Published in final edited form as:

Anal Chim Acta. 2017 May 22; 968: 40–49. doi:10.1016/j.aca.2017.03.003.

Improving Data Quality and Preserving HCD-Generated Reporter Ions with EThcD for Isobaric Tag-based Quantitative Proteomics and Proteome-wide PTM Studies

Qing Yu¹, Xudong Shi², Yu Feng¹, K. Craig Kent², and Lingjun Li^{1,3,*}

¹School of Pharmacy, University of Wisconsin, Madison, WI 53705, USA

²Department of Surgery, School of Medicine and Public Health, University of Wisconsin, Madison, WI 53705, USA

³Department of Chemistry, University of Wisconsin, Madison, WI 53706, USA

Abstract

Mass spectrometry (MS)-based isobaric labeling has undergone rapid development in recent years due to its capability for high throughput quantitation. Apart from its originally designed use with collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD), isobaric tagging technique could also work with electron transfer dissociation (ETD), which provides complementarity to CID and is preferred in sequencing peptides with post-translational modifications (PTMs). However, ETD suffers from long reaction time, reduced duty cycle and bias against peptides with lower charge states. In addition, common fragmentation mechanism in ETD results in altered reporter ion production, decreased multiplexing capability, and even loss of quantitation capability for some of the isobaric tags, including custom-designed dimethyl leucine (DiLeu) tags. Here, we demonstrate a novel electron-transfer/higher-energy collision dissociation (EThcD) approach that preserves original reporter ion channels, mitigates bias against lower charge states, improves sensitivity, and significantly improves data quality for quantitative proteomics and proteome-wide PTM studies. Systematic optimization was performed to achieve a balance between data quality and sensitivity. We provide direct comparison of EThcD with ETD and HCD for DiLeu- and TMT-labeled HEK cell lysate and IMAC enriched phosphopeptides. Results demonstrate improved data quality and phosphorylation localization accuracy while preserving sufficient reporter ion production. Biological studies were performed to investigate phosphorylation changes in a mouse vascular smooth muscle cell line treated with four different conditions. Overall, EThcD exhibits superior performance compared to conventional ETD and offers distinct advantages compared to HCD in isobaric labeling based quantitative proteomics and quantitative PTM studies.

* Address reprint requests to: Dr. Lingjun Li, School of Pharmacy & Department of Chemistry, University of Wisconsin, 777 Highland Ave, Madison, WI 53705, USA, lingjun.li@wisc.edu. Phone: +1-(608)265-8491, Fax: +1-(608)262-5345.

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Supplemental Information. Supplemental information associated with this article can be found in the online version.

Graphical abstract

Keywords

ETD; isobaric tag; DiLeu; TMT; quantitative proteomics; post-translational modification

1. Introduction

The advancement of modern MS instrumentation has enabled nearly complete characterization of the proteome from a model organism.¹ Various fragmentation techniques exist, including collision-induced dissociation (CID), higher-energy collisional dissociation (HCD) and electron-transfer dissociation (ETD). In the CID/HCD process, uniform backbone fragmentation heavily relies on one or several mobile protons.^{2–4} However, the presence of internal basic residues (i.e., Arg, Lys or His) can sequester protons, eliminate random protonation and bias cleavage sites.^{5,6} That inherent drawback makes CID preferentially work well with smaller peptide pieces without internal basic residues, such as tryptic peptides. Additionally, peptides possessing post-translational modifications (PTMs) can often undergo lower energy fragmentation pathways to cleave the labile PTM first, instead of peptide amide bond. Peptides containing phosphorylation are examples of the latter case, where phosphate competes with backbone amide for protonation and CID often readily promotes elimination of phosphoric acid without breaking peptide backbones. The resulting MS² are scarcely informative.^{7,8} Elevated collision energies used in HCD, or beam-type CID, shortens activation time, allows Orbitrap detection, greatly mitigates such problem and produces high-quality spectra.⁹ To further overcome such obstacles in peptide fragmentation and PTM studies, ETD has been demonstrated as powerful alternative fragmentation scheme to CID/HCD, as it occurs in a sequence-independent manner and is able to preserve labile PTMs like phosphorylation.^{8,10,11} Despite several advantages, ETD occurs on the millisecond timescale, significantly impairs instrument duty cycle and produces dominant charge-reduced precursor ions without further fragmentation that lowers database search scores, whereas CID/HCD only takes microseconds to complete with predominant fragment ions and minimum residual precursor ions after MS². Moreover, CID/HCD is favorable for doubly charged peptides that constitute the majority of peptide contents of enzymatically digested proteins. Based on these observations, various studies employed a combined use of CID/HCD and ETD to take advantage of their complementarity.^{12,13} However, not a single MS² mode can be universally applied to all peptides and pinpointing phosphosite remains challenging, especially with multiple potential phosphosites in a peptide sequence.

