

# Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) for Studying Dynamics of Protein Abundance and Posttranslational Modifications

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**Abstract**

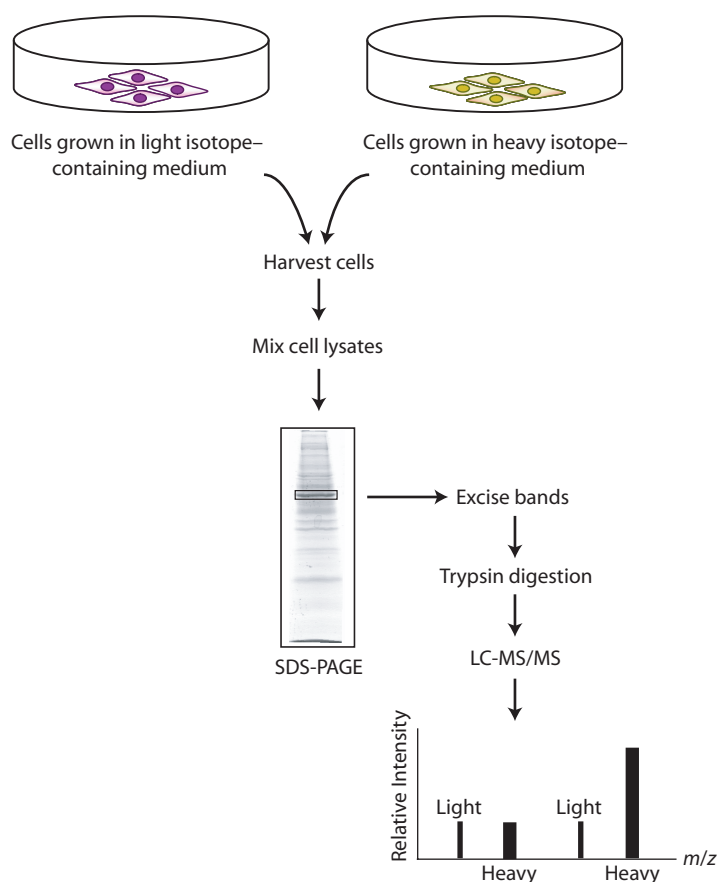
**Stable isotope labeling with amino acids in cell culture (SILAC) is a simple and straightforward approach for in vivo incorporation of a tag into proteins for relative quantitation by mass spectrometry. SILAC is a simple, yet powerful, method for investigating the dynamics of protein abundance and posttranslational modifications. Here, we provide detailed instructions for using this method to study protein complexes, protein-protein interactions, and the dynamics of protein abundance and posttranslational modifications. We expect that SILAC will become a routine technique because of its applicability to most areas of cell biology. We have also developed a Web site (<http://www.silac.org>) to provide researchers with updated information about this method and related resources.**

**Introduction**

Mass spectrometry has emerged as a powerful tool in proteomics. Until recently, however, it was mostly used for qualitative purposes, such as identification of proteins and posttranslational modifications. Development of a number of novel techniques has extended the application of mass spectrometry to relative quantitation as well. Relative quantitation is usually achieved by in vitro methods that involve modification of specific amino acids or N or C termini of proteins or peptides. Such methods include the introduction of tags containing heavy atoms such as  $^{13}\text{C}$  by chemical derivatization (1–3) or by digestion of the peptide with trypsin in the presence of  $^{18}\text{O}$ -labeled water (4). A potential alternative is to label proteins in vivo by growing cells in media containing heavy isotopes or heavy isotope-containing amino acids (5, 6). One limitation is that the in vivo labeling methods can only be used in cell culture-based systems.

Stable isotope labeling with amino acids in cell culture (SILAC) is a simple and accurate approach that relies on the incorporation of amino acids containing substituted stable isotopic nuclei (for example, deuterium,  $^{13}\text{C}$ ,  $^{15}\text{N}$ ) into proteins in living cells (6). Thus, in a typical experiment (Fig. 1), two cell populations are grown in culture media that are identical except that one medium contains a “light,” and the other a “heavy,” form of a particular amino acid (for example,  $^{12}\text{C}$ - and  $^{13}\text{C}$ -labeled L-lysine, respectively). Stable isotope-containing amino acids are commercially available and can be used for this purpose. When the stable isotope-containing amino acid is supplied to cells instead of the natural amino acid, it is incorporated into newly synthesized proteins by the growing cells. After a number of cell divisions, the naturally occurring amino acid is completely replaced by its isotopically labeled analog (Fig. 2). Because there is very little chemical difference between the isotopically labeled amino acid and its naturally occurring counterpart, the cells grown in stable isotope-containing amino acids behave exactly like the control cell population. SILAC requires no chemical manipulation, because it relies on the normal biosynthetic machinery of cells. This method is efficient and reproducible, because the incorporation of the isotope label is 100%. Further, the amount of labeled protein required for analysis using the SILAC method is far less than that required by in vitro chemical methods. Here, we present SILAC as a simple approach to quantitative proteomics for the investigation of protein dynamics in cell-based systems.

The proteome is a dynamic entity and is tightly regulated to maintain homeostasis at the cell, tissue, and organ levels. Proteomics has traditionally been associated with methods that result in displaying a large number of proteins from samples on two-dimensional polyacrylamide



**Fig. 1.** Experimental strategy for the SILAC method for relative quantitation of proteins or posttranslational modifications. Cells are grown in media containing either light or heavy isotope. Cells grown in the heavy medium are treated with a factor (for example, calyculin A, EGF, or PDGF) and then the lysates are mixed. The mixed lysate can be resolved directly by SDS-PAGE, as shown, or after immunoprecipitation of the protein of interest. The bands are excised and digested with trypsin, and the peptide mixture is analyzed by LC-MS/MS. The schematic of a mass spectrum shows the expected mass difference between the light and heavy labeled peptides because of the incorporation of the heavy amino acid. An intensity difference is seen only if there is a difference in the abundance of the peptide.

gels. With the routine use of mass spectrometry-based methods, proteomics has come to include large-scale functional analysis of gene products, studies of protein-protein interaction, and measurement of protein dynamics, as well (7). Quantitation of mRNA levels is traditionally used as a surrogate measure of protein levels; however, mRNA levels do not necessarily correlate with protein abundance (8, 9). Thus, protein-based methods are necessary to complement such mRNA-based measurements. In this respect, SILAC can be applied to scenarios involving investigation of protein dynamics. We will briefly discuss some of these applications and provide detailed protocols using prototypical examples.

### Relative Quantitation of Protein Abundance

One application of SILAC is the quantitation of protein abundance that is up- or down-regulated by a stimulus. Expression of genes and gene products is generally tissue-specific, and the abundance of proteins is subject to modulation by various growth factors and other stimuli. SILAC is an effective strategy for studying protein synthesis and turnover in response to stimuli. Furthermore, protein abundance can be monitored in a temporal fashion with the SILAC method by using a different isotopic version of an amino acid for each time point.

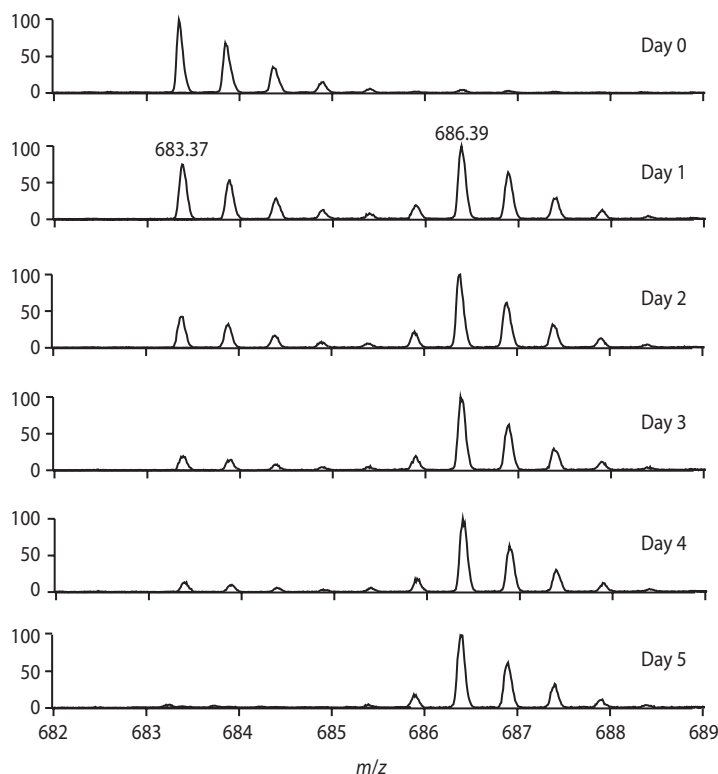
SILAC may also be used to perform proteomic profiling (for example, of cancer cell lines). Microarrays have become popular for large-scale analyses of genes involved in cancers. Cell lines are often good model systems for studying cancer mechanisms. Cancer cell lines can therefore be compared to their normal counterparts using SILAC, as shown for prostate cancer (10).

