

Chapter 2

Stable Isotope Labeling by Amino Acids Applied to Bacterial Cell Culture

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Abstract

Stable isotope labeling by amino acids in cell culture (SILAC) is a widely used approach in quantitative proteomics; however, due to limitations such as required auxotrophy for the amino acids employed for labeling, it was thus far rarely employed in bacteria. Although limitations of SILAC in microbiological applications are significant and restrict its use exclusively to cells cultured in minimal media, we and others have successfully used it to fully label proteomes of model bacteria and measure their relative expression dynamics under different experimental conditions. Here we provide a brief overview of applications of SILAC in bacteria and describe a detailed protocol for SILAC labeling of *Escherichia coli* and *Bacillus subtilis* cells in culture, which in many cases can be applied to other members of both gram-positive and gram-negative bacterial species.

Key words SILAC, Quantitative proteomics, Mass spectrometry, Bacteria, Prokaryotes

1 Introduction

1.1 Quantitative Proteomics in Bacteria

Since its onset in the early 1990s, mass spectrometry-based proteomics has been revolutionizing biomedical research while also having a significant impact in the microbiology field [1]. Bacteria are especially suitable for global quantitative gene expression analyses, due to their relatively simple and small genomes and proteomes; for example, the theoretical proteome of *E. coli* consists of approximately 4,100 protein-coding genes, of which about 2,700 are estimated to be expressed under standard laboratory growth conditions [2].

Traditional proteomics approaches relied on 2D gel electrophoresis for protein separation and quantification, whereas more modern quantitative proteomic approaches are mostly gel-free and use stable (nonradioactive) isotope labeling to quantify directly from mass spectra [3]. The stable isotope labeling approaches in quantitative proteomics have been extensively reviewed elsewhere [4–6]. Briefly, two major strategies for introducing a stable isotope

label into the proteome exist: chemical labeling, where a labeled compound is attached by a chemical reaction (in vitro) to specific reactive groups on protein/peptide termini or side chains of amino acids (e.g., N-terminal protein/peptide amino groups or thiol group on cysteines), and metabolic labeling, where the cell, tissue, or organism is grown in a defined medium with a single stable isotope-labeled source, and thus metabolically incorporates the label. In both approaches, protein samples from two states that are to be compared are labeled with either the unlabeled (“light”) or labeled (“heavy”) version of a compound. The differentially labeled samples are mixed and digested prior to MS measurement, in which intensities and fragmentation patterns of peptides are measured and used for subsequent identification and quantitation. If applicable, metabolic labeling, which includes ^{15}N labeling and SILAC, is preferred due to the high efficiency of labeling and straightforward sample preparation which minimizes quantitation errors due to sample handling. Application of ^{15}N labeling to bacteria is relatively straightforward and involves culturing of the cells on a sole source of nitrogen (usually ammonium salt) in a minimal medium and has previously been applied to bacteria [7, 8]. It does not require the creation of auxotrophic strains, but data analysis and interpretation are rather difficult due to the variable mass difference between labeled peptide pairs (difference depends on the number of N atoms in each peptide) and different isotope distributions between two members of a pair. Conversely, SILAC-labeled samples are easier to analyze and quantify than ^{15}N -labeled samples, as SILAC introduces a label of equal size (mass) in all peptides and thus keeps the isotope patterns of heavy and light signals almost identical. Every peptide is detected by a mass spectrometer system with high resolution capabilities in the form of two MS peaks (light and heavy) and relative quantitation is achieved by calculating the ratio between the measured signals (Fig. 1a). Protein samples can also be extended to three states which are labeled with either unlabeled (“light”) or labeled (“medium-heavy” and “heavy”) versions of a compound (Fig. 1b).

