Chapter 2

Metabolic Labeling of Model Organisms Using Heavy Nitrogen (^{15}N)

Joost W. Gouw, Bastiaan B.J. Tops, and Jeroen Krijgsveld

Abstract

Quantitative proteomics aims to identify and quantify proteins in cells or organisms that have been obtained from different biological origin (e.g., "healthy vs. diseased"), that have received different treatments, or that have different genetic backgrounds. Protein expression levels can be quantified by labeling proteins with stable isotopes, followed by mass spectrometric analysis. Stable isotopes can be introduced in vitro by reacting proteins or peptides with isotope-coded reagents (e.g., iTRAQ, reductive methylation). A preferred way, however, is the metabolic incorporation of heavy isotopes into cells or organisms by providing the label, in the form of amino acids (such as in SILAC) or salts, in the growth media. The advantage of in vivo labeling is that it does not suffer from side reactions or incomplete labeling that might occur in chemical derivatization. In addition, metabolic labeling occurs at the earliest possible moment in the sample preparation process, thereby minimizing the error in quantitation. Labeling with the heavy stable isotope of nitrogen (i.e., ^{15}N) provides an efficient way for accurate protein quantitation. Where the application of SILAC is mostly restricted to cell culture, ^{15}N labeling can be used for micro-organisms as well as a number of higher (multicellular) organisms. The most prominent examples of the latter are *Caenorhabditis elegans* and *Drosophila* (fruit fly), two important model organisms for a range of regulatory processes underlying developmental biology. Here we describe in detail the labeling with ^{15}N atoms, with a particular focus on fruit flies and *C. elegans*. We also describe methods for the identification and quantitation of ^{15}N-labeled proteins by mass spectrometry and bioinformatic analysis.

Key words: Stable isotope labeling, proteomics, nitrogen, model organism, mass spectrometry, protein quantitation, *Drosophila*, *C. elegans*.

1. Introduction

Labeling of proteins with stable isotopes has provided a strong impetus to quantitative proteomics over the past few years (1). It entails the incorporation, either in vitro or in vivo, of stable

K. Gevaert, J. Vandekerckhove (eds.), *Gel-Free Proteomics*, Methods in Molecular Biology 753,
DOI 10.1007/978-1-61779-148-2_2,

isotope-labeled atoms like ^{2}H, ^{13}C, ^{15}N, or ^{18}O into proteins, resulting in a mass increase that can be detected by mass spectrometry. In mixtures of differentially labeled samples, spectral intensities of light and heavy peptides directly reflect their relative abundance, thus providing a measure for the relative expression levels of proteins. Proteins can be derivatized in vitro (e.g., by methods such as iTRAQ (2), ICAT (3), or reductive dimethylation (4)) or in vivo by the metabolic incorporation of the label during growth of the target organism. Isotope-tagged amino acids have been widely used for the labeling of cell cultures (SILAC; (3)) and (lower) organisms that can be cultured in defined media (5). Labeling with heavy nitrogen, replacing all naturally occurring ^{14}N by ^{15}N atoms, provides an alternative means with applications in (multicellular) organisms. While unicellular organisms (bacteria, yeast) can be readily cultured in ^{15}N-enriched media (6), we and others have demonstrated the labeling of *Caenorhabditis elegans* (7, 8), fruit flies (7, 9), plants (10), and mammals (11) with ^{15}N atoms. We have applied metabolic labeling of fruit flies to analyze the mother-to-zygote transition, uncovering both maternal- and zygote-specific proteins (12). In *C. elegans*, we have compared protein expression in male and female animals, disclosing expression of sex-specific proteins (13). The same approach has been used by others to analyze insulin signaling (8). Here we provide a step-wise protocol for metabolic labeling with heavy nitrogen of *Escherichia coli*, yeast, *C. elegans*, and *Drosophila*, along with procedures for protein analysis by mass spectrometry. Finally, methods are provided for bioinformatic interpretation of the data, including protein identification and quantitation.

2. Materials

Deionized water, 18 MΩ, needs to be used for all solutions and buffers.

