65	-0.00	0	39	1	EAGMQPQLQIR + Arg_heavy

Continued on next page

Protein Identification and Quantitation, Continued

Protein Quantitation

Once protein identification is complete using Mascot or other instrument specific software, perform quantitation for differential protein expression.

Protein quantitation is performed using either of the following two methods and depends on the type of software available with your MS instrument:

- GPS Explorer™ software available for ICAT® reagent analysis capability using the AB/MDS Sciex Family of MALDI TOF/TOF® Analyzers (see below for details) is suited to perform fully automated analysis of SILAC™ raw data for protein identification and quantitation

OR

- Manual quantitation (see page 57 for details) of SILAC™ raw data if the MS instrument does not support ICAT® reagent quantitation.

GPS Explorer™

GPS Explorer™ 3.0 software is innovative applications software that supports many biological workflows such as traditional in-gel digestion, MDLC/MS/MS (LC MALDI), and PTM discovery all with intelligent results dependent analysis using RDA™ software feature.

Invitrogen has currently approved only the GPS Explorer™ software (Applied Biosystems) available for ICAT® reagents as suitable for quantitation of SILAC™ data. The raw data files from the AB/MDS Sciex Family of MALDI TOF/TOF® Analyzers are processed using GPS Explorer™ software with Mascot to quantitate differential protein expression.

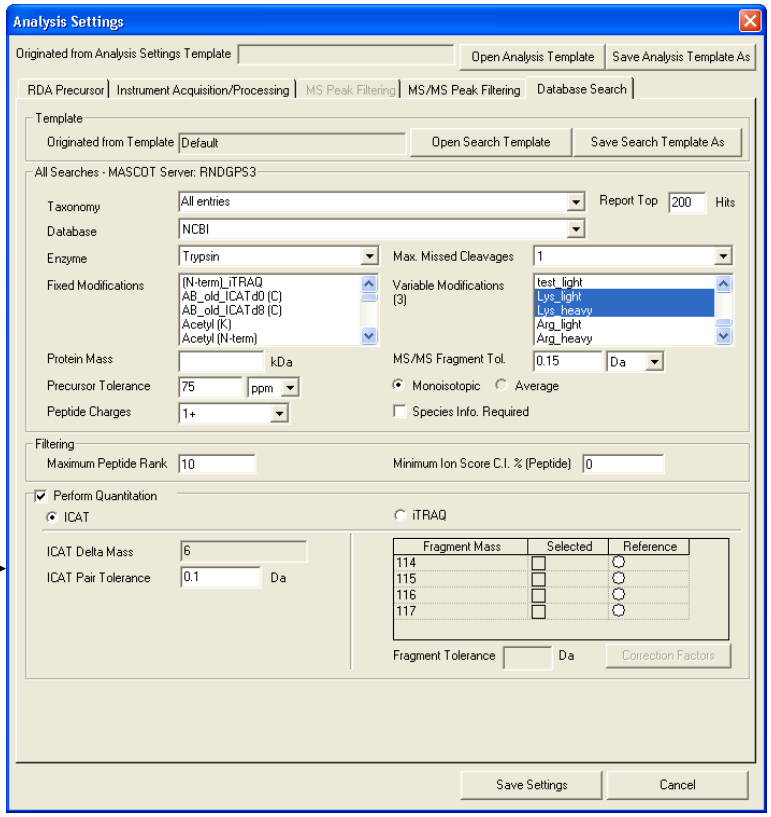
Currently, only one pair of light and heavy Lys or Arg at a time can be selected for quantification using GPS Explorer™ software.

Continued on next page

Using GPS Explorer for Protein Quantitation

Brief instructions are provided below to set up the GPS Explorer software for SILAC™ data analysis. For details on using the software, follow the manufacturer's instructions.

