Instructions

SILAC Protein Quantitation Kits

CIL Catalog No. **Description**

DMEM-LYS-C **SILAC Protein Quantitation Kit DMEM (Dulbecco's Modified Eagle's Medium)**

Kit contains:

- SILAC DMEM Media, 2 x 500 mL
- Dialyzed FBS, 2 x 50 mL
- L-Lysine•2HCl (U-$^{13}C_6$, 99%), 50 mg
- L-Lysine•2HCl, 50 mg
- L-Arginine•HCl, 2 x 50 mg

RPMI-LYS-C **SILAC Protein Quantitation Kit RPMI 1640**

Kit contains:

- SILAC RPMI 1640 Media, 2 x 500 mL
- Dialyzed FBS, 2 x 50 mL
- L-Lysine•2HCl (U-$^{13}C_6$, 99%), 50 mg
- L-Lysine•2HCl, 50 mg
- L-Arginine•HCl, 2 x 50 mg

Storage: Upon receipt store media at 4°C and the Dialyzed Fetal Bovine Serum (FBS) at -20°C. Store all other components at room temperature. The Dialyzed FBS is shipped separately with dry ice; all other components are shipped at ambient temperature.

Table of Contents

Introduction…………………………………………………………………………………………2
Important Product Information…………………………………………………………………2
Additional Materials Required…………………………………………………………………3
Procedure for Stable Isotope Labeling………………………………………………………4
Troubleshooting……………………………………………………………………………………5
Related Products……………………………………………………………………………………9
References ……………………………………………………………………………………………9

Introduction

Cambridge Isotope Laboratories' (CIL) SILAC Protein Quantitation Kits with DMEM or RPMI 1640 contain all reagents necessary for successful isotope metabolic protein labeling, enabling quantitation of protein expression levels from differentially treated cell populations. Stable isotope labeling with amino acids in cell culture (SILAC) is a simple and accurate method to quantify differential changes in the proteome (1-4). SILAC uses metabolic incorporation of nonradioactive ^{13}C, ^{15}N, D enriched amino acids (commonly referred to as "labeled" or "heavy") into proteins using specially formulated DMEM or RPMI 1640. Typical experiments involve growing two cell populations that are identical except that one contains the natural amino acid (commonly referred to as "unlabeled" or "light") and the other population containing one or more "heavy" amino acids. One population will be treated in some fashion (e.g., through chemical treatment or genetic manipulation) whereas the other population is not treated and thus serves as a control.

The SILAC workflow is shown schematically in Figure 1. Equal concentrations of cell lysate from both cell populations are combined for sample processing and subsequent protein separation by SDS-PAGE. Proteins are digested with trypsin to generate peptides for mass spectrometry (MS) and quantitation of isotopic peptide pairs. CIL's SILAC Kits allow MS identification and quantitation of low-abundance proteins, cell-surface or organelle-specific proteins, and post-translational modifications such as phosphorylation or glycosylation.

