A six-plex proteome quantification strategy reveals the dynamics of protein turnover

Fangjun Wang*, Kai Cheng*, Xiaoluan Wei*, Hongqiang Qin, Rui Chen, Jing Liu & Hanfa Zou

CAS Key Lab of Separation Sciences for Analytical Chemistry, National Chromatographic Research and Analysis Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China.

MS1 full scan based quantification is one of the most popular approaches for large-scale proteome quantification. Typically only three different samples can be differentially labeled and quantified in a single experiment. Here we present a two stages stable isotope labeling strategy which allows six different protein samples (six-plex) to be reliably labeled and simultaneously quantified at MS1 level. Briefly in the first stage, isotope lysine-d0 (K0) and lysine-d4 (K4) are in vivo incorporated into different protein samples during cell culture. Then in the second stage, three of K0 and K4 labeled protein samples are digested by lysine C and in vitro labeled with light (2CH3), medium (2CD2H), and heavy (213CD3) dimethyl groups, respectively. We demonstrated that this six-plex isotope labeling strategy could successfully investigate the dynamics of protein turnover in a high throughput manner.

Results

Quant-ArMone software for the proteome quantification of MS1 results. Currently, no software is available for the quantitative analysis of six-plex datasets. Therefore, we developed a new software for quantitative proteomics called Quant-ArMone (Fig. 1 and Fig. S2). This proteome quantification software builds upon the previous proteomics data processing platform termed ArMone[11]. A detail description of the algorithms at the core of
Quant-ArMone is provided in the methods section. We firstly tested whether Quant-ArMone is comparable to other quantification software. In particular, Quant-ArMone was compared with MSQuant, which is widely used for proteome quantification analyses based on stable isotope labeling\(^1\). Briefly, 25 \(\mu\)g of tryptic digests of proteins extracted from mouse brain were labeled using light (2CH\(_3\)) and heavy (2\(^13\)CD\(_3\)) dimethylation labeling reagents, respectively. As well, 100 \(\mu\)g of the tryptic digests was labeled with medium (2CD\(_2\))H labeling reagent. Then, the three samples were combined and analyzed by strong cation exchange-reversed phase (SCX-RP) online two-dimensional (2D) LC-MS/MS system. Mascot was used to identify peptides and proteins present in the samples. Then, MSQuant and Quant-ArMone were both used to quantify the proteins identified by Mascot. Respectively, 930 and 916 distinct proteins were quantified using MSQuant and Quant-ArMone, and 893 (97\%) proteins were quantified by both software. The log\(_2\) ratio distribution profiles obtained by these two quantification software were both symmetrically distributed around the theoretically values (Fig. S3), and the quantification accuracy was comparable. Overall, Quant-ArMone provides similar quantitative results to the established MSQuant software in three-plex MS1 quantification. However, Quant-ArMone is the only software that can handle six-plex MS1 quantification currently.

**Performance of proteome quantification by the Six-plex isotope labeling strategy.** Two types of replicate analyses were performed to evaluate the performance of the six-plex quantification strategy. In the first type of replicate analysis, HeLa cells were fully labeled with medium containing K0 or K4 amino acids in two plates. 15 \(\mu\)g of identical protein samples extracted from K0 or K4 labeled Hela cells were digested and labeled with light, medium, or heavy dimethyl groups, respectively. Then, these six protein samples were equally mixed together and analyzed by SCX-RP 2D LC-MS/MS system. Protein identification was performed by Mascot whereas six-plex proteome quantification was performed by Quant-ArMone (Fig. 1 and Fig. S2). Finally, 5306 unique peptides were positively identified, among which 3786 (71\%) unique peptides were successfully quantified for all of the six protein samples, corresponding to 1239 distinct protein groups. Accurate quantification was achieved across all of the six isotopic forms with \(\sim\)96\% of peptides and proteins without significant changes (within the range of \([0.5, 2]\) relative ratio) (SI S2). Furthermore, log\(_2\) ratio distributions of peptides and proteins are both normally and symmetrically distribute around the theoretic values (Fig. 2a and b). Therefore, all of the six protein samples come from two different cell lines (K0 and K4 labeled) can be accurately quantified by using Quant-ArMone.