The translocation of proteins in response to stimuli can also be analyzed by SILAC. In response to certain stimuli, proteins translocate to different subcellular compartments (for example, from the cytoplasm to the plasma membrane or nucleus). Such translocation is difficult to monitor on a proteomic scale. SILAC can be used in combination with cell fractionation techniques to study alterations in the subcellular localization of proteins.

SILAC can be used to identify the components of inducible protein complexes. Proteins do not usually function in isolation. Reversible protein complexes transmit signals in cellular responses to stimuli (11). SILAC can be used to identify components of protein complexes that are formed upon activation of signaling pathways, as has been shown for Grb2 in epidermal growth factor (EGF) receptor signaling (12).

### Relative Quantitation of Posttranslational Modifications

Protein dynamics are governed not only by the rates of protein biosynthesis and degradation, but also by the extent of posttranslational modifications. The extent of a posttranslational modification can be monitored by SILAC, as was shown recently for phosphorylation of the Frigg protein (13).



**Fig. 2.** Incorporation of  $^{13}\text{C}_6$  L-lysine into proteins at various time points. NIH 3T3 cells were switched to  $^{13}\text{C}_6$  L-lysine-containing medium on day 0 and samples were obtained on days 0, 1, 2, 3, 4, and 5 during the adaptation process. The lysates were resolved by SDS-PAGE, a band of 80 kD was excised from each time point, and LC-MS/MS analysis was performed on the eluted tryptic peptides. The doubly charged peaks at  $m/z$  683.3 and 686.4 correspond to a peptide from heat shock protein 84 and exhibit a mass difference of 6 daltons because of incorporation of one heavy lysine residue. The panels show the extent of incorporation of  $^{13}\text{C}_6$  L-lysine into this peptide at the indicated times. Complete incorporation of  $^{13}\text{C}_6$  L-lysine into the peptide was clearly observed in cell lysates harvested on day 5.

## Materials

### Amino Acids

$^{13}\text{C}_6$  L-Arginine hydrochloride (Cambridge Isotope Laboratories, Andover, MA, #CLM-2265).

*Note:  $^{13}\text{C}_6$  L-arginine is a stable isotope of  $^{12}\text{C}_6$  L-arginine and is 6 daltons heavier than  $^{12}\text{C}_6$  L-arginine.*

$^{13}\text{C}_6$ ,  $^{15}\text{N}_4$  L-Arginine hydrochloride (Cambridge Isotope Laboratories, #CNLM-539).

*Note:  $^{13}\text{C}_6$ ,  $^{15}\text{N}_4$  L-arginine is a stable isotope of  $^{12}\text{C}_6$ ,  $^{14}\text{N}_4$  L-arginine and is 10 daltons heavier than  $^{12}\text{C}_6$ ,  $^{14}\text{N}_4$  L-arginine.*

$^{13}\text{C}_6$  L-Lysine dihydrochloride (Cambridge Isotope Laboratories, #CLM-2247).

*Note:  $^{13}\text{C}_6$  L-lysine is a stable isotope of  $^{12}\text{C}_6$  L-lysine and is 6 daltons heavier than  $^{12}\text{C}_6$  L-lysine.*

$^{13}\text{C}_9$  L-Tyrosine (Cambridge Isotope Laboratories, #CLM-2263).

*Note:  $^{13}\text{C}_9$  L-tyrosine is a stable isotope of  $^{12}\text{C}_9$  L-tyrosine and is 9 daltons heavier than  $^{12}\text{C}_9$  L-tyrosine.*

L-Arginine (Sigma-Aldrich, St. Louis, MO, #A-8094)

L-Lysine dihydrochloride (Sigma-Aldrich, #L-5751)

L-Tyrosine disodium salt (Sigma-Aldrich, #T-1145)

### Cell Culture Reagents

Dialyzed fetal bovine serum (Invitrogen, Carlsbad, CA, #26400-044)

Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 deficient in lysine, arginine, and tyrosine (Invitrogen)

*Note: These medium compositions are available from Invitrogen as a custom service. The custom-synthesized media have exactly the same composition as the regular media (DMEM, #11965-092; RPMI 1640, #11875-093, both from Invitrogen), except that they are deficient in the specified amino acids.*

Penicillin-streptomycin (10,000 U of penicillin and 10,000  $\mu\text{g}/\text{ml}$  of streptomycin) (Invitrogen, #15140-122)

Trypsin-ethylenediamine tetraacetic acid (EDTA) (Invitrogen, #25300-054)

### Agonists and Inhibitors

3-isobutyl-1-methylxanthine (Sigma-Aldrich, #I-5879)

Calyculin A (EMD Biosciences, La Jolla, CA, #208851)

Dexamethasone (Sigma-Aldrich, #D-8893)

Human recombinant EGF [Upstate Biotechnology, #01-107 (<http://www.upstate.com>)]

Human recombinant platelet-derived growth factor (PDGF) BB (Upstate Biotechnology, #01-305)

Human insulin (Sigma-Aldrich, #I-1507)

### Cell Lines

HEK 293T cell line (ATCC, Manassas, VA, #CRL-11268)

HeLa cell line (ATCC, #CCL-2)

NIH 3T3 cell line (ATCC, #CRL-1658)

NIH 3T3-L1 cell line (ATCC, #CL-173)

### Cell Lysis and Immunoprecipitation Reagents

- Agarose-conjugated antiphosphotyrosine mouse monoclonal antibody (clone 4G10) (Upstate Biotechnology, #16-101)
- Antibody to Flag M2 affinity gel (Sigma-Aldrich, #A-2220)
- Antibody to Shc (Upstate Biotechnology, #06-203)
- Biotin-conjugated RC20 antiphosphotyrosine monoclonal antibodies (BD Biosciences, Palo Alto, CA, #610022)
- Complete Protease Inhibitor Cocktail Tablets (protease inhibitor tablets) [Roche Diagnostics, #1697498 (<http://www.roche-diagnostics.com>)]
- DC Protein Assay Kit I (Bio-Rad, Hercules, CA, #500-011)
- NP-40 (EMD Biosciences, #492015)
- Phenyl phosphate (Sigma-Aldrich, #P-7751)
- Phosphate-buffered saline (PBS) (Invitrogen, #14190-144)
- Protein A-agarose beads (Sigma-Aldrich, P-3476)
- Sodium deoxycholate (Sigma-Aldrich, #D-6750)
- Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, #L-5750)
- Streptavidin-agarose beads (Upstate Biotechnology, #16-126)
- Sodium vanadate (Sigma-Aldrich, #S-6508)

### Gel Staining Reagents

- 2-Mercaptoethanol (2-ME)
- Acetic acid, glacial [Fisher, #A38-500 (<https://www1.fishersci.com>)]
- Acetonitrile [JT Baker, #9017-03 (<http://www.jtbaker.com>)]
- Colloidal blue staining kit (Invitrogen, #LC6025)
- Formaldehyde (JT Baker, #2106-01)
- Methanol (EMD Chemicals, #MX0475P-4)
- Silver nitrate (EMD Chemicals, #SX0205-5)
- Sodium carbonate (Sigma-Aldrich, #S-2127)
- Sodium thiosulfate (EMD Chemicals, #SX0820-1)

### In-Gel Reduction, Alkylation, and Digestion Reagents

- Ammonium bicarbonate (Sigma-Aldrich, #09830)
- Dithiothreitol (DTT) (Sigma-Aldrich, #43815)
- Formic acid (Sigma-Aldrich, #06450)
- Iodoacetamide (Sigma-Aldrich, #I-6125)
- Modified trypsin, sequencing grade [Promega, #V5111 (<http://www.promega.com>)]
- Scapel/No. 10 surgical blade (Feather Safety Razor, Japan)

## Materials for Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Sample Preparation

Emitters for liquid chromatography (LC) with tip inner diameter  $10 \pm 1 \mu\text{m}$  (New Objective, Woburn, MA, #FS-360-20-10-N-20)

Formamide (super pure grade) (Fisher Chemicals, #BP228-100)

Fused-silica capillary tubing for LC:  $75 \mu\text{m}$  ID,  $365 \mu\text{m}$  OD (Polymicro Technologies, Phoenix, AZ, #TSP075375)

Gel loader tips (Invitrogen) or purification capillaries (Proxeon A/S, Odense, Denmark)

Heptafluorobutyric acid (HFBA) (Sigma-Aldrich, #H-7133)

Kasil 1 (potassium/silicate solution, PQ) (Astro Chemicals, Springfield, MA, #25110),

Nanoelectrospray capillaries (Proxeon A/S)

Protein standard for optimizing LC-MS/MS, such as trypsin-digested bovine serum albumin (BSA)

Reversed-phase column material for LC: ODS-AQ, pore size 12 nm, particle size  $5/15 \mu\text{m}$  (YMC, Kyoto, Japan).

