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Quantitative proteomics using reductive dimethylation for stable isotope labeling

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Title: Quantitative proteomics using reductive dimethylation for stable isotope labeling

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Keywords: quantitative proteomics; mass spectrometry; stable isotope; reductive dimethylation; peptide labeling; LC-MS/MS

Short Abstract

Stable isotope labeling of peptides by reductive dimethylation (ReDi labeling) is a rapid, inexpensive strategy for accurate mass spectrometry-based quantitative proteomics. Here we demonstrate a robust method for preparation and analysis of protein mixtures using the ReDi approach that can be applied to nearly any sample type.

Long 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.

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. ^{15}N labeling[1] and SILAC[2] introduce isotopic labels metabolically *in vivo,* whereas iCAT[3], iTRAQ[4], and reduction dimethylation[5] 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 (Fig 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 (Fig 2). We discuss advantages and limitations of using ReDi labeling for quantitative proteomics.

Protocol

This method was previously described[12].

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 minutes 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. Aklylate 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 h 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 h 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.

6.1 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.

6.2 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.

6.3 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 Na_2HPO_4, 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.

7.1 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.

7.2 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.

7.3 Stop vacuum and elute labeled peptides first with 2 ml 40% ACN, 0.5% acetic acid, then with 2 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. 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.

8.1 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).

8.2 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 µl 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 ºC.

9. Purify peptides by STop And Go Extraction (StageTips): Prepare C18-StageTip[7] microcolumns by packing 200 µl 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 µl of methanol, then 130 ul 80% ACN, 0.5% acetic acid. Equilibrate StageTips with 130 µl 0.1% TFA. Transfer peptide mixture to StageTips and wash with 130 µl 0.1% TFA, then 40 µl 0.1% TFA, then 40 µl 0.5% acetic acid. Elute peptides first with 20 µl 40% ACN, 0.5% acetic acid, then 20 µl 80% ACN, 0.5% acetic acid. Combine eluates and dry by vacuum filtration.

10. Microcapillary LC-MS/MS.

10.1 Dissolve peptides in 1-5 µl 5% formic acid, 5% ACN to a concentration of approximately 1 µg/µl. Resolve ~1 µg peptides on a 100 µm × 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 ~300 nl min^{-1}.

10.2 Identify peptides by using an LTQ Orbitrap Velos[12] 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 1×10^6 for the full MS and 2,000 for MS/MS. Set maximum ion accumulation times to 1000 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 SEQUEST[8] using these parameters (Table I, jove2014_step11.xls).

Insert table from jove2014_step11.xls here.

11.1 Filter peptides to a 1% false discovery rate with a method such as the target-decoy[9] 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) ratios[10]. 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 phytofermentan*s 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 (Fig 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 (Fig 4A). Protein fold changes for duplicate pairs of cultures (glucose versus cellulose) were also highly correlated (r^2=0.82), (Fig 4B). *S. cerevisiae* comparisons (Fig 3) are from a single culture and thus show technical reproducibility of ReDi based quantitative proteomics. *C. phytofermentans* measurements (Fig 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.

Figures

Fig 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.

Fig 2 Overview of protocol for quantitative proteomics by reductive dimethylation. Red numbers above arrows correspond to steps described in the protocol.

Fig 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.

Fig 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.

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 (Fig 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 microbes[12] for which few mutant strains are available[13] and human stem cells[14], among others[15].

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 quantified[16]. 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 Da[17] and ReDi labeling has recently been extended to 5 multiplexed samples[18]. 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 spectrometry[5]. 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 well[19]. In addition to proteome quantification, ReDi labeling is being applied for isoform determination[20] and can be combined with other methods to analyze post-translational modifications such as phosphorylation[11,18] acetylation[21], and glycosylation[22]. Because of its versatility and inexpensive, quantitative chemistry, ReDi labeling will be increasingly applied to quantitative proteomics in new and exciting ways.