2.1. Labeling of E. coli

1. *E. coli*, OP50 strain (streak plate or glycerol stock) (Caenorhabditis Genetics Center, University of Minnesota)
2. Media for labeling: Spectra 9-U medium (unlabeled) and Spectra 9-N medium (^{15}N-labeled) (Cambridge Isotope Laboratories)

2.2. Labeling of C. elegans

1. *C. elegans* (Caenorhabditis Genetics Center, University of Minnesota)

2. Pellets of labeled (^{14}N and ^{15}N) OP50 *E. coli* (from a freshly grown 400 ml culture)
3. 1 M $CaCl_2$
4. 1 M $MgSO_4$
5. 5 mg/ml cholesterol in ethanol
6. 1 M potassium phosphate buffer, pH 6.0: dissolve 108.3 g KH_2PO_4 and 35.6 g K_2HPO_4 in 1 l of water
7. Agarose
8. Media for labeling: Spectra 9-U and Spectra 9-N media
9. M9 buffer: 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, add water to 1 l
10. Petri dishes (preferably 15 cm diameter)

2.3. Labeling of Yeast

1. Minimal medium: dissolve 1.7 g yeast nitrogen base without amino acids and ammonium sulfate (Difco), 20 g sucrose (nitrogen-free), and 5 g ^{15}N-labeled ammonium sulfate (Cambridge Isotope Laboratories) in 1 l of water (*see* **Note 1**).
2. *Saccharomyces cerevisiae* type II (Sigma).
3. Phosphate-buffered saline (PBS): dissolve 8 g NaCl, 200 mg KCl, 1.44 g Na_2HPO_4, and 240 mg KH_2PO_4 in 900 ml of water. Adjust the pH to 7.4 using HCl or NaOH and add water to 1 l.
4. Falcon tubes, 50 ml.
5. Sterile flasks of 50 ml and 2.5 l.

2.4. Labeling of Drosophila

1. Larva box that contains enough openings to allow for ventilation. However, all openings should be covered with fine gauze to prevent the escape of hatched flies (*see* **Note 2**).
2. Fly collection cage (cylindrical) with one side covered by fine gauze and the other side by a Petri dish (*see* **Note 3**).
3. Petri dishes that can be mounted onto the fly collection cage (i.e., the diameter should be similar to the fly collection cage, see above).
4. 10% (w/v) Tegosept: dissolve 5 g of *p*-hydroxybenzoic acid methyl ester in 50 ml of 95% ethanol.
5. Ampicillin (50 mg/ml): dissolve 0.5 g ampicillin in 10 ml of water.
6. Larva box mixture: dissolve 9 g sucrose (nitrogen-free) and 9 g of dry weight yeast in 70 ml of water (if fresh yeast culture is used instead of dry yeast, then the volume of water should be corrected accordingly). Add 37.5 μl of propionic

acid, 240 µl of phosphoric acid, 840 µl of Tegosept, and 60 µl of ampicillin to this sucrose/yeast mixture.

7. Sheet(s) of cotton wool.
8. Whatman #1 filter paper, about 3 cm in diameter.
9. Food and collection plates: dissolve 6.25 g sucrose (nitrogen-free) and 7.5 g pure agarose in 375 ml of water and autoclave this solution (this will also dissolve the agar). Cool down to 55°C and add 1.88 ml of Tegosept and 1.88 ml of ethyl acetate. Dispense 20 ml of this solution into the Petri dishes and let this solidify (15–30 min). Store the plates in plastic bags at 4°C.

2.5. Analyzing the Level of ^{15}N-Enrichment and Optimizing the Mixing Ratio of Labeled and Unlabeled Peptides

1. Polypropylene tubes (e.g., 1.5 ml from Eppendorf).
2. Micropestles (Eppendorf) or ultrasonic homogenizer (e.g., the LABSONIC® M from Sartorius Stedim).
3. 50 mM ammonium bicarbonate (it is not necessary to adjust the pH).
4. Lysis buffer: 8 M urea and 2 M thiourea in 50 mM ammonium bicarbonate supplemented with a Protease Inhibitor Cocktail tablet (Roche).
5. 200 mM dithiothreitol in 50 mM ammonium bicarbonate.
6. 200 mM iodoacetamide in 50 mM ammonium bicarbonate. This solution must be prepared fresh and should be kept in the dark.
7. Trypsin (sequencing-grade modified trypsin, Promega) in aliquots of 20 µg in 40 µl of 50 mM acetic acid per tube (0.5 µg/µl) should be kept at –20°C.