However, SILAC labeling also has notable limitations. In order to fully incorporate the labeled amino acid, the cell must not be able to synthesize it endogenously. Due to a robust bacterial metabolism, this effectively means that most of the bacterial species must be rendered auxotrophic for a specific amino acid prior to the SILAC experiment, e.g., by knocking out one or more genes involved in its biosynthesis pathway (*E. coli* is a notable exemption, see below). SILAC is almost completely inapplicable to photosynthetic bacteria, although some approaches applied to SILAC labeling of plant cells cultured in the dark [9] may be also applicable to bacteria. To ensure that there is only one source of the labeled amino acid in a cell’s environment, minimal media must be employed in all bacterial SILAC cell cultures. This obviously prevents

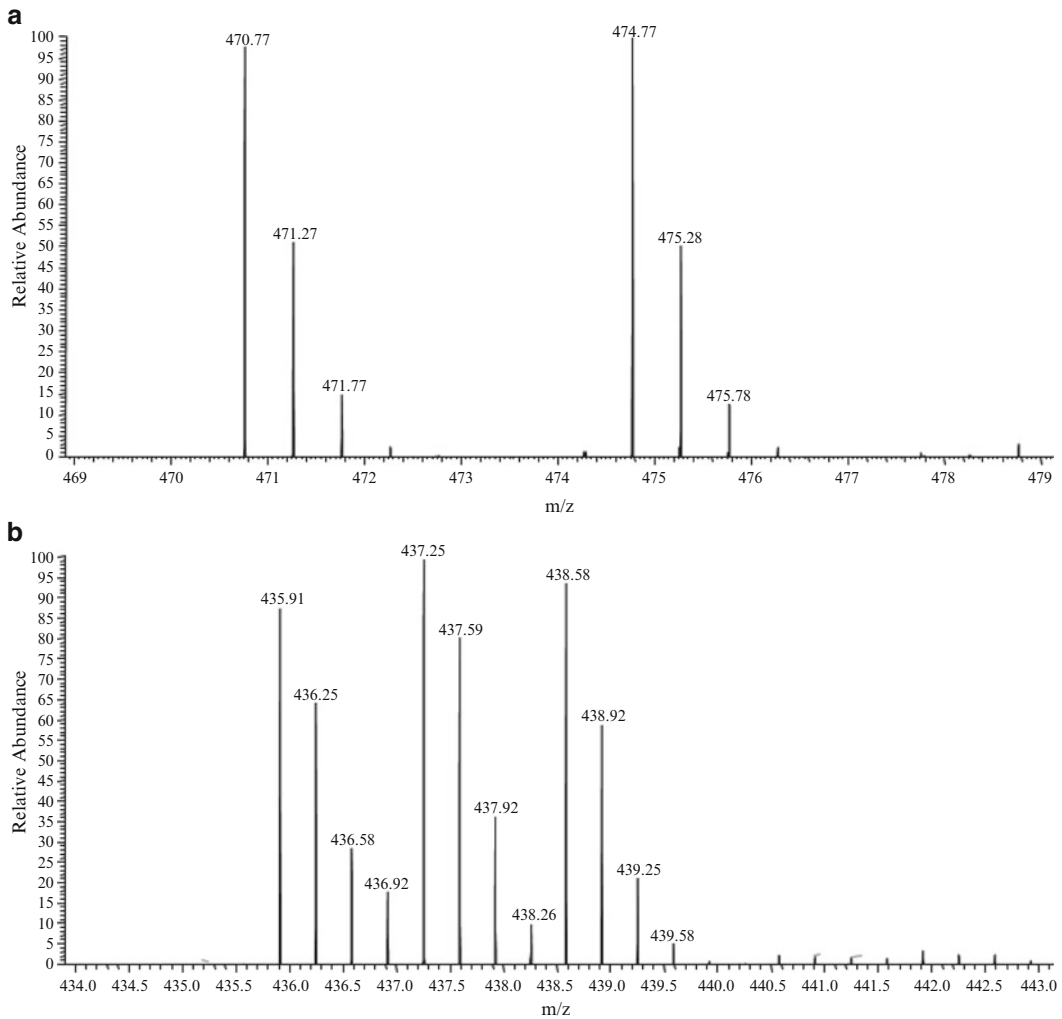


Fig. 1 Typical MS spectra of SILAC signals. **(a)** MS full scan corresponding to a double SILAC labeling experiment using Lys0 and Lys8 amino acids resulting in a mass shift of 8 Da between signal peaks. **(b)** MS full scan corresponding to a triple SILAC labeling experiment using Lys0, Lys4, and Lys8 amino acids resulting in a mass shift of 4 Da between signal peaks

