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-13C₆, 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-13C₆, 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.

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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.

![SILAC Workflow Diagram](image)

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.
• 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)
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-13C6, 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-13C6, 99%; 15N4, 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-13C6, 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).
**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 x 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-13C6, 99%) or L-Arginine•HCl (U-13C6; 15N4) 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 x 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 x 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 x 10^6.
cells are typically required. When isolating membrane proteins using Mem-PER Reagent or nuclear proteins using NE-PER Reagent, use 1-2 × 10⁷ cells. Greater than 2 × 10⁸ 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⁻¹³C₆, 99%) labeled and L-Arginine•HCl (U⁻¹³C₆, 99%; ¹⁵N₄, 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.
Figure 3. Schematic of SILAC Ratio Quantitation. Compared to light peptides, +2 ionized, heavy isotope peptides containing L-Lysine•HCl (U-\textsuperscript{13}C\textsubscript{6}) or L-Arginine•HCl (U-\textsuperscript{13}C\textsubscript{6}; \textsuperscript{15}N\textsubscript{4}) will be shifted by 3 and 5 m/z, respectively. Schematic illustrates representative examples of an L-Lysine•HCl (U-\textsuperscript{13}C\textsubscript{6}) labeled peptide that decreased in half compared to a corresponding light peptide and a L-Arginine•HCl (U-\textsuperscript{13}C\textsubscript{6}; \textsuperscript{15}N\textsubscript{4}) labeled peptide with relative abundance that increased three times compared to a corresponding light peptide.
# Troubleshooting

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<thead>
<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peaks observed only for peptides containing light amino acids</td>
<td>Did not use SILAC media and/or dialyzed serum</td>
<td>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.</td>
</tr>
<tr>
<td>Incomplete incorporation of heavy amino acids into proteins</td>
<td>Incubate rapidly growing (log phase) cells with heavy amino acids for at least five doublings. Confirm cell growth and viability before cell lysis.</td>
<td></td>
</tr>
<tr>
<td>Improper mixing of light and heavy samples</td>
<td>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.</td>
<td></td>
</tr>
<tr>
<td>Keratin contamination in samples</td>
<td>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.</td>
<td></td>
</tr>
<tr>
<td>Treatment of heavy sample reduced protein levels below limit of MS detection</td>
<td>Enrich for proteins of interest before MS analysis.</td>
<td></td>
</tr>
<tr>
<td>Peptides for protein of interest not detected</td>
<td>Insufficient protein in cell lysates</td>
<td>Increase amount of cells used to generate cell lysate. Increase amount of sample analyzed by MS. Enrich for protein/peptide of interest before MS analysis.</td>
</tr>
<tr>
<td>Protein is of low abundance</td>
<td>Peptide identification score is low</td>
<td>Ensure MS instrument is calibrated correctly. Verify database search criteria.</td>
</tr>
<tr>
<td>Heavy proline detected in peptides from cells labeled with heavy arginine</td>
<td>High arginase activity in cell line</td>
<td>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.</td>
</tr>
</tbody>
</table>
Related Products

<table>
<thead>
<tr>
<th>Catalog No.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLM-2247-H</td>
<td>L-Lysine•2HCl (U-$^{13}$C$_6$, 99%)</td>
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<tr>
<td>CNLM-291-H</td>
<td>L-Lysine•2HCl (U-$^{13}$C$_6$, 99%; $^{15}$N$_2$, 99%)</td>
</tr>
<tr>
<td>DLM-2640</td>
<td>L-Lysine•2HCl (4,4,5,5-D$_4$, 96-98%)</td>
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<tr>
<td>CLM-2265-H</td>
<td>L-Arginine•HCl (U-$^{13}$C$_6$, 99%)</td>
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<tr>
<td>CNLM-539-H</td>
<td>L-Arginine•HCl (U-$^{13}$C$_6$, 99%; $^{15}$N$_4$, 99%)</td>
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<tr>
<td>ULM-8347</td>
<td>L-Arginine•HCl (unlabeled)</td>
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<tr>
<td>ULM-8766</td>
<td>L-Lysine•2HCl (unlabeled)</td>
</tr>
<tr>
<td>ULM-8333</td>
<td>L-Proline (unlabeled)</td>
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Please visit [www.isotope.com](http://www.isotope.com) for a complete listing of products for quantitative proteomics.

References


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Current versions of product instructions are available at [www.isotope.com](http://www.isotope.com). For a faxed copy, call 800-322-1174 or contact your local representative at 1-800-ISOTOPE.