Reversed-phase column material for LC: Zorbax SB-C18, pore size  $300 \text{ \AA}$ , particle size  $5 \mu\text{m}$  (Agilent Technologies, Palo Alto, CA)

Snake skin dialysis tubing, 3500 kD molecular weight cutoff (Pierce, Rockford, IL, #68035)

Software program for estimating peptide pair intensity, such as ProteinLynx Global Server (version 2.0) (Micromass, Manchester, UK)

Software program for identifying proteins, such as Mascot (Matrix Science, Boston, MA), SEQUEST [Thermo Electron (<http://www.thermo.com>)], SpectrumMill (Agilent Technologies), or ProteinLynx (Waters-Micromass)

Standard solution for calibrating the Q-TOF instrument: [Glu<sup>1</sup>]-Fibrinopeptide B, human, synthetic (Sigma, Steinheim, Germany, #F-3261)

## Equipment

Eppendorf Microcentrifuge 5415D (Brinkmann Instruments, Westbury, NY)

High-pressure vessel for nano-LC column packing (Proxeon A/S, #SP035)

LC equipment (all from Agilent Technologies): 1100 Series HPLC system equipped with a capillary pump, micro-vacuum degasser, microwell-plate sampler, and a control module

Mass spectrometer: Q-TOF API-US (Micromass)

*Note: Any tandem mass spectrometer with sufficient resolution can be used.*

Nanoelectrospray ion source for Micromass Q-TOF (Proxeon A/S, # ES028A/ES028B)

Vacuum concentrator microcentrifuge (vacufuge)

Vortexer

## Recipes

### Recipe 1: DMEM with $^{13}\text{C}_6$ L-Arginine

Prepare a concentrated stock of the amino acids (0.398 mM  $^{13}\text{C}_6$  L-arginine hydrochloride, 0.798 mM L-lysine dihydrochloride, and 0.398 mM L-tyrosine disodium salt) in 20 ml of DMEM deficient in arginine, lysine, and tyrosine. Dissolve completely, then filter the medium through a 0.22- $\mu\text{m}$  filter. Add the entire 20 ml of sterilized amino acid stock, 10% dialyzed fetal bovine serum, and 1% penicillin-streptomycin. Add amino acid-deficient DMEM to 1 liter. Medium should be prepared in a laminar flow hood. Store the medium at 4°C until use.

*Note: Media containing any combination of stable isotope-containing amino acids can be prepared using DMEM or RPMI 1640 as a base. The stable isotope-containing amino acids are added to 20 ml of medium along with the remaining amino acids in their nonisotopic forms. RPMI or DMEM deficient in arginine, lysine, and tyrosine can be used (Table 1). It is possible to use lower concentrations of stable isotope-containing amino acids (for example, 50% of the recommended amount) in the preparation of SILAC media if the cost is a consideration, as long as the cells are monitored. However, in such cases, it is advisable to grow the control cells with similarly reduced concentrations of naturally occurring amino acids, as well. It is essential to use dialyzed serum to avoid adding amino acids with the serum.*

Amino Acids (Light and Heavy)	Amount (Concentration) in RPMI-1640	Amount (Concentration) in DMEM
L-Arginine	200 mg/L (1.15 mM)	69.3 mg/L (0.398 mM)
L-Lysine hydrochloride	40 mg/L (0.274 mM)	146 mg/L (0.798 mM)
L-Tyrosine disodium salt	29 mg/L (0.111 mM)	104 mg/L (0.398 mM)
$^{13}\text{C}_6$ L-Lysine dihydrochloride	46.4 mg/L	169.6 mg/L
$^{13}\text{C}_6$ , $^{15}\text{N}_4$ L-Arginine hydrochloride	248 mg/L	86.1 mg/L
$^{13}\text{C}_6$ L-Arginine hydrochloride	248 mg/L	86.1 mg/L
$^{13}\text{C}_9$ L-Tyrosine	23.3 mg/L	83.6 mg/L

**Table 1.** Amounts and molar concentrations of light and heavy amino acids to be used in the preparation of media for SILAC experiments.

### Recipe 2: Complete DMEM

Regular commercial DMEM (Invitrogen) can be used for growing control cells. Alternatively, custom-made, amino acid-deficient DMEM supplemented with appropriate concentrations of amino acids can be used, as follows. Prepare a concentrated stock of the amino acids (0.398 mM L-arginine, 0.798 mM L-lysine dihydrochloride, and 0.398 mM L-tyrosine disodium salt) in 20 ml of DMEM deficient in arginine, lysine, and tyrosine. Dissolve completely, then filter the medium through a 0.22- $\mu\text{m}$  filter. Add the sterilized stock, 10% dialyzed fetal bovine serum, and 1% penicillin-streptomycin, and amino acid-deficient DMEM to 1 liter. Medium should be prepared in a laminar flow hood. Store the media at 4°C until use.

### Recipe 3: Preadipocyte Differentiation Medium

0.5 mM 3-isobutyl-1-methylxanthine

1  $\mu\text{M}$  dexamethasone

167 nM insulin

Add to 1 ml of DMEM with  $^{13}\text{C}_6$  L-Arginine (Recipe 1). Make just before use.

### Recipe 4: Adipocyte Growth Medium

Prepare 167 nM insulin in 1 ml of DMEM with  $^{13}\text{C}_6$  L-Arginine (Recipe 1). Make just before use.

### Recipe 5: DMEM with $^{13}\text{C}_6$ , $^{15}\text{N}_4$ L-Arginine

Prepare a concentrated stock of the amino acids (0.398 mM  $^{13}\text{C}_6$ ,  $^{15}\text{N}_4$  L-arginine hydrochloride, 0.798 mM L-lysine dihydrochloride, and 0.398 mM L-tyrosine disodium salt) in 20 ml of DMEM deficient in arginine, lysine, and tyrosine. Dissolve completely, then filter the medium through a 0.22- $\mu\text{m}$  filter. Add the entire 20 ml of sterilized amino acid stock, 10% dialyzed fetal bovine serum, and 1% penicillin-streptomycin. Add amino acid-deficient DMEM to 1 liter. Medium should be prepared in a laminar flow hood. Store the medium at 4°C until use.

### Recipe 6: PDGF-BB Stock Solution

Prepare 100  $\mu\text{g}$  of PDGF-BB in 100  $\mu\text{l}$  of sterile, serum-free culture DMEM (without antibiotics) and store at 4°C.

### Recipe 7: Sodium Orthovanadate

Prepare a 100-mM solution of sodium orthovanadate, adjust pH to 10.0 with 1N HCl, and bring to boil once in the microwave. It should turn colorless.

### Recipe 8: Lysis Buffer

150 mM NaCl  
50 mM Tris-HCl, pH 7.4  
1% NP-40

Combine components in 500 ml of deionized water and store at room temperature. Immediately before use, add protease inhibitor tablets (one per 50 ml) and 1 mM sodium orthovanadate from the 100 mM Orthovanadate Stock (Recipe 8).

### Recipe 9: DMEM with $^{13}\text{C}_6$ L-Arginine and $^{13}\text{C}_6$ L-Lysine

Prepare a concentrated stock of the amino acids (0.398 mM  $^{13}\text{C}_6$  L-arginine hydrochloride, 0.798 mM  $^{13}\text{C}_6$  L-lysine dihydrochloride, and 0.398 mM L-tyrosine disodium salt) in 20 ml of DMEM deficient in arginine, lysine, and tyrosine. Dissolve completely, then filter the medium through a 0.22- $\mu\text{m}$  filter. Add the entire 20 ml of sterilized amino acid stock, 10% dialyzed fetal bovine serum, and 1% penicillin-streptomycin. Add amino acid-deficient DMEM to 1 liter. Medium should be prepared in a laminar flow hood. Store the medium at 4°C until use.

### Recipe 10: Calyculin A Stock

Prepare 10  $\mu\text{g}$  of Calyculin A in 1 ml of ethanol to make a 10- $\mu\text{M}$  stock.

### Recipe 11: Modified RIPA Buffer

150 mM NaCl  
50 mM Tris-HCl, pH 7.4  
1% NP-40  
0.25% Sodium deoxycholate  
1 mM EDTA

Immediately before use, add protease inhibitor tablets (one per 50 ml) and 1 mM sodium orthovanadate [from stock of Sodium Orthovanadate (Recipe 8)], 10 mM sodium fluoride, 10 mM glycerophosphate, and 5 mM sodium pyrophosphate. Prepare fresh each time.

### Recipe 12: EGF Stock Solution

Prepare 100  $\mu\text{g}$  of EGF in 100  $\mu\text{l}$  of sterile serum-free culture medium (without antibiotics) and store at 4°C.

### Recipe 13: Phenyl Phosphate Solution

Prepare 100 mM phenyl phosphate in PBS. Prepare fresh before use.

### Recipe 14: 5× Sample (Laemmli) Buffer

0.3125 M Tris-HCl, pH 6.8

10% SDS

25% v/v 2-ME

50% glycerol

0.016% bromophenol blue

Add to samples at a 1:5 ratio for a 1× final solution.

### Recipe 15: Fixing Solution

Prepare 100 ml of solution with a methanol:acetic acid:water ratio of 50:5:45 (v/v/v).

### Recipe 16: Sensitizing Solution

Prepare 0.02% sodium thiosulfate in 100 ml of distilled water.