the use of SILAC in quantitative proteomic analysis of environmental samples and clinical isolates (note that this limitation also applies to ^{15}N labeling). Despite of these obvious limitations, we and others have shown that a full SILAC labeling of a bacterium is possible and can be used to address fundamental molecular physiology processes, such as starvation or exchange of carbon sources [7, 10]. Once applicable to study a bacterial organism and process of interest, SILAC is a simple and cost-effective way to perform quantitation of a bacterial proteome to an unprecedented depth. It can be performed in several ways, such as super-SILAC [11], pulsed SILAC [12],

and dynamic SILAC [13] and, together with appropriate data processing software, enables global, comprehensive quantitation of the complete bacterial proteome or sub-proteome (e.g., phosphoproteome, acetylome, membrane proteome).

1.2 Overview of the SILAC Labeling Protocol

Here we describe SILAC labeling of two commonly used bacterial model organisms, *E. coli* and *B. subtilis*. In order to avoid metabolic loss of label and quantitation bias due to conversion of arginine (Arg) to proline (Pro), in our laboratory we perform SILAC labeling of bacterial cultures through lysine (Lys) only; however, we note that several approaches to avoid Arg-Pro conversion in animal cells have been described (e.g., addition of unlabeled proline to cell cultures or knockout (KO) of Arg and Pro biosynthesis) [14, 15]. The labeling process itself is quite straightforward and requires replacing the unlabeled amino acid with the same amount of the labeled amino acid in the minimal medium and letting the cells divide in such a medium at least 5–6 times. In case of *B. subtilis*, we use a LysA KO mutant strain that is auxotroph for lysine. Creation of such auxotrophic strains is a prerequisite for SILAC experiments in most bacterial species; however, in case of *E. coli* we and others have observed that the full SILAC labeling is possible even in the wild-type (WT) strain [16]. To ensure full incorporation of the labeled amino acid, we first inoculate a small SILAC pre-culture, which we run into mid-logarithmic phase and use to inoculate the main SILAC culture used in the experiment. In every SILAC experiment, we perform two critical steps of quality control: the incorporation level of the SILAC label (to ensure complete proteome labeling) and mixing check (to minimize possible quantitation error due to erroneous mixing). Finally, we use the MaxQuant software suite [17–19] to perform identification and quantification of the data.

2 Materials

2.1 Bacterial Strains

In order to ensure the highest level of incorporation of the amino acid isotope, the bacterial strain employed should be an auxotrophic mutant of the corresponding amino acid biosynthetic pathway. However, in some cases, for example in the lysine biosynthetic pathway in some strains of *E. coli*, efficient incorporation is achieved also in the WT prototrophic strain [16]. Preliminary incorporation tests should be performed in order to determine if an auxotrophic mutant is required or not.

2.2 SILAC Amino Acids

In principle, every amino acid can be used for SILAC labeling, but Arg and Lys are preferred due to compatibility with trypsin and LysC digestion protocols. If a bacterial strain is an auxotrophic mutant for lysine, then typically Lys4 and Lys8 are used.

Moreover, the conventional SILAC format can easily be extended to a triple labeling format (e.g., Lys0, Lys4, and Lys8). Common suppliers of arginine and lysine amino acids as well as their stable isotopes can be purchased from many companies (*see Note 1*).

2.3 Bacterial Growth Media

Several different types of chemically defined minimal media exist for the growth of bacteria. The Belitsky Minimal Medium (BMM) [20] with minor modifications and the M9 medium are routinely used in our laboratories for *B. subtilis* and *E. coli*, respectively. The minimal medium chosen will depend on the specific type of bacteria being studied, and therefore its respective optimal minimal medium should be used.