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Disclosures

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

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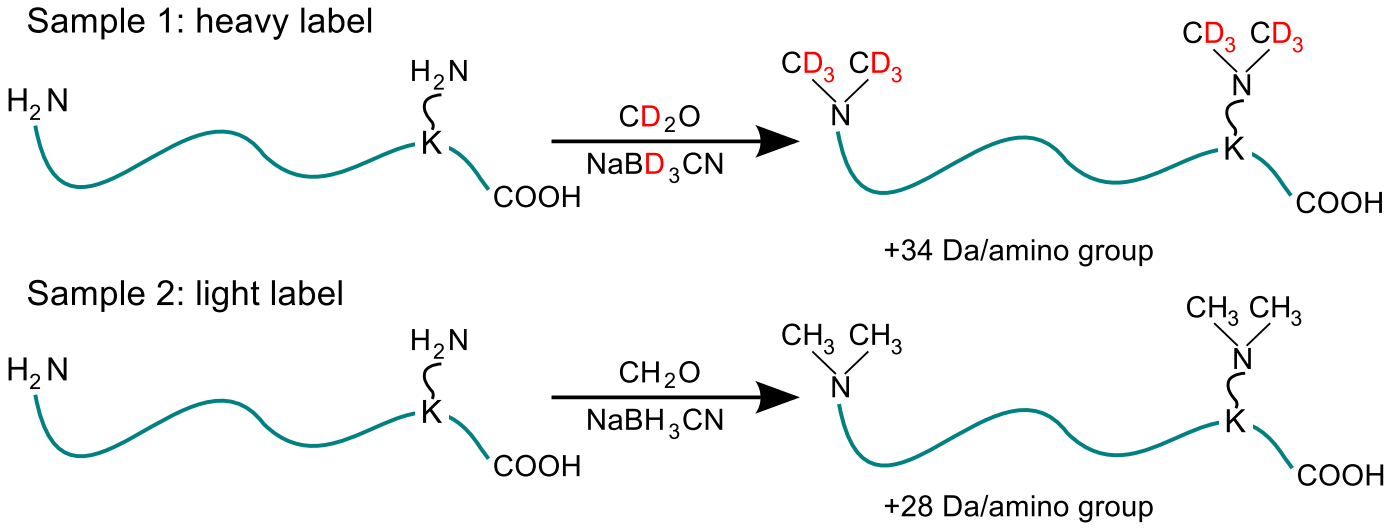

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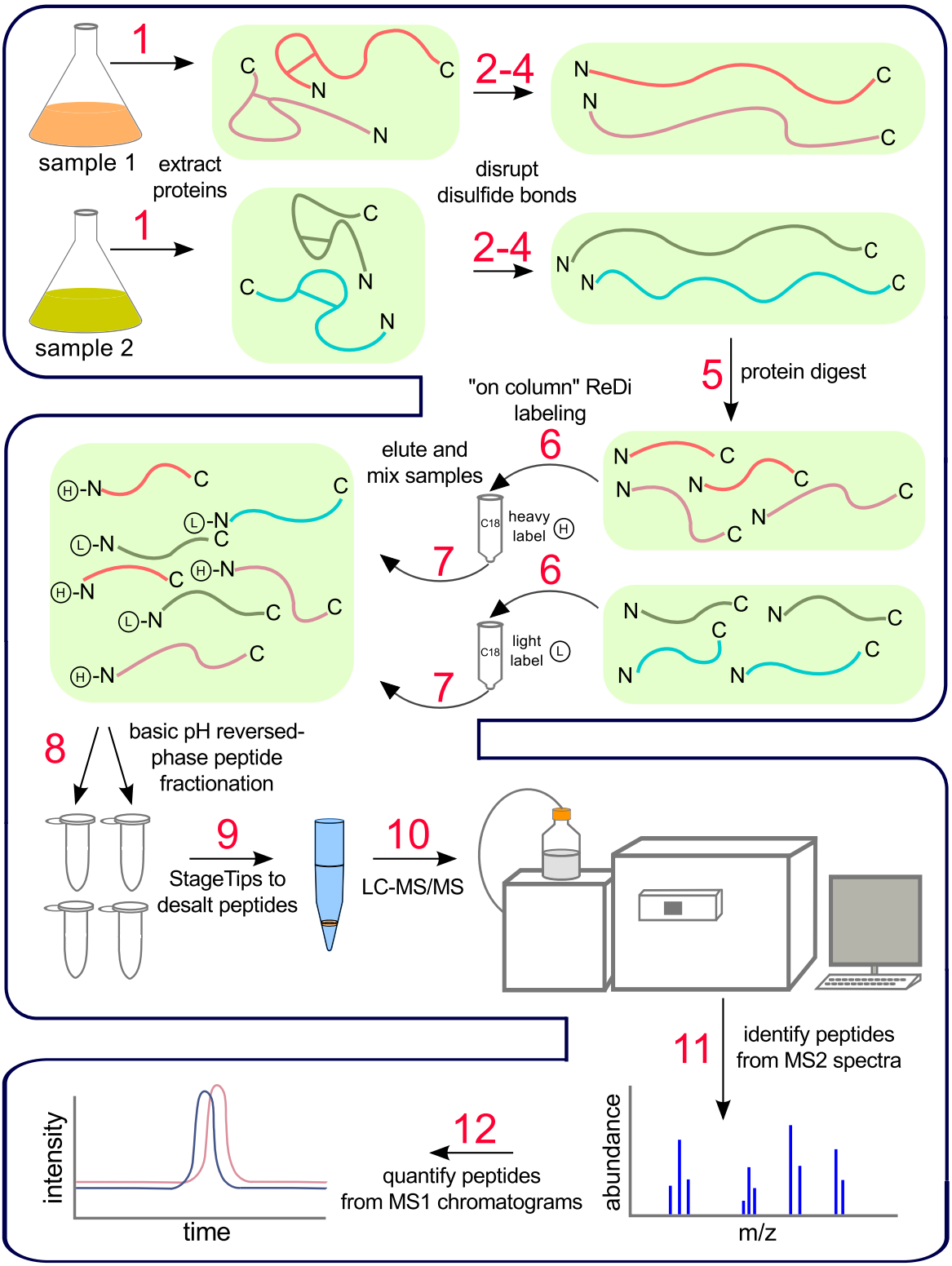