2.6. Analysis of Labeled Proteins by LC-MS/MS

1. High-performance liquid chromatography (HPLC)-grade acetonitrile and acetic acid.
2. HPLC buffer A: 0.1 M acetic acid in water or 0.1% (v/v) formic acid in water.
3. HPLC buffer B: 0.1 M acetic acid (or 0.1% (v/v) formic acid) in 8/2 (v/v) acetonitrile/water.
4. Nanoflow liquid chromatography system (e.g., Agilent 1200 system consisting of an optional vacuum degasser, binary (nano) pump, and (micro) autosampler).
5. Reversed-phase C_{18} analytical column (e.g., in-house packed; 50 µm × 20 cm fused-silica capillary (Polymicro) with ReproSil-Pur C18-AQ 3 µm particles, 200 Å pore size (Dr. Maisch GmbH)). Other columns (50–100 µm internal diameter, packed or monolithic) will work as well.
6. Reversed-phase C_{18} trap column (e.g., in-house packed; 100 µm × 2 cm fused-silica capillary (Polymicro) with Aqua C_{18} 5 µm particles, 200 Å pore size (Phenomenex)).

7. High-resolution electrospray hybrid mass spectrometer (e.g., Q-TOF, LTQ-Orbitrap, LTQ-FT).
8. The HPLC, columns, and mass spectrometer are connected essentially as described previously (14).

3. Methods

3.1. Labeling of E. coli

1. Inoculate a small volume (5 ml) of bacterial culture medium in a Falcon tube. For ^{14}N and ^{15}N labeling of bacteria, Spectra 9-U and Spectra 9-N media are used, respectively.
2. Culture bacteria for 8–15 h (overnight) at 37°C with shaking at 250 rpm.
3. Inoculate a large volume (400 ml) of the appropriate culture medium with 0.1–1 ml of the overnight culture.
4. Culture bacteria for 8–15 h (overnight) at 37°C with shaking at ~250 rpm (*see* **Note 4**).
5. Harvest bacteria by spinning at 2,400×*g* for 10 min.

3.2. Labeling of C. elegans

C. elegans animals are typically cultured on nematode growth medium (NGM) agar seeded with OP50 *E. coli*. NGM agar contains NaCl, bacterial nutrients (peptone), cholesterol, buffering salts (phosphate buffer, pH 6.0), and agar. To ensure optimal isotopic labeling of *C. elegans*, all nitrogen-containing ingredients of the NGM medium should be replaced either by the Spectra-9 medium or by nitrogen-free substitutes. Plates used to metabolically label *C. elegans* are therefore prepared with agarose (1% w/v) instead of agar and contain Spectra-9 medium (25% v/v) instead of peptone and NaCl. Preparation for 1 l:

1. Mix 10 g of agarose and 250 ml of Spectra-N medium in a flask or bottle. Add 725 ml of water. Autoclave for 15 min at 121°C (agarose cannot be autoclaved too long due to caramelization).
2. Cool flask in a water bath or stove at 55°C.
3. Add 1 ml of 1 M $CaCl_2$, 1 ml of 5 mg/ml cholesterol in ethanol, 1 ml of 1 M $MgSO_4$, and 25 ml of 1 M phosphate buffer.
4. Swirl to mix well.
5. Dispense the solution into Petri dishes (*see* **Note 5**).
6. Leave plates at room temperature for 1–2 days before use to allow excess moisture to evaporate.
7. Re-suspend equal amounts of pelleted ^{14}N- and ^{15}N-labeled *E. coli* in M9 buffer (typically 400 ml of bacterial culture is pelleted and re-suspended in 50 ml M9 buffer).

8. Put 2–3 ml of the bacterial suspension on the plates and spread using a glass rod.
9. Allow excess moisture to evaporate and the OP50 *E. coli* lawn to grow by leaving the plates overnight at room temperature.
10. At this point the plates can be used to culture *C. elegans* or be stored at 4°C for several weeks. To ensure optimal labeling of *C. elegans*, culture animals for at least four generations (preferably more) on the isotope plates before protein extracts are generated (*see* **Note 6**).
11. When sufficient numbers of animals are cultured, harvest the animals by rinsing the plates with M9 medium and spinning the animals at 400×*g* for 2 min. To remove excess bacteria, re-suspend pelleted *C. elegans* in M9 buffer in a 50 ml Falcon tube and allow to settle on ice (the bacteria will remain in suspension).

3.3. Labeling of Yeast

1. Re-suspend a couple of yeast grains in 5 ml of water.
2. Use this yeast to inoculate 5 ml minimal media in a 50 ml flask.
3. Grow overnight at 30°C with shaking at 230–270 rpm.
4. Inoculate 1 l of minimal media in a 2.5 l flask with 500 μl of the overnight culture.
5. Grow overnight at 30°C with shaking at 230–270 rpm.
6. Collect yeast in 500 ml GSA bottles and centrifuge at 2,400×*g* for 20 min at 4°C.
7. Decant the supernatant. The supernatant can be incubated overnight (at 30°C and shaking at 230–270 rpm) to collect additional yeast.
8. Re-suspend the remaining yeast (pellet) in 20 ml of PBS, transfer to a 50 ml Falcon tube, and centrifuge at 2,400×*g* for 20 min at 4°C. Remove the PBS and use this tube to pool subsequent yeast (e.g., from the incubated supernatant). If the supernatant is incubated, collect the yeast and pool this with the previously collected yeast.
9. Store the Falcon tube at 4°C for direct use or at –80°C for long-term storage (*see* **Note 7**).

3.4. Labeling of Drosophila

The following instructions assume the use of newly grown wild-type *Drosophila melanogaster* embryos (*see* **Note 8**).

1. Collect per larva box 20–30 mg of 0–12 h-old embryos and rinse them extensively with water, followed by a brief rinse with 70% ethanol (note that 100% ethanol will dehydrate the embryos). Store the embryos at room temperature until the larva box is ready.

2. Prepare the larva box by placing a layer of cotton on the bottom of the box and soak the cotton with the larva box mixture. The cotton should be completely soaked, but without leaving any pools of liquid in the box. Close the box until the embryos are ready.
3. Spray the Whatman filter with 70% ethanol, place the collected and washed embryos onto the filter, and distribute them evenly using a fine brush.
4. Place the Whatman filter with the embryos in the center of the larva box and close the box (*see* **Note 9**).
5. Prepare the Petri dishes required for the fly collection cage. Note that these dishes must be replaced when they are depleted of yeast or when (staged) embryos are to be collected.
6. When a significant amount of flies have hatched, transfer them to the fly collection cage. Prepare the collection cage by adding some labeled or unlabeled yeast to the Petri dish (*see* **Note 10**).
7. Transfer the hatched flies to the fly collection cage by holding the larva box upside down and filling the box with carbon dioxide gas. When holding the larva box upside down, be very careful that flies do not get wet due to condensed water. The CO_2 will anesthetize the flies making it easier to transfer them. Optionally, during this step, the volume of flies can be determined using a measuring cylinder. A total of 30 mg of embryos yield around 5 ml of flies.
8. Directly after transferring the flies to the collection cage, use the Petri dish to cover one side of the fly collection cage (the other side is covered by fine gauze).
9. Put the larva box to 25°C and 80% humidity to allow hatching of the remaining pupae. These flies can then be added to the previously collected flies using Step 8.

3.5. Analyzing the Level of ^{15}N-Enrichment

There are two separate, yet equally important, phenomena that contribute independently to the final percentage of the stable isotope in proteins: (1) the purity of the stable isotope that is obtained from the supplier (e.g., 99% ^{15}N) and (2) the degree of incorporation (efficiency) of that stable isotope into proteins. Although labeling with ^{15}N can be very efficient, even an incorporation efficiency as high as 98% causes some challenges for proper peak selection by the mass spectrometer, peptide identification, and quantitation (15). It is therefore recommended to use the highest purity heavy nitrogen (i.e., 99+% ^{15}N) that is available and to minimize other sources of unlabeled nitrogen throughout metabolic incorporation of the label. The final enrichment level should be determined to ensure complete incorporation

of the label, which can be determined by LC-MS as follows (*see* **Note 11**):