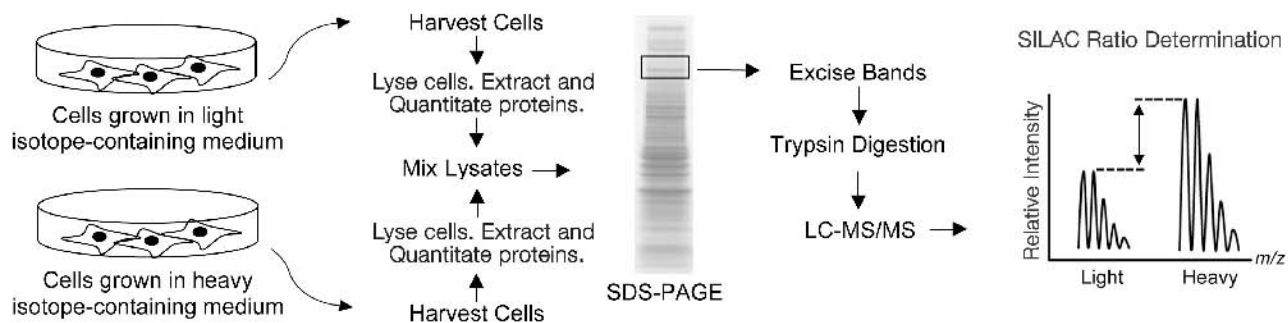

Figure 1. Schematic of SILAC Workflow

Additional labeled amino acids containing ^{13}C, ^{15}N, D, are conveniently listed on page__ of this manual. The addition of other labeled amino acids is often added to increase the labeled peptide coverage. For example, the metabolic incorporation of labeled Arginine and Lysine will label about twice the number of tryptic peptides as compared to using labeled Lysine alone.

Important Product Information

- CIL's SILAC Kits are supplied with L-Lysine•2HCl (U-$^{13}C_6$, 99%), to quantify lysine-containing peptides. To increase peptide coverage for MS analysis, substitute light L-Arginine with L-Arginine•HCl (U-$^{13}C_6$, 99%; $^{15}N_4$, 99%) (CIL Catalog No. CNLM-539-H).

- Isotope-labeled L-Lysine•2HCl (U-$^{13}C_6$, 99%), and L-Arginine•HCl (U-$^{13}C_6$, 99%; $^{15}N_4$, 99%) are biochemically identical to their natural analogs. Therefore, cell growth, morphology and signaling are not affected when incubated with heavy amino acids compared to cells grown in media containing an equivalent amount of light amino acids.

Cambridge Isotope Laboratories, Inc 50 Frontage Road , Andover, MA 01810 978-749-8000 800-322-1174 (N. America) 978-749-2768 fax

- Both labeled and unlabeled L-Lysine•2HCl and L-Arginine•HCl cause a temporary change in media color (red to yellow) upon dissolving. This effect is caused by a brief reduction in media pH that is reversed upon complete mixing and buffering of amino acids with the entire volume of media.

- The final concentration upon dissolving 50 mg of L-Lysine•2HCl and 50 mg L-Arginine•HCl in 500 mL of medium is 0.46 mM and 0.47 mM, respectively. Some cell lines may require more Lysine or Arginine for optimal cell growth and should be supplemented accordingly.

- To maintain sterility, dissolve labeled and unlabeled L-Lysine•2HCl and L-Arginine•HCl in media according to instructions and sterile-filter with a 0.22 µm filter.

- Media supplements, such as L-glutamine and antibiotics (e.g., penicillin, streptomycin), may be used to maintain media performance and sterility.

- To avoid contamination of MS samples, always wear gloves when handling samples and gels. Use ultrapure MS-grade reagents to prepare peptides. Perform sample preparation in a cleaned work area using new polypropylene tubes and razor blades.

Additional Materials Required

- Rapidly growing cell line adapted to DMEM or RPMI 1640 media
- 0.22 µm sterile filters
- Phosphate-buffered saline (PBS): 0.1 M phosphate, 0.15 M sodium chloride; pH 7.2 (Thermo Scientific Product No. 28372 or equivalent)
- Cell lysis reagent such as RIPA Lysis and Extraction Buffer (Thermo Scientific Product No. 89901 or equivalent), M-PER® Mammalian Protein Extraction Reagent (Product No. 78501) NE-PER® Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific Product No. 78833 or equivalent) or Mem-PER® Membrane Protein Extraction Reagent Kit (Thermo Scientific Product No. 89826 or equivalent)
- Pierce BCA Protein Assay Kit (Thermo Scientific Product No. 23227 or equivalent)
- Reducing Sample Buffer (Thermo Scientific Product No. 39000 or equivalent)
- Polyacrylamide gel for SDS-PAGE (Thermo Scientific Product No. 25204 or equivalent)
- GelCode™ Blue Stain Reagent (Thermo Scientific Product No. 24590 or equivalent)
- In-Gel Tryptic Digestion Kit (Thermo Scientific Product No. 89871 or equivalent)
- Formic Acid, 99+% (Thermo Scientific Product No. 28905 or equivalent)
- Protease and phosphatase inhibitors (Thermo Scientific Product No. 78410 and 78420 or equivalent, respectively)

Cambridge Isotope Laboratories, Inc 50 Frontage Road , Andover, MA 01810 978-749-8000 800-322-1174 (N. America) 978-749-2768 fax

Procedure for Stable Isotope Labeling

The following protocol is an example application for this product. Specific applications will require optimization or a different workflow.

