

HOTMAQ: a multiplexed absolute quantification method for targeted proteomics

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Abstract

Mass spectrometry-based targeted proteomics enables absolute quantification of multiple proteins in a single assay, but the sample throughput of this technique is rather low. To improve this, we have developed a hybrid offset-triggered multiplex absolute quantification (HOTMAQ) strategy that combines cost-effective mass difference and isobaric tags to enable simultaneous construction of an internal standard curve, real-time identification of peptides, and mass offset-triggered quantification of target proteins in up-to twelve unfractionated samples via a single liquid chromatography-mass spectrometry (LC-MS) run with excellent sensitivity and accuracy.

Selected reaction monitoring (SRM) or parallel reaction monitoring (PRM) coupled with stable isotope-mass spectrometry (MS), in which peptide standards are spiked into samples in known amounts to determine absolute abundances of target peptides via signal intensity ratios, presents as a reference method for targeted proteomics¹. To improve acquisition efficiency of SRM/PRM, spiked-in isotopic internal standards have been used to prompt real-time measurement of analytes and on-the-fly adjustment of acquisition parameters². Similarly, a method termed TOMAHAQ has utilized synthetic peptides to trigger quantification of multiplexed targets based on a known mass offset³. However, the dependence on single-point calibration for absolute quantification in TOMAHAQ may provide inaccurate estimates when the amounts of target peptides span a large dynamic range, particularly in preclinical and clinical biofluids. The routine application of TOMAHAQ would also be hindered by high cost of isotope labels used in this method.

Stable isotope labels can be categorized into two types: (i) mass difference labels that introduce mass shifts of several daltons onto precursor ions, permitting their direct relative and absolute quantification in full MS (MS1) spectra⁴⁻⁶; and (ii) isobaric labels that impart a single nominal mass shift onto precursors in MS1 spectra, but produce discrete reporter ions for relative quantification of peptides in tandem (MS/MS) spectra⁷⁻⁹. We have developed our own cost-effective amine-reactive *N,N*-dimethyl leucine (DiLeu) tags that offer the flexibility to employ either approach for multiplexed quantification of many samples in a single LC-MS/MS experiment. Isotopic DiLeu (iDiLeu) tags enable 5-plex mass difference quantification through the use of 3 Da mass differences between tags¹⁰, while DiLeu isobaric tags enable up to 12-plex quantification via reporter ions using high-resolution MS/MS acquisition¹¹. Both variants share the same chemical structure, differing only in their composition and number of heavy stable isotopes (¹³C, ²H, ¹⁵N, and ¹⁸O). Herein, we aim to develop a novel hybrid offset-triggered multiplex absolute

quantification (HOTMAQ) strategy to combine isobaric tags (DiLeu) and mass difference tags (iDiLeu) to enable accurate absolute quantification of targeted peptides across multiple complex samples.

An overview of this quantification method is outlined in **Figure 1**. Synthetic peptides are labeled with iDiLeu tags (d0, d6, d9, d12) to impart mass additions of 141.1, 147.1, 150.2, 153.2 Da to peptides, respectively. Individual peptide samples of interest are concurrently labeled with 12-plex isobaric DiLeu tags, introducing nominal mass addition of 145.1 Da per tag, as a substitution for the d3 iDiLeu tag (mass addition of 144.1 Da). The 4-plex iDiLeu-labeled synthetic peptides are diluted in a series of concentrations and spiked into 12-plex DiLeu-labeled samples to construct standard curves for simultaneous absolute quantification in a single LC-MS run. Because iDiLeu and DiLeu tags are identical in chemical structure, the multiplexed synthetic peptides and target peptides have the same chromatographic elution profiles, while their differences in stable isotope configurations make them distinct in mass from one another by 4, 6, 9, and 12 Da, enabling determination of total amounts of multiplexed isobaric DiLeu-labeled target peptides via the iDiLeu standard curve in the MS1 scan. In addition to generating iDiLeu standard curves, the d0-labeled synthetic peptides also function as real-time monitors by matching MS2 spectrum to a product mass inclusion list, to trigger acquisition of 12-plex DiLeu labeled target peptides based on their known offset mass of 4.01 Da. To maximize effective time for measuring multiplexed target peptides in a scheduled time window, MS2 acquisition alternates between two modes: a fast low-resolution mode for d0-labeled synthetic peptides and a high-resolution mode for 12-plex DiLeu labeled target peptides. While quantification accuracy and precision of reporter ion-based methods suffer due to ratio distortion from co-isolated and co-fragmented near-isobaric peptides, pre-selection of fragment ions for targeted synchronous precursor selection (SPS)-MS3

analysis can mitigate this effect and enable accurate quantification³. The relative abundance of each 12-plex DiLeu-labeled peptide is determined by SPS-MS3 acquisition, and the absolute amounts of target peptides in each sample are quantified by integrating the total amount obtained using the standard curve.