### Recipe 17: Staining Solution

Prepare 0.1% silver nitrate in 100 ml of distilled water. Chill to 4°C just before use.

### Recipe 18: Developing Solution

Prepare 0.04% formaldehyde in 2% sodium carbonate dissolved in 500 ml of distilled water.

### Recipe 19: Quenching Solution

Prepare 1% acetic acid in 100 ml of distilled water.

### Recipe 20: Wash Buffer

Prepare 50 mM ammonium bicarbonate in 100 ml of distilled water.

### Recipe 21: Reduction Buffer

Prepare 10 mM DTT in 25 mM ammonium bicarbonate. Aliquots of 1 ml can be stored at -20°C.

### Recipe 22: Alkylation Buffer

Prepare 55 mM iodoacetamide in 25 mM ammonium bicarbonate. Aliquots of 1 ml can be stored at -20°C in brown tubes.

### Recipe 23: Digestion Buffer

Prepare a stock solution containing 20 µg of trypsin in 200 µl of 1 mM HCl (this can be stored at –20°C). Just before use, dilute this stock solution in 25 mM ammonium bicarbonate to a final trypsin concentration of 6.5 ng/µl.

### Recipe 24: Mobile Phase B for Liquid Chromatography

0.4% acetic acid

0.005% HFBA

Prepare 2 liters of this solution in acetonitrile:water (90:10 v/v).

### Recipe 25: Mobile Phase A for Liquid Chromatography

0.4% acetic acid

0.005% HFBA

Prepare 2 liters of this solution in deionized water.

## Instructions

For all SILAC procedures, the cells must be adapted to the stable isotope-containing medium before the experiment to achieve complete incorporation of the isotope in the cellular proteins. Five passages in the stable isotope-containing medium are sufficient to achieve adaptation. In addition, for each type of experiment described, stable isotope-labeled cells must be compared to unlabeled cells grown in medium containing naturally occurring amino acids at the same concentrations as those used for the heavy amino acid medium.

The instructions cover three main areas: (i) sample preparation, (ii) sample separation by SDS–polyacrylamide gel electrophoresis (PAGE) and in-gel trypsin digestion, and (iii) LC-MS/MS.

## Sample Preparation for the Relative Quantitation of Protein Abundance

This section describes the preparation of samples for quantifying proteins secreted in response to a stimulus. Relative quantitation of secreted proteins from NIH 3T3-L1 preadipocytes before and after their differentiation into adipocytes are the model described (6, 14), but the method can be adapted to analyze secretion from any cultured cell.

After analysis by LC-MS/MS, the intensities of the peptide ion signals from the light and heavy peptides representing proteins secreted during and after differentiation provide a relative measure of the abundance of the proteins.

1. Adapt one population of NIH 3T3-L1 preadipocytes to DMEM with <sup>13</sup>C<sub>6</sub> L-Arginine (Recipe 1) at 37°C, 5% CO<sub>2</sub> for five passages. Grow one population of cells in Complete DMEM (Recipe 2) as a control.
2. Divide the <sup>13</sup>C<sub>6</sub> L-arginine-labeled cells and the unlabeled control cells at an equal ratio into three 10-cm dishes and grow until they are about 70% confluent (about 36 hours).
3. Induce the <sup>13</sup>C<sub>6</sub> L-arginine-labeled preadipocytes to differentiate by replacing the medium with 10 ml of Preadipocyte Differentiation Medium (Recipe 3) and grow the cells for 2 days (48 hours).

*Note: Replace the medium on the control cells with Complete DMEM (Recipe 2). The day differentiation is started is Day 0.*

4. On day 2, replace the medium on the <sup>13</sup>C<sub>6</sub> L-arginine-labeled cells with 10 ml of Adipocyte Growth Medium (Recipe 4) and grow the cells for 2 days (48 hours).
5. On day 4, withdraw insulin and grow the cells for 5 days more, replacing the medium every second day with 10 ml of DMEM with <sup>13</sup>C<sub>6</sub> L-Arginine (Recipe 1) for the labeled differentiated cells or 10 ml of Complete DMEM (Recipe 2) for the control cells.

*Note: Take extreme care not to disrupt the cells while changing media or washing steps.*

6. On day 9, collect the supernatants from both differentiated and undifferentiated cells separately.
7. Centrifuge the supernatants at 1500g for 10 min at room temperature.
8. Filter the supernatants using a 0.2- $\mu$ m filter.

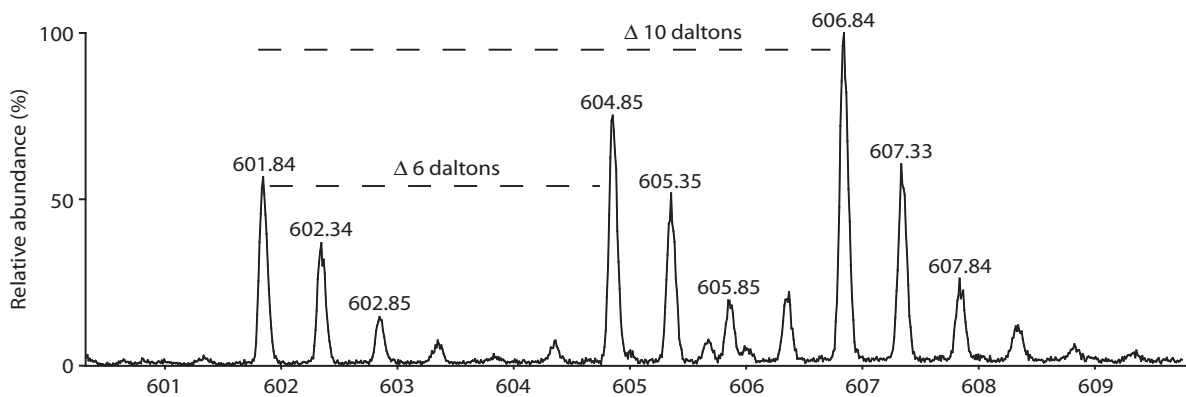
*Note: The supernatants should now be devoid of the cells.*

9. Quantitate the total protein content in each using the Bio-Rad DC Protein Assay Kit I (Folin-Lowry method) according to the manufacturer's instructions.
10. Mix equal amounts of the protein (about 500  $\mu$ g) from differentiated stable isotope-labeled supernatant with the undifferentiated, unlabeled control supernatant.

*Note: The samples can be frozen at  $-80^{\circ}\text{C}$  at this point or analyzed immediately by proceeding to the instructions detailed under Electrophoresis and Silver Staining.*

### Sample Preparation for Monitoring Components of a Protein Complex

Shc serves as an adaptor molecule in receptor tyrosine kinase (RTK) signaling by docking to tyrosine phosphorylated molecules through its phosphotyrosine-binding and Src-homology 2 (SH2) domains (15). Here, we describe the steps for immunoprecipitation of the Shc protein complex from NIH 3T3 cells stimulated with PDGF for different times to monitor the interaction of Shc with other proteins in the complex. Three different states are measured simultaneously. After analysis with LC-MS/MS, the relative abundance of the proteins in the Shc complex can be determined in stimulated and unstimulated cells; see Fig. 3 for an example.



**Fig. 3.** Monitoring components of a protein complex in three states using SILAC. NIH 3T3 cells adapted to  $^{13}\text{C}_6$  L-arginine and  $^{13}\text{C}_6$ ,  $^{15}\text{N}_4$  L-arginine were grown as three different cell populations: light (unlabeled amino acids), medium ( $^{13}\text{C}_6$  L-arginine, 6 daltons heavier), and heavy ( $^{13}\text{C}_6$  $^{15}\text{N}_4$  L-arginine, 10 daltons heavier). Cells growing with unlabeled amino acids were left untreated. Cells grown in medium and heavy isotope-labeled media were treated with PDGF for 5 min and 30 min, respectively. Cell lysates from all three conditions were mixed, and the Shc protein complex was immunoprecipitated using an antibody against Shc. The immunoprecipitated proteins were resolved by SDS-PAGE. The mass spectrum shown is derived from the tryptic digest of one of the bands. The relative change in binding of the protein Nexilin to Shc can be visualized on the basis of the relative intensities of a peptide derived from the three different states. The doubly charged peaks at  $m/z$  604.85 and 606.84 represent a mass difference of 6 daltons and 10 daltons from the unlabeled species of  $m/z$  601.84. The trend of increasing intensity after 5 or 30 min of PDGF treatment indicates that this protein is increasing in abundance within the Shc protein complex at these time points.

1. Adapt one population of NIH 3T3 cells to DMEM with  $^{13}\text{C}_6$  L-Arginine (Recipe 1) (medium-labeled cells), one population to DMEM with  $^{13}\text{C}_6$ ,  $^{15}\text{N}_4$  L-Arginine (Recipe 5) (heavy-labeled cells), and one in Complete DMEM (Recipe 2) (light cells) as a control at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ .

*Note: During adaptation, NIH 3T3 cells should be carefully monitored for their growth rates and any morphological changes.*

2. Expand each population of cells into five 15-cm dishes, and grow until 90% confluent.

3. Wash cells once in 5 ml of serum-free DMEM (without antibiotics), suspend in 5 ml of the same medium, and allow the cells to “starve” overnight (12 hours maximum) at 37°C, 5% CO<sub>2</sub>.