1. Start GPS Explorer™ software on the MS instrument (AB/MDS Sciex Family of MALDI TOF/TOF® Analyzers).
2. Navigate to the Data Analysis screen.
3. Select the following as variable modifications in the Analysis Settings Screen as shown in the figure below:
   - For a single label experiment with *Lys, select a pair of light and heavy Lys as variables. Ensure the mass difference of 6 Da shows up at the bottom of the screen in ICAT® Delta Mass under ICAT® Settings.
   - For a double label experiment with *Lys and *Arg, select a pair of light and heavy Lys or a pair of light and heavy Arg. Simultaneous selection of both labeled Lys and Arg does not work. Ensure the following mass difference shows up at the bottom of the screen in ICAT® Delta Mass under ICAT® Settings (indicated with an arrow in the figure below):
     - 6 Da mass difference when *Lys is selected **or**
     - 10 Da mass difference when *Arg is selected
4. Make sure the ICAT® Quantification box is checked and the ICAT® Pair Tolerance is set to 150 ppm under ICAT® Settings (see figure below).



An example of results using the GPS Explorer™ software is shown on the next page.

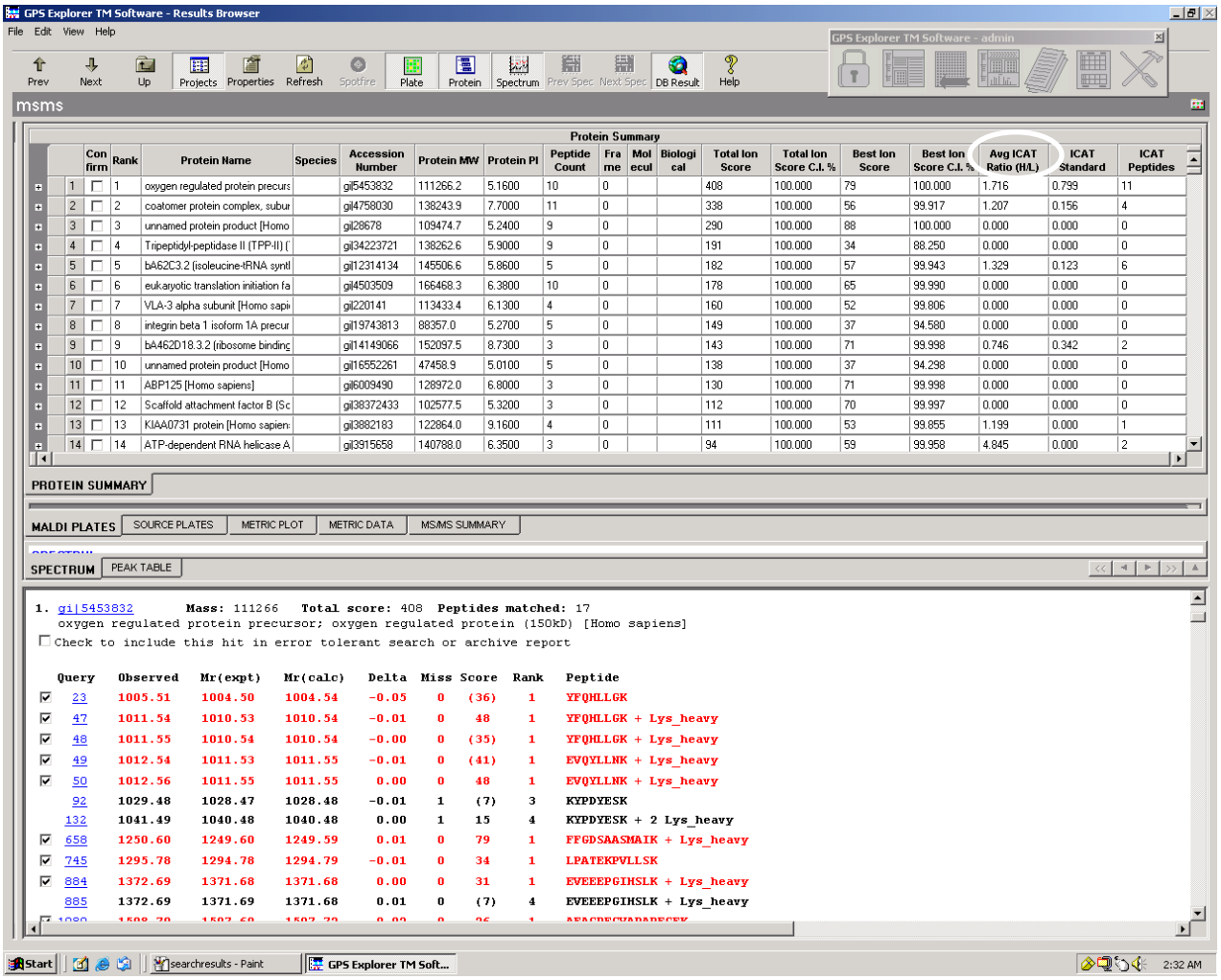
Continued on next page

Protein Identification and Quantitation, Continued

Example of GPS Explorer™ Analysis Results

An example of quantitation result based on a pair of light and heavy Lys peptides obtained after analysis using GPS Explorer™ software is shown below.

To view the quantitation results, review the data in the column Avg ICAT® Ratio (H/L) indicated with a circle in the figure below. For down regulated proteins, the ratio will be less than 1 and for up regulated proteins, the ratio will be greater than 1. See next page for details on interpreting the results.



Continued on next page

Interpreting the Results

To analyze differential protein expression results with SILAC™ experiments, review the data in the column, Avg ICAT® Ratio (H/L) as shown in the example of GPS Explorer™ results (indicated with a circle) on the previous page.

The ratio indicates up regulation or down regulation for various proteins analyzed. For example, the protein oxygen regulated protein precursor shows an Avg ICAT® Ratio (H/L) of 1.76 suggesting that this protein may be up regulated while the protein bA462D18.3.2 (ribosome binding protein) shows a ratio of 0.746 suggesting that this protein may be down regulated.

An Avg ICAT® Ratio (H/L) ratio of zero indicates the following:

- Only 1 peak from the peak pair was identified and therefore the software was unable to calculate a ratio. This happens when the signal for the peptide is very low (for low abundant peptides) and is sometimes occluded by background or if the ratio of peak pair relative abundance is very high (>10) or very low (<0.3). For such peptides, it is important to go back to the original raw data file and confirm manually.