2.3.1 BMM Synthetic Growth Medium for *B. subtilis*

The medium consists of 8 mM MgSO₄, 27 mM KCl, 7 mM sodium citrate, 50 mM Tris-HCl pH 7.5, 2 mM CaCl₂, 0.6 mM KH₂PO₄, 15 mM (NH₄)₂SO₄, 10 μM MnSO₄, 1 μM FeSO₄, 0.5 % glucose, 250 μM thymine (optional: proline, *see Note 2*), 0.025 % light amino acid for light medium, and 0.025 % heavy amino acid for labeled medium.

2.3.2 M9 Minimal Medium for Growth of *E. coli*

The medium consists of M9 Minimal Salts (5×, Sigma), 1 % thiamine, 1 mM MgSO₄, 10 μM CaCl₂, 0.5 % glucose (optional: proline, *see Note 2*), 0.025 % light amino acid for light medium, and 0.025 % heavy amino acid for labeled medium.

2.3.3 LB Plates

The LB plates were prepared from LB media (Roth) and 15 g/l of Agar (Roth).

2.4 Bacterial Lysis

1. Y-PER™ Yeast Protein Extraction Reagent (Thermo Scientific).
2. Lysozyme (chicken egg white isolate).
3. Sonifier.

2.5 Chloroform–Methanol Protein Precipitation

1. Milli-Q water.
2. Chloroform (>99.8 % GC grade).
3. Methanol (>99.8 % GC grade).

2.6 Determination of Protein Concentration

1. Bradford Protein Assay Dye Reagent Concentrate (Bio-Rad).
2. Folded filter paper.
3. BSA or lysozyme can be used as a protein standard.

2.7 In-solution Protein Digestion

1. Denaturation buffer: 6 M urea, 2 M thiourea, 1 % (w/v) *n*-octylglucoside, 10 mM HEPES, pH 8.0 (*see Note 3*).
2. Reduction buffer: 1 M dithiothreitol (DTT) in 50 mM ammonium bicarbonate (ABC) (*see Note 4*).
3. Alkylation buffer: 550 mM iodoacetamide (IAA) in 50 mM ABC.

4. Lysyl Endopeptidase LysC.
5. Trypsin, sequencing grade, modified.
6. 20 mM ABC.

2.8 StageTips

1. Methanol (>99.8 % GC grade).
2. Solvent A*: 2 % acetonitrile (ACN)/1 % trifluoroacetic acid (TFA).
3. Solvent A: 0.5 % acetic acid.
4. Solvent B: 80 % ACN in 0.5 % acetic acid.
5. Empore™ 47 mm C18 Disk (3 M, Cat. No. 2215).

2.9 Liquid Chromatography Mass Spectrometry

1. HPLC solvent “A”: 0.5 % acetic acid.
2. HPLC solvent “B”: 80 % ACN in 0.5 % acetic acid.
3. HPLC loading solvent: 2 % ACN/1 % TFA.
4. 15 cm fused silica column emitters with inner diameter of 75 µM (New Objective).
5. Reversed phase material for nano-HPLC: Reprosil-Pur C18-AQ, 3 µM resin (Dr. Maisch GmbH).
6. EASY-nLC II system (Thermo).
7. High resolution MS instrument. For example, LTQ Orbitrap Elite MS instrument (Thermo).

2.10 Data Analysis

1. MaxQuant Software suite [17–19].

3 Methods

3.1 Bacterial Growth in SILAC Media

1. Streak out bacteria for single colonies on a standard LB plate (*see* **Note 5**).
2. Generate bacterial pre-cultures as follows: inoculate the minimal medium with a single colony containing 0.025 % of the appropriate SILAC amino acid (“heavy”) (*see* **Note 6**), and in a separate flask, inoculate the minimal medium with a single colony containing 0.025 % of the non-labeled form of the amino acid (“light”).
3. Grow bacterial pre-cultures under appropriate growth conditions to an OD600 of 0.3–0.5 (corresponding to a growth phase between early to mid-exponential growth).
4. Use pre-cultures in early to mid-exponential growth phase (*see* **Note 7**) to inoculate minimal media containing 0.025 % of either heavy or light amino acids to a starting OD600 between 0.03 and 0.05 and grow to desired OD600.

