



HAL
open science

Quantitative Proteomics Using Reductive Dimethylation for Stable Isotope Labeling

Andrew C Tolonen, Wilhelm Haas

► **To cite this version:**

Andrew C Tolonen, Wilhelm Haas. Quantitative Proteomics Using Reductive Dimethylation for Stable Isotope Labeling. Journal of visualized experiments : JoVE, 2014, 89, 10.3791/51416 . cea-04646696

HAL Id: cea-04646696

<https://cea.hal.science/cea-04646696>

Submitted on 12 Jul 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Video Article

Quantitative Proteomics Using Reductive Dimethylation for Stable Isotope Labeling

Andrew C. Tolonen^{1,2,3}, Wilhelm Haas⁴¹CEA, DSV, IG, Genoscope²CNRS-UMR8030, Évry, France³Université d'Évry Val d'Essonne⁴Massachusetts General Hospital Cancer CenterCorrespondence to: Andrew C. Tolonen at atolonen@genoscope.cns.fr, Wilhelm Haas at whaas@mgh.harvard.eduURL: <https://www.jove.com/video/51416>DOI: [doi:10.3791/51416](https://doi.org/10.3791/51416)

Keywords: Chemistry, Issue 89, quantitative proteomics, mass spectrometry, stable isotope, reductive dimethylation, peptide labeling, LC-MS/MS

Date Published: 7/1/2014

Citation: Tolonen, A.C., Haas, W. Quantitative Proteomics Using Reductive Dimethylation for Stable Isotope Labeling. *J. Vis. Exp.* (89), e51416, doi:10.3791/51416 (2014).

Abstract

Stable isotope labeling of peptides by reductive dimethylation (ReDi labeling) is a method to accurately quantify protein expression differences between samples using mass spectrometry. ReDi labeling is performed using either regular (light) or deuterated (heavy) forms of formaldehyde and sodium cyanoborohydride to add two methyl groups to each free amine. Here we demonstrate a robust protocol for ReDi labeling and quantitative comparison of complex protein mixtures. Protein samples for comparison are digested into peptides, labeled to carry either light or heavy methyl tags, mixed, and co-analyzed by LC-MS/MS. Relative protein abundances are quantified by comparing the ion chromatogram peak areas of heavy and light labeled versions of the constituent peptide extracted from the full MS spectra. The method described here includes sample preparation by reversed-phase solid phase extraction, on-column ReDi labeling of peptides, peptide fractionation by basic pH reversed-phase (BPRP) chromatography, and StageTip peptide purification. We discuss advantages and limitations of ReDi labeling with respect to other methods for stable isotope incorporation. We highlight novel applications using ReDi labeling as a fast, inexpensive, and accurate method to compare protein abundances in nearly any type of sample.

Video Link

The video component of this article can be found at <https://www.jove.com/video/51416/>

Introduction

Measuring concentration differences of many proteins between complex samples is a central challenge in proteomics. Increasingly, this is being done by labeling proteins in each sample with different isotopic tags, combining the samples, and using mass spectrometry to quantify concentration differences. Several methods exist for stable isotopic labeling of proteins and peptides. ¹⁵N labeling¹ and SILAC² introduce isotopic labels metabolically *in vivo*, whereas iCAT³, iTRAQ⁴, and reduction dimethylation⁵ add stable isotope tags after protein extraction and digestion. Among these methods, reductive dimethylation (ReDi labeling) is gaining popularity as an inexpensive, reproducible method to quantify protein concentration differences in nearly any type of sample.

ReDi labeling involves reacting peptides with formaldehyde to form a Schiff base, which is then reduced by cyanoborohydride. This reaction dimethylates free amino groups on N-termini and lysine side chains and monomethylates N-terminal prolines. The protocol described here methylates peptides in sample 1 with a "light" label using reagents with hydrogen atoms in their natural isotopic distribution and sample 2 with a "heavy" label using deuterated formaldehyde and cyanoborohydride (**Figure 1**). Each dimethylated amino group on a peptide results in a mass difference of 6.0377 Da between light and heavy forms, which is employed to distinguish between the two forms using a mass spectrometer. Specifically, relative peptide abundances are quantified as the ratio of MS1 extracted ion chromatogram areas (MS1 peak area ratio) of light and heavy version for each peptide ion pair. The relative abundance of a protein is calculated as the median MS1 peak area ratio among all peptides in the protein. In this report, we describe a robust protocol for conducting ReDi labeling experiments by LC-MS/MS that includes reversed-phase peptide solid-phase extraction, on-column ReDi labeling, peptide fractionation by basic pH reversed phase (BPRP) chromatography, and purification of peptide mixtures using StageTips (**Figure 2**). We discuss advantages and limitations of using ReDi labeling for quantitative proteomics.