- The cell treatment may have lowered the expression of the peptide in treated cells such that the software is unable to identify the signal as a significant signal.

The results are significant if the coefficient of variance (CV) is <30%. A significant variance amongst the peptides correlated to the same protein may indicate the following:

- The protein identification was incorrect
- Co-elution of an unrelated isobaric peptide distorted the peak profile
- Certain residues occurring in the peptide outlier are subject to metabolic interconversion (for example, Arg to Pro).

Under these conditions, check the profile manually and dismiss the peptide from the analysis, if appropriate.

Troubleshooting

Introduction

Review the table below to troubleshoot your experiments using SILAC™ Protein Identification and Quantitation Kits.

For troubleshooting MS, refer to the manual supplied with the MS instrument or contact the core facility.

Problem	Cause	Solution
Protein ID scores are low or poor data quality after MS	Insufficient cells used	Be sure to determine the number of cells required for analysis as described on page 17. Use 5-fold more cells to obtain a good signal after MS for low abundant proteins.
	Improper MS analysis	• Ensure the MS instrument was properly tuned and calibrated prior to sample analysis. • Check that the correct database, organism taxonomy, peptide modifications, labeled amino acid, and enzyme were selected during data analysis.
	Loss of phosphopeptides after PiMAC™ Resin	Follow the instructions on page 41 to properly prepare the PiMAC™ column in the gel loading tip, and perform chromatography to avoid any loss of phosphopeptides.
	Loss of peptides after trypsin digestion	**Do not** concentrate the peptides to dryness after trypsin solution. If the peptides are concentrated to dryness, the peptides are difficult to resuspend resulting in loss of peptides.
Peaks observed for unlabeled (light) amino acid for the protein	Incomplete incorporation of heavy amino acid	Perform the labeling for at least 6 doublings to ensure complete incorporation of the label. Be sure to use log-phase with >90% viability.
	Additional supplements added to the medium may contain amino acids	Always use dialyzed FBS to prepare the medium. **Do not** use regular FBS or use any other media supplements that may contain free amino acids.
	Amino acid prepared in complete medium	Prepare the amino acid using basal, unsupplemented D-MEM or RPMI 1640 supplied with the kit as described on page 23. Do not use any other complete medium to prepare the amino acids.