Table 1
Volume of Y-PER reagent (Thermo Scientific) to add per milligram of cell pellet

Wet cell pellet weight (mg)	Y-PER reagent volume (μl)
50	125–250
100	250–500
250	625–1,250
500	1,250–2,500

3.2 Bacterial Lysis

This protocol is slightly modified and optimized for efficient protein extraction of *B. subtilis* and *E. coli* cells based on the Y-PER™ Yeast Protein Extraction Reagent protocol. However, it is also effective for both gram-positive and gram-negative bacteria in general. There are various other types of lysis buffers available, but caution must be taken with regard to the amount of common enzymes (lysozyme, DNase) present in large amounts in many of these buffers. They can lead to a false estimation of the amount of bacterial proteins in the sample.

1. Pellet cells by centrifugation at approximately $3,000\times g$ for 5 min at 4 °C (*see Note 8*).
2. Resuspend the cells in an appropriate amount of Y-PER Reagent as indicated in Table 1. Pipette up and down until the mixture is homogeneous.
3. Prior to the addition of the Y-PER™ reagent to the cell pellet, add lysozyme to a final concentration of 50 μg/ml. This can be added from a lysozyme stock solution (e.g., a 5 mg/ml lysozyme stock solution) (*see Notes 9 and 10*).
4. Agitate the mixture at 37 °C for 20 min.
5. Sonicate the mixture for 30 s at an amplitude of 40 %.
6. Pellet the cell debris by centrifuging at $14,000\times g$ for 30 min and remove supernatant immediately after end of centrifugation to avoid contamination with cellular debris.

3.3 Chloroform–Methanol Protein Precipitation (See Note 11)

1. Mix the sample with 4 volumes of methanol and vortex.
2. Add 1 volume of chloroform and vortex.
3. Add 3 volumes of water and vortex.
4. Spin 1 min at maximum speed (*see Note 12*).
5. Discard aqueous phase (*see Note 13*).
6. Add 4 volumes of methanol and vortex.
7. Spin for 2 min at maximum speed.

8. Remove methanol and let the precipitate dry.
9. Dissolve the protein sample in the lowest volume of denaturation buffer possible (optimally, this should be around 1–2 µg of protein per µl of denaturation buffer).

3.4 Determination of Protein Concentration

1. Dilute Bradford Protein Assay-stock solution 1:5 with water.
2. Filter through a folded filter (*see Note 14*).
3. Prepare standard calibration curve and proceed with standard Bradford procedures (*see Notes 15 and 16*).

3.5 In-solution Digestion

This protocol is just one of many possible methods of digestion. Other possibilities include in-gel digestion and further in-solution fractionation through isoelectric focusing of the bacterial peptides.

1. Add reduction buffer to the sample to a final concentration of 1 mM DTT; incubate 1 h at room temperature (*see Note 17*).
2. Add alkylation buffer to the sample to a final concentration of 5.5 mM IAA; incubate for 1 h at room temperature in the dark.
3. Check the pH (should be 8.0); adjust with NaOH or HCL if necessary.
4. Add 1 µg of LysC per 100 µg of protein and incubate for 3 h at room temperature (*see Note 18*).
5. Dilute sample with 4 volumes of 20 mM ABC.
6. Check the pH (should be 8.0); adjust if necessary (*see Note 19*).
7. Add additional 1 µg of LysC per 100 µg of sample protein and incubate overnight at room temperature (*see Note 20*).

3.6 Quality Control: Incorporation of the SILAC Amino Acid and Mixing Check

1. Determine the efficiency of incorporation of the amino acid isotope by performing MS analysis (*see Subheading 3.8*). Calculate the median value of the heavy/light ratios by using the formula: $\text{Incorporation} = [\text{H/L}]_{\text{median}} / (1 + [\text{H/L}]_{\text{median}})$. Multiply by 100 to get median incorporation in percentage (Fig. 2). To achieve optimal conditions for SILAC experiments, the labeling efficiency should be more than 95 %.
2. For mixing check, mix heavy and light samples in a 1:1 ratio (based on Bradford measurements) followed by MS analysis and data processing to verify accurate 1:1 mixing of heavy and light samples (Fig. 3).