Protocol

NOTE: This method was previously described¹².

1. Protein Isolation

Prepare 1 mg of cellular protein by lysing cells, preferably by physical methods such as French press, bead beating, or sonication. Avoid lysozyme-mediated cell lysis because the enzyme will confound mass spectrometry measurements.

2. TCA Precipitation of Proteins

Add 1 volume trichloroacetic acid (TCA) to 4 volumes protein and chill on ice for 10 min to precipitate proteins. Centrifuge at 12,000 x g for 5 min at 4 °C and remove the supernatant. Resuspend the pellet in 1 ml of ice-cold acetone and centrifuge at 12,000 x g for 5 min at 4 °C. Remove the supernatant and invert the tube on the bench to dry the pellet for 15 min. Store protein pellets at -80 °C.

3. Denature Proteins and Reduce Disulfide Bonds

Resuspend proteins to ~2 mg/ml in 500 µl denaturation and reduction buffer (either 4 M urea or 3% SDS in 50 mM HEPES pH 8.5, 5 mM DTT). Optionally, include a protease inhibitor in the buffer. Incubate proteins for 30 min at 56 °C, followed by 10 min at room temperature.

4. Alkylate Free Sulfhydryl Groups to Irreversibly Disrupt Disulfide Bond Formation

Prepare fresh 0.3 M iodoacetamide in water. CAUTION! Iodoacetamide is highly toxic. Add 25 µl 0.3 M iodoacetamide (15 mM final concentration) to 500 µl protein and incubate for 20 min in the dark at room temperature. Quench iodoacetamide by adding 10 µl of 300 mM DTT (5 mM final DTT concentration). Store alkylated proteins at -80 °C.

5. Protein Digestion

TCA precipitate proteins (as described in step 2) and resuspend in 1 ml of 50 mM HEPES (pH 8.2), 1 M urea. Prepare a stock solution of Lysyl endoproteinase (Lys-C) in water at a concentration of 2 µg/µl and add 5 µl to the protein solution. Incubate the mixture for 16 hr at room temperature. Ensure the final Lys-C concentration is 10 ng/µl and the protein-to-LysC ratio (w/w) is 1/50 to 1/200. Resuspend 20 µg sequencing grade trypsin in 40 µl of 50 mM acetic acid, add 5 µl (10 µg trypsin) to the Lys-C digest, and incubate for 6 hr at 37 °C. Use the same protease concentration and protease-to-protein ratios for Lys-C as used for trypsin.

6. Reversed-phase Peptide Extraction

1. Acidify peptides by adding trifluoroacetic acid (TFA) to a final concentration of 0.5% (pH=2). Attach a C18 column to an extraction manifold. Use the highest possible flow rate for all steps except loading and elution of the peptides.
2. Wet column with 6 ml acetonitrile (ACN). Wash column with 6 ml 80% ACN, 0.1% TFA, then equilibrate with 6 ml 0.1% TFA. Do not allow the column to run dry between steps.
3. Stop vacuum pressure and load 500 µg of peptides onto the column at a flow rate of approximately 1 ml/min. Once peptides have bound to the column, restart vacuum and wash with 6 ml 0.1% TFA, then with 3 ml of citric acid buffer (0.09 M citric acid, 0.23 M Na₂HPO₄, pH 5.5).
Note: Higher peptide amounts can be labeled but the C18 column binding capacity should be at least two-fold higher than the peptide quantity to avoid sample loss.

7. On-column Peptide Labeling by Reductive Dimethylation (ReDi Labeling)

Perform this step under a chemical hood as hydrogen cyanide is released in low concentration during the labeling process.

