Five-plex dimethyl labeling for quantitative proteomics

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Supporting Information

Experimental

Reagents and Materials

Endoproteinase Lys-C was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). RPMI 1640 medium was purchased from Invitrogen (Carlsbad, CA, USA). Acetonitrile (ACN, HPLC grade) was provided by Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Amresco (Solon, OH, USA). Formaldehyde (CH_2O), formaldehyde-D_2 (CD_2O) and formaldehyde-$^{13}CD_2$ ($^{13}CD_2O$), sodium cyanoborohydride ($NaBH_3CN$), sodium cyanoborodeuteride ($NaBD_3CN$), dithiothreitol (DTT), iodoacetamide (IAA) and other chemical reagents were purchased from Sigma (St. Louis, MO, USA). All the water in the experiments was purified by a Milli-Q system from Millipore Company (Bedford, MA, USA). Fused silica capillaries with 75 μm i.d. were provided by Polymicro Technologies (Phoenix, USA), and with 200 μm i.d. were purchased from Yongnian Optical Fiber Factory (Hebei, China). Daisogel ODS-AQ (3 μm, 120 Å) HPLC packing materials were purchased from DAISO Chemical CO., Ltd. (Osaka, Japan).

Cell Culture, protein extraction and In-solution Quintuplex Isotope Dimethylation Labeling

HeLa cells were cultured, harvested and the proteins were extracted as previously described.[1] Briefly, cells were grown in 1640 medium containing 10% (v/v) bovine serum and 100 U penicillin/streptomycin at 37 ^{o}C in a humidified incubator under 5% CO_2. After harvesting, ice-cold Lysis buffer (8 M urea, 50 mM Tris-HCl (pH = 7.4), 65 mM DTT, 1% protease cocktail (v/v), 1% Triton X-100 (v/v), 1 mM EDTA, 1 mM EDGA, 1 mM PMSF, 1 mM NaF and 1 mM Na_3VO_4) was added. The obtained mixtures were homogenized with sonication. Then, cell debris was removed by centrifugation at 20,000 g for 15 min at 4 °C, and the proteins within the supernatant were precipitated with 4 volumes of ice-cold acetone/ethanol/acetic acid (v/v/v = 50/50/0.1) at -20 °C. The purified proteins were resuspended in the denaturing buffer (pH 8.0) containing 8 M urea and 100 mM triethylammonium bicarbonate (TEAB), and the protein concentration was

determined by Bradford assay. Then, the protein solution was reduced by 20 mM dithiothreitol (DTT) at 60 °C for 1 h and alkylated by 40 mM iodoacetamide (IAA) in darkness at room temperature for 40 min. After proteolytic digestion by Lys-C (with substrate : enzyme = 50:1, w/w), the peptides were separately labeled with mass differential isotopical dimethylation reagents by the in-solution labeling procedures.[2] The isotopically labeled peptides were mixed together, desalted by a C18 solid-phase extraction column, lyophilized to powder and re-dissolved in 0.1% FA/H_2O for the following LC-MS/MS analysis.

Phosphopeptides Enrichment

Phosphopeptides were enriched by using self-developed Ti^{4+}-IMAC microspheres[2] after dimethyl labeling. The microspheres were suspended in the sample loading buffer containing 80% (vol/vol) ACN and 6% (v/v) TFA, and mixed with proteins with a ratio of 10:1 (w/w), followed by violent vibration for 30 min. After removing the supernatant by centrifugation, the microspheres were washed with washing buffer 1 (50% (v/v) ACN, 0.5% (v/v) TFA containing 200 mM NaCl) and washing buffer 2 (50% (v/v) ACN and 0.1% (v/v) TFA) for 20 min, respectively. Finally, the phosphopeptides were eluted by adding 10% (v/v) ammonia-water and lyophilized to powder for following analysis.