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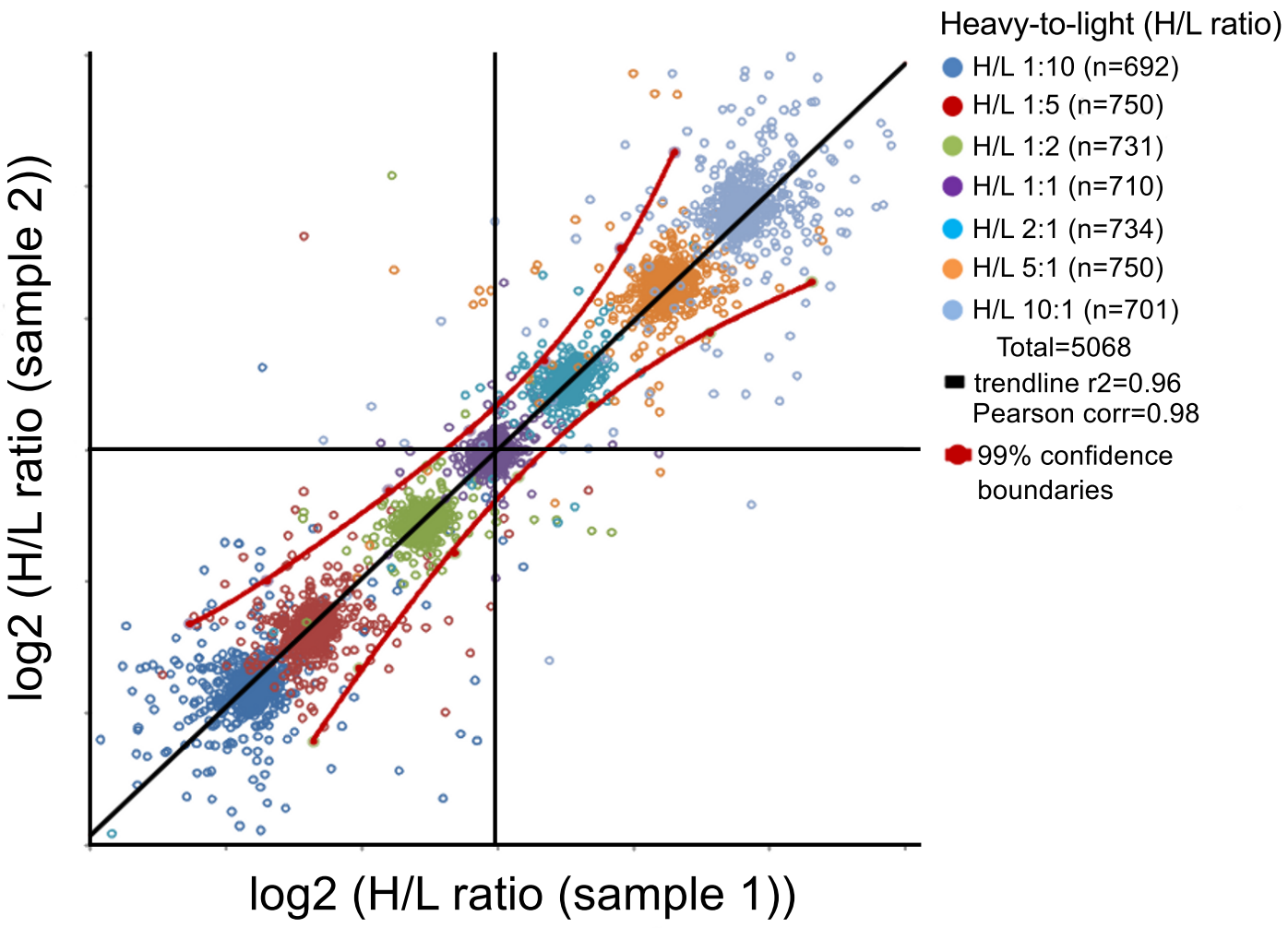