*Note: Avoid any isotope contamination by washing cells with their respective serum-free growth media into which they were adapted.*

4. Add PDGF-BB Stock Solution (Recipe 6) to the two arginine-labeled cell populations to a final concentration of 50 ng/ml, and incubate the dishes at 37°C, 5% CO<sub>2</sub> for different time points (for example, incubate the <sup>13</sup>C<sub>6</sub> L-arginine-labeled cells with PDGF-BB for 5 min and the <sup>13</sup>C<sub>6</sub>, <sup>15</sup>N<sub>4</sub> L-arginine-labeled cells for 30 min). The control cells do not receive PDGF-BB.

*Note: The PDGF receptor (PDGFR) consists of two subunits,  $\alpha$  and  $\beta$ , which form homo- and heterodimers. PDGF also exists as two different polypeptides, A and B, and can form homo- and heterodimers. PDGF AA binds specifically to PDGFR  $\alpha\alpha$ , and AB binds to PDGFR  $\alpha\alpha$  and  $\alpha\beta$ . But PDGF BB binds to all the forms of PDGFR— $\alpha\alpha$ ,  $\alpha\beta$ , and  $\beta\beta$ —and is most potent in terms of biological activity (16).*

5. Aspirate the medium and add 1 ml of Lysis Buffer (Recipe 8) to each dish and place on ice for 15 min.

*Note: Sodium orthovanadate is a protein tyrosine phosphatase inhibitor.*

6. Scrape the cells off the dishes and combine into separate centrifuge tubes for light, medium-labeled, and heavy-labeled samples.
7. Vortex for 30 s and leave on ice for 30 min with occasional mixing.
8. Centrifuge the whole cell lysates in a Sorvall SS-34 rotor at 10,000 rpm (12,000g) for 30 min at 4°C.
9. Measure the protein content by Bio-Rad DC Protein Assay Kit I (Folin-Lowry method).
10. Mix equal amounts of the three different lysates from the three different states (about 750 to 1000 mg of lysate for each condition).
11. Rock the lysate overnight with 500  $\mu$ l of protein A-agarose at 4°C to reduce nonspecific binding of proteins to protein A and beads.

*Note: Preclearing lowers the amount of nonspecific contaminants in the cell lysate and removes proteins with high affinity for protein G or protein A.*

12. Add 150  $\mu$ g of antibody to Shc to the lysate mixture along with 300  $\mu$ l of protein A-agarose, and rock for 6 hours at 4°C to immunoprecipitate the Shc signaling complex.

*Note: Any antibody can be added at this step. Antibodies that are covalently coupled to the matrix are preferable, because heavy chains of antibodies do not interfere with the analysis.*

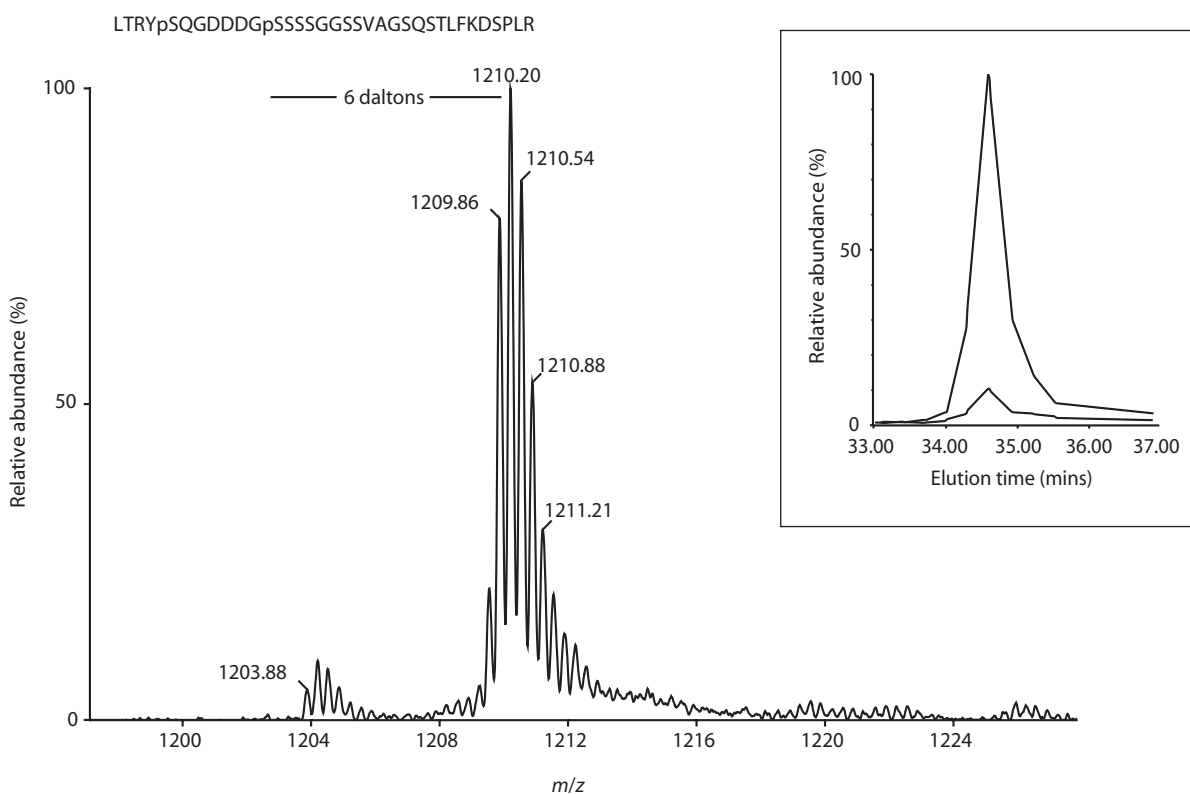
13. Wash the antibody-bound Shc signaling complex with 1 ml of Lysis Buffer (Recipe 8) three times, centrifuging at 400 rpm in a sorvall SS-34 rotor after each wash to pellet the beads.

*Note: The samples can be frozen at –80°C at this point or analyzed immediately by proceeding to the instructions detailed under Electrophoresis and Silver Staining.*

## Sample Preparation for Relative Quantitation of Posttranslational Modifications

Posttranslational modifications of proteins regulate their activity and function. Here, we describe an immunoprecipitation experiment for relative quantitation of serine phosphorylation in a protein tagged with a Flag epitope that is expressed in human embryonic kidney 293T (HEK 293T) cells by transient transfection. The use of transfected cells expressing an epitope-tagged version of the protein is essential when studying molecules against which antibodies are not available commercially. Proteins can also be tagged with other epitopes [for example, hemagglutinin (HA) or Myc]; then the corresponding antibodies should be used. Endogenous proteins can also be studied with this method, provided that an antibody is available that recognizes the protein. In such cases, the methods described below would be applicable to any adherent cell line, and the transient transfection step can be omitted and the antibody that recognizes the endogenous protein would be used for immunoprecipitation instead of the anti-Flag antibody.

An example of the change in the relative abundance of phosphorylated Frigg protein obtained following LC-MS/MS is shown in Fig. 4.



**Fig. 4.** Relative quantitation of phosphorylation using SILAC. HEK 293T cells overexpressing a Flag epitope-tagged Frigg protein were grown in media containing either unlabeled or  $^{13}\text{C}_6$ -labeled L-lysine. The cells grown in the heavy isotope were treated for 30 min with calyculin A, and Frigg was immunoprecipitated using anti-Flag antibodies. The band corresponding to Frigg was excised, digested with trypsin, and subjected to LC-MS/MS. A relative ratio of 1:9 between the triply charged ions at  $m/z$  1203.88 and 1209.86 is observed (see inset for the total ion chromatogram). This ratio reflects an increase of phosphorylated species upon 30 min of calyculin A treatment as compared to the resting state. MS/MS sequencing of heavy and light peptides produces the same fragmentation pattern with two phosphorylation sites on the indicated serine residues in the peptide LTRYpSQGDDDDGpSSSSGGSSVAGSQSTLFKDSPLR.

1. Adapt one population of HEK 293T cells to DMEM with  $^{13}\text{C}_6$  L-Lysine and  $^{13}\text{C}_6$  L-Arginine (Recipe 9) (heavy cells) and one population to Complete DMEM (Recipe 2) (control cells) for five passages at 37°C, 5%  $\text{CO}_2$ .

*Note: The use of labeled arginine and lysine ensures complete peptide coverage so that peptides containing the posttranslational modification are not missed.*

2. Expand each cell population to five 10-cm dishes and grow to 70% confluence at 37°C, 5%  $\text{CO}_2$ .
3. Transfect both populations of cells with 15  $\mu\text{g}$  of the cDNA encoding the epitope-tagged protein of interest using the standard calcium phosphate method.
4. At 24 hours after transfection, wash heavy cells once in 10 ml of serum-free DMEM with  $^{13}\text{C}_6$  L-Lysine and  $^{13}\text{C}_6$  L-Arginine (Recipe 9) without antibiotics, suspend in 5 ml of the same medium with  $^{13}\text{C}_6$  L-Lysine and  $^{13}\text{C}_6$  L-Arginine (Recipe 9), and allow the cells “starve” overnight (12 hours maximum) at 37°C, 5%  $\text{CO}_2$ . Wash control cells in Complete DMEM (Recipe 2).