Strong-Cation Exchange-Reversed Phase Two Dimensional Liquid Chromatography Separation Coupled with Tandem Mass Spectrometry (SCX-RP 2D LC-MS/MS) Analysis

SCX-RP 2D LC-MS/MS analysis was performed with SCX monolithic column (200 μm, i.d., 7 cm) prepared as previously described.[5] The capillary analytical column (75 μm, i.d.) was in-house packed to about 15 cm length with C18 particles (3 μm, 120 Å). The flow rate was adjusted to about 200 nL/min. The mobile phase A was 0.1% (v/v) FA/H_2O, and mobile phase B was 0.1% (v/v) FA/ACN. For proteome analysis, a series of salt steps (50, 150, 250, 400 and 1000 mM NH_4AC) was performed to fractionate the SCX monolithic column-trapped peptides to the analytical column, followed by RP gradient separation. The RP gradient was set as follows: from 1-10% solvent B in 2 min, 10-35% in 105 min, 35-90% in 2 min, after washing with 90% solvent B for 3 min, the system was equilibrated with 100% solvent A for 10 min. For phosphoproteome analysis, the salt steps were set as 20, 48, 100, 250 and 1000 mM NH_4AC. The RP gradient was set as follows: from 1-5% solvent B in 2 min, 5-25% in 70 min, 25-35% in 10 min, 35-80% in 2 min, after washing with 80% solvent B for 10 min, then the system was equilibrated with 100% solvent A for 24 min.

An LTQ-Orbitrap Velos (Thermo, San Jose, CA) mass spectrometry was utilized for quantitative proteome and phosphoproteome analyses. The mass spectrometer was set as follows: ion transfer capillary 250 °C, spray voltage 1.8 kV, full MS scan from m/z 400 to 2000 with a resolution of 60000 in centroid mode. Data-dependent MS/MS scans were performed by selecting the 20 most intense ions in the full MS scan for CID with 35.0% normalized collision energy. The dynamic exclusion was set as follows: repeat count 1, repeat duration 30 s, and exclusion duration 120 s. In addition, for the identification of phosphopeptides, the multi-stage activation (MSA) strategy was used and m/z 24.50000, 32.67000, 38.67000, 49.00000, 58.00000, 98.00000 were set as neutral loss masses.

Data Processing

Data analysis was accomplished by using MaxQuant software (http://maxquant.org/, version 1.3.0.3) against IPI human database (v3.80)). The quantification of five-plex isotope labeled samples was achieved by combing two different MaxQuant searching groups as it cannot handle

over three labels (Fig. S1). #1 (+28.0313 Da), #3 (+32.0564 Da), #5 (+36.0757 Da) labeling forms were set in one group, while #1(+28. 0313 Da), #2 (+30.0439 Da), #4 (+34.0690 Da) were set in the other. And the searching results were combined by MaxQuant automatically (Fig. S5). Cysteine residue (+57.0215) was set as static modification and oxidation (+15.9949 Da) was set as variable modification, and both oxidation and phosphorylation of Ser, Thr, and Tyr (+79.96633 Da) were set as variable modifications for the phosphoproteome analysis. Peptides were processed using fully Lys-C cleavage and up to two missed cleavage sites were allowed. Main search tolerance was 6 ppm, and the first search tolerance was 20 ppm. Both the protein and peptides identification false discovery rates (FDR) were <1%. The rest of the parameters follow the default settings of MaxQuant software.

Mascot software (http://www.matrixscience.com/, version 2.3.0) was also used to examine the labeling efficiency. All the *.raw files were firstly converted to *.mgf files by DTASupercharge (v2.0a7). The generated *.mgf files were searched against IPI human database (v3.80) via Mascot (version 2.3.0). Cysteine residues (+57.021464 Da) were set as fixed modification. The identification of labeled peptides was processed by combining two separate searching due to a maximum of only 9 variable modifications was allowed. Dimethyl labeling #1 (+28.0313 Da) at K and N-terminus, #3 (+32.0564 Da) at K and N-terminus, #4 (+34.0690 Da) at K and N-terminus, #5 (+36.0757 Da) at K and N-terminus, and oxidation (+15.9949 Da) were set as variable modifications in one search, and #1 (+28.0313 Da) at K and N-terminus, #2 (+30.0439 Da) at K and N-terminus, #4 (+34.0690 Da) at K and N-terminus, #5 (+36.0757 Da) at K and N-terminus, and oxidation (+15.9949 Da) were set in the other. Peptides were processed using fully Lys-C cleavage and up to two missed cleavage sites were allowed. Peptide mass tolerance was 10 ppm, and fragment mass tolerance was 0.8 Da. Peptides having Mascot score ≥25 (rank 1, P ≤0.05) were used to examine the labeling efficiency.