3.7 Peptide Cleanup via StageTips

1. Activate StageTips with 200 µl of methanol.
2. Wash/equilibrate with 200 µl of solvent A*.
3. Add sample about 10 % of solvent A* can be added to the sample (*see Notes 21 and 22*).
4. Wash with 200 µl of solvent A (*see Note 23*).

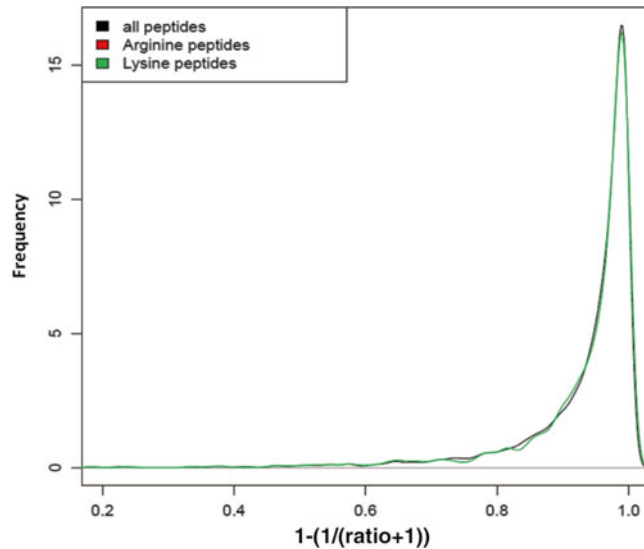


Fig. 2 Efficiency of Incorporation. The incorporation efficiency of the amino acid stable isotopes can be determined on all identified SILAC peptides (pairs) using the following formula: $1-(1/(\text{ratio}+1))$. As seen above, if the level of incorporation is near to 100 %, the median incorporation level will be close to 1. This calculation should be done separately for arginine- and lysine-containing peptides because the arginine and lysine stable isotopes may have different labeling efficiencies. In the example above, there are no ratios for arginine containing peptides due to the fact that only Lys8 was used

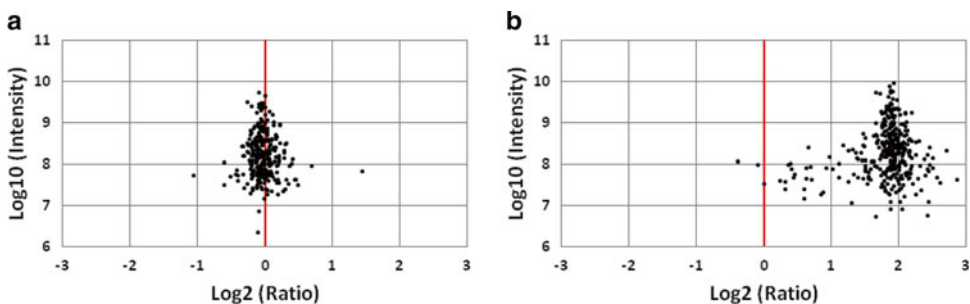


Fig. 3 Importance of accurate mixing of differentially labeled SILAC samples. **(a)** Scatterplot of peptide ratios versus intensities illustrates an example of accurate 1:1 mixing of heavy to light amino acids. **(b)** Scatterplot of peptide ratios versus intensities illustrates a poor example of heavy to light amino acid mixing with an approximately fourfold higher level of heavy compared to light amino acid; while relatively small mixing errors (<30 %) are routinely dealt with by processing software, extreme mixing errors will result in wrong quantitation values

5. Elute peptides with 50 μl of solvent B directly into an Eppendorf tube.
6. Dry peptides in a SpeedVac for approx. 10–12 min to approx. 5 μl of sample (*see Note 24*).
7. Add 1 μl of solvent A* to the sample.

3.8 Mass Spectrometry Analysis

Peptide samples are analyzed using online nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS). In our laboratory, nanoLC-MS/MS experiments are performed on an EASY-nLC II system connected to an LTQ-Orbitrap Elite through a nano-electrospray ion source; however, almost any other LC-MS setup (including those using MALDI sources) can be used for analysis.