A. Supplementation of Media

1. Remove 50 mL of media from each bottle and replace with 50 mL of thawed Dialyzed FBS.

2. Dissolve 50 mg of L-Lysine• 2HCl (U-$^{13}C_6$, 99%) (labeled) and 50 mg of L-Arginine-HCl (unlabeled) using 1 mL of media and mix thoroughly.
   Note: If using L-Arginine•HCl (U-$^{13}C_6$, 99%; $^{15}N_4$, 99%) available separately, CIL Catalog No. CNLM-539-H for double labeling, substitute it for L-Arginine•HCl.

3. Add dissolved amino acids to one 500 mL bottle of media containing Dialyzed FBS and mix thoroughly. If required for a specific cell line, include additional antibiotics and media supplements.

4. Sterile-filter media containing dissolved amino acids using a 0.22 µm filter.

5. Label the bottle containing L-Lysine• 2HCl (U-$^{13}C_6$, 99%) "Labeled"

6. Repeat steps 2-4 using 50 mg of L-Lysine•2HCl (unlabeled) and 50 mg of L-Arginine•HCl (unlabeled). Label this second bottle of supplemented media "Unlabeled."
   Note: After supplementing media with dialyzed serum, media stability is less than 6 months. Store media at 4°C protected from light.

B. Incorporation of Isotopic Labeled Amino Acids

1. Depending on cell type, split 1-2 × 10^5 of cells adapted to grow in DMEM or RPMI 1640 media into two tissue culture flasks or plates, one containing heavy and one containing light SILAC media.
   Note: For suspension cells grown in T-25 flasks, use 8 mL of each media. For adherent cells grown in 60 × 15 mm plates, use 4 mL of each media.

2. Passage both cell populations for at least five cell doublings by changing medium or splitting cells as appropriate every 2-3 days. Maintain density so that cells are actively growing in log phase (between 30-90% confluency).

   Note: Cells grown in labeled and unlabeled SILAC media should have similar cell growth and viability.

3. After five cell doublings, incorporation of heavy L-Lysine and/or L-Arginine should be > 95% (Figure 2). Harvest 10^6 cells from each sample (light and heavy) to determine incorporation efficiency (Section C).

4. Once full isotope incorporation has been determined continue to culture light- and heavy-labeled cells to desired cell number required for subsequent cell treatment and lysis (Section D).
   Note: Light- and heavy-labeled cells can be frozen using the appropriate freezing medium (e.g., 10% DMSO in SILAC media).

Cambridge Isotope Laboratories, Inc 50 Frontage Road , Andover, MA 01810 978-749-8000 800-322-1174 (N. America) 978-749-2768 fax

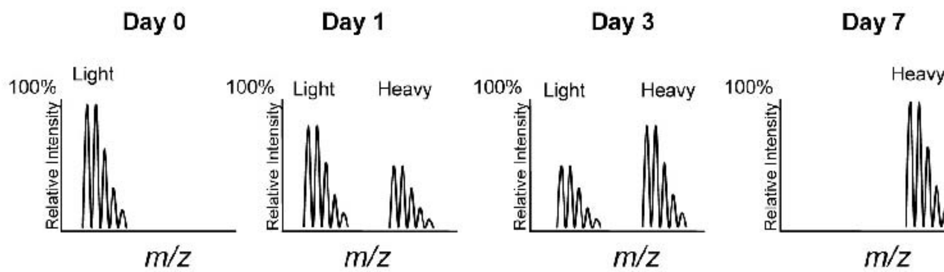

Figure 2. Schematic of label incorporation time course.

C. Determination of Isotope Incorporation Efficiency

1. Lyse a portion (e.g., 10^6 cells) of both heavy and light SILAC cells with 500 µL of 1X reducing sample buffer. Boil samples for 5 minutes and clarify by centrifuging at 14,000 × *g* for 1 minute.