Details of protein spiking-in Experiment

E.coli (bl21) was grown in Luria-Bertani medium (with 1mM kanamycin) with shaking at 37 °C overnight. After harvesting, bacteria were washed three times with PBS and lysed following the above handling of HeLa cells. We spiked three protein standards, ovalbumin, myoglobin and BSA, into five E. coli protein extraction samples (5×100 μg) with different concentrations. The proteins were spiked into the E. coli samples to obtain standard/sample ratios (w/w) of 1:200, 2:200, 5:200, 10:200, 20:200 (BSA) and 20:200, 10: 200, 5:200, 2:200, 1:200 (ovalbumin and myoglobin). After treatment with DTT/IAA, digestion and dimethyl labeling (according to above description), the mixture of the five samples were analyzed by LC-MS. Peptides were loaded on a C18 capillary trap column (200 μm i.d. × 4 cm) packed with C18 AQ beads (5 μm, 120 Å). The separation was performed on an analytical column (75 μm i.d. × 15 cm) packed with C18 AQ beads (3 μm, 120 Å). The RP gradient was set as follows: from 0-5% solvent B in 5 min, 5-35% in 195 min, 35-80% in 5 min, after washing with 80% solvent B for 10 min, the system was equilibrated with 100% solvent A for 20 min. MS conditions of an LTQ-Orbitrap Velos follows the above description.

Figures and Tables

Fig. S1. Over lapped isobaric clusters of tryptically digested peptides without lysine (2 Da mass difference).

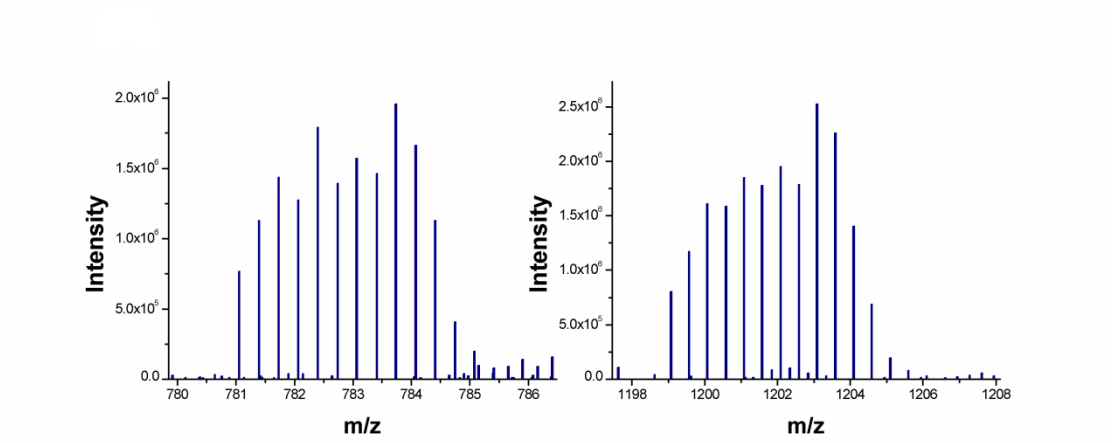

Figure S2. Log_2 ratio distributions of the replicated experiment. (a) quantified peptide and (b) quantified protein distributions of the sample mixed at 1:1:1:1:1 ratio. Log_2 ratio distributions of (c) quantified peptide and (d) quantified protein distributions of the sample mixed at 1:2:4:2:1 ratio. Log_2 ratio distributions of (e) quantified phosphopeptide and (f) quantified phosphorylation site distributions of the sample mixed at 1:1:1:1:1 ratio.