Continued on next page

Problem	Cause	Solution
The relative abundance between most of the heavy to light labeled proteins is not 1:1	Error in mixing cells	Count the cells prior to mixing and adjust the number of cells harvested to ensure the cells from two populations are mixed in a 1:1 ratio by cell number. Be sure to use log-phase cells with >90% viability.
Poor amino acid incorporation (more apparent when labeling with lysine)	Arginine terminating peptides cause ionization suppression effects that impair the detection of peak pairs	Perform analysis using LC-MS or include a simple fractionation step with a ZipTip® with C_{18} resin (use only a 30% elution step) prior to MS analysis.
Sequence database search identifies keratin as the top candidate	Samples contaminated with keratin	Always wear gloves while handling the gels and use ultrapure proteomics grade reagents for in-gel trypsin digestion. Perform all gel manipulations in a clean dust-free environment away from a door or window and always use a fresh razor blade to excise the gel bands.
MS spectra contaminated with peaks at regular interval (*e.g.,* 44 Da repeats of polyethylene glycol)	Samples contaminated by polymer	Be sure to use polypropylene microcentrifuge tubes and HPLC grade solvents.
The Coefficient of Variance (CV) for the protein quantitation within one experiment exceeds 30%	Improper MS analysis	• Ensure the MS instrument was properly tuned and calibrated prior to sample analysis. • Ensure the chromatographic separation was effective and the columns used for separation were free of contaminants from prior separations. Always run blanks between chromatographic separations to avoid any contaminations. Use clean MALDI plates for analysis.

Continued on next page

Problem	Cause	Solution
Inconclusive identification and quantitation of phosphoproteins due to poor data quality	Loss of phosphorylation	• To avoid loss of phosphorylation, process the samples immediately after collection and perform all steps at 4°C. Do not store the samples for prolonged periods of time. • Use the SILAC™ Phosphoprotein Lysis Buffer A or B to prepare lysates for phosphoprotein analysis. The Lysis Buffer A or B contain the tyrosine phosphatase inhibitor preventing the loss of phosphorylation. • Follow the instructions on page 41 to properly prepare the PiMAC™ column in the gel loading tip, and perform chromatography to avoid any loss of phosphopeptides. • **Do not** concentrate the peptides to dryness after trypsin solution. If the peptides are concentrated to dryness, the peptides are difficult to resuspend resulting in loss of peptides.
	No phosphoprotein enrichment step performed	To enable proper data analysis and identification of phosphorylated proteins, always perform enrichment of phosphoproteins prior to analysis as described on page 34.

Appendix

Protein Quantitation Using Manual Calculations

Introduction

Instructions to perform manual quantitation of raw data are described in this section.

You need to perform quantitation for at least 3–5 peptides for each protein to evaluate the protein expression level.

An experienced user can perform ~4 queries in ~10 minutes.

Outline

For manual quantitation, generate an EIC (extracted ion chromatogram) or SIC (selected ion chromatogram) from the heavy and light peptide peak pairs of interest. Use the area under each peak to calculate the relative intensity differences for the peptide pairs.

Manual Protein Quantitation

Quantification of peptide pair is done and validated manually by examining the MS/MS spectrum.