In the second type of replicate analysis, three HeLa cell cultures were separately labeled with medium containing K0 amino acids and three others labeled with K4 amino acids for a total of six plates. The remaining processing steps are the same as described above except the six protein samples are mixed with 1:2:3:6:1:2 ratio before analysis. After data processing, 4552 (73\%) unique peptides were successfully quantified from 6246 identified peptides for all of the six protein samples, corresponding to 1388 distinct protein groups. Accurate quantification results were also obtained across all of the six biological replicate samples with \(\sim\)93\% of the Log\(_2\) ratios of both peptides and proteins are within one fold change from theoretical ratios (Fig. 2c and d, SI S3). Therefore, high throughput and high accurate proteome quantification can be achieved in both technological and biological replicate analyses by the six-plex labeling strategy.

**High-throughput protein turnover analysis by the six-plex isotope labeling strategy.** The steady state levels of proteins are maintained through a balance between protein synthesis and degradation. Disturbances in this balance are often related to abnormal physiological and pathological status, such as cancer\(^1\). Investigating the dynamics of protein synthesis and degradation, as well as the protein turnover time can enhance our understanding of biological processes\(^4\). Pulsed SILAC labeling strategy has been extensively applied to study the dynamics of protein turnover in different biological systems\(^15\). Usually, more than five time points are necessary to measure protein turnover dynamics. Unfortunately, the current pulsed SILAC approach is time consuming as each time point requires a quantitative proteome analysis. In contrast, our six-plex isotope labeling strategy can be used to obtain protein turnover dynamics by doing only two experiments to monitor six time points (Fig. 3), which greatly simplifies the processes protein turnover analysis. Here, this approach is exemplified for the study of protein turnover dynamics in HeLa cells across six time points (Table S2). Briefly, six plates of HeLa cells cultures in K4 SILAC were transferred into K0 SILAC for different length of time as described in methods section.

---

**Figure 1 | Schematic diagram for processing the datasets of six-plex isotope labeling and protein turnover analyses.** All acquired raw files were converted to *.mgf files using DTASupercharge (v2.0a7), followed with database searching with Mascot Version 2.3.0 (Matrix Science). Then, Quant-ArMone was used to determine the position of the identified peptide in its belonged six-plex isotopic forms and the corresponding precursor masses and charge of six-plex in MS1 can be calculated and used for feature detection. Isotope Pattern Calculator was implemented to calculate the relative intensities of isotope peaks for deconvolution. In a MS1 scan, one label form with at least three isotope peaks will be seen as a significant signal, and peak hill over the retention time range present in over five consecutive scan was used for quantification. In protein turnover analyses, relative isotope abundance (RIA) \(K_d/(K_0 + K_4)\) for each time point was calculated. The six-plex XICs in one experiment represent three time points, and three RIA can be calculated.
Decreases over time in the signals for the K4 SILAC labeled peptides are related to protein degradation whereas increases in the K0 SILAC labeled peptides are related to new protein syntheses. Protein turnover dynamics were measured for 4878 unique peptides from 1365 distinct proteins observed in at least three time points. The 50% turnover time, defined as the time point at which the intensities of the K4 and K0 are equal, was calculated for each of the 1365 proteins. The average 50% turnover time for all the proteins is 20 hrs, and 91% of the proteins are within the range from 10 to 30 hrs. The 50% turnover time of 822 proteins in our study were also obtained in previous reports by using conventional double pulsed SILAC strategy, and the coefficient of variations (CVs) of 655 proteins (80%) are ≤0.3 in these two analyses. Therefore, the protein turnover results obtained by the six-plex isotope labeling strategy are consistent to conventional SILAC strategy. However, protein samples at three time points can be simultaneously quantified in one experiment in our six-plex strategy, in contrast to separate quantification experiment is needed for each time point in conventional strategy.