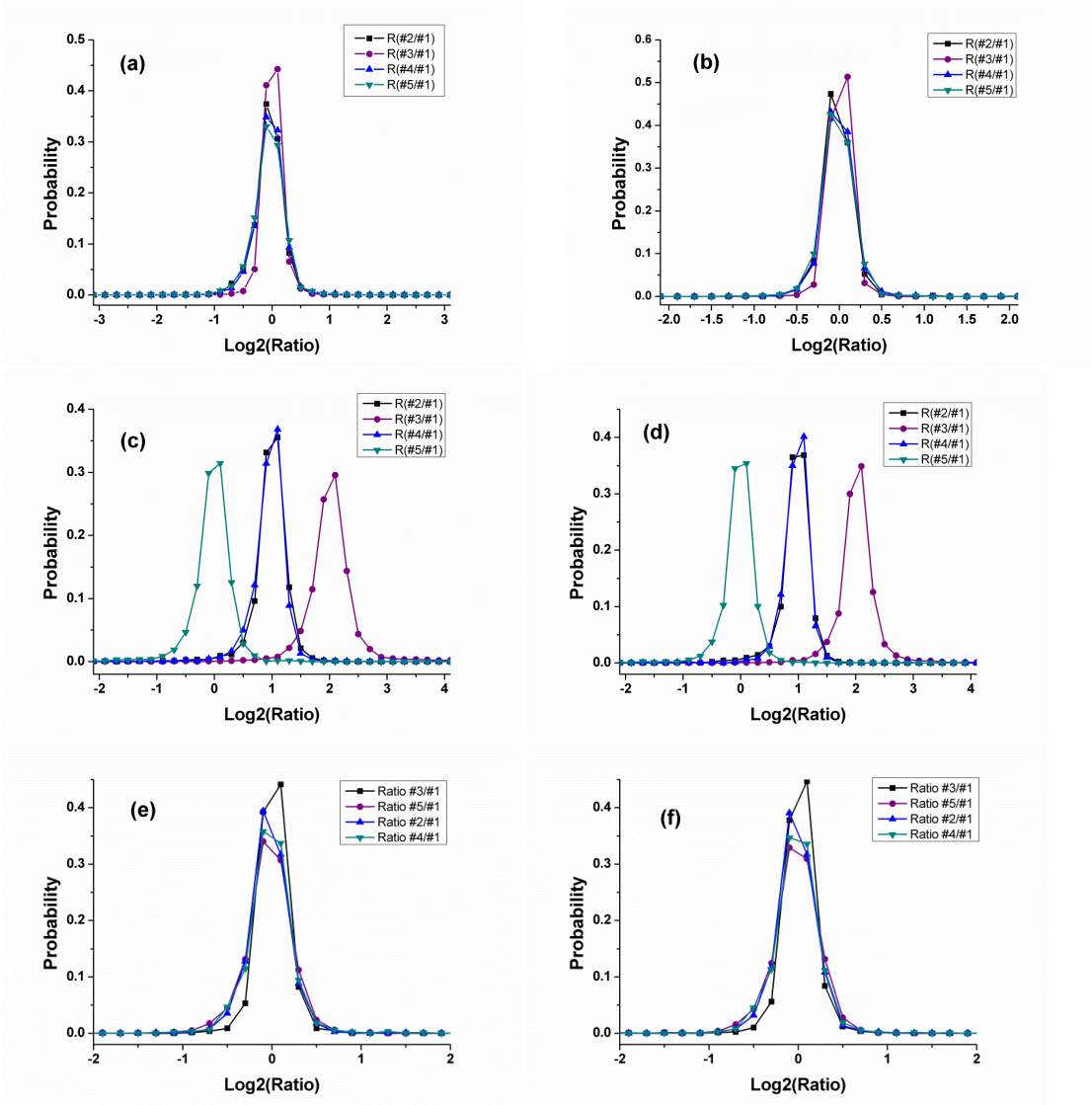

Figure S3. The quantification of individual peptides of the protein standards.

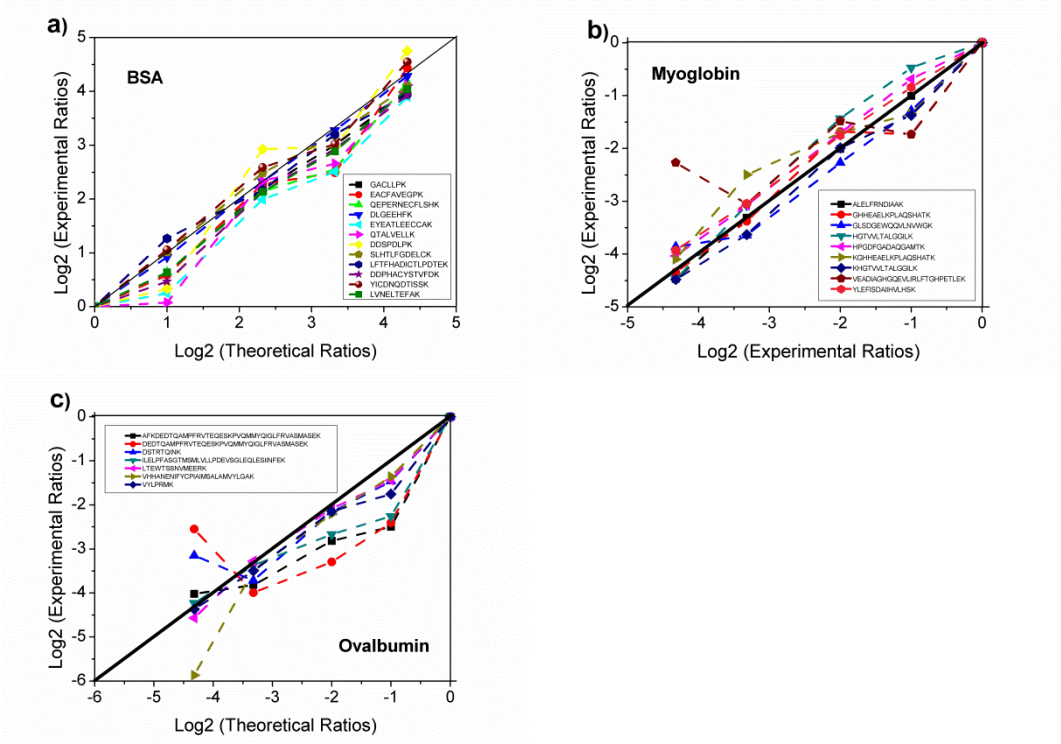

Figure S4. Comparison of proteome and phosphoproteome quantification accuracy of five-plex and conventional double or triple dimethyl isotope labeling strategies in our previous works (the proteins samples were mass differentially labeled and equally mixed). The log2(Ratio) distribution of peptides (a) and proteins (b) obtained by using five-plex (R (#2/#1), R (#3/#1), R (#4/#1) and R (#5/#1)) and double isotope dimethyl labeling (R(M/L)).[3] And the log2(Ratio) distribution of phosphopeptides (c) and phosphorylation sites (d) obtained by using five-plex (R (#2/#1), R(#3/#1), R (#4/#1) and R (#5/#1)) and triple isotope dimethyl labeling (R(M/L) and R(H/L)).[4]

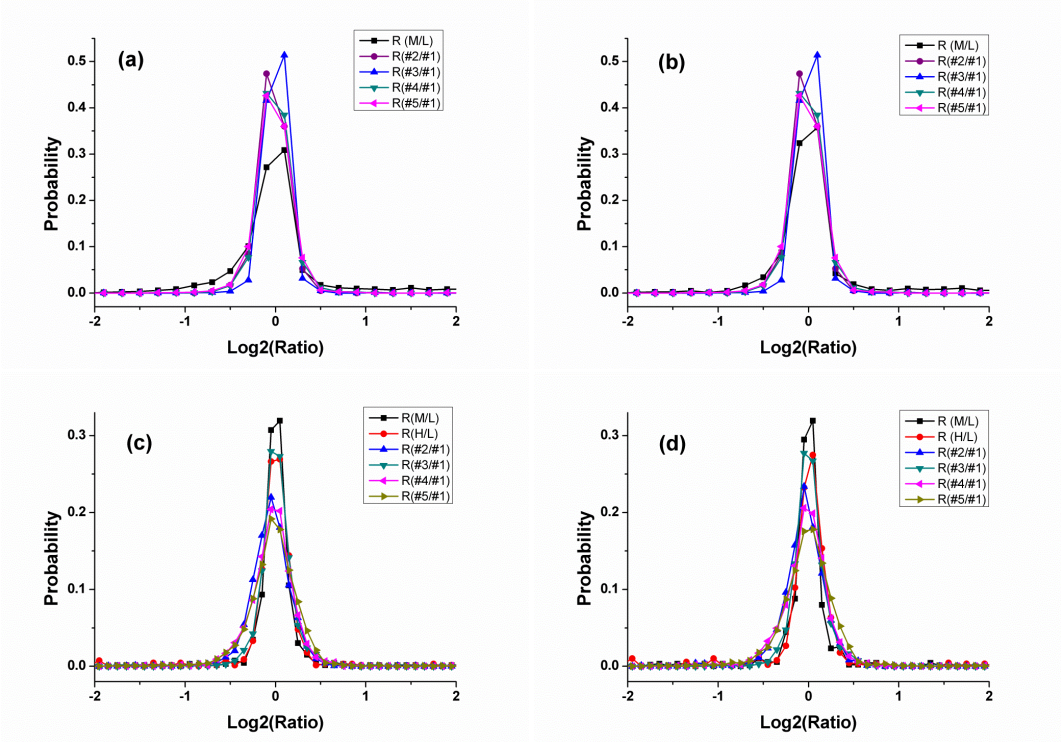

Figure S5. Function of “parameter group” in MaxQuant software. Two sets of parameters can be set in two groups, with simultaneous processing.

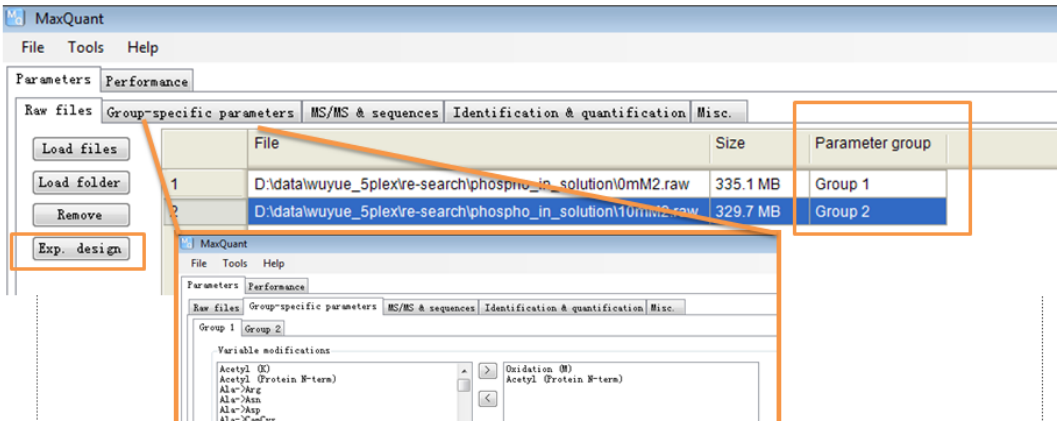

Table S1. The quantification deviations and numbers of identified/quantified peptides/proteins of the parallel experiments.

Experiments	CV% (quantific-ation deviation)	Replicate 1 (quantified num./identified num.)	Replicate 2 (quantified num./identified num.)
Proteome quantification 1:1:1:1:1 (peptides)	5.36	4530/5359	4059/5244
Proteome quantification 1:1:1:1:1 (proteins)	5.06	1190/1297	1117/1270
Proteome quantification 1:2:4:2:1 (peptides)	11.0	3876/5809	4014/6078
Proteome quantification 1:2:4:2:1 (proteins)	9.85	1370/1709	1442/1833
Phosphoproteome quantification (sites)	6.95	2047/3952	1976/2554

References

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