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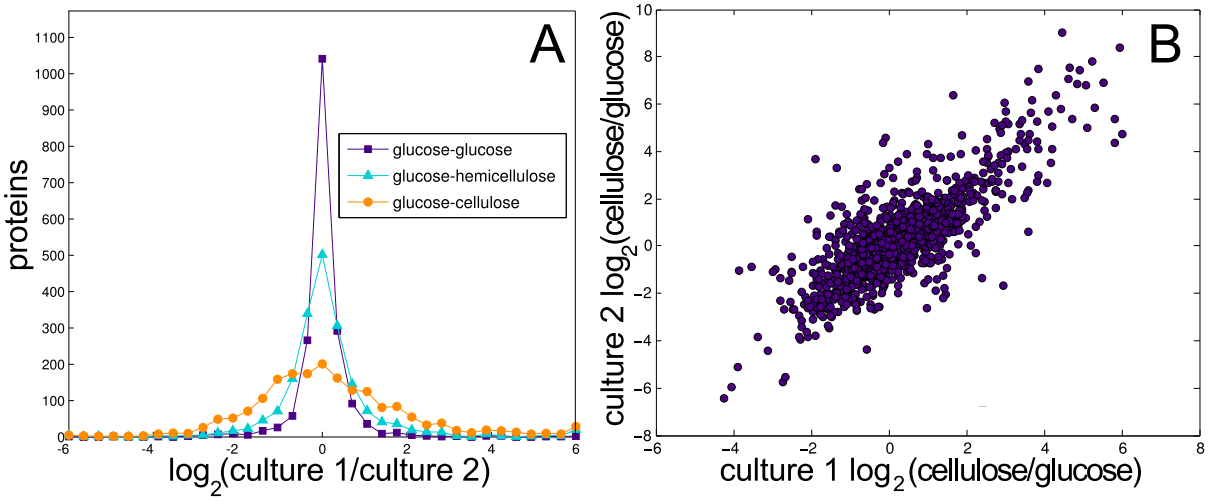

Table
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SearchParameters

Peptid
General Parameters
Static Modifications
Dynamic Modifications

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

Excel Spreadsheet- Table of Materials/Equipment
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Name of Material/Equipment	Company
trichloroacetic acid	Sigma-Aldrich
acetone	Sigma-Aldrich
Sodium dodecyl sulfate	Sigma-Aldrich
sodium hydroxide	Sigma-Aldrich
DL-Dithiothreitol	Sigma-Aldrich
protease Inhibitor Complete Mini Cocktail	Roche
iodoacetamide	Sigma-Aldrich
HEPES	Sigma-Aldrich
calcium chloride	Sigma-Aldrich
Lysyl Endoprotease	Wako Chemicals
sequencing grade trypsin	Promega
acetic acid	Sigma-Aldrich
trifluoroacetic acid	Sigma-Aldrich
tC18 Sep-Pak C18 cartridges	Waters
extraction manifold	Waters
acetonitrile	Sigma-Aldrich
formaldehyde	Sigma-Aldrich
cyanoborohydride	Sigma-Aldrich
deuterated formaldehyde	Sigma-Aldrich
sodium cyanoborodeuteride	CDN isotopes
MES	Sigma-Aldrich
C18-HPLC column (4.6 x 250 mm, 5 µm particle size)	Agilent
formic acid	Sigma-Aldrich
C18 Empore Disks	3M
methanol	Sigma-Aldrich

Catalog Number	Procedure
T9159	protein precipitation
650501	protein precipitation
71736	denature, reduce protein
S8045	denature, reduce protein
43816	denature, reduce, alkylate protein
4693124001	denature, reduce protein
I6125	alkylate protein
H7523	resuspend, extract, label protein
C5670	resuspend protein
129-02541	protein digestion
V5111	protein digestion
320099	protein digestion
299537	Reversed-phase peptide extraction
WAT054960	Reversed-phase peptide extraction
WAT200609	Reversed-phase peptide extraction
14261	various
252549	“light” peptide labeling
71435	“light” peptide labeling
492620	“heavy” peptide labeling
D-1797	“heavy” peptide labeling
M3671	peptide labeling
770450-902	basic pH reversed-phase chromatography
399388	various
14-386-3	STAGE tips
494437	STAGE tips