**Discussion**

We have developed a six-plex strategy for quantitative proteomics. As well, we have developed a software called Quant-ArMone for the quantitative analysis of the six-plex datasets. Quant-ArMone appears to be as performant as conventional algorithms such as MSQuant for the quantitative analysis of proteomic results. Furthermore, Quant-ArMone is the only software capable of handling the six-plex datasets. When six identical protein samples were used to investigate the performance of this six-plex strategy, ~96% of the peptides and proteins were accurately quantified among the six protein samples with relative ratios in the range from 0.5 to 2. The quantification accuracy of this six-plex labeling strategy is comparable to conventional dimethylation and SILAC strategies as described in our previous works. More complex biological replicate protein samples with a short dynamic range were further exemplified, and high quantification accuracy were still achieved.

The six-plex labeling strategy and Quant-ArMone were used to study protein turnover dynamics in HeLa cells over a 48 hours period. The bulk of the proteins had a 50% turnover time of ~20 hrs. Interestingly, the multicopy maintenance (MCM) proteins initially thought to be only involved in DNA replications are also involved in gene expression regulation, damage response and chromatin remodeling. Although their levels are not expected to change during the cell cycle, they are known to be polyubiquitinated and to be degraded by the proteasome. We obtained 50% turnover values of ~15 hrs for six members of the MCM protein family, indicating faster than average protein turnover, especially MCM7 exhibited a 50% turnover time ~10 hrs. This is likely a reflection of the need to rapidly degrade MCM proteins following their involvement in chromosomal processes. Furthermore, although some of the histones were reported as long-lived proteins in rat brain, the 50% turnover values of the 22 detected histone proteins are all closed to 21 hrs in Hela cells, which might be due to the relatively rapid turnover of cancer cell lines.
Figure 3  Schematic diagram for investigating the dynamics of protein synthesis and degradation by using six-plex isotope labeling strategy. As both SILAC and dimethylation isotope labeling methods were feasibly combined in the six-plex isotope labeling strategy, pulsed SILAC samples in three time points could be labeled with light, medium, and heavy dimethyl groups and compared in one experiment. And six times points could be feasibly monitored in two LC-MS/MS experiments.

In summary, a six-plex stable isotope labeling strategy was successfully developed and six different protein samples can be differentially labeled and quantified at MS1 level in a single experiment. Because all of the quantification information is obtained from a single MS1 scan, variations due to LC separation, ionization, and MS detection can be reduced. Finally, the six-plex isotope labeling strategy was successfully applied to investigate the dynamics of protein turnover in Hela cells. We believe that the six-plex labeling strategy can be applied to different types of high-throughput proteome quantification analyses.

Methods

Cell culture, harvest, and lysis. To investigate the dynamics of protein synthesis and degradation, six plates of Hela cells were cultured in lysine-depleted 1640 medium supplemented with heavy lysine-d4 (K4) and 10% dialyzed fetal bovine serum (FBS), and were incubated at 37 °C in a humidified atmosphere of 5% CO2. After six doublings, the “heavy” medium was removed and the cells were washed twice with sterile PBS. The cells were then incubated in normal 1640 medium containing light Lysine-d0 (K0) for 0, 3, 6, 12, 24, and 48 hrs (Fig. S4). Then the cells were washed with ice-cold PBS, pelleted by centrifugation. And the same amount (1 × 10^6 cells) of harvested Hela cells at each time point were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.2 + 8 M urea + protease inhibitor cocktail, pH 8.2) and sonicated three times for 30 (200 W) with at least 1 min on ice between two pulses. The cell lysate was centrifuged at 25,000 g, and the supernatant was kept for following preparation.

For six-plex isotope labeling strategy testing, Hela cells were cultured in lysine-depleted 1640 medium supplemented with either K0 or K4 in two or six plates. After six doublings, the “light” and “heavy” labeled cells were harvested and lysed, respectively, as described above.