1. Transfer 1 mg of heavy-labeled sample (*E. coli*, yeast, flies, *C. elegans*) into an appropriately labeled tube. Add 100 μl of ice-cold lysis buffer and lyse the organisms on ice by sonication or by using micropestles.
2. Clear the mixture by centrifugation for 20 min at 20,000×*g* and 4°C.
3. Transfer the supernatant to a new tube and add dithiothreitol to a final concentration of 2 mM (1 μl of dithiothreitol stock solution per 100 μl of sample solution).
4. Incubate at 56°C for 25 min.
5. After cooling to room temperature, add iodoacetamide to a final concentration of 4 mM (2 μl of iodoacetamide stock solution per 100 μl of sample solution) and incubate for 30 min in the dark at room temperature.
6. Dilute the sample four times with ammonium bicarbonate (*see* **Note 12**), add 2 μl (1 μg) of trypsin, and incubate overnight at 37°C.
7. Purify the peptides by reversed-phase solid phase extraction (*see* **Note 13**).
8. Analyze around 1 μg of peptides by LC-MS/MS (*see* **Section 3.7**).
9. Search the data in a database search engine to identify the peptides (*see* **Note 14**).
10. Select several (i.e., 20) high-scoring, abundant peptides that differ in mass and hence chemical composition and determine their chemical formulae using their amino acid sequence, charge, and modifications if present (e.g., oxidized methionine, *see* **Note 15**).
11. Simulate (with software like IsoPro 3.0, *see* **Note 16**) isotope distributions using the peptide's chemical formula and decreasing ^{15}N-enrichments. Start with 100% ^{15}N and decrease this in a step-wise manner (e.g., 0.5% steps). Compare each distribution (i.e., the height of all of the isotopes) to the summed experimental isotope distribution of the peptide and the best "fit" is the peptide's actual ^{15}N-enrichment.
12. The actual ^{15}N-enrichment of each of the 20 peptides should be very similar. If this is not the case, this most likely indicates that labeling is not complete yet and that extended labeling (i.e., the next generation) is required. If, on the contrary, the actual enrichment of all the peptides is very similar, and if the average of these enrichments is close to the purchased purity of the label, full incorporation is reached (*see* **Note 17**).

3.6. Optimizing the Mixing Ratio of Labeled and Unlabeled Proteins

A critical aspect in quantitative proteomics is the mixing of two or more differentially labeled samples. Preferably, proteins that do not change in abundance between conditions should be present in equal amounts (i.e., a 1:1 ratio). Therefore, one should aim for mixing samples in a 1:1 ratio based on protein content. When samples are mixed in suboptimal ratios, quantitation might be difficult for regulated proteins due to the limited dynamic range of mass spectrometers. There are several ways to mix differentially labeled samples which can be as simple as combining an equal number of cells or embryos. Other methods are based on absolute protein amounts estimated by protein assays or the intensity of separated proteins on a 1D gel using SDS-PAGE. These provide good approximations, but are not accurate enough to achieve an exact 1:1 ratio. To prepare a 1:1 mixture of unlabeled and ^{15}N-labeled protein the following procedure can be used:

1. Add 1 mg of unlabeled sample (*C. elegans* or *Drosophila*) to 3 mg of the corresponding labeled sample (tube 1).
2. In a second tube, add 1 mg of labeled sample (*C. elegans* or *Drosophila*) to 3 mg of the corresponding unlabeled sample (tube 2).
3. Add 100 μl of ice-cold lysis buffer to both samples and lyse them on ice by sonication or by using micropestles.
4. For the samples in both tubes, follow Steps 2–6 described in **Section 3.5**.
5. Analyze both samples by LC-MS by injecting 0.5 μl of each sample.
6. Search the data in a database search engine and quantify a number of abundant proteins (i.e., proteins with a high number of peptides) that are expected not to change in abundance using quantification software to determine the actual mix ratio. The ratio of these proteins should be similar and the average ratio represents the actual mix ratio in each of the tubes.
7. Based on these actual mixing ratios, the volumes can be calculated that need to be combined from tubes 1 and 2 to achieve a 1:1 ratio. Mix in a new tube calculated volumes of samples 1 and 2 using the following formula (*see* **Note 18**):

$$V_{\text{sample 1}} = V_{\text{total}} \frac{R_{\text{desired}} - R_{\text{sample 2}}}{R_{\text{sample 1}} - R_{\text{sample 2}}}$$

where $V_{\text{sample 1}}$ is the volume of sample 1 to be added to a new tube, V_{total} is the total volume of the new tube, R_{desired} is the desired mix ratio (usually 1), $R_{\text{sample 1}}$ and $R_{\text{sample 2}}$ are the actual mix ratios of samples 1 and 2, respectively. After calculating the volume of sample 1, the volume of sample 2

($V_{total} - V_{sample\ 1}$) should be added to get the desired mix ratio.

8. Purify the peptides by reversed-phase solid phase extraction (*see* **Note 13**).
9. This sample is ready for further proteomic analysis (LC-MS/MS) (*see* **Section 3.7**).

3.7. Analysis of Labeled Proteins by LC-MS/MS

Mixtures of labeled and unlabeled peptides are best analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for protein identification and quantitation. This process involves the online separation of peptides by reversed-phase LC, electrospray ionization of peptides, and fragmentation of detected peptides in the mass spectrometer. This setup can be achieved on multiple platforms (i.e., various types of LC systems coupled to a range of mass spectrometers), and the exact protocol depends on available instrumentation (for reviews, *see* (16, 17)). Therefore, we will describe the workflow only in general terms, highlighting some aspects that should be kept in mind for optimal performance for a quantitative analysis.

1. A chromatographic system should be chosen that can deliver flow rates at 100–300 nl/min, either as a splitless nanoflow system or as a conventional system running at 100–300 μl/min and passive splitting to the desired flow rate.
2. Choose a reversed-phase column (either pre-packed or homemade) that efficiently captures both hydrophilic and hydrophobic peptides. The internal diameter should be in the range of 50–100 μm; length can vary between 10 and 40 cm depending on gradient length and flow rate, but 15–20 cm is a good starting point. Slope and length of the gradient should be optimized for the sample and column system. For complex samples, a typical gradient can be generated by raising the concentration of acetonitrile from 5% to approximately 40% over a 2 h period (*see* **Note 19**).
3. The amount of sample (peptide mixture) injected into the system should not exceed the capacity of the column. Overloading will cause peak broadening and (potentially) saturation of the detector in the mass spectrometer. Both will compromise proper quantitation.
4. Ideally, a high-resolution mass spectrometer should be used. Resolution should be sufficient to determine the charge state of the peptide and the mass of the mono-isotopic peak. Enhanced resolution also helps in distinguishing labeled from non-labeled peptides based on isotope pattern. High resolution (such as in TOF, Orbitrap, or FT instruments) is usually coupled to high mass accuracy, aiding in the identification process.

5. After processing raw data to peaklists (often by vendor-specific software), proteins can be identified by a range of database search algorithms. Most, but not all of them, have the option to identify ^{15}N-labeled proteins (*see* **Note 14**).
6. Protein quantitation is a critical process that is supported by an increasing number of software packages. Although standardization of mass spectral data formats is steadily progressing (e.g., mzXML, mzML), the use of most software packages for protein quantitation is often dictated (and limited) by the data format of the mass spectrometer that was used. There are a number of options for quantitation of ^{15}N-labeled proteins. MSQuant (18) and Census (19) support a range of data formats and are available free of charge. Mascot distiller (www.matrixscience.com) is a commercial package supporting nearly all data formats.

4. Notes

1. The minimal media should not contain any unlabeled nitrogen (in the form of amino acids or residual salts) and it is advised to use highly ^{15}N-enriched (>99%) ammonium sulfate. This will result in the highest possible enrichment in yeast and (eventually) in *Drosophila*, thereby enhancing the accuracy of quantitation by mass spectrometry.
2. The larva box used in these experiments had the dimensions of 20 × 15 × 10 cm and the amount of embryos for this box can vary between 10 and 500 mg.
3. When a cylindrical fly collection cage is used that fits on a Petri dish, flies can easily be fed by replacing the Petri dishes (with labeled or unlabeled yeast). In addition, (staged) embryos can also be collected simply by replacing the Petri dish at desired intervals. A fly cage with an approximate diameter of 9 cm and height of 10 cm can hold an optimal amount of 5 ml of flies.
4. *E. coli* tends to grow slightly slower in Spectra-9 N medium than in Spectra-9 U medium.
5. Since a rather large amount of *C. elegans* animals is needed for proteomic experiments, 15 cm ø Petri dishes are best suited.
6. Depending on the *C. elegans* strain(s) used, different culturing conditions might be required, but animals are usually cultured at 15–20°C. It will take approximately 12–15 days to culture four generations at 20°C. Since these

animals have a life span of 2–3 weeks, it is best to pick individual (young) animals when transferring animals to a new plate to get rid of the older partially metabolically labeled animals.

7. Yeast can be used for *Drosophila* labeling experiments after 12 months of storage at –80°C.
8. We have used the *D. melanogaster* strain Oregon-R, but this protocol should be adaptable to many other fly strains.
9. When the larva boxes are kept at 25°C and 80% humidity throughout larval and pupal developmental stages, flies hatch after approximately 9 days.
10. A small amount of labeled or unlabeled yeast is added to the Petri dish and serves as the food source for the flies. When the dish is depleted of yeast, it should be replaced with a fresh one. The amount of yeast spread on the plate depends on the amount of time the plate is left in the fly cage. For shorter periods of time (0–3 h), a medium strip of yeast (about 3 cm in diameter and 0.5 cm deep) is sufficient, but more yeast is required for longer periods (e.g., overnight).
11. Alternatively, if no LC-MS/MS system is available, the level of incorporation can be determined by MALDI-TOF mass spectrometry. To this end, labeled proteins should be separated by SDS-PAGE gel electrophoresis, preferably on a large (15 cm) gel. After Coomassie staining, a number of intense bands can be digested with trypsin, followed by MALD-TOF analysis and protein identification. The disadvantage of this approach is that it is very likely that even a single gel band will still contain multiple proteins, which might hamper the protein identification process.
12. The urea concentration needs to be 2 M or less to prevent inhibitory effects on trypsin activity.
13. Protein digests can be desalted using ZipTips (Millipore) or homemade tips. To create your own, pack a small plug of C_{18} material (3 M Empore C18 extraction disk) into a GELoader tip (Eppendorf) similar to what has been described previously (20). The tip (homemade or ZipTips) is first washed with 2 × 20 μl of acetonitrile, followed by 20 μl of 8/2 (v/v) acetonitrile/water with 0.1 M acetic acid and finally equilibrated with 2 × 20 μl of 0.1 M acetic acid. The sample is then added onto the material in steps of 20 μl, followed by washing with 20 μl of 0.1 M acetic acid. The peptides are eluted and collected in a 0.5 ml tube with 2 × 20 μl of 8/2 (v/v) acetonitrile/water with 0.1 M acetic acid and dried using a vacuum centrifuge.

14. There are several search algorithms that allow identification of ^{15}N-labeled proteins. In Mascot (http://www.matrixscience.com/), select "^{15}N metabolic" in the "quantitation" drop-down menu. In The GPM (http://human.thegpm.org), check the box "all ^{15}N amino acids." In Sequest, you need to define a new set of amino acids in which all ^{14}N masses are replaced by their ^{15}N-isotope.
15. MS-Isotope (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msisotope) is a useful tool to calculate the chemical composition of peptides and their modifications. It also provides the isotope distribution of (unlabeled) peptides.
16. The software tool IsoPro 3.0 can be used to simulate isotope distributions based on varying abundances of natural occurring elements. IsoPro used to be available as freeware, but we noticed that the Website has been discontinued recently. Ask the authors (J.W.G or J.K.) for availability.
17. If the actual enrichment of the peptides is very similar and if the average of these enrichments is not close to the purchased purity of the label, this is most likely caused by incomplete labeling, and longer periods of labeling might still be required. Alternatively, other sources of unlabeled nitrogen can affect the purity of the label and introduce similar effects. Be absolutely certain that the chemicals used in the labeling experiments are nitrogen-free (e.g., use nitrogen-free sucrose instead of sugar to prepare the larva box mixture). Starting from a 99% pure source of ^{15}N, incorporation efficiencies of 97–99% should be achievable.
18. This method of mixing is based on the assumption that several abundant proteins do not change in abundance between both conditions. If there are differences expected between these proteins, another method to mix is recommended.
19. The easiest way to check the condition of your HPLC system and reversed-phase columns is by injecting a standard peptide mixture (e.g., tryptic digest of bovine serum albumin).

References

1. Ong, S. E., and Mann, M. (2005) Mass spectrometry-based proteomics turns quantitative, *Nat Chem Biol 1*, 252–262.
2. Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlet-Jones, M., He, F., Jacobson, A., and Pappin, D. J. (2004) Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents, *Mol Cell Proteomics 3*, 1154–1169.
3. Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999)

Quantitative analysis of complex protein mixtures using isotope-coded affinity tags, *Nat Biotechnol 17*, 994–999.

4. Boersema, P. J., Raijmakers, R., Lemeer, S., Mohammed, S., and Heck, A. J. (2009) Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics, *Nat Protoc 4*, 484–494.
5. Everley, P. A., Bakalarski, C. E., Elias, J. E., Waghorne, C. G., Beausoleil, S. A., Gerber, S. A., Faherty, B. K., Zetter, B. R., and Gygi, S. P. (2006) Enhanced analysis of metastatic prostate cancer using stable isotopes and high mass accuracy instrumentation, *J Proteome Res 5*, 1224–1231.
6. Oda, Y., Huang, K., Cross, F. R., Cowburn, D., and Chait, B. T. (1999) Accurate quantitation of protein expression and site-specific phosphorylation, *Proc Natl Acad Sci USA 96*, 6591–6596.
7. Krijgsveld, J., Ketting, R. F., Mahmoudi, T., Johansen, J., Artal-Sanz, M., Verrijzer, C. P., Plasterk, R. H., and Heck, A. J. (2003) Metabolic labeling of *C. elegans* and *D. melanogaster* for quantitative proteomics, *Nat Biotechnol 21*, 927–931.
8. Dong, M. Q., Venable, J. D., Au, N., Xu, T., Park, S. K., Cociorva, D., Johnson, J. R., Dillin, A., and Yates, J. R., 3rd. (2007) Quantitative mass spectrometry identifies insulin signaling targets in *C. elegans*, *Science 317*, 660–663.
9. Findlay, G. D., Yi, X., Maccoss, M. J., and Swanson, W. J. (2008) Proteomics reveals novel *Drosophila* seminal fluid proteins transferred at mating, *PLoS Biol 6*, e178.
10. Kim, J. K., Harada, K., Bamba, T., Fukusaki, E.-i., and Kobayashi, A. (2005) Stable isotope dilution-based accurate comparative quantification of nitrogen-containing metabolites in Arabidopsis thaliana T87 Cells using in vivo ^{15}N-isotope enrichment, *Biosci Biotechnol Biochem 69*, 1331–1340.
11. Wu, C. C., MacCoss, M. J., Howell, K. E., Matthews, D. E., and Yates, J. R., 3rd. (2004) Metabolic labeling of mammalian organisms with stable isotopes for quantitative proteomic analysis, *Anal Chem 76*, 4951–4959.
12. Gouw, J. W., Pinkse, M. W., Vos, H. R., Moshkin, Y. M., Verrijzer, C. P., Heck, A. J. R., and Krijgsveld, J. (2009) In vivo stable isotope labeling of fruit flies reveals post-transcriptional regulation in the maternal-to-zygotic transition, *Mol Cell Proteomics 8*, 1566–1578.
13. Krijgsveld, J., Tops, B. B., Gauci, S., and Heck, A. J. (2009) Worms from Venus and Mars: Proteomics profiling of sexual differences in *Caenorhabditis elegans* using in vivo 15 N isotope labeling, *J Proteome Res 9*, 341–351.
14. Meiring, H. D., van der Heeft, E., ten Hove, G. J., and de Jong, A. P. J. M. (2002) Nanoscale LC-MS$^{(n)}$: Technical design and applications to peptide and protein analysis, *J Sep Sci 25*, 557–568.
15. Gouw, J. W., Tops, B. B. J., Mortensen, P., Heck, A. J. R., and Krijgsveld, J. (2008) Optimizing identification and quantitation of ^{15}N-labeled proteins in comparative proteomics, *Anal Chem 80*, 7796–7803.
16. Motoyama, A., and Yates, J. R., 3rd. (2008) Multidimensional LC separations in shotgun proteomics, *Anal Chem 80*, 7187–7193.
17. Qian, W. J., Jacobs, J. M., Liu, T., Camp, D. G., 2nd, and Smith, R. D. (2006) Advances and challenges in liquid chromatography-mass spectrometry-based proteomics profiling for clinical applications, *Mol Cell Proteomics 5*, 1727–1744.
18. Mortensen, P., Gouw, J. W., Olsen, J. V., Ong, S. E., Rigbolt, K. T., Bunkenborg, J., Cox, J., Foster, L. J., Heck, A. J., Blagoev, B., Andersen, J. S., and Mann, M. (2010) MSQuant, an open source Platform for mass spectrometry-based quantitative proteomics, *J Proteome Res 9*, 393–403.
19. Park, S. K., Venable, J. D., Xu, T., and Yates, J. R., 3rd. (2008) A quantitative analysis software tool for mass spectrometry-based proteomics, *Nat Methods 5*, 319–322.
20. Rappsilber, J., Ishihama, Y., and Mann, M. (2003) Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics, *Anal Chem 75*, 663–670.

http://www.springer.com/978-1-61779-147-5

Gel-Free Proteomics
Methods and Protocols
Gevaert, K.; Vandekerckhove, J. (Eds.)
2011, XII, 400 p., Hardcover
ISBN: 978-1-61779-147-5
A product of Humana Press