*Note: Avoid any isotope contamination by washing cells with their respective growth media into which they were adapted.*

*Note: It is necessary to starve these cells overnight to reduce the basal levels of phosphorylation.*

5. Add Calyculin A Stock (Recipe 10) to a final concentration of 100 nM to the heavy cells and incubate for 30 min at 37°C. Control cells are left untreated.

*Note: Calyculin A is a serine-threonine phosphatase inhibitor and is used to induce hyperphosphorylation on serine and threonine residues.*

6. Aspirate the medium after calyculin A treatment, add 1 ml of cold Modified RIPA Buffer (Recipe 11) to each dish, and place the dishes on ice for 15 min.

*Note: Sodium fluoride, glycerophosphate, and sodium pyrophosphate are inhibitors of serine-threonine phosphatases.*

7. Scrape the heavy and control cells into separate 50-ml centrifuge tubes.
8. Vortex for 30 s and leave on ice for 30 min with occasional mixing.
9. Centrifuge the whole-cell lysates in a Sorvall SS-34 rotor at 10,000 rpm (12,000g) for 30 min at 4°C.
10. Measure the protein content by Bio-Rad DC Protein Assay Kit I (Folin-Lowry method) according to the manufacturer's instructions.
11. Mix equal amounts of clarified lysates (about 5 mg) from the five dishes of untreated control cells and five dishes of treated heavy cells in a fresh 50-ml tube.

*Note: Lysates for SILAC are usually mixed at this stage before preclearing of the lysates with protein A-agarose beads. However, the cell lysates can also be mixed after preclearing and before immunoprecipitation of molecule(s) of interest.*

12. Rock the lysates with 500 µl of protein A-agarose beads and incubate for 4 hours to overnight at 4°C to reduce nonspecific binding of proteins during immunoprecipitation.
13. Pellet the protein A-agarose beads by spinning in a Sorvall SS-34 rotor at 4000 rpm (2000g) for 2 min at 4°C, and transfer the supernatant to a fresh tube.
14. Combine the precleared lysates into a single tube, add 30 µg of anti-Flag M2 affinity gel, and rock for 6 hours to overnight at 4°C.
15. Centrifuge 1 min at maximum speed in a microcentrifuge and discard the supernatant.
16. Wash the beads three times with 1 ml of Modified RIPA Buffer (Recipe 11), pelleting the beads by centrifugation after each wash.

*Note: The samples can be frozen at -80°C at this point or analyzed immediately by proceeding to the instructions detailed under Electrophoresis and Silver Staining.*

### Sample Preparation for the Identification of Tyrosine Kinase Substrates

RTKs initiate signaling by transmitting extracellular signals into the cell by means of phosphorylation of their substrates on tyrosine residues (17). Here, we describe a simple *in vivo* approach to identify tyrosine kinase substrates using the EGF receptor (EGFR) signaling pathway in HeLa cells as an example.

1. Adapt one population of HeLa cells to DMEM with <sup>13</sup>C<sub>6</sub> L-Lysine and <sup>13</sup>C<sub>6</sub> L-Arginine (Recipe 9) (heavy cells) and one population to Complete DMEM (Recipe 2) (control cells) for five passages at 37°C, 5% CO<sub>2</sub>.
2. Expand each cell population to 20 15-cm dishes each and grow to 90% confluence at 37°C, 5% CO<sub>2</sub>.
3. Wash the cells once with 5 ml of serum-free medium, replace with 10 ml of serum-free medium, and allow the cells to "starve" overnight (12 hours maximum) at 37°C, 5% CO<sub>2</sub>.

*Note: Starving the cells overnight reduces the basal levels of phosphorylation. The cells must be washed in corresponding serum-free media: serum-free DMEM with <sup>13</sup>C<sub>6</sub> L-Lysine and <sup>13</sup>C<sub>6</sub> L-Arginine (Recipe 9) or serum-free Complete DMEM (Recipe 2).*

4. Stimulate the heavy cells with EGF Stock Solution (Recipe 12) at a final concentration of 100 ng/ml and incubate at 37°C for 5 min.
 

*Note: Do not add EGF to the control cells.*
5. Aspirate the medium from each dish, add 1 ml of Modified RIPA buffer (Recipe 11) to each dish, and place the dishes on ice for 15 min.
6. Scrape the cells off the dishes into separate tubes for heavy and control.
7. Vortex for 30 s and leave on ice for 30 min with occasional mixing.
8. Centrifuge the whole-cell lysates in a Sorvall SS-34 rotor at 10,000 rpm (12,000g) for 30 min at 4°C.
9. Combine the supernatants from the heavy and control samples in a fresh 50-ml tube and mix.
10. Rock the supernatant with 500 µl of protein A-agarose beads for 4 hours to overnight at 4°C.

*Note: This preclearing step is essential to ensure less nonspecific binding of proteins during immunoprecipitation step. When precipitation is done using a glutathione-S transferase (GST) fusion protein, we recommend preclearing with GST bound to glutathione-Sepharose beads.*

11. Pellet the protein A-agarose beads by spinning in a Sorvall SS-34 rotor at 4000 rpm (2000g) for 2 min at 4°C, and transfer the supernatant to a fresh 50-ml tube.

12. Add 400 µg of agarose-conjugated 4G10 monoclonal antiphosphotyrosine antibody, 200 µg of RC20 biotin-conjugated antiphosphotyrosine antibody, and 300 µl bead volume of streptavidin-conjugated agarose beads to the supernatant. Incubate for 6 hours to overnight at 4°C with gentle rocking.

*Note: Because different monoclonal antiphosphotyrosine antibodies immunoprecipitate slightly different sets of tyrosine phosphorylated proteins, we routinely use a mixture of two antiphosphotyrosine antibodies.*

13. Wash the agarose beads three times with 5 ml of Modified RIPA buffer (Recipe 11), centrifuging at 10,000 rpm for 1 min and discarding the supernatant after each wash.
14. Add 1 bed volume of Phenyl Phosphate Solution (Recipe 13) to the washed beads and incubate for a total of 10 min at 37°C, with gentle mixing after the first 5 min.
15. Transfer the eluate to a fresh 10-ml tube and repeat step 14 once or twice more, pooling the eluates.
16. Dialyze the pooled eluates using dialysis tubing (molecular weight cut off 3500) twice against 4 liters of distilled water for a minimum of 4 hours each time to remove the phenyl phosphate.
17. Reduce the volume of the dialyzed eluate in a vacufuge to about 100 to 150 µl.

*Note: The samples can be frozen at -80°C at this point or analyzed immediately by proceeding to the instructions detailed under Electrophoresis and Silver Staining.*

## **Electrophoresis and Silver Staining of Polyacrylamide Gels**

Samples from each of the procedures mentioned above are separated by SDS-PAGE and then visualized with either silver staining (for low-abundance samples) or by colloidal blue staining (18). Colloidal blue staining should be performed according to the manufacturer's instructions. Methods for silver staining are described below.

1. Boil samples in tightly closed microcentrifuge tubes in 5× Sample (Laemmli) Buffer (Recipe 14) for 5 min.

*Note: For the samples for the relative quantitation of protein abundance, use 50 µg of protein. For monitoring a protein complex and determining the relative quantitation of posttranslational modifications, the beads containing the immunoprecipitated proteins are the samples. For identifying tyrosine kinase substrates, the eluate and the beads are separate samples, and the beads are used to monitor the efficiency of elution.*

2. Run on a vertical electrophoresis apparatus with a 4% stacking gel, 10% resolving gel.

*Note: We prefer to use 14 × 16 cm gels of 1.5 mm thickness, because they provide good resolution and have relatively high loading capacity. Minigels can be used when large amounts of complex protein mixtures are not being analyzed and when resolution is not a problem. The percentage of acrylamide in the running gel will determine the molecular weight range of most effective separation. This should be changed based on the specific needs of the experiment.*

3. Fix the gel by incubating it in Fixing Solution (Recipe 15) for 20 to 30 min.

*Note: All volumes should be sufficient to fully submerge the gel and will depend on the size of the gel and the container.*

4. Rinse the gel slab with water (two changes, 2 min per change) and then leave it in water for 1 hour on a shaking platform.

*Note: Extended washing time helps to eliminate the yellowish background usually observed after developing of the gel.*

5. Incubate the gel in Sensitizing Solution (Recipe 16) for 1 to 2 min.

6. Discard Sensitizing Solution and quickly rinse the gel slab with two changes of water (10 s each).

*Note: Agitate gently to make sure that the gel slab is covered evenly.*

7. Incubate the gel in prechilled Staining Solution (Recipe 17) for 30 min at 4°C.

8. Discard the staining solution and quickly rinse the gel with two changes of water (30 s per change).

9. Incubate the gel in Developing Solution (Recipe 18). Discard the developing solution as soon as it turns yellow, and replace it with fresh solution.