Protein sample preparation and dimethylation isotope labeling. The proteins within the supernatant were precipitated by chloroform/methanol precipitation. After washing with methanol, the pellets were resuspended in 1 mL denaturing buffer containing 50 mM TEAB (pH 8.1) and 8 M urea and the protein concentration was determined by Bradford assay. The protein samples were reduced by DTT at 37 °C for 2 hrs and alkylated by iodoacetamide in dark at room temperature for 40 min. Then, the sample solution was diluted to 1 M urea by using 5 M TEAB (pH 8.1), and lysine C was added with weight ratio of enzyme to protein at 1/50 and incubated at 37 °C overnight. 100 µg of the protein digest was loaded onto a 100 mg homemade C18 solid phase extraction (SPE) column and labeled by 5 mL of light (0.2% CH2O and 30 mM NaH2CN), medium (0.2% CD2O and 30 mM NaH2CN), or heavy (0.2% 13CD2O and 30 mM NaBD3CN) dimethylation reagents. After a wash with 1 mL 0.1% formic acid (FA) aqueous solution, the isotopic labeled peptides were eluted from the C18 SPE column using 1 mL 80% ACN. Then, the comparative samples were mixed together with specific ratio and lyophilized (Fig. S1).

Online two-dimensional LC-MS/MS analysis. The lyophilized sample was redissolved into 0.1% FA solution, and loaded onto a 200 μm i.d. × 7 cm strong cation exchanged (SCX) monolithic column for online multidimensional analysis24. 0.1% formic acid in water (solvent A) and 0.1% formic acid in ACN (solvent B) were used for RP gradient separation, and the RP binary gradient was set as follows: from 1–10% solvent B in 5 min, from 10–35% solvent B 15 min, from 35–90% solvent B 5 min, after flush with 90% solvent B for 8 min the system was equilibrated by solvent A for 12 min. Then, a series of salt steps elution were applied to fractionate the SCX monolith trapped peptides to the separation column. The NH4Ac (pH 2.7) salt steps were as follows: 50, 100, 150, 200, 250, 300, 350, 400, 500, and 1000 mM (for protein synthesis and degradation analysis, two more fractions 450 and 700 mM were added). Each salt step lasted 8 min (the final one 10 min) with a flow rate 300 μL/min, followed with a RP binary gradient separation and MS detection as described above. The LTQ-Orbitrap XL (Thermo, San Jose, CA) was used for MS detection. The temperature of the ion transfer capillary was 200 °C, the electrospray voltage was +1.8 kV, and the normalized collision energy was 35%. The MS full scan was acquired from m/z 400 to 2000 in Orbitrap with a resolution of 60,000 in centroid mode, and the MS/MS scan was acquired in LTQ linear ion trap. All MS and MS/MS spectra were acquired in the data dependent analysis (DDA) mode, in which the 6 most intense ions with MS scan were selected for MS/MS scan by collision induced dissociation (CID). Preview mode was enabled to exclude the precursors with unknown gain control (AGC) and maximum ion injection time were set to 1 × 10^4 and 500 ms, and 1 × 10^4 and 50 ms for MS and MS/ MS scan, respectively. The dynamic exclusion function was: repeat count 1, repeat duration 60 s, and exclusion duration 180 s.

Data processing. All acquired raw files were converted to .mzXML files using DTA Supercharge (v2.04). Then the .mzXML files were searched against a recently released NCBI human protein database (10 March 2013, 35922 entries) downloaded from ftp://ftp.ncbi.nih.gov/refseq/H_sapiens/mRNA_Prot/human.protein.faa.gz, using Mascot Version 2.3.0 (Matrix Science) in decoy searching mode to evaluate the search results23. The stand alone tool of Mascot Percolator (version 2.00)24 and Percolator (version 2.04) were downloaded from http://www.sanger.ac.uk/resources/software/mascotpercolator/. Automatic decoy database search in mascot was performed, so the generated result file (.dat) contains the entire target and decoy matches. The program cli.MascotPercolator -rankdelta 1 -target filepath -decoy filepath -out filepath'', where the target, decoy, and output file paths were substituted by the correct path. The generated result file (.dat) contains a log file, a target peptide list and a decoy peptide list, and for each peptide the q-value was calculated. The program was executed with “java -Xmx1024 m -cp MascotPercolator.jar cli.MascotPercolator -rankdelta 1 -target filepath -decoy filepath” -out filepath”, where the “-target” and “-decoy” shared the same path of the .dat file. Result files were generated contain a log file, a target peptide list and a decoy peptide list, and for each item in the peptide lists a q-value was reported. In this work we used the identified peptides with q-value < 0.05 were reserved for quantitative analysis.