1. Load peptides directly onto a 15 cm long 75 μm -inner diameter analytical column packed with reversed-phase C18 Reprosil AQUA-Pur 3 μm particles using the “Intelliflow” setting (variable flow rate up to a maximum pressure of 280 bar).
2. Reduce flow rate to 200 nl/min after loading, and then separate peptides with a segmented linear gradient of ACN from 5 to 33 to 50 % in 0.5 % acetic acid for either 60, 100, 150, or 240 min (*see Note 25*).
3. Use the following MS settings on the LTQ-Orbitrap Elite: target values 1E6 charges (Orbitrap analyzer) and 5E3 charges (linear ion trap). Operate the instrument in the positive ion mode with the following acquisition cycle: a full scan recorded in the Orbitrap mass analyzer at a resolution R of 120,000 followed by MS/MS (CID Rapid Scan Rate) of the 20 most intense peptide ions in the LTQ mass analyzer.

3.9 Data Processing and Analysis

1. Download the latest version of the MaxQuant software (*see Note 26*).
2. Load the appropriate RAW files (*see Note 27*).
3. Open the Experimental Design tab. This will write a folder located in the same location as where the RAW files are and will be named “Combined”. This folder will contain an experimentaldesigntemplate.txt file (*see Note 28*).
4. Set number of threads located at the bottom left hand corner of the MaxQuant software (*see Note 29*).
5. Select the Group-Specific parameters tab and specify the following: variable modifications of interest (if any), the multiplicity (corresponding to how many amino acid isotopes were used), and the specific protease used for the experiment. All other parameters in this tab should initially be left to their default settings unless further specific changes are required.
6. Open the MS/MS & Sequences tab. Specify the appropriate FASTA file corresponding to the organism being studied by selecting the Add File tab (*see Note 30*). The MS/MS and all other parameters should be left at their default settings unless desired otherwise.
7. Open the Identification & Quantification tab. Open “Select file” under the experimental design tab and select the experimental design file that was created earlier. All other parameters

should be left at their default settings unless desired otherwise (*see Note 31*).

8. Open the performance tab and then click the start tab located at the bottom of the MaxQuant screen. If the setup was successful, the analysis shall begin. The entire progress of the analysis can be monitored under the performance tab. When the analysis is complete, MaxQuant will indicate this with a “Done” button. All data is located in the combined folder inside the txt folder. All of the .txt files can be opened with Microsoft Excel™.

4 Notes

1. SILAC amino acids can be purchased from several companies such as EURISO-TOP, Sigma-Aldrich, Silantes, and Cambridge Isotope Laboratories.
2. As previously documented in eukaryotes, there is also the possibility of arginine to proline conversion in bacteria [14, 15]. This can cause difficulties in the quantitation due to the abundance of non-quantifiable satellite peaks caused from the proline signal. In order to circumvent this, proline may be added to the minimal media (amount must be optimized according to the bacterial strain used) in order to discourage the bacteria to convert arginine to proline since proline would already exist at a saturated level in the minimal media.
3. All solvents used in this procedure should be prepared with Milli-Q water. pH of Buffers can be adjusted with NaOH or HCl until the correct pH is obtained.
4. The denaturation, reduction, and alkylation buffers may be stored in aliquots at -20 °C and remain stable for several months.
5. LB Plates are prepared using standard 25 g/l of LB media and 15 g/l of agar. Mixture is then autoclaved, and once cooled (to approximately 55 °C), the mixture is poured into petri dishes, allowed to harden, and stored at 4 °C in the dark until use. Incubation temperatures vary according to the type of bacteria used. Furthermore, antibiotics may also be used if necessary (e.g., if bacteria employed contains antibiotic resistance markers).
6. It was established in *B. subtilis* and *E. coli* that a final amino acid concentration of 0.025 % does not compromise bacterial growth.
7. Pre-cultures need to be in early to mid-exponential growth to ensure efficient and accurate reproducibility between experiments; an “overgrown” stationary overnight bacterial culture contains a mixed bacterial cell population composed of dormant, living,

and dead bacterial cells, whereas a culture in the exponential growth phase contains a higher proportion of healthy, synchronized bacterial cells, and therefore improving reproducibility between biological replicates of experiments.