2. Load 25-50 µL of heavy and light samples into two separate wells of a polyacrylamide gel and separate proteins by electrophoresis.

3. Stain gel using GelCode Blue Stain Reagent according to the product instructions. Excise the same protein band from each gel lane.

4. Digest proteins to MS-compatible peptides using the In-Gel Tryptic Digestion Kit or other suitable method.

5. Verify incorporation efficiency using MS analysis of peptides from light- and heavy-labeled proteins (Section E). Compared to light peptides, +2 ionized, heavy isotope peptides containing L-Lysine• 2HCl (U-$^{13}C_6$, 99%) or L-Arginine•HCl (U-$^{13}C_6$; $^{15}N_4$) will be shifted by 3 and 5 *m/z*, respectively.

D. Cell Treatment, Lysis and SDS-PAGE

After verifying that > 95% of the heavy isotope label was incorporated, the remaining cells prepared in Section B4 are ready for treatment to alter protein abundance in one cell population. Types of treatment include cell differentiation induction, siRNA knockdown of target proteins, environmental stress or drug treatment. For best results treat only the heavy-labeled cells because keratin contaminants are more easily detected when the light sample is used as the control; keratin contaminants contain only unlabeled amino acids.

1. After cell treatment, harvest and count both unlabeled and labeled (treated) cells.
   Note: In some cases it may be desirable to repeat the treatment of the light cells to check for any bias.

2. Pellet cells by centrifuging for 5-10 minutes at 500 × *g*. Remove media, wash cells with five cell-pellet volumes of PBS and pellet cells again.

3. Lyse cells on ice using an appropriate lysis buffer containing protease and phosphatase inhibitors. Lyse cells with minimal lysis buffer to obtain protein concentrations of 2-10 mg/mL. Centrifuge samples at 14,000 × *g* for 5 minutes to pellet the cell debris.

   Note: The total number of cells to use for lysis depends on sample preparation, enrichment and instrumentation

   sensitivity. To obtain 50-100 µg of total protein using whole cell lysis buffers, such as M-PER Reagent or RIPA, 1-2 × 10^6

Cambridge Isotope Laboratories, Inc 50 Frontage Road , Andover, MA 01810 978-749-8000 800-322-1174 (N. America) 978-749-2768 fax

cells are typically required. When isolating membrane proteins using Mem-PER Reagent or nuclear proteins using NE-PER Reagent, use 1-2 × 10^7 cells. Greater than 2 × 10^8 cells may be needed to isolate low abundance proteins from organelles (mitochondria, lysosomes, etc) or when using affinity enrichment strategies such as immunoprecipitation or post-translational modification (phosphorylation, glycosylation) capture.

4. Determine protein concentration of each sample in triplicate using a BCA Protein Assay Kit (Thermo Scientific No. 23227 or equivalent) and a standard curve generated with bovine serum albumin (BSA).

5. Mix equal protein amounts of each cell lysate in a new tube.
   Note: Additional protein enrichment techniques may be performed on equally mixed lysates before SDS-PAGE.

6. Dilute equally mixed sample to 2 mg/mL with 2X reducing sample buffer. Boil samples for 5 minutes and clarify by centrifuging at 14,000 × *g* for 1 minute.

7. Load 50-100 µg (25-50 µL) of clarified sample in one well of a gel and separate proteins by SDS-PAGE.

8. Stain gel using GelCode Blue Stain Reagent (Thermo Scientific No. 24590 or equivalent). Excise protein bands from lane by cutting 8-12 gel slices (0.5 cm × 0.5 cm) using new razor blades.

9. Generate MS-compatible peptides using the In-Gel Tryptic Digestion Kit (Thermo Scientific No. 89871 or equivalent) or other suitable method.

E. MS Analysis and Quantitation of SILAC Peptides

Typical LC-MS/MS analysis of peptides is performed using a C18 reverse-phase column (e.g., 3 µm, Hypersil Gold C18, Thermo Scientific Product No. 25002-05303 or equivalent).