Recently, Frese et al. developed a dual fragmentation technique, coined as electron-transfer/higher-energy collision dissociation (EThcD).¹⁴ This method submits ETD-generated ion species to an additional HCD activation and generates both b/y and c/z fragment ion series yielding informative MS² fragmentation spectra. This hybrid fragmentation method enables phosphosite localization through the production of site-specific fragment ions and is in general beneficial for all qualitative proteomics studies.^{15–18}

Besides qualitative characterization, MS-based relative quantitation has become a powerful tool enabling routine comparative proteomics studies. One of the most popular methods for relative quantitation through MS is isobaric labeling of proteins prior to analysis which permits the simultaneous comparison of multiple samples. All currently available isobaric tags, including TMT, iTRAQ and DiLeu, are originally designed for use with CID or HCD.^{19–21} Under these fragmentation conditions, reporter ions are preferentially released to generate quantitative information.^{20–22} As expected, distinct fragmentation mechanism of ETD also alters reporter ion production of isobaric tag-labeled peptides and results in loss of quantitation channels (e.g., TMT and iTRAQ). Consequently, ETD requires inclusion of other isotopic variants such that HCD and ETD quantitation cannot be performed with the same batch of prepared sample.^{23–25} Furthermore, the custom-made DiLeu tags lose quantitation capability due to the fact that ETD-generated reporters are below 50 m/z detection limit of an Orbitrap mass analyzer.

Here we demonstrate a fine-tuned EThcD approach for universal isobaric tag-based quantitative studies that can preserve the original CID/HCD type of reporter ions, which is extremely useful for DiLeu-enabled quantitation. Moreover, this hybrid fragmentation technique produces abundant b/y and c/z ions in the resulting tandem MS spectra with significantly improved data quality for confident identification, benefiting PTM characterization. We present systematic comparisons of ETD, HCD and EThcD with both DiLeu- and TMT-labeled peptides and demonstrate that our approach is superior to conventional ETD in acquisition speed, identification rate and phosphosite localization. Additionally, our evaluation suggests that such method provides comparable (or even better) performance to HCD in global quantitative proteomics but clearly outperforms HCD in phosphopeptide identification and phosphosite localization. Finally, we demonstrate the utility of our new approach by studying phosphoproteomic changes of mouse smooth muscle cells (SMC) with different treatments.

2. Materials and methods

2.1 Sample preparation

HEK293 and mouse aortic smooth muscle (MOVAS) cells were harvested, lysed in 8 M urea buffer and digested with trypsin or Lys-C. 100 µg peptides were then labeled with DiLeu or TMT for experiments of global proteomics. Phosphopeptides were enriched with IMAC from 3 mg labeled HEK293 or 2.4 mg MOVAS protein digest and fractionated by high-pH reversed-phase chromatography. Detailed description of the sample preparation is available in the Supplemental Information.