1. Perform the analysis using the MS instrument software. Start the software and navigate to the chromatogram screen.
2. Start the quantitation with the Mascot Peptide Summary list (see page 52 for an example of Mascot Peptide Summary list) or equivalent.
3. Select the protein for analysis.
4. Select a Query/peptide for analysis. Make sure the quality control parameters (such as Delta, missed, score) are reasonable. If not, select another query with reasonable parameters.
5. Generate a SIC (selected ion chromatogram) for the query.
6. Select an elution profile in SIC chromatogram.
7. Create a MS spectrum for the selected profile, and confirm the precursor m/z and fragment assignments for at least 3 major fragments. If you cannot confirm these parameters, select another profile in the SIC chromatogram.
8. For the selected profile, confirm the retention time, protein identification, and ensure the light and heavy peptide pair is distinguishable. If you cannot distinguish the light and heavy peptide pair or the peptide pair is obscured by unrelated isobaric peptides, select another query as described on Step 4.
9. Measure the relative intensity for the peptides.
10. Perform at least 3–5 queries (Steps 4–9) for each protein.
11. Calculate the average and standard deviation for all queries for the selected protein. **The resulting value is the relative abundance ratio.** The results are significant if the coefficient of variance (CV) is <30% within one experiment. A coefficient of variance >30% within one experiment, indicates missed identification or poor quality of MS spectra (see page 53 for details).
12. Repeat the procedure for the next protein.

Accessory Products

Media Components

The table below lists cell culture media products available separately from Invitrogen. For more information about these products, visit www.invitrogen.com or call Technical Support (page 60).

Product	Quantity	Catalog no.
SILAC™ [U-$^{13}C_6$, $^{15}N_4$]-L-Arginine (*Arg)	100 mg	MS10009
SILAC™ [U-$^{13}C_6$,]-L-Arginine (*Arg)	100 mg	MS10011
Trypan Blue Stain	100 mL	15250-061
Penicillin-Streptomycin, liquid	100 mL	15070-063
Fetal Bovine Serum (FBS)	500 mL	16000-044
Fetal Bovine Serum, Dialyzed	100 mL	26400-036
D-MEM	1000 mL	11965-084
RPMI 1640	1000 mL	11875-085
IMDM	1000 mL	12440-046
Advanced D-MEM/F-12	500 mL	12634-010
L-Glutamine (100X), 200 mM	20 mL	25030-149
Sodium Pyruvate, 100 mM (100X)	100 mL	11360-070
Phosphate Buffered Saline (PBS), 1X	500 mL	10010-023
GlutaMAX™-I Supplement	100 mL	35050-061
Recovery™ Cell Culture Freezing Medium	50 mL	12648-010
TrypLE™ Express Stable Trypsin Replacement Enzyme (1X) without Phenol Red	100 mL	12604-013
	500 mL	12604-021
	20 × 100 mL	12604-039
TrypLE™ Express Stable Trypsin Replacement Enzyme (1X) with Phenol Red	100 mL	12605-010
	500 mL	12605-028
	20 × 100 mL	12605-036

Continued on next page

Accessory Products, Continued

Additional Reagents

The table below lists additional products available separately from Invitrogen. For more information about these products, visit www.invitrogen.com or call Technical Support (page 60).

Product	Quantity	Catalog no.
Recombinant Protein G Agarose	5 mL	15920-010
SimplyBlue™ SafeStain	1 L	LC6060
SilverQuest™ Silver Staining Kit	1 kit	LC6070
NuPAGE® Novex® 4–12% Bis-Tris Gel, 10 well, 1.0 mm	1 box of 10 gels	NP0321BOX
NuPAGE® MOPS SDS Running Buffer (20X)	500 mL	NP0001
NuPAGE® MES SDS Buffer (20X)	500 mL	NP0002
NuPAGE® Antioxidant	15 mL	NP0005
NuPAGE® LDS Sample Buffer (4X)	10 mL	NP0007
NuPAGE® Sample Reducing Agent (10X)	250 µL	NP0004
XCell *SureLock*™ Mini-Cell	1 unit	EI0001
Invitrosol™ LC/MS Protein Solubilizer (5X)	5 mL	MS10007
1-D PAGE Cleavable ICAT® Reagent Application Kit	1 kit	MS10012
Trypsin	1 kit	MS10015

Antibodies

A large variety of high-quality antibodies against various proteins is available from Invitrogen. Visit our website for details or contact Technical Support (page 60).