10. When a sufficient degree of staining has been obtained, quench further staining by discarding the developing solution and leaving the gel in Quenching Solution (Recipe 19).

*Note: The gel can be stored at 4°C in Quenching Solution for a few months.*

## In-Gel Trypsin Digestion

1. Rinse the gel with distilled water for 10 min to remove any particulate matter.
2. Excise bands of interest with a clean scalpel.  
*Note: Cut as close to the edge of the band as possible to reduce the amount of "background" gel.*
3. Chop the excised bands into pieces of about 1 mm × 1 mm and transfer the gel particles into a 0.5-ml microcentrifuge tube.
4. Wash the gel particles with 100 µl of Wash Buffer (Recipe 20) for 5 min.
5. Add 100 µl of acetonitrile and incubate for 10 min until the gel pieces shrink. Spin down the gel particles at maximum speed in a microcentrifuge for 10 s, and discard all liquid. Repeat one time.  
*Note: The total liquid volume should correspond to about 3 to 4 times the total volume of gel pieces.*
6. Swell the gel pieces in 50 µl of Reduction Buffer (Recipe 21) and incubate for 30 min at 56°C to reduce the proteins.
7. Spin down the gel particles at maximum speed in a microcentrifuge for 10 s, and discard all liquid.
8. Shrink the gel pieces with 50 µl of acetonitrile, spin down the gel particles at maximum speed in a microcentrifuge for 10 s, and discard all liquid.
9. Add 50 µl of Alkylation Buffer (Recipe 22) to block reactive cysteines, and incubate for 20 min at room temperature in the dark.
10. Spin down the gel particles at maximum speed in a microcentrifuge for 10 s, and discard all liquid.
11. Wash the gel particles with 100 µl of Wash Buffer (Recipe 20) for 10 min, spin down the gel particles at maximum speed in a microcentrifuge for 10 s, and discard all liquid.
12. Add 100 µl of acetonitrile and incubate for 5 min until the gel pieces shrink, spin down the gel particles at maximum speed in a microcentrifuge for 10 s, and discard all liquid.
13. Repeat step 12 two more times.
14. Rehydrate the gel particles in Digestion Buffer (Recipe 23) on ice for 30 to 45 min. After 15 to 20 min, check the samples and add more Digestion Buffer (Recipe 23) if all liquid has been absorbed by the gel pieces.  
*Note: A simplified alternative is to rehydrate the gel slices in sufficient Digestion Buffer (Recipe 24) at room temperature to cover gel pieces followed by incubation at 37°C overnight. The method also applies to in-gel digestions with other commonly used and specific proteases, such as chymotrypsin, lys-C, or glu-C.*
15. Remove and discard any remaining supernatant.
16. Add sufficient Digestion Buffer (Recipe 23) to cover gel pieces (5 to 25 µl) and to keep them wet during enzymatic cleavage (37°C overnight).
17. Add 10 to 15 µl of 25 mM ammonium bicarbonate, vortex briefly, and incubate at room temperature for 10 min.
18. Spin in a microcentrifuge at maximum speed for 10 s, and transfer the supernatant to a fresh microcentrifuge tube.
19. Add acetonitrile (one to two times the volume of the gel particles) to the gel particles, and incubate at 37°C for 15 min with agitation.
20. Spin in a microcentrifuge at maximum speed for 10 s, and pool the supernatant with the extract from step 18.
21. Add 40 to 50 µl of 5% formic acid to the gel particles, and incubate for 15 min at 37°C with agitation.
22. Spin in a microcentrifuge at maximum speed for 10 s, and pool the supernatant with the extract from steps 18 and 20.
23. Add acetonitrile (one to two times the volume of the gel particles) to the gel particles, incubate at 37°C for 15 min with agitation.
24. Spin in a microcentrifuge at maximum speed for 10 s, and pool the supernatant with the extract from steps 18, 20, and 22.
25. Dry down the pooled extracts in a vacufuge.

*Note: Dry digests can be stored in a freezer at -20°C for months.*

## Reversed Phase Column Preparation

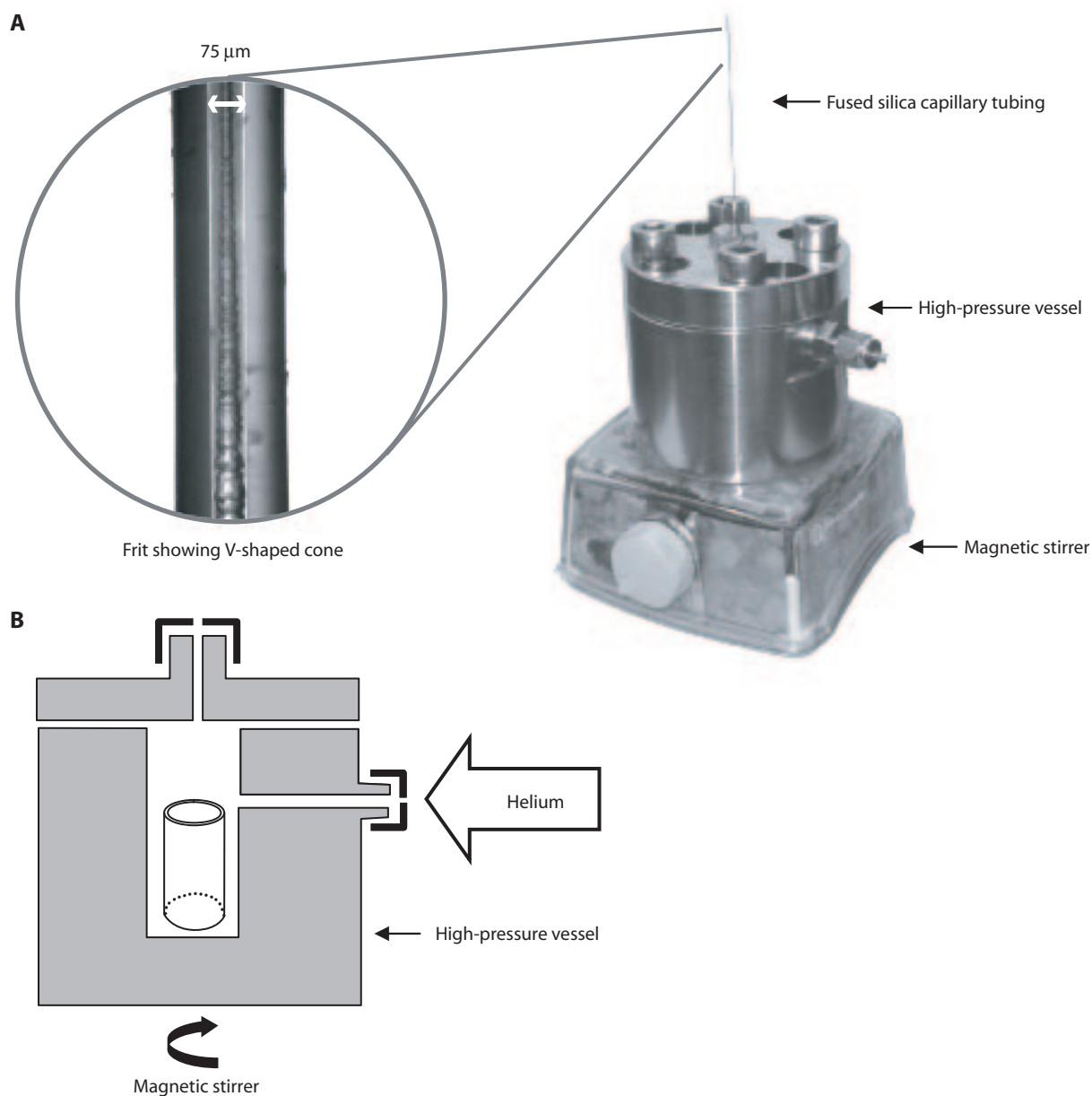
There are two major steps to preparing the column for LC-MS/MS. First, a frit restrictor is prepared, and then the column is packed. Prepacked columns are also commercially available and can be purchased from various vendors, such as Michrom Bioresources (Auburn, CA), New Objective, and Grace Vydac (Hesperia, CA). Depending on the type of sample to be analyzed, one can choose from two different column setups, one with columns in tandem (a precolumn followed by an analytical column) or one with a single analytical column. The tandem column setup is preferred if a large volume (10 to 40  $\mu\text{l}$ ) is to be analyzed, because the precolumn material allows one to load the sample with a high flow rate (4 to 5  $\mu\text{l}/\text{min}$ ). The sample can subsequently be desalted (washed) for additional time if needed before it is eluted onto the analytical column and into the mass spectrometer. This setup is very robust and can be used for most types of samples. One drawback is that sensitivity may be lost because of broadened LC peaks.