2.2 LC-MS²

All LC-MS experiments were performed using an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) interfaced with a Dionex Ultimate 3000 UPLC system (Thermo Fisher Scientific). A binary solvent system composed of H₂O containing 0.1% formic acid (A) and ACN containing 0.1% formic acid (B) was used for all analysis. Peptides were loaded and separated on a 75 $\mu\text{m} \times 15\text{ cm}$ self-fabricated column packed with 1.7 μm , 150 $\text{\AA}$, BEH C18 material obtained from a Waters UPLC column (part no. 186004661). Samples were loaded with 3% Solvent B, and a 90-minute gradient was used during which solvent B was linearly ramped to 30%. Survey scans were performed with a scan range from 300 to 1500 m/z at a resolving power of 60K with an AGC target of 1×10^5 . The top 15 precursors were selected for MS² with an isolation window of 1 Da, a resolving power of 15K, an AGC target of 5×10^4 and a lower mass limit of 110 m/z . Dynamic exclusion was set at 45 s with a 10 ppm tolerance. For optimization of EThcD conditions, precursor ions were repeatedly selected and fragmented with different EThcD reaction times or normalized collision energies (NCE) respectively. ETD experiments were performed with calibrated ETD reaction time enabled. HCD experiments were performed with a NCE of 30 for DiLeu-labeled samples or 35 for TMT-labeled samples. For quantitation of MOVAS phosphopeptides, optimized EThcD parameters (Table S1, Supplemental Information) were applied and fractions were analyzed.

2.3 Data Processing

Mass spectra were processed using Proteome Discoverer 2.1 (Thermo Scientific). Raw files were searched against the UniProt *H. sapiens* or *M. musculus* protein database (April 12, 2016; 16764 entries) using the Sequest HT algorithm. b/y ions were enabled for searching data acquired with HCD and c/z ions were searched for ETD. Both ion series were enabled in the search of EThcD data. Lys-C or trypsin was selected as enzyme with maximum of two missed-cleavages. A precursor tolerance of 50 ppm and a fragment ion tolerance of 0.02 Da were allowed. Static modifications consisted of DiLeu or TMT labels on peptide N-termini and lysine residues and carbamidomethylation of cysteine residues. Dynamic modifications consisted of oxidation of methionine residues for all experiments and phosphorylation on serine, threonine and tyrosine for enriched phosphopeptide experiments. Identifications were validated with 1% PSM and 1% protein FDR using percolator. Quantitation was performed with biological triplicates in Proteome Discoverer with a reporter ion integration tolerance of 20 ppm for the most confident centroid. Reporter ion ratio values for protein groups were exported to Excel workbook and corrections were performed followed by statistical analysis with student t-test.^{19,26}

3. Results and Discussion

3.1 Optimization of EThcD parameters for DiLeu-labeled peptides

Similar to commonly used commercial isobaric tags, such as TMT and iTRAQ, the structure of the DiLeu isobaric labeling reagent is composed of a reporter group, a balance group, and an amine-reactive group that enables selective modification of peptide N-termini and lysine side chains.^{22,27} Although they were originally developed based on a CID platform, it is noted that CID causes loss of labile PTMs prior to fragmentation of the peptide backbone,

often preventing correct peptide identification, localization of the modified site, and as a result, relative quantitation. ETD has recently become popular as it is capable to preserve labile PTMs such as phosphorylation and *O*-linked glycosylation.⁸ The elevated interest in studying PTMs has drawn efforts to apply isobaric labeling strategy with an ETD approach. Previous studies suggest that TMT and iTRAQ-labeled peptides produce a unique set of ETD-generated reporter ions.^{23,25} For example, instead of six-plex reporters, 6-plex TMT⁶ only produces four unique reporter ions.^{24,28} However following the same pattern, DiLeu produces ETD reporters under m/z 50 by cleaving the N-C $_{\alpha}$ bond, whose detection is not possible due to the Orbitrap detection limit set above m/z 50 (Figure 1). As shown in Figure 1A, the ETD spectrum of triply charged peptide *GEFVTTVQQRGA*VI*K (* denotes the DiLeu labeled sites) is dominated by its charge-reduced precursor at m/z 994 and a peak at m/z 971. This 45 Da mass difference is caused by the loss of the proposed reporter ion (Figure 1B). