INSTRUCTIONS

SILAC Protein Quantitation Kits

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A33969 A33970 A33971 A33972 A33973

Number	Description
A33969	**SILAC Protein Quantitation Kit (LysC) – DMEM** **Kit Contents:** **DMEM for SILAC,** 2 × 500mL **$^{13}C_6$ L-Lysine-2HCl,** 50mg **L-Lysine-2HCl,** 50mg **L-Arginine-HCl,** 2 × 50mg **Dialyzed Fetal Bovine Serum,** 1 × 100mL
A33970	**SILAC Protein Quantitation Kit (LysC) – DMEM:F12** **Kit Contents:** **DMEM:F12 for SILAC,** 2 × 500mL **$^{13}C_6$ L-Lysine-2HCl,** 50mg **L-Lysine-2HCl,** 50mg **L-Arginine-HCl,** 2 × 50mg **Dialyzed Fetal Bovine Serum,** 1 × 100mL
A33971	**SILAC Protein Quantitation Kit (LysC) – RPMI 1640** **Kit Contents:** **RPMI 1640 Medium for SILAC,** 2 × 500mL **$^{13}C_6$ L-Lysine-2HCl,** 50mg **L-Lysine-2HCl,** 50mg **L-Arginine-HCl,** 2 × 50mg **Dialyzed Fetal Bovine Serum,** 1 × 100mL
A33972	**SILAC Protein Quantitation Kit (Trypsin) – DMEM** **Kit Contents:** **DMEM:F12 for SILAC,** 2 × 500mL **$^{13}C_6$ $^{15}N_2$ L-Lysine-2HCl,** 50mg **L-Lysine-2HCl,** 50mg **$^{13}C_6$ $^{15}N_4$ L-Arginine-HCl,** 50mg **L-Arginine-HCl,** 50mg **Dialyzed Fetal Bovine Serum,** 1 × 100mL

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A33973 **SILAC Protein Quantitation Kit (Trypsin) – RPMI 1640**

Kit Contents:

DMEM:F12 for SILAC, 2 × 500mL

$^{13}C_6{}^{15}N_2$ L-Lysine-2HCl, 50mg

L-Lysine-2HCl, 50mg

$^{13}C_6$ $^{15}N_4$ L-Arginine-HCl, 50mg

L-Arginine-HCl, 50mg

Dialyzed Fetal Bovine Serum, 1 × 100mL

Storage: Upon receipt store media at 4°C, Dialyzed Fetal Bovine Serum (FBS) at -20°C and amino acids 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

The Thermo Scientific™ SILAC Protein Quantitation Kits with DMEM, DMEM:F12 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- or ^{15}N-labeled amino acids, referred to as "heavy" amino acids, into proteins using specially formulated media supplemented with dialyzed fetal bovine serum.

Typical experiments involve growing two cell populations using cell culture media that are identical except that one contains the natural amino acid, referred to as "light", and the other contains the heavy form (e.g., $^{12}C_6$ and $^{13}C_6$ L-lysine, respectively). Heavy L-arginine, available separately (Product No. 88210, 89990) or part of SILAC kits (Product No. A33972, A33973), is often added to enhance peptide isotope label coverage when using trypsin for protein digestion. Protein levels in one sample are then altered through chemical treatment or genetic manipulation. Equal concentrations of protein cell lysate from both cell populations are then combined and digested with Lys C or trypsin using in-gel (Product No. 89871) or in-solution (Product No. 84840) sample preparation methods to generate peptides for mass spectrometry (MS) and quantitation of isotopic peptide pairs (Figure 1). Alternatively, the untreated sample can be used as an internal standard to compare multiple samples with different treatments. When combined with Thermo Scientific™ Protein/Peptide Sample Enrichment Products, the Pierce 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.

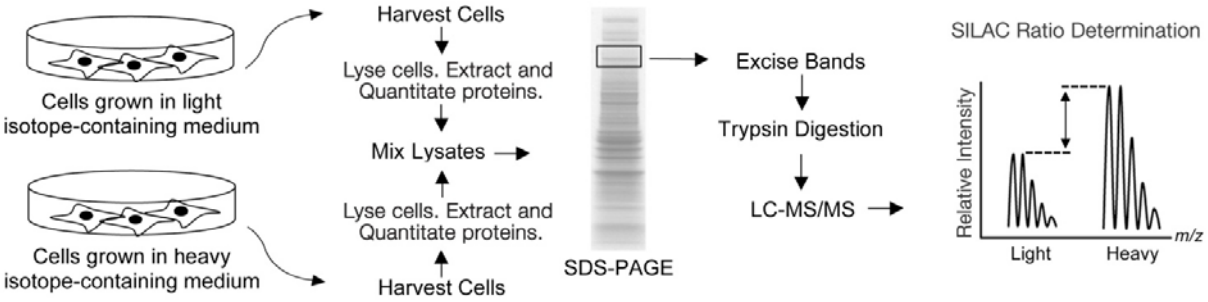

Figure 1. Schematic of SILAC workflow.

Although duplex SILAC experiments are most common, up to three different experimental conditions can be readily analyzed with different heavy isotopes of lysine and arginine. For lysine three-plex experiments, 4,4,5,5-D_4 L-lysine and $^{13}C_6$ $^{15}N_2$ L-lysine are used to generate peptides with 4- and 8-Da mass shifts, respectively, compared to peptides generated with light lysine (See Additional Information). For arginine three-plex experiments, $^{13}C_6$ L-arginine and $^{13}C_6$ $^{15}N_4$ L-arginine are used to generate peptides with 6- and 10-Da mass shifts, respectively, compared to peptides generated with light arginine. L-leucine is commonly used for SILAC labeling because of its prevalence in protein sequences and ability to allow for quantitation of non-tryptic peptides. Proline is a non-essential amino acid that is sometimes added to SILAC media to prevent the metabolic conversion of heavy arginine to heavy proline in mammalian cell lines with high arginine dehydrogenase activity.[5]

Important Product Information

- LysC SILAC Kits are supplied with $^{13}C_6$ L-Lysine-2HCl to label and quantify lysine-containing peptides from LysC protein digests. LyC SILAC kits can be converted to a trypsin SILAC kit by substituting light L-arginine with $^{13}C_6$ L-Arginine-HCl (Product No. 88210) or $^{13}C_6$ $^{15}N_4$ L-Arginine-HCl (Product No. 89990). Note the heavy L-lysine-2HCl isotope is different ($^{13}C_6$ vs. $^{13}C_6$ $^{15}N_4$) between the LysC and trypsin SILAC kits.
- Trypsin SILAC kits are supplied with $^{13}C_6$ $^{15}N_2$ L-Lysine-2HCl and $^{13}C_6$ $^{15}N_4$ L-Arginine-HCl to label and quantify lysine-containing and arginine-containing peptides from trypsin protein digests.
- Stable isotope-labeled amino acids 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. Cells cultured using SILAC media supplemented with Dialyzed FBS (Product No. 26400036) may have some reduction in growth compared to cells grown in media supplemented with standard FBS.
- Both heavy and light 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 50mg of L-Lysine-2HCl and 50mg of L-Arginine-HCl in 500mL of medium is 0.46mM and 0.47mM, respectively. Some cell lines may require more lysine or arginine for optimal cell growth and should be supplemented accordingly.
- To maintain sterility, dissolve heavy and light L-Lysine-2HCl and L-Arginine-HCl in media according to instructions and sterile-filter with a 0.22µm 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 and perform sample preparation in a cleaned work area.

Additional Materials Required

- Rapidly growing cell line adapted to DMEM, DMEM:F12 or RPMI 1640 media
- 0.22µm sterile filters
- Phosphate-buffered saline (PBS): 0.1M phosphate, 0.15M sodium chloride; pH 7.2 (Product No. 28372)
- Cell lysis reagent such as Thermo Scientific™ RIPA Lysis and Extraction Buffer (Product No. 89901), M-PER™ Mammalian Protein Extraction Reagent (Product No. 78501) or Pierce™ IP Lysis Buffer (Product No. 87787)
- Protease and phosphatase inhibitors [Product No. 87786 and 78420, respectively or 78440 (combination)]
- Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Product No. 23227)
- Reducing Sample Buffer (Product No. 39000)
- Polyacrylamide gel for SDS-PAGE (Product No. 25204)
- Thermo Scientific™ GelCode™ Blue Stain Reagent (Product No. 24590)
- Thermo Scientific™ Pierce™ In-Gel Tryptic Digestion Kit (Product No. 89871)
- LC-MS Grade Acetonitrile (Product No. 51101)
- Formic Acid, 99+% (Product No. 28905)

Procedure for Stable Isotope Labeling

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

A. Supplementation of Media

1. Remove 50mL of media from each bottle and replace with 50mL of thawed Dialyzed FBS.
2. For the LysC SILAC kit, dissolve 50mg of $^{13}C_6$ L-Lysine-2HCl (heavy) and 50mg of L-Arginine-HCl (light) using 1mL of media and mix thoroughly.
3. For the trypsin SILAC kit, dissolve 50mg of $^{13}C_6$ $^{15}N_2$ L-Lysine-2HCl (heavy) and 50mg of $^{13}C_6$ $^{15}N_4$ L-Arginine-HCl (heavy) using 1mL of media and mix thoroughly.
4. Add dissolved amino acids to one 500mL bottle of media containing Dialyzed FBS and mix thoroughly. If required for a specific cell line, include additional antibiotics and media supplements.
5. Sterile-filter media containing dissolved amino acids using a 0.22µm filter.
6. Label the bottle containing stable isotope amino acids as "Heavy."
7. Repeat steps 2-5 using 50mg of L-Lysine-2HCl (light) and 50mg of L-Arginine-HCl (light). Label this second bottle of supplemented media "Light."

   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 cells adapted to grow in DMEM, DMEM:F12 or RPMI 1640 media into two tissue culture flasks or plates with one containing heavy and one containing light SILAC media.

   Note: For suspension cells grown in T-25 flasks, use 8mL of each media. For adherent cells grown in 60 × 15mm plates, use 4mL 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).
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 expand 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).

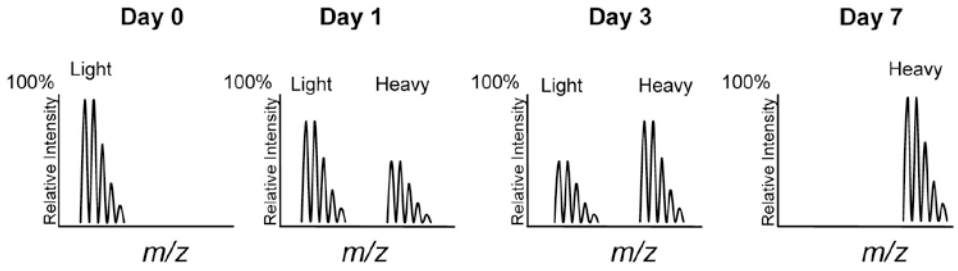

Figure 2. Schematic of label incorporation time course.

C. Determination of Isotope Incorporation Efficiency

1. Lyse a portion (e.g., 10^6 cells) of both heavy and light SILAC cells with 500µL of 1X reducing sample buffer. Boil samples for 5 minutes and clarify by centrifuging at 14,000 × *g* for 1 minute.
2. Load 25-50µL 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 (see the Tech Tip on the website for the modified kit protocol) or other suitable method.
5. Verify incorporation efficiency using MS analysis of peptides from light- and heavy-labeled proteins (Section E).

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.

1. After cell treatment, harvest and count both light- and heavy-labeled (treated) cells.
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 2-10mg/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 50-100µg of total protein using whole cell lysis buffers, such as M-PER Reagent, RIPA or Pierce IP Lysis Buffer, 1-2 × 10^6 cells are typically required. When isolating membrane proteins using Thermo Scientific™ Mem-PER™ Reagent or nuclear proteins using Thermo Scientific™ 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 the Pierce BCA Protein Assay Kit 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 fractionation or enrichment techniques may be performed on equally mixed lysates before SDS-PAGE.
6. Dilute equally mixed sample to 2mg/mL with 2X reducing sample buffer. Boil samples for 5 minutes and clarify by centrifuging at 14,000 × *g* for 1 minute.
7. Load 50-100µg (25-50µL) of clarified sample in one well of a gel and separate proteins by SDS-PAGE.

8. Stain gel using GelCode Blue Stain Reagent. Excise protein bands from lane by cutting 8-12 gel slices (0.5cm × 0.5cm) using new razor blades.
9. Generate MS-compatible peptides using the In-Gel Tryptic Digestion Kit 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., 3µm, Hypersil™ Gold C18, Thermo Scientific Product No. 25002-05303) using a high resolution mass spectrometer such as a hybrid linear ion trap-Orbitrap™ (Thermo Scientific) or equivalent is recommended for MS analysis.

1. Fractionate peptides with a 5-40% acetonitrile gradient containing 0.1% formic acid at 200µL/minute for 1-3 hrs.
2. Protein identification can be performed by searching the human IPI database or equivalent with the Thermo Scientific™ Proteome Discoverer™ software (v1.2 or later).
3. Peptide SILAC ratios can be calculated using Proteome Discoverer Software (v1.2 or later) Precursor Ions Quantifier node using a pre-built Quantification Method included with the software.

   Note: Custom quantification methods can be created by editing the SILAC two-plex or three-plex method.
4. Determine protein SILAC ratios by averaging all peptide SILAC ratios from peptides identified of the same protein.

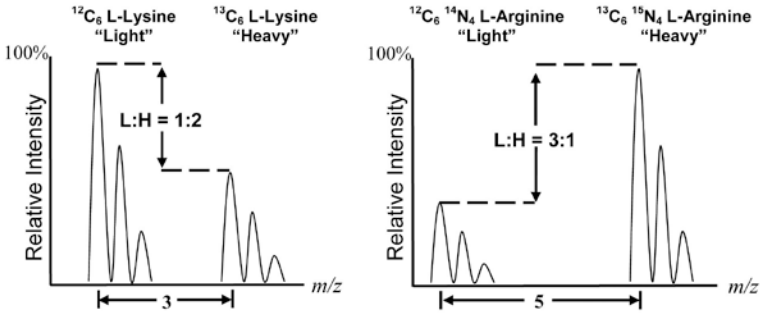

Figure 3. Schematic of SILAC Ratio Quantitation. Compared to light peptides, +2 ionized, heavy isotope peptides containing $^{13}C_6$ L-Lysine or $^{13}C_6$ $^{15}N_4$ L-Arginine will be shifted by 3 and 5 *m/z*, respectively. Schematic illustrates representative examples of a $^{13}C_6$ L-Lysine-labeled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

Troubleshooting

Problem	Possible Cause	Solution
Spectra for only light peptides observed	Did not use SILAC media and/or dialyzed serum	Use Dialyzed FBS to supplement SILAC media
	Incorrect modifications used for software quantitation	Verify Precursor Ions Quantifier method and isotope monoisotopic mass (See Additional Information)
	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 was in low abundance	Increase amount of sample analyzed by MS
		Enrich for protein/peptide of interest before MS analysis
	Peptide identification score was 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
		Supplement SILAC media with additional L-proline
		Reduce heavy arginine concentration in media

Additional Information

A. Light and heavy amino acid molecular weights (MW) and monoisotopic mass increase

Amino Acid	MW	Monoisotopic Mass Increase
L-Arginine-HCl	210.66	N/A
$^{13}C_6$ L-Arginine-HCl	216.62	6.020129
$^{13}C_6$ $^{15}N_4$ L-Arginine-HCl	220.59	10.008269
L-Leucine	131.17	N/A
$^{13}C_6$ L-Leucine	137.13	6.020129
L-Lysine-2HCl	219.11	N/A
4,4,5,5-D_4 L-Lysine-2HCl	223.13	4.025107
$^{13}C_6$ L-Lysine-2HCl	225.07	6.020129
$^{13}C_6$ $^{15}N_2$ L-Lysine-2HCl	227.05	8.014199
3,3,4,4,5,5,6,6-D_4 L-Lysine-2HCl	227.16	8.05021
L-Proline	115.13	N/A

B. Information available from our website

- SILAC Protein Quantitation Kits FAQ
- Tech Tip #60: Prepare SILAC peptides using the In-Gel Tryptic Digestion Kit

Reordering Information

88364	**DMEM for SILAC**, 500mL
88365	**RPMI-1640 Medium for SILAC**, 500mL
88370	**DMEM:F12 for SILAC**, 500mL
26400036	**Dialyzed Fetal Bovine Serum**, 100mL

Supplemental Amino Acid Product Numbers

Amino Acid	Light	D_4	$^{13}C_6$	D_8	$^{13}C_6$ $^{15}N_2$	$^{13}C_6$ $^{15}N_4$
L-Arginine-HCl	89989 (50mg) 88427 (500mg)	N/A	88210 (50mg) 88433 (500mg)	N/A	N/A	89990 (50mg) 88434 (500mg)
L-Leucine	88428 (500mg)	N/A	88435 (50mg) 88436 (500mg)	N/A	N/A	N/A
L-Lysine-2HCl	89987 (50mg) 88429 (500mg)	88437 (50mg) 88438 (500mg)	89988 (50mg) 88431 (500mg)	A33613 (50mg) A33614 (500mg)	88209 (50mg) 88432 (500mg)	N/A
L-Proline	88211 (115mg) 88430 (500mg)	N/A	N/A	N/A	N/A	N/A

Cited References

1. Mann, M. (2006). Functional and quantitative proteomics using SILAC. *Nat Rev* **7:**952-9.
2. Everly, P.A., *et al*. (2004). Quantitative 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. and Mann, M. (2006). Protein interaction screening by quantitative immunoprecipitation combined with knockdown (QUICK). *Nat Methods* 3**(12):**981-3.
4. Amanchy, R., *et al*. (2005). Stable isotope labeling with amino acids in cell culture (SILAC) for studying dynamics of protein abundance and posttranslational modifications. *Science STKE* **267:**1-20.
5. Brendall, S.C., *et al*. (2008). Prevention of amino acid conversion in SILAC experiments with embryonic stem cells. *Mol Cell Proteomics* 7(9):1587-97.

Stable isotope-labeled amino acids are supplied by Cambridge Isotope Laboratories, Inc., Cambridge, MA.

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