8. Cells may be processed immediately after centrifugation or the cell pellet may be frozen at -20°C or -80°C .
9. For lysozyme stock solutions, make a 5 mg/ml lysozyme stock solution with 50 mM Tris-HCl, pH 7.5, and split into 100 μl aliquots. Store these aliquots at -20°C until ready for use.
10. At this step, protease inhibitors may be added.
11. Alternative methods for precipitation/purification may be used.
12. Spin at a minimum of $4,000 \times g$ in order to ensure good separation of the protein mixture.
13. Proteins will remain between the hydrophobic and hydrophilic phases.
14. Keep diluted and filtered Bradford reagent in a glass bottle (at room temperature, the reagent can be kept for approximately 2 weeks).
15. Standard calibration curves may be generated with known amounts of a standard protein such as bovine serum albumin.
16. Other methods of protein determination may be employed such as the BCA kit, NanoDrop technologies etc.
17. Avoid heating up the samples during protein solubilization and digestion due to the fact that the high concentration of urea will lead to carbamylation of the free amino groups present.
18. Other proteases may also be used depending on the desired protein cleavage. Note that a combination of heavy lysine SILAC labeling and LysC protease digestion results in one labeled amino acid per fully cleaved peptide (i.e., all peptides except for the protein C-termini are quantifiable).
19. The pH may be adjusted accordingly with a very low volume of 1 M Tris-HCl.
20. If cells are labeled with lysine only (like presented in this protocol), LysC is used as the protease for the overnight digestion step. LysC cleaves at the C terminal end of lysine residues, allowing for quantitation of all resulting peptides. However, if arginine and lysine SILAC amino acids are used for labeling, trypsin should be utilized as the protease for the overnight digestion step.
21. Solvent A* contains TFA that is a good ion pairing reagent and increases the binding efficiency of the peptides to the C18 material in the stage tip.

22. Take extra caution to ensure that the StageTip does not become dry (critical until the sample is loaded onto the tip).
23. StageTips can be stored at 4 °C until measurement.
24. Ensure that the sample is not dried completely.
25. The total acquisition time can be varied typically in a gradient range within 100 or 240 min depending on the desired level of peptide coverage/identifications. For example, during an incorporation check, a shorter MS method shall suffice (60 min) due to the fact that detection of approximately 1,000 peptides will be enough for the accurate determination of the level of the SILAC amino acid incorporation.
26. MaxQuant software can be downloaded at www.maxquant.org. The following publications provide both relevant and useful information that pertains to the theory and usage of this software [17–19, 21, 22]. The protocol instructions described for the MaxQuant software suite is based on version 1.3.0.5. It is important to note that there are other programs available that could be used as an alternative for downstream data analysis.
27. The RAW files should be copied to a local folder where the processing is to occur.
28. The experimental designtemplate.txt file can be opened with the Microsoft Excel™ program and modified according to which experiment(s) correspond to which RAW file(s). Slices (fractions) may also be designated if further fractionation methodologies were performed (examples include off gel fractionation or in gel fractionation).
29. The number of threads may be set according to the number of RAW files. However, the total number of threads should not exceed the total capacity of the operating computer (number of cores). For each thread used, approximately 1.5 GB of RAM is required. For optimal processing performance, at least twice the size of the total size of all RAW files being analyzed should be available on the hard disk space. Further information may be found at: <http://www.maxquant.org/requirements.htm>.
30. The FASTA files of the organism which correspond to all known annotated genes of the organism must be added and therefore the microorganism being studied must be fully sequenced.
31. The Misc tab contains advanced features of the MaxQuant software that will not be discussed in this chapter with one exception: When performing incorporation and mixing checks for quality control purposes, the Re-quantify option should be unchecked as it could skew the true value of the incorporation rate if left checked.

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