Measuring 12-plex DiLeu-labeled peptides at an appropriate point along the iDiLeu standard curve is a prerequisite for accurate quantitative measurements by the HOTMAQ method, so we firstly evaluated its quantitative performance paired with iDiLeu d0- and 12-plex DiLeu-labeled synthetic peptide standards in a background of iDiLeu d0- and 12-plex DiLeu-labeled yeast tryptic peptides, which were combined at unity ratios in all proof-of principle experiments. Three synthetic peptides were labeled with iDiLeu d0 and 12-plex DiLeu, spiked at known concentrations into the labeled yeast background. Because trigger peptides in low abundance are not able to adequately initiate acquisition of target peptide precursors, and those present in extremely high abundance could reduce quantitative accuracy through greater isotopic interference at any MS level, the optimal ratio between trigger peptides and target peptides were determined initially by testing samples at 10:1, 20:1, 50:1, 100:1 mixing ratios, maintaining the unity ratios for 12-plex DiLeu-labeled target peptides. The ratio of 20:1 was determined to be optimal with a relative error within 10% and coefficient of variation (CV) below 7% (**Supplementary Fig. 1**).

To demonstrate high sensitivity and accuracy of HOTMAQ, 12-plex DiLeu-labeled peptide standards were prepared by combining at ratios of 1:1:1:1:1:1:1:1:1:1 and 1:1:2:2:5:10:10:5:2:2:1:1 (115a – 118d) with 100 amol on column. As an example, a d0 iDiLeu- and 12-plex DiLeu-labeled peptide (THLGEALAPLSK) observed as two distinct m/z clusters with a 2.67 Da mass difference (**Fig. 2a**), had near-identical retention time at 62.2 min. Upon real-time identification of the d0-labeled peptide, acquisition of the 12-plex DiLeu-labeled peptide follows

(**Fig. 2b**). A target product mass inclusion list was constructed for targeted SPS-MS3 analysis as interference ions may be more abundant than target product ions, leading to ratio distortion in standard SPS-MS3. After isotopic interference correction, the 12-plex DiLeu ratios (in triplicate) were plotted against each other (**Supplementary Fig. 2**). Across all channels, the median ratios were within 10% and 18% of the expected values with average CVs of 6.3% and 13.1% for 1:1 and 10:1 ratios, respectively. We also compared the quantitative accuracy of this method with conventional PRM and standard data-dependent SPS-MS3 at a ratio of 1:1:2:2:5:10:10:5:2:2:1:1 spanning from 100 amol to 1 fmol in the labeled yeast background (**Fig. 2c**). The CVs for all three quantification methods were below 20%. As shown in radar plots, the relative errors at ratios of 2, 5, and 10 were within 10% for HOTMAQ method, but were up to 22% for the other two quantification methods. The accuracy of PRM-MS2 method suffered due to interference of near-isobaric yeast contaminant ions that were isolated and fragmented together with target ions. SPS-MS3 improved quantitative precision, but remaining fragment ion interference caused an underestimation of the mixing ratios. Next, we evaluated the absolute quantification accuracy of the HOTMAQ method using 4-plex iDiLeu- and 12-plex DiLeu-labeled peptide standards. 12-plex DiLeu-labeled peptides were combined at a ratio of 1:1:2:2:5:10:10:5:2:2:1:1 with the lowest amount at 100 amol in the labeled yeast background (**Fig. 2d**). Each of the three peptide standards were quantified with excellent linearity ($R^2 = 0.999$). By incorporating 12-plex DiLeu ratios measured by targeted SPS-MS3, the final amounts for each channel were 0.08, 0.09, 0.21, 0.2, 0.38, 0.88, 0.95, 0.38, 0.18, 0.20, 0.08, and 0.09 fmol (115a-118d) with an average relative error of 12.3% and CV of 8.3%. These results illustrate that the overall accuracy and precision for absolute quantification by HOTMAQ is excellent in a multiplexed experiment.