Since this is a novel six-plex isotope labeling strategy, there is no appropriate software available at present. Therefore, we developed a new quantitative analysis platform termed Quant-AR-Mone to fulfill this requirement (Fig. 1 and Fig. S2). In addition to the basic function of isotope labeling based quantification, protein turn-over analysis related features were also contained. This software is free for academic use and the latest version can be downloaded at http://www.bioanalysis.dipac.cn/proteomics/software/Quant-AR-Mone.html.

Quant-AR-Mone mainly deals with MS data obtained by high resolution mass spectrometer, such as LTQ Orbitrap. The supported spectra data formats were .mzXML and .mzData. In theory, peptide contains lysine residues may display a
six-plex XIC (extracted ion chromatogram) in our labeling strategy. According to the isotope labels in an identified peptide, we can determine the position of this peptide in its belonged six-plex. Then the corresponding precursor masses and charge of six-plex can be calculated and used for feature detection. The molecular mass difference between the nearest isotopic forms is 4 Da for peptides with one lysine amino acid, which means that regardless of the charge state of the precursor ions the 5th isotope peak of a isotopic form will be overlapped with the monoisotopic peak of the next near isotopic cluster. Therefore for each isotopic label form the first four peaks were used for calculated the intensity. The influence of overlapped isotope clusters was also considered. A software tool named Isotope Pattern Calculator (IPC) download from http://omics.pnl.gov/software/IPC.php was used to calculate the relative intensities of isotope peaks and the overlapped portion were deducted25. In a MS1 scan one label of this peak was used to calculate the peptide ratio. Typically chromatographic shifts of isotope labels will not be large, but different peptide ions overlapped in m/z in adjacent retention time will increase the deviation. Therefore if the distance between two centroids of peaks is greater than the peak widths, this quantification is probably not precise and will be discarded. From our limited experience this method shows favorable simplicity, effectiveness and robustness. Normalization of ratios can be selected to correct the errors of loading amount of protein samples. Results of multiple salt gradients in a same experiment will be combined. Protein ratio can be calculated (Figure 1). For the dynamic analysis of protein turnover, four integrate quantitative results were obtained (0 h, 3 h, 6 h; 0 h, 3 h, 48 h; 6 h 12 h, 24 h; 12 h, 24 h, 48 h, Table S2), each of which contained information of three time point, and general every time point had a technology replication. The RIA of protein was calculated as the median of all the peptide RIA. Only protein detected in three or more time point were used for further analyses. In theory, the RIA at each time point will fit to an exponential function26:

\[
\text{RIA}_t = \text{RIA}_0 + (\text{RIA}_0 - \text{RIA}_\infty) \exp(-k_{\text{loss}} \times t)
\]

The fitting curve for every protein was calculated to match to this function. The ratio point with max offset was discarded and recalculated. Then fittings with correlation coefficient less than 0.8 were discarded. Finally the corresponding 50% turnover time and \(k_{\text{loss}}\) were obtained (Fig. 1 and SI S4).

The fitting curves for all detected proteins (SI S6) can be obtained at http://www.bioanalysis.dicp.ac.cn/proteomics/software/Quant-ArMone.html.

1. Nilsson, T. et al. Mass spectrometry in high-throughput proteomics: ready for the big time. Nat. Methods 7, 681–685 (2010).
2. Ning, Z., Zhou, H., Wang, F., Abu-Farha, M. & Figgeys, D. Analytical aspects of proteomics: 2009–2010. Anal. Chem. 83, 4407–4426 (2011).
3. Ong, S.-E. & Mann, M. Mass spectrometry-based proteomics turns quantitative. Nat. Chem. Biol. 1, 252–262 (2005).
4. Ong, S. E. et al. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol. Cell. Proteomics 1, 376–386 (2002).
5. Ross, P. L. et al. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol. Cell. Proteomics 3, 1154–1169 (2004).
6. Boersema, P. J., Rajmakers, R., Lemeer, S., Mohammed, S. & Heck, A. J. R. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. Nat. Protoc. 4, 484–494 (2009).
7. Werner, T. et al. High-Resolution Enabled TMT 8-plexing. Anal. Chem. 84, 7188–7194 (2012).
8. Dephoure, N. & Gygi, S. P. Hyperplexing: a method for higher-order multiplexed quantitative proteomics provides a map of the dynamic response to rapamycin in yeast. Sci. Signal. 5, r2 (2012).
9. Wenger, C. D. et al. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. Nat. Methods 8, 933–935 (2011).
10. Ting, L., Rad, R., Gygi, S. P. & Haas, W. MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. Nat. Methods 8, 937–940 (2011).
11. Doherty, M. K. & Whitfield, P. D. Proteomics moves from expression to turnover: update and future perspective. Expert. Rev. Proteomics 8, 325–334 (2011).
12. Edén, E. et al. Proteome half-life dynamics in living human cells. Science 331, 764–768 (2011).
13. Ahmed, Y., Boisvert, F. M., Lundberg, E., Uhlen, M. & Lamond, A. I. Systematic analysis of protein pools, isoforms, and modifications affecting turnover and subcellular localization. Mol. Cell. Proteomics 11, M111 013680 (2012).
14. Wang, F., Yue, M., Cheng, K. & Zou, H. ArMone: a software suite specially designed for processing and analysis of phosphoproteome data. J. Proteome Res. 9, 2743–2751 (2010).
15. Mortensen, P. et al. MSQuant, an open source platform for mass spectrometry-based quantitative proteomics. J. Proteome Res. 9, 393–403 (2010).
16. Doherty, M. K. & Whitfield, P. D. Proteomics moves from expression to turnover: update and future perspective. Expert. Rev. Proteomics 8, 325–334 (2011).
17. Ahmad, Y., Boisvert, F. M., Lundberg, E., Uhlen, M. & Lamond, A. I. Systematic analysis of protein pools, isoforms, and modifications affecting turnover and subcellular localization. Mol. Cell. Proteomics 11, M111 013680 (2012).
18. Wang, F. et al. A Fully Automated System with Online Sample Loading, Isotope Dimethyl Labeling and Multidimensional Separation for High-Throughput Quantitative Proteome Analysis. Anal. Chem. 82, 3007–3015 (2010).
19. Wang, F. et al. Combination of online enzyme digestion with stable isotope labeling for high-throughput quantitative proteome analysis. Proteomics 12, 3129–3137 (2012).
20. Meierhofer, D., Wang, X., Huang, L. & Kaiser, P. Quantitative analysis of global ubiquitination in HeLa cells by mass spectrometry. J. Proteome Res. 7, 4566–4576 (2008).
21. Cambridge, S. B. et al. Systems-wide proteomic analysis in mammalian cells reveals conserved, functional protein turnover. J. Proteome Res. 10, 5275–5284 (2011).
22. Wang, F., Dong, J., Jiang, X., Ye, M. & Zou, H. Capillary trap column with strong cation-exchange monolith for automated shotgun proteome analysis. Anal. Chem. 79, 6599–6606 (2007).
23. Kall, L., Canterbury, J. D., Weston, J., Noble, W. S. & MacCoss, M. J. Semi-supervised learning for peptide identification from shotgun proteomics datasets. Nat. Methods 4, 923–925 (2007).
24. B rooft, M., Yu, L., Hubbard, T. & Choudhary, J. Accurate and sensitive peptide identification with Mascot Percolator. J. Proteome Res. 8, 3176–3181 (2009).
25. Cappadona, S. et al. Deconvolution of overlapping isotopic clusters improves quantification of stable isotope-labeled peptides. J. Proteomics 74, 2204–2209 (2011).
26. Pratt, J. M. et al. Dynamics of protein turnover, a missing dimension in proteomics. Mol. Cell. Proteomics 1, 579–591 (2002).