1. Fractionate peptides with a 5-90% acetonitrile gradient containing 0.1% formic acid at 200 µL/minute for 200-400 minutes.
   Note: A hybrid linear ion trap-Orbitrap Mass Spectrometer (Finnigan LTQ-FT, Thermo Scientific) or equivalent is recommended for MS analysis.

2. Protein identification can be performed by searching the human IPI database (ipi.Human.v3.18.fasta) with SEQUEST in the Thermo Scientific BioWorks 3.3.1 software suite.

3. Peptide SILAC ratios can be calculated using BioWorks 3.3.1 PepQuan SILAC function using the following settings: target ions = residue = K, Mass = 6.0, mass tolerance = 0.1, minimum threshold = 10, no smoothing, and perform calculation base on area.

4. For L-Lysine• 2HCl (U-$^{13}C_6$, 99%) labeled and L-Arginine•HCl (U-$^{13}C_6$, 99%; $^{15}N_4$, 99%) labeled peptides, compare peak intensities to peptides separated by 3 *m/z* and 5 *m/z*, respectively (see Figure 3 for examples).

5. Determine protein SILAC ratios by averaging all peptide SILAC ratios from peptides identified of the same protein.

Cambridge Isotope Laboratories, Inc 50 Frontage Road , Andover, MA 01810 978-749-8000 800-322-1174 (N. America) 978-749-2768 fax

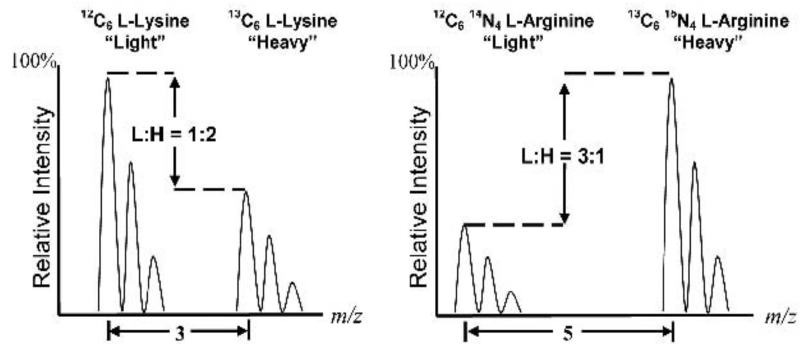

Figure 3. **Schematic of SILAC Ratio Quantitation.** Compared to light peptides, +2 ionized, heavy isotope peptides containing L-Lysine• 2HCl (U-$^{13}C_6$) or L-Arginine•HCl (U-$^{13}C_6$; $^{15}N_4$) will be shifted by 3 and 5 m/z, respectively. Schematic illustrates representative examples of an L-Lysine• 2HCl (U-$^{13}C_6$) labeled peptide that decreased in half compared to a corresponding light peptide and a L-Arginine•HCl (U-$^{13}C_6$; $^{15}N_4$) labeled peptide with relative abundance that increased three times compared to a corresponding light peptide.

Cambridge Isotope Laboratories, Inc 50 Frontage Road , Andover, MA 01810 978-749-8000 800-322-1174 (N. America) 978-749-2768 fax

Troubleshooting

Problem	Possible Cause	Solution
Peaks observed only for peptides containing light amino acids	Did not use SILAC media and/or dialyzed serum	Use only SILAC DMEM or RPMI 1640 media supplemented with dialyzed serum
		Always compare cell populations grown in media supplemented with equivalent amounts of light and heavy amino acids
	Incomplete incorporation of heavy amino acids into proteins	Incubate rapidly growing (log phase) cells with heavy amino acids for at least five doublings
		Confirm cell growth and viability before cell lysis
	Improper mixing of light and heavy samples	Mix equal amounts (1:1) of heavy- and light-labeled cell lysates
		Lyse equal amounts of heavy- and light-labeled cells to ensure equal amounts of protein for mixing
	Keratin contamination in samples	Always wear gloves when handling samples and gels
		Use ultrapure MS grade reagents to prepare peptides
		Perform sample preparation in clean work area using new polypropylene tubes and razor blades
	Treatment of heavy sample reduced protein levels below limit of MS detection	Enrich for proteins of interest before MS analysis
Peptides for protein of interest not detected	Insufficient protein in cell lysates	Increase amount of cells used to generate cell lysate
	Protein is of low abundance	Increase amount of sample analyzed by MS
		Enrich for protein/peptide of interest before MS analysis
	Peptide identification score is low	Ensure MS instrument is calibrated correctly
		Verify database search criteria
Heavy proline detected in peptides from cells labeled with heavy arginine	High arginase activity in cell line	Sum peak intensities for peptides containing both heavy proline and heavy arginine to determine total heavy relative peak intensity for SILAC ratio
		Reduce heavy arginine concentration in media
		Use a cell line with low arginase activity