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Title of Article: Quantitative mass spectrometry-based proteomics using stable isotope labeling by reductive dimethylation

Author(s): Andrew C Tolonen, Wilhelm Haas

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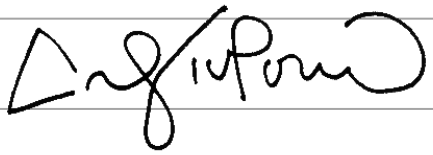
Signature:	 Date: 15 july 2013

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Dear Dr Kinahan,

Thank you very much for sending the reviews for our manuscript “Quantitative proteomics using reductive dimethylation for stable isotope labeling”. We have revised the manuscript to incorporate the reviewer's comments. Here we provide the manuscript as a .doc file in which we tracked changes relative to the file downloaded from the JoVE website. In addition, we respond point-by-point to the reviewer's comments below. Text written by the reviewers is shown in blue and our responses are in black.

Sincerely,
Andrew Tolonen and Wilhelm Haas

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Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The manuscript provides a detailed step by step protocol for stable isotope labeling of peptides with reductive di-methylation. The protocol touches on how to identify labeled peptides and subsequently quantify proteins.

Major Concerns:
N/A

Minor Concerns:
The authors might **include an overview depicting the complete workflow from sample**

preparation to final data analysis.

This is an excellent idea. We now provide a complete overview of the protocol as Fig 2.

The manuscript explicitly mentions another publication for which the method was used. Thus a careful cross comparison of the content/wording may be needed to avoid a conflict of interest with the publisher of the afore mentioned publication.

We have written the manuscript text to assure that there is no conflict of interest with our previous publication.

The detailed steps in the protocol should be checked for completeness. For example **how did the authors elute the peptides from the C18 column in step 9**?

We have clarified in the text that the peptides were eluted with 40% acetonitrile, 1% formic acid.

While the method doesn't need to be complete in every detail, certain key explanations may be helpful for understanding the protocol: **One example represents the separation of peptides with immobilized pH gradient isoelectric focussing. The purpose of this step remains unclear.** The manuscript should be revised accordingly.

We have replaced the IPG-IEF fractionation with a newer method, basic pH reversed phase (BPRP) chromatography. We provide a comprehensive description of BPRP chromatography in step 8.

Reviewer #2:
Manuscript Summary:
In the present work, Tolonen and Haas provide a detailed protocol of their earlier published work on low pH reductive dimethylation for quantitative proteomics, and apply it to the analysis of two model organisms. Accurate, inexpensive and versatile methods for quantitative proteomics are urgently needed; thus, there is compelling rationale for this study.

Major Concerns:
I have no major concerns with the protocol as described. The reduction and alkylation steps seem a bit odd - **it isn't clear why the authors chose to reduce and alkylate in such a small volume (40 mg/ml protein?)**.

While we had no problems reducing and alkylating in this small volume, we modified the protocol to perform these steps in a greater volume at 2 mg/ml.

But given how easy, simple and straightforward this method has been shown by many others to be, I have few reservations about their approach or the quality of their results.

Reviewer #3:
Manuscript Summary:
This manuscript by Tolonen et. al. describes a protocol for reductive dimethylation of peptides. The step-by-step protocol described here has a few improvements to the original method. The

authors have also evaluated the protocol by using whole cell lysates from a fungus (Saccharomyces cerevisiae) and a bacterium (Clostridium phytofermentans).

Major Concerns:
N/A

Minor Concerns:
1. The authors need to **provide the details of instrumentation used for the LC-MS/MS analysis**. In the protocol described, the authors state that a linear ion trap/Orbitrap mass spectrometer may be used (lines 175 and 176). However, it is not clear which instrument was used for the representative study.

We now specify that peptides were identified using an LTQ Orbitrap Velos in our previous study (Tolonen et al, 2011), but other similar instruments can be used.

2. The number of proteins quantified in one of the two studies is mentioned (line 212). The authors should also **mention the number of proteins identified and quantified in the two representative studies**.

As noted by the reviewer, the number of identified *C. phytofermentans* proteins is in the text. The number of identified yeast proteins is shown in Fig 3.

3. The authors have not mentioned whether labeling efficiency was tested to determine the percentage of peptides that were labelled. **The labeling efficiency needs to be stated.**

We thank the reviewer for pointing this out. We now describe in the Representative Results section how we determined that the ReDi labeling efficiency was 98%.

SW figure 1

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SW figure 2

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