### Preparation of a frit restrictor

1. Cut a 15-cm piece of fused silica capillary tubing.
2. Mix 44  $\mu\text{l}$  of Kasil 1 with 8  $\mu\text{l}$  of formamide and vortex for 1 to 3 min.  
*Note: The mixed solution becomes viscous.*
3. Touch one end of the capillary gently into the mixture for 1 to 2 s. The solution will move upward into the tube by capillary action.  
*Note: The solution should not fill the capillary, but only needs to travel about 2 mm up the tubing.*
4. Wipe off the excess solution outside the capillary.
5. Leave it to polymerize overnight at room temperature (25°C) in a vertical position.
6. Check the frit under a microscope to verify that it is about 2 mm in length and presents a V-shaped cone tool that points outward, as shown in Fig. 5.  
*Note: The frit may be trimmed if it is too long, just retaining the V-shaped cone portion.*
7. Insert the capillary into a high-pressure vessel for nano-LC column packing (Proxeon A/S) and wash it with 100% acetonitrile.  
*Note: The acetonitrile wash also ensures that the frit is tightly affixed to the capillary.*

### Packing the column

1. Add the reversed phase material (about 4 mg) to a glass microvial with a small magnetic stirring rod, and add 500  $\mu\text{l}$  of methanol to make a slurry.
2. Place the glass microvial containing the slurry into the high-pressure vessel.
3. Insert the fused silica capillary tubing (frit end should be pointing outward) into the high-pressure vessel for nano-LC column packing so that the end of the capillary is in the slurry but does not touch the bottom of the microvial.
4. Lay the pressure vessel on a stir plate and turn on the magnetic stirring at low speed.
5. Turn on the gas (helium) pressure up to 50 bars (800 psi). The gas pressure may be increased to 1000 psi as the column packing progresses.  
*Note: If the column clogs during packing, try packing a new column using a lower pressure (100 to 500 psi).*
6. Stop packing when the bed volume reaches 5 cm (for the precolumn) or 10 to 15 cm (for the column).
7. Connect the capillary column to the HPLC system and flush it with Mobile Phase B (Recipe 24) for 1 hour (at a flow rate of 1  $\mu\text{l}/\text{min}$ ) followed by Mobile Phase A (Recipe 25) for 2 hours.
8. Perform at least three chromatography separations with a standard protein (such as 100 fmol/ $\mu\text{l}$  trypsin-digested BSA) to evaluate and optimize the LC-MS/MS system and to saturate the active sites in the column material.  
*Note: This should be done before analyzing any of the experimental samples.*
9. Run a blank gradient to evaluate if there is any carry-over from previous runs.



**Fig. 5.** Packing of reversed-phase columns for peptide separation. **(A)** A high-pressure vessel into which a fused silica capillary (with a frit restrictor) has been inserted is placed on a magnetic stirrer. A magnified view of a well-packed fused silica capillary (the frit) is shown. **(B)** A schematic showing the cross section of the high-pressure vessel into which a flat-bottomed glass vial is inserted. The glass vial contains the reversed-phase matrix (in slurry form), and the fused silica capillary is inserted into it for column packing from the top.

## LC-MS/MS Analysis

The flow rate from the pumps varies from 1 to 5  $\mu\text{l}/\text{min}$  depending on the system. During sample loading, the flow rate is high, whereas during peptide separation, the flow rate is decreased. After separation, for column equilibration, the flow rate may be increased to the loading flow rate.

We have acquired the mass spectra on a Micromass Q-TOF API-US in positive-ion mode with the data processing done on a MassLynx Windows XP PC data system (version 4). Before analysis of the experimental samples, the instrument must be calibrated using a standard solution of 1  $\text{pmol}/\mu\text{l}$  [Glu<sup>1</sup>]-fibrinopeptide B, in accordance with the manufacturer's instructions.

1. Centrifuge the supernatant from the in-gel digestion step at 14,000 rpm for 2 min at 4°C in a microcentrifuge.

*Note: This step prevents any small pieces of gel from being loaded onto the well plate and consequently blocking the capillary tubing.*

2. Dry down the supernatant in a vacufuge if the volume exceeds that required for the auto-sampler loading loop.

*Note: The maximum loading volume depends on the “loading loop” in the auto-sampler. One should select a “loading loop” that is appropriate for the particular analysis. We use a 40 µl “loading loop,” which allows one to load 1 to 40 µl of peptide mixture.*

3. Use the auto-sampler to automatically load the sample using a linear gradient of 5 to 10% Mobile Phase B (Recipe 24) onto the column.

*Note: The flow rate should be 4 to 5 µl/min for a tandem column setup and 1 to 1.5 µl/min for a single analytical column setup.*

4. Separate and elute the peptides from the column using a flow rate of 250 nl/min. Apply a 10 to 40% linear gradient of Mobile Phase B (Recipe 24) for 34 min, followed by a 40 to 90% linear gradient of Mobile Phase B (Recipe 24) for 3 min, and then followed by 90% Mobile Phase B (Recipe 24) for 1 to 2 min.

*Note: The gradient program stated here is only a guide and can be modified as required.*

5. Equilibrate the column with a 5% Mobile Phase B (Recipe 24) for 1 to 2 min at the loading flow rate (4 to 5 µl/min for a tandem column setup and 1 to 1.5 µl/min for a single analytical column setup).

6. For ionization of the molecules, apply a potential of 2.7 kV to the emitter in the nanoelectrospray ion source, and apply a voltage of 35 V to the cone.

7. Identify the proteins by searching the mass spectrometry data using a software program such as Mascot, SEQUEST, SpectrumMill, or ProteinLynx.

8. Estimate the peptide pair intensity ratios manually or using appropriate software, such as ProteinLynx Global Server (version 2.0).

*Note: For manual estimation, the extracted ion chromatogram (EIC) is generated from the peaks corresponding to the peptide pairs (light and heavy) of interest, which can then be used to calculate the difference of intensity between peptide pairs (ion ratios) based on the area under the peaks.*

## Troubleshooting

During an LC-MS/MS run, peaks corresponding to different peptides with similar physicochemical properties can overlap. This could result in errors in measurement of the intensity ratios. It is difficult to avoid this problem if very complex protein mixtures are analyzed. Better fractionation by purification of the protein or protein complex (for example, by two-dimensional LC) or the use of higher resolution mass spectrometers such as Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR-MS) should be tried in such cases.

For experiments in which resting cells are compared to stimulated cells, the peptide signal in the resting state might be completely missing and, therefore, no peptide pairs will be observed for comparison purposes. To avoid this problem, we treat a fraction (10%) of cells grown in the light medium (the resting cells) with the stimulus.

The dialyzed serum used to prepare the culture media lacks proteins and polypeptides less than 10 kD. Some cells might require factors that may not be available in the dialyzed serum, so these cells do not survive or propagate in media supplemented with dialyzed serum. In such cases, another cell line that adapts to the medium supplemented with dialyzed serum should be used.

## Notes and Remarks

The SILAC method cannot be used for labeling of harvested tissue samples because they cannot be grown in cell culture.

Choosing the proper combination of heavy isotopes is important. Arginine and lysine are the preferred choices as heavy isotopes, because trypsin cleaves after arginine and lysine residues, so every peptide ending in arginine or lysine can be quantitated in comparison to the light state. For routine quantitative analyses, only one of these isotopes needs to be used. However, for complete coverage of proteins, both arginine and lysine heavy isotopes should be used. This is crucial when relative quantitation of posttranslational modifications is performed. Tyrosine labeling can be used in special cases, such as identification of tyrosine kinase substrates by “marking” tyrosine-containing peptides (19).

We have tested several adherent (NIH 3T3, HEK 293T, HeLa, HepG2, 3T3L1) and suspension (HeLa S3, Jurkat, BaF3, PC-12) cell lines. We find that SILAC labeling has no deleterious effects on these cells' growth and division, morphology, or biological responses.

Alternative labeling options using deuterium instead of  $^{13}\text{C}$  and  $^{15}\text{N}$  have been described for the SILAC method; for example, SILAC was originally described using deuterated leucine (6). However, during reversed-phase chromatography, deuterated species are separated from nondeuterated analogs, and thus elute at slightly different times. To overcome this drawback, fully substituted  $^{13}\text{C}$ -labeled and  $^{15}\text{N}$ -labeled amino acids, such as arginine and lysine, are preferable. Peptides labeled with these molecules present the same chromatographic retention times, or coelute, in LC-MS/MS methods (20). Therefore, the intensity ratios of peak pairs are more accurately assessed.

In the MS/MS, using a broader detection window allows fragmentation of multiple ions at the same time. A narrow window of 2 dalton should be set to prevent the selection of two labeled ions to be fragmented simultaneously, ensuring fragmentation of the heavy and light isotope peaks separately. In the MS/MS mode, inclusion of the entire cluster corresponding to the same precursor ion (isotope peaks), instead of unique selection of the second or third isotopes, ensures good quality collision-induced dissociation (CID) spectra. In Q-TOF instrument, a setting of 2 daltons was used for the MS/MS mode. This setting is an absolute mass value that will extract a 2-dalton range of the  $m/z$  scale and will identify the most intense peak (candidate precursor) within this  $m/z$  range.

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