1. Prepare 12 ml of "light" and "heavy" ReDi buffers to methylate peptide free amines. Light ReDi buffer consists of 0.8% formaldehyde and 0.12 M sodium cyanoborohydride carrying hydrogens in their natural isotopic distributions in citric acid buffer. Heavy ReDi buffer consists of 0.8% deuterated formaldehyde and 0.12 M deuterated sodium cyanoborohydride in citric acid buffer.
2. Incubate column containing peptides by adding 10 ml either light or heavy ReDi buffer to the peptide-containing columns at flow rate of 1 ml/min and repeat to ensure complete labeling. Wash column with 6 ml 0.1% TFA and then with 1 ml 0.5% acetic acid.
3. Stop vacuum and elute labeled peptides first with 1 ml 40% ACN, 0.5% acetic acid, then with 1 ml 80% ACN, 0.5% acetic acid using a flow rate of approximately 0.5 ml/min. If desired, measure the labeling efficiency of individual samples by mass spectrometry before mixing heavy and light samples (see "Representative Results"). Mix 1:1 heavy and light-labeled peptide samples to be quantified by mass spectrometry.

8. Separate Peptide Mixture by Basic pH Reversed Phase (BPRP) Chromatography

Basic pH reversed phase (BPRP) chromatography to separate the peptide mixture into multiple fractions, which are independently analyzed by LC-MS/MS to increase proteome coverage.

1. Fractionate the peptide mixture on a C18-HPLC column by applying a gradient of increasing ACN concentration in 10 mM ammonium bicarbonate (pH 8). Start with 5% (v/v) ACN for 5 min, increase to 35% ACN in 60 min, and then to 90% ACN in 1 min. Retain the 90% ACN for 4 min before reducing the ACN to 5% to re-equilibrate the column for 9 min. Collect 96 fractions of equal volume in a 96-well plate (A1 to H12). Monitor the fractionation using a UV detector at 220 nm while peptides are eluting off the column (10-70 min for the conditions described here).

- Combine fractions from wells A1, C1, E1, and G1 (fraction A1), from wells B1, D1, F1, and H1 (fraction B1), from wells A2, C2, E2, and G2 (fraction A2) and accordingly for the remaining fractions. Remove the solvent using a vacuum centrifuge. Resuspend peptides from fractions A1, B2, A3, B4, A5, B6, A7, B8, A9, B10, A11, and B12 in 130 μ l of 1 M urea/0.5% TFA and purify using StageTips as described in step 9. Store fractions B1, A2, B3, A4, B5, A6, B7, A8, B9, A10, B11, and A12 at -20 $^{\circ}$ C.

9. Purify Peptides by SStop and Go Extraction (StageTips)

Prepare C18-StageTip⁷ microcolumns by packing 200 μ l pipette tips with two C18 disks with an internal diameter (ID) of 1.07 mm. Put Stage Tips into Eppendorf tubes. Use a microcentrifuge to wash tips with 130 μ l of methanol, then 130 μ l 80% ACN, 0.5% acetic acid. Equilibrate StageTips with 130 μ l 0.1% TFA. Transfer peptide mixture to StageTips and wash with 130 μ l 0.1% TFA, then 40 μ l 0.1% TFA, then 40 μ l 0.5% acetic acid. Elute peptides first with 20 μ l 40% ACN, 0.5% acetic acid, then 20 μ l 80% ACN, 0.5% acetic acid. Combine eluates and dry by vacuum filtration.

10. Microcapillary LC-MS/MS

- Dissolve peptides in 1-5 μ l 5% formic acid, 5% ACN to a concentration of approximately 1 μ g/ μ l. Resolve \sim 1 μ g peptides on a 100 μ m \times 20 cm C18-reversed phase HPLC column with a gradient of 6-22% ACN in 0.125% formic acid applied over 75 or 100 min at a flow rate of \sim 300 nl/min.
- Identify peptides by using an LTQ Orbitrap Velos¹² or similar liquid chromatography-mass spectrometry platform with a mass spectrometer providing high-resolution and high mass accuracy. Operate the mass spectrometer in data-dependent mode with a full MS scan (resolution of 60,000) acquired in the Orbitrap analyzer. Generate linear ion trap MS/MS spectra for the 20 most abundant ions detected in the full MS spectrum. Set automatic gain control (AGC) targets to 1×10^5 for the full MS and 2,000 for MS/MS. Set maximum ion accumulation times to 1,000 msec for MS and 150 msec for MS/MS. Exclude fragmented peptide precursor ions from further selection from MS/MS for 20–60 sec.