We further benchmarked the HOTMAQ method by analyzing cerebrospinal fluid (CSF) collected from 11 individuals at preclinical stage of Alzheimer's disease (AD) and 11 healthy controls enrolled in Wisconsin Alzheimer's Disease Research Center (**Supplementary Fig. 3**). The preclinical phase of AD featured as starting to accumulate neuropathological abnormalities but remaining normal cognition. This asymptomatic phase begins many years before clinical dementia, which would provide critical opportunity for early diagnosis via reliable biomarkers^{12,13}. We applied HOTMAQ method to verify three candidate protein biomarkers, transthyretin (TTR), neurosecretory protein VGF (VGF), and apolipoprotein E (apoE) across the 22 CSF samples in triplicates¹⁴. All the samples were analyzed in less than three hours, owing to the 12-fold gain in analytical throughput by HOTMAQ. The linear dynamic range of each target protein was optimized to include the endogenous abundance in the standard curve (**Fig. 2e, Supplementary Fig. 4a-b**). We observed that each of the three targeted proteins exhibited down-regulation in preclinical AD stage, which is consistent to the results previously reported for AD dementia¹⁵⁻¹⁷, but there is no clear consensus that apoE is negatively associated with AD dementia¹⁸ (**Fig. 2f, Supplementary Fig. 4c-d**). Analysis of student's t-test yielded *p*-value of 0.03 for apoE, indicating significant difference of apoE amount between healthy controls and preclinical AD patients (decreased 17.4%), but not for TTR (*p* = 0.83), and VGF (*p* = 0.26). The apoE-encoding gene *APOE* has three major polymorphic alleles, ϵ 2, ϵ 3, and ϵ 4, all of which differently modulate amyloid beta aggregation and clearance in AD pathogenesis. The *APOE* ϵ 4 allele is strongly associated with an earlier age of AD onset and increased risk of late-onset AD¹⁹. ϵ 4 carriers exhibited lower amount of apoE than ϵ 4 non-carriers in healthy subjects (**Fig. 2g**). Given sex differences in the risk of AD²⁰, we observed that the mean apoE level was lower in female healthy

controls compared with male healthy controls. (**Fig. 2h**). If replicated, the reason for this potential difference should be examined in future studies.

In summary, the novel HOTMAQ strategy increases analytical throughput of absolute quantification up to 12-fold with high accuracy. The unparalleled advantage of HOTMAQ is that a single injection can achieve three aims: 1) An internal standard curve can be constructed specifically for each target peptide through mass difference labeling; 2) Real-time identification of trigger peptide prompts on-the-fly detection and quantification of target peptide in a scheduled time window; 3) Targeted SPS-MS3 analysis enables accurate determination of 12-plex DiLeu reporter ion abundances. The HOTMAQ strategy ideally bridges the gap between the discovery and verification phases for candidate biomarkers from large cohorts of clinical specimens. Using the HOTMAQ strategy to firstly investigate preclinical AD, we observed significant down-regulation of apoE in agreement with previous studies of AD dementia, and these results may provide support to the development of early-stage diagnostic tools and therapeutic interventions to delay the onset of dementia. The utility of this new strategy goes beyond AD CSF biomarker verification; its greatly enhanced throughput and quantitative performance, paired with sample flexibility, should be useful in targeted peptidomics, proteomics, and phosphoproteomics in general.

METHODS

Methods and any associated references are available in supplementary information.

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AUTHOR CONTRIBUTIONS

X.Z., F.M., and L.L. designed the research. X.Z., Q.Y., and D.F. performed experiments. L.L. wrote software program for data analysis. X.Z., L.L., and Z.C. analyzed data. H.Z., C.C., and O.O. collected and provided the CSF specimens. X.Z., D.F., and L.L. prepared the manuscript and all authors provided revisions.

COMPETING FINANCIAL INTEREST

The authors declare no competing financial interests.