CIL

Related Products

Catalog No.	Description
CLM-2247-H	L-Lysine•2HCl (U-$^{13}C_6$, 99%)
CNLM-291-H	L-Lysine•2HCl (U-$^{13}C_6$, 99%; $^{15}N_2$, 99%)
DLM-2640	L-Lysine•2HCl (4,4,5,5-D_4, 96-98%)
CLM-2265-H	L-Arginine•HCl (U-$^{13}C_6$, 99%)
CNLM-539-H	L-Arginine•HCl (U-$^{13}C_6$, 99%; $^{15}N_4$, 99%)
ULM-8347	L-Arginine•HCl (unlabeled)
ULM-8766	L-Lysine•2HCl (unlabeled)
ULM-8333	L-Proline (unlabeled)

Please visit www.isotope.com for a complete listing of products for quantitative proteomics.

References

1. Mann, M. **2006.** Functional and quantitative proteomics using SILAC. Nat Rev Mol Cell Biol, 7*(12)*, 952-958.

2. Everly, P.A., *et al*. **2004**. Quanitative cancer proteomics: Stable isotope labeling with amino acids (SILAC) as a tool for prostate cancer research. Mol Cell Proteomics *3(7)* ,729-35.

3. Selbach, M., Mann, M. **2006.** Protein interaction screening by quantitative immune precipitation combined with knockdown (QUICK). Nature Methods, 3*(12)*, 981-983.

4. Amanchy, R., Kalume. D.E., Pandey, A. **2005.** Stable isotope labeling with amino acids in cell culture (SILAC) for studying dynamics of protein abundance and posttranslational modifications. Sci STKE, *267*, 1-20.

SILAC Media and Dialyzed FBS are manufactured by Thermo Fisher Scientific, Inc. SILAC Media is provided by Thermo Fisher Scientific under license from the University of Washington and protected by U.S. Patent 6,653,076, for research use only. Purchase of this product conveys a non-transferable license to the Purchaser to use this product in methods protected under U.S Patent 6,653,076 (owed by University of Washington) for research purposes only.

This product (“Product”) is warranted to operate or perform substantially in conformance with published Product specifications in effect at the time of sale, as set forth in the Product documentation, specifications and/or accompanying package inserts (“Documentation”) and to be free from defects in material and workmanship. Unless otherwise expressly authorized in writing, Products are supplied for research use only. No claim of suitability for use in applications regulated by FDA is made. The warranty provided herein is valid only when used by properly trained individuals. Unless otherwise stated in the Documentation, this warranty is limited to one year from date of shipment when the Product is subjected to normal, proper and intended usage. This warranty does not extend to anyone other than the original purchaser of the Product (“Buyer”).

No other warranties, express or implied, are granted, including without limitation, implied warranties of merchantability, fitness for any particular purpose, or non infringement. Buyer’s exclusive remedy for non-conforming Products during the warranty period is limited to replacement of or refund for the non-conforming Product(s).
There is no obligation to replace Products as the result of (i) accident, disaster or event of force majeure, (ii) misuse, fault or negligence of or by Buyer, (iii) use of the Products in a manner for which they were not designed, or (iv) improper storage and handling of the Products.

Current versions of product instructions are available at *www.isotope.com*.
For a faxed copy, call 800-322-1174 or contact your local representative at 1-800-ISOTOPE.