11. MS/MS Data Acquisition

Identify peptides by comparing MS/MS spectra RAW files to a theoretical database with an algorithm such as SEQUEST⁸ using these parameters (Table 1).

Table 1. Peptide Database Search Parameters.

General Parameters	fully tryptic digestion with up to 2 missed cleavages
	25 ppm precursor ion tolerance
	1.0 Da fragment ion tolerance
Static Modifications	+57.02146 Da on cysteine, carboxyamidomethylation
	+28.03130 Da on lysine and the peptide N-terminus, light dimethylation label
Dynamic Modifications	+15.99491 Da on methionine, oxidation
	+6.03766 Da on lysine and the peptide N-terminus, heavy dimethylation label

- Filter peptides to a 1% false discovery rate with a method such as the target-decoy⁹ strategy using a database of open reading frames in the actual and reversed orientations.

12. Peptide Quantification

Calculate the areas of heavy and light pairs of MS1 extracted ion chromatograms (MS1 peak areas) and peptide signal-to-noise (S/N) ratios¹⁰. Include peptide pairs only when their average signal-to-noise ratio is above five. Quantify relative abundance of a peptide in the two samples as the ratio of MS1 peak areas of heavy and light versions of the same peptide (MS1 peak area ratio). Calculate relative protein abundances as the median MS1 peak area ratio for all peptides in the protein.

Representative Results

We evaluated the accuracy, precision, and reproducibility of ReDi labeling using *Saccharomyces cerevisiae* and *Clostridium phytofermentans* whole cell lysates. We first quantified the ReDi labeling efficiency of a mix of *C. phytofermentans* protein lysates from cellulose (heavy labeled, H) and glucose (light label, L) cultures. When filtered to a 1% peptide false discovery rate, this sample contained 11,194 unique peptide sequences with a 98% ReDi labeling efficiency. Unfractionated *S. cerevisiae* protein lysate was similarly labeled with H or L reagents, mixed at various ratios, and analyzed. Protein expression differences (\log_2 (median MS1 peak areas)) reproducibly reflect the ratios at which the H and L samples were mixed across a wide range of mixing ratios (Figure 3). Specifically, the fold-change of 99% of the proteins were measured as being smaller than 1.6 fold for the 1:1 mixed samples. In the 1:10 and 10:1 samples, 99% of proteins were within 3.8 fold of the expected ratio, showing an increase in the standard deviation at greater distance from a 1:1 mixture. When ReDi labeling was applied to the *Clostridium phytofermentans* proteome, we quantified more than 2,000 proteins with 94% proteins measured within 2-fold levels for replicate cultures growing on glucose (Figure 4A). Protein fold changes for duplicate pairs of cultures (glucose versus cellulose) were also highly correlated ($r^2=0.82$), (Figure 4B). *S. cerevisiae* comparisons (Figure 3) are from a single culture and thus show technical reproducibility of ReDi based quantitative proteomics. *C. phytofermentans* measurements (Figures 4A, B) compare replicate cultures so expression differences represent both measurement error

and biological variation between cultures. Together, these experiments support that ReDi proteomics is an accurate and reproducible method to quantify protein expression differences between complex samples.

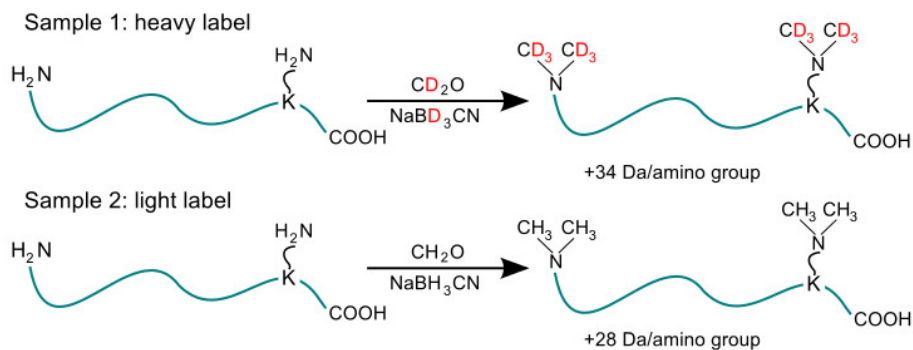


Figure 1. Reductive dimethylation of peptides using heavy and light reagents to dimethylate free amines. The same peptide labeled with heavy versus light reagents has a +6.0377 Da mass shift per free amine. The peptide shown here is labeled both at the N-terminus and on a lysine side chain. Image adapted from Reference 11. [Please click here to view a larger version of this figure.](#)

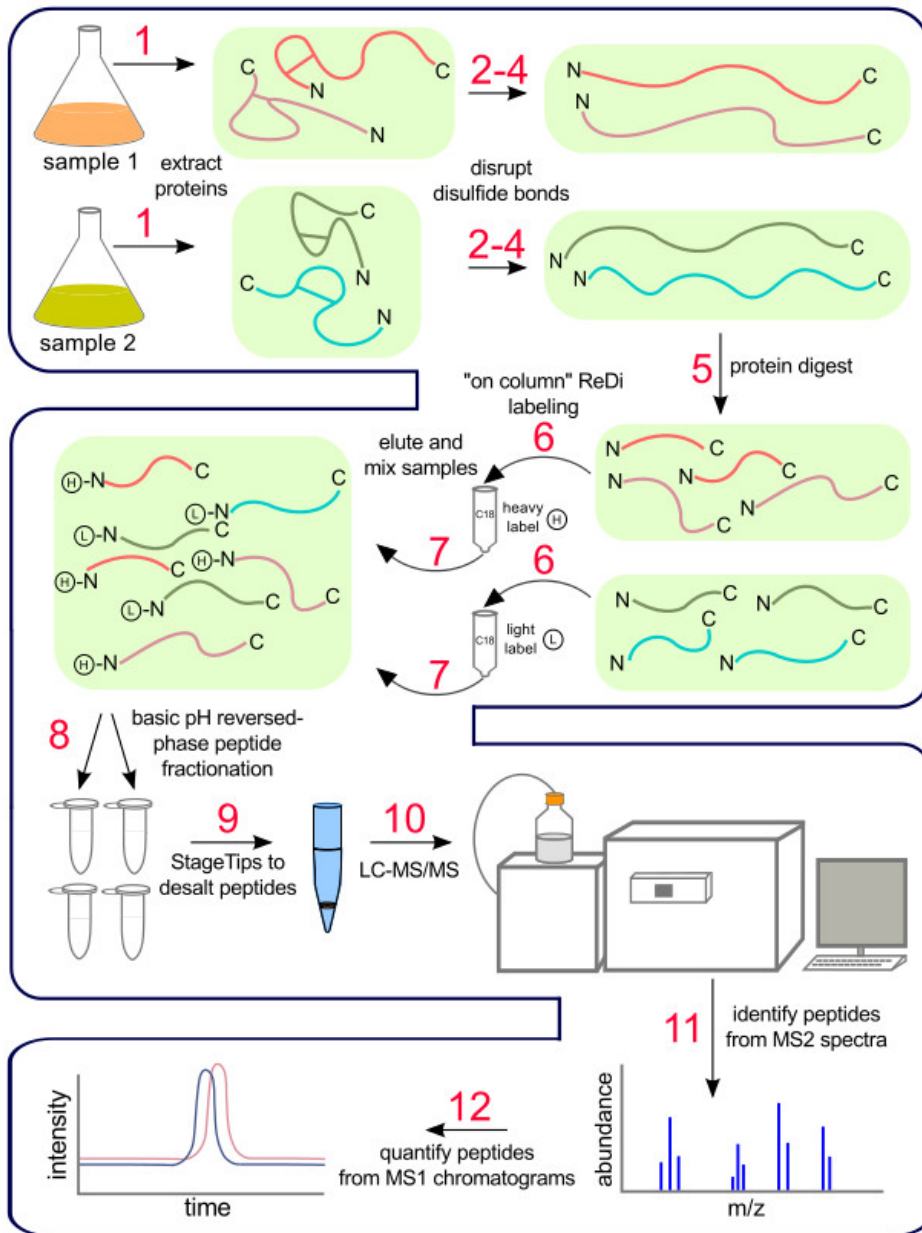


Figure 2. Overview of protocol for quantitative proteomics by reductive dimethylation. Red numbers above arrows correspond to steps described in the protocol. [Please click here to view a larger version of this figure.](#)

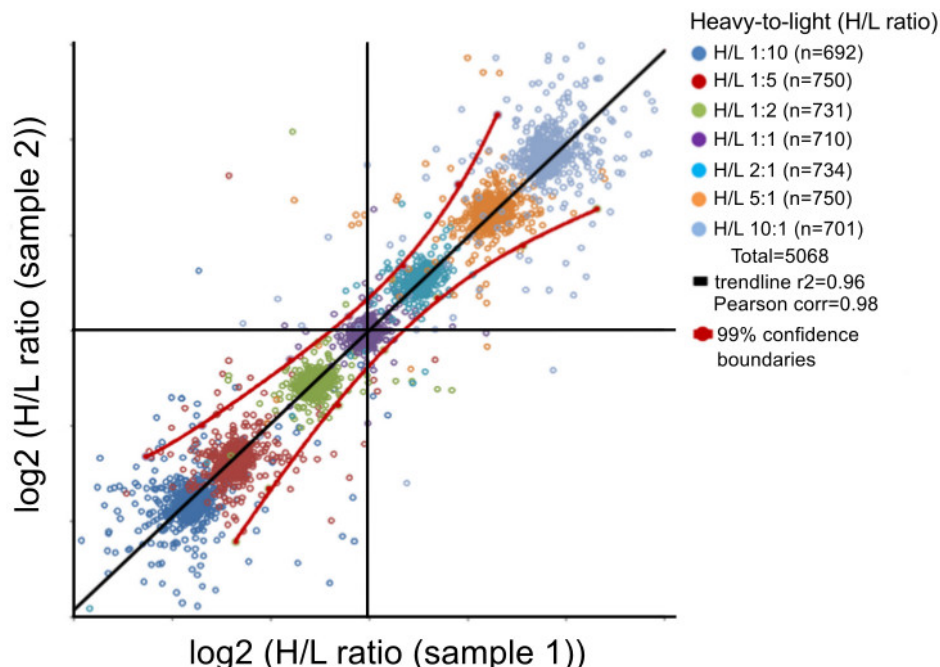


Figure 3. Evaluation of ReDi labeling using *Saccharomyces cerevisiae* whole cell lysates. Samples from a single culture were labeled with either heavy (H) and light (L) reagents, mixed at various ratios (1H:10L, 1H:5L, 1H:2L, 1H:1L, 2H:1L, 5H:1L, and 10H:1L), and protein differences between H and L samples were quantified as \log_2 median MS1 peak area ratios (\log_2 H/L ratio). The R^2 value of a linear trend line through all data points was 0.96 (black line); the Pearson correlation was 0.98. Confidence boundaries (red lines) show the absolute perpendicular distance from the trend line within which 95% of the data points were found for each sample. Image adapted from Reference 12. [Please click here to view a larger version of this figure.](#)

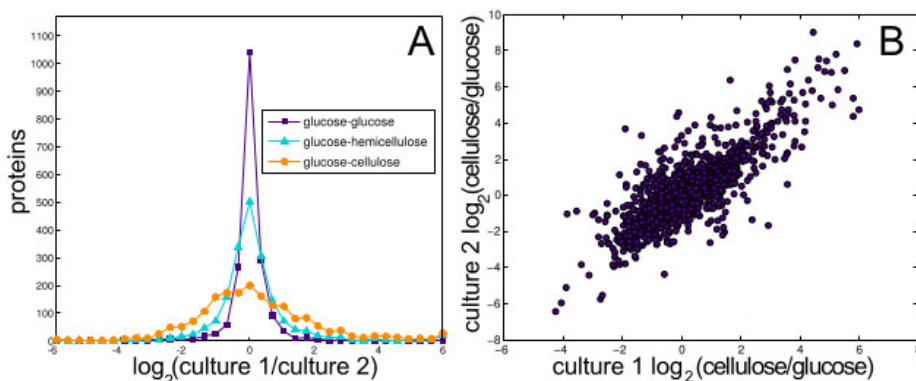


Figure 4. Quantification of ReDi-labeled *Clostridium phytofermentans* proteins from cultures growing of different carbon sources. A) Protein expression in a glucose culture relative to a replicate glucose culture, a hemicellulose culture, and a cellulose culture. The fraction of proteins expressed within twofold levels: glucose-glucose (94%), glucose-hemicellulose (80%), and glucose-cellulose (49%). **B)** Fold changes in protein expression for glucose versus cellulose cultures are highly correlated ($r^2=0.82$) for duplicate pairs of cultures. Images adapted from Reference 12. [Please click here to view a larger version of this figure.](#)

Discussion

Several points make stable isotope labeling of peptides using reductive dimethylation (ReDi labeling) an attractive method for quantitative proteomics: inexpensive labeling reagents (reagents cost less than \$1 per sample), fast reaction rate (~10 min), absence of side products, high reproducibility (**Figures 3, 4**), stable reaction products, ability to use any protease, and high ionization efficiency of labeled peptides. Chemical labeling by ReDi is also advantageous relative to metabolic labeling since it does not require strains or cell lines with specific amino acid auxotrophies or growth on a synthetic medium. As such, ReDi can be applied to nearly any type of protein sample including novel microbes¹² for which few mutant strains are available¹³ and human stem cells¹⁴, among others¹⁵.

A limitation of ReDi labeling is a lower ability to multiplex samples relative to other methods such as isobaric labeling (e.g., iTRAQ or TMT), for which currently up to 8 samples can be simultaneously quantified¹⁶. The method we describe here allows the quantitative comparison of two differentially labeled samples. Additional isotopic combinations of formaldehyde and cyanoborohydride can be used to produce up to 3 labels that differ by at least 4 Da¹⁷ and ReDi labeling has recently been extended to 5 multiplexed samples¹⁸. Another challenge of ReDi labeling is that

deuterated peptides elute slightly before light ones when using reversed-phase chromatography. To control for this "deuterium effect", peptide quantification should always be based on the entire extracted ion chromatogram (MS1 peak area) instead of intensities from one scan.

The ReDi proteomics protocol described here includes numerous improvements^{11,15,17} made since the first description of reduction dimethyl labeling of peptides for mass spectrometry⁵. We described an "on-column" labeling method to allow higher peptide amounts and the labeling efficiency can be verified before sample mixing, but "in solution" and "online" labeling methods exist as well¹⁹. In addition to proteome quantification, ReDi labeling is being applied for isoform determination²⁰ and can be combined with other methods to analyze post-translational modifications such as phosphorylation^{11,18}, acetylation²¹, and glycosylation²². Because of its versatility and inexpensive, quantitative chemistry, ReDi labeling will be increasingly applied to quantitative proteomics in new and exciting ways.

Disclosures

The authors declare no competing financial interests or other conflicts of interest.

Acknowledgements

We thank SP Gygi and GM Church for help and guidance. This work was supported by a CNRS chaire d'excellence to ACT.

References

1. Washburn, M. P., Ulaszek, R., Deciu, C., Schieltz, D. M., & Yates III, J. R. Analysis of quantitative proteomic data generated via multidimensional protein identification technology. *Analytical chemistry*. **74** (7), 1650-1657 (2002).
2. Ong, S.-E., Blagoev, B., *et al.* Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Molecular & cellular proteomics: MCP*. **1** (5), 376-386 (2002).
3. Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., & Aebersold, R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nature biotechnology*. **17** (10), 994-999, doi:10.1038/13690 (1999).
4. Ross, P. L. *et al.* Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Molecular & cellular proteomics: MCP*. **3** (12), 1154-1169, doi:10.1074/mcp.M400129-MCP200 (2004).
5. Hsu, J.-L., Huang, S.-Y., Chow, N.-H., & Chen, S.-H. Stable-isotope dimethyl labeling for quantitative proteomics. *Analytical Chemistry*. **75** (24), 6843-6852, doi:10.1021/ac0348625 (2003).
6. Chick, J. M., Haynes, P. A., Molloy, M. P., Bjellqvist, B., Baker, M. S., & Len, A. C. L. Characterization of the rat liver membrane proteome using peptide immobilized pH gradient isoelectric focusing. *Journal of Proteome Research*. **7** (3), 1036-1045, doi:10.1021/pr700611w (2008).
7. Rappsilber, J., Ishihama, Y., & Mann, M. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Analytical chemistry*. **75** (3), 663-670 (2003).
8. Eng, J. K., McCormack, A. L., & Yates III, J. R. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *Journal of the American Society for Mass Spectrometry*. **5** (11), 976-989, doi:10.1016/1044-0305(94)80016-2 (1994).
9. Elias, J. E., & Gygi, S. P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nature methods*. **4** (3), 207-214, doi:10.1038/nmeth1019 (2007).
10. Bakalarski, C. E. *et al.* The impact of peptide abundance and dynamic range on stable-isotope-based quantitative proteomic analyses. *Journal of Proteome Research*. **7** (11), 4756-4765, doi:10.1021/pr800333e (2008).
11. Wilson-Grady, J. T., Haas, W., & Gygi, S. P. Quantitative comparison of the fasted and re-fed mouse liver phosphoproteomes using lower pH reductive dimethylation. *Methods (San Diego, Calif.)*, doi:10.1016/j.ymeth.2013.03.031 (2013).
12. Tolonen, A. C., Haas, W., Chilaka, A. C., Aach, J., Gygi, S. P., & Church, G. M. Proteome-wide systems analysis of a cellulosic biofuel-producing microbe. *Molecular Systems Biology*. **7**, 461, doi:10.1038/msb.2010.116 (2011).
13. Tolonen, A. C., Chilaka, A. C., & Church, G. M. Targeted gene inactivation in *Clostridium phytofermentans* shows that cellulose degradation requires the family 9 hydrolase Cphy3367. *Molecular Microbiology*. **74** (6), 1300-1313, doi:10.1111/j.1365-2958.2009.06890.x (2009).
14. Munoz, J. *et al.* The quantitative proteomes of human-induced pluripotent stem cells and embryonic stem cells. *Molecular systems biology*. **7**, 550, doi:10.1038/msb.2011.84 (2011).
15. Kovanich, D., Cappadona, S., Raijmakers, R., Mohammed, S., Scholten, A., & Heck, A. J. R. Applications of stable isotope dimethyl labeling in quantitative proteomics. *Analytical and bioanalytical chemistry*. **404** (4), 991-1009, doi:10.1007/s00216-012-6070-z (2012).
16. McAlister, G. C. *et al.* Increasing the multiplexing capacity of TMTs using reporter ion isotopologues with isobaric masses. *Analytical chemistry*. **84** (17), 7469-7478, doi:10.1021/ac301572t (2012).
17. Boersema, P. J., Aye, T. T., Van Veen, T. A. B., Heck, A. J. R., & Mohammed, S. Triplex protein quantification based on stable isotope labeling by peptide dimethylation applied to cell and tissue lysates. *Proteomics*. **8** (22), 4624-4632, doi:10.1002/pmic.200800297 (2008).
18. Wu, Y. *et al.* Five-plex isotope dimethyl labeling for quantitative proteomics. *Chemical communications (Cambridge, England)*, doi:10.1039/c3cc47998f (2014).
19. Boersema, P. J., Raijmakers, R., Lemeer, S., Mohammed, S., & Heck, A. J. R. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nature Protocols*. **4** (4), 484-494, doi:10.1038/nprot.2009.21 (2009).
20. She, Y.-M., Rosu-Myles, M., Walrond, L., & Cyr, T. D. Quantification of protein isoforms in mesenchymal stem cells by reductive dimethylation of lysines in intact proteins. *Proteomics*. **12** (3), 369-379, doi:10.1002/pmic.201100308 (2012).
21. Chen, S.-H., Chen, C.-R., Chen, S.-H., Li, D.-T., & Hsu, J.-L. Improved N(α)-acetylated Peptide Enrichment Following Dimethyl Labeling and SCX. *Journal of proteome research*. **12** (7), 3277-3287, doi:10.1021/pr400127j (2013).
22. Sun, Z. *et al.* Capture and Dimethyl Labeling of Glycopeptides on Hydrazide Beads for Quantitative Glycoproteomics Analysis. *Analytical Chemistry*. **84** (20), 8452-8456, doi:10.1021/ac302130r (2012).