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Figures

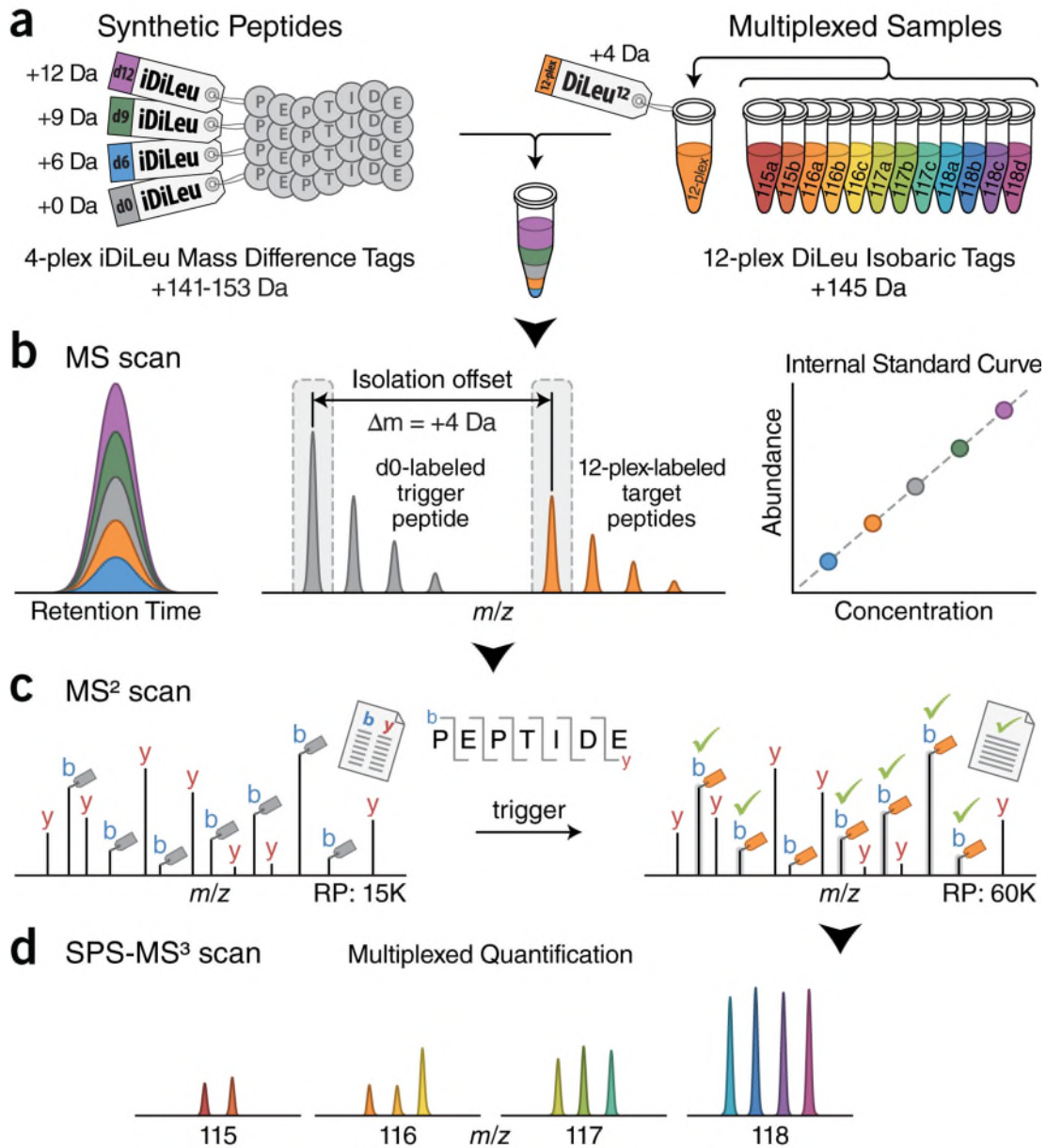


Figure 1. Schematic illustration for HOTMAQ method. (a) Synthetic peptides are labeled with 4-plex iDiLeu at different concentrations and spiked into 12-plex DiLeu-labeled analytes. (b) Labeled peptides are detected with identical chromatographic elution profiles as five precursor ion clusters. The 4-plex iDiLeu labeled-synthetic peptides are used to generate internal calibration

curves to quantify the total amount of multiplexed target peptides. iDiLeu d0-labeled synthetic trigger peptides and multiplexed DiLeu target peptides are separated in MS1 spectra by a mass offset of 4.01 Da, which enables synthetic trigger peptides to initiate quantitative analysis of target peptides regardless of target peptide precursor abundances. (c) Real-time MS2 analysis of d0-labeled synthetic peptides by matching MS2 spectrum to a product mass inclusion list, to unambiguously triggers fragmentation of 12-plex DiLeu-labeled target peptides in a predefined monitoring window. Acquisition parameters are alternating between low-resolution mode for monitoring d0-labeled trigger peptides and high-resolution mode for quantifying 12-plex DiLeu-labeled target peptides. Fragment ions of 12-plex DiLeu-labeled target peptides are selected for synchronous precursor selection (SPS)-MS3 analysis. (d) The relative abundance of each 12-plex DiLeu-labeled peptide is accurately determined by targeted SPS-MS3 acquisition at a resolving power of 60K (at m/z 200). The absolute amounts of target peptides are quantified by integrating the total amount obtained using the standard curve.

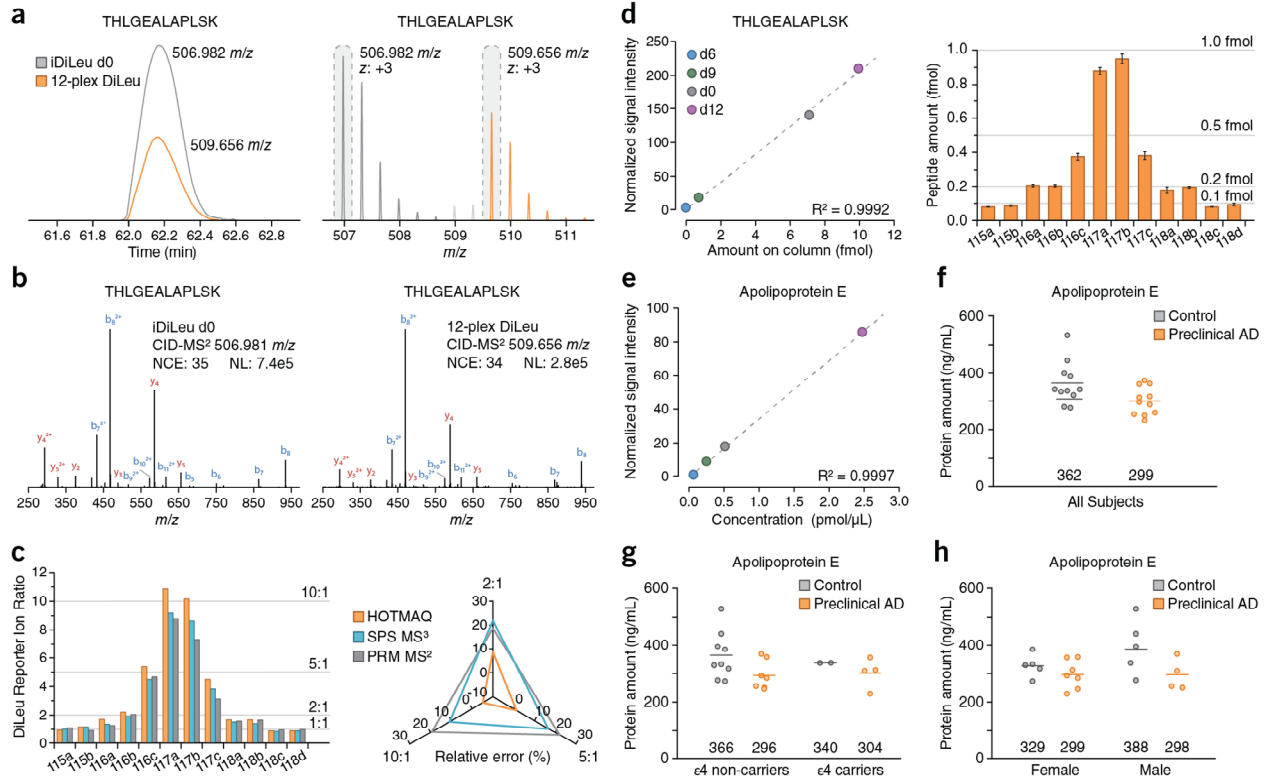


Figure 2. HOTMAQ feasibility and quantitative results. (a) iDiLeu d0 and 12-plex DiLeu-labeled peptides (THLGEALAPLSK) co-elute with an identical retention time of 62.2 min. The two precursor ion clusters (z : +3) are separated by 2.67 m/z , as the peptide carry two tags at both N-terminal and lysine side chain. (b) An example of an iDiLeu d0-labeled peptide successfully triggering fragmentation of 12-plex DiLeu-labeled targeted peptides. (c) Comparison of quantification accuracy for PRM, standard SPS, and HOTMAQ. 12-plex DiLeu-labeled peptide standards were combined at ratios of 1:1:2:2:5:10:10:5:2:2:1:1 (115a – 118d). In radar plot of relative errors at the average ratio of 2, 5 and 10, the greater the distance of each tested condition from relative error of 0, the worse the quantification accuracy of this quantification method. (d) Calibration curve constructed for peptide (THLGEALAPLSK) with exceptional linearity of $R^2=0.9992$. The HOTMAQ method demonstrated exceptional absolute quantification accuracy at ratios of 1:1:2:2:5:10:10:5:2:2:1:1 (115a – 118d) with the minimal loading amount at 100 amol.

(e) Twenty-two CSF samples from preclinical AD and control subjects were labeled with 12-plex DiLeu. The spiked-in 4-plex iDiLeu-labeled synthetic peptides displayed excellent linearity for ApoE peptides. (f) Compared to healthy controls (grey), apoE was observed to be down-regulated in preclinical AD patients (orange). (g) ApoE amount for healthy (grey) and preclinical (orange) subjects were measured in *APOE* ϵ 4 non-carriers and carriers. (h) ApoE amount for healthy (grey) and preclinical (orange) subjects were measured in female and male categories.