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, SDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
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Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our website (www.invitrogen.com).

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SDS

Safety Data Sheets (SDSs) are available on our website at www.invitrogen.com/sds.

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References

Amanchy, R., Kalume, D. E., and Pandey, A. (2005) Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) for Studying Dynamics of Protein Abundance and Posttranslational Modifications. Science's STKE *267*, p12

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994) *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Interscience, New York

Bae, W., and Chen, X. (2004) Proteomic Study for the Cellular Responses to Cd2+ in Schizosaccharomyces pombe through Amino Acid-Coded Mass Tagging and Liquid Chromatography Tandem Mass Spectrometry. Mol. Cell. Proteomics. *3*, 596-607

Blagoev, B., Kratchmarova, I., Ong, S. E., Nielsen, M., Foster, L. J., and Mann, M. (2003) A Proteomics Strategy to Elucidate Functional Protein-Protein Interactions Applied to EGF Signaling. Nat. Biotechnol. *21*, 315-318

Chambers, J. L., Christoph, G. G., Krieger, M., Kay, L., and Sroud, R. M. (1974) Silver Ion Inhibition of Serine Proteases. Biochemical and Biophysical Research Communications *59*, 70-74

Chen, X., Smith, L. M., and Bradbury, M. E. (2000) Site-Specific Mass Tagging with Stable Isotopes in Proteins for Accurate and Efficient Protein Identification. Anal. Chem *72*, 1134-1143

Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W., and Wingfield, P. T. (1998) *Current Protocols in Protein Science*. Current Protocols (Chanda, V. B., Ed.), John Wiley and Sons, Inc., New York

Everley, P. A., Krijgsveld, J., Zetter, B. R., and Gygi, S. P. (2004) Quantitative Cancer Proteomics: Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) as a Tool for Prostrate Cancer Research. Mol. Cell. Proteomics *3*, 729-735

Helmann, U., Wernstedt, C., Gonez, J., and Heldin, C. (1995) Improvement of an In Gel Digestion Procedure for the Mircopreparation of Internal Protein Fragments for Amino Acid Sequencing. Anal. Biochem. *224*, 451-455

Ibarrola, N., Kalume, D. E., Gronborg, M., Iwahori, A., and Pandey, A. (2003) A Proteomic Approach for Quantitation of Phosphorylation Using Stable Isotope Labeling in Cell Culture. Anal Chem *75*, 6043-6049

Oda, Y., Huang, K., Cross, F. R., Cowburn, D., and Chait, B. T. (1999) Accurate Quantitation of Protein Expression and Site-Specific Phosphorylation. Proc. Nat. Acad. Sci. *96*, 6591-6596

Ong, S. E., Blagoev, B., Kratchmarova, I., Kristensen, D. B., Steen, H., Pandey, A., and Mann, M. (2002) Stable Isotope Labeling by Amino Acids in Cell Culture, SILAC, as a Simple and Accurate Approach to Expression Proteomics. Molecular and Cellular Proteomics *1*, 376-386

Peter, J. (2000) *Proteome Research Mass Spectrometry.* (Peters, J., Ed.), Springer Verlag, Berlin

Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass Spectrometric Sequencing of Proteins from Silver-stained Polyacrylamide Gels. Anal. Chem *68*, 850-858

Simpson, R. J. (2003) *Proteins and Proteomics: A Laboratory Manual.*, Cold Spring Harbor Press, New York

Speicher, D. (2004) *Proteome Analysis: Interpreting The Genome.* (Speicher, D., Ed.), Elsevier Press, Oxford

Zhu, H., Hunter, T. C., Pan, S., Yau, P. M., Bradbury, M. E., and Chen, X. (2002) Residue-specific Mass Signatures for the Efficient Detection of Protein Modifications by Mass Spectrometry. Anal. Chem *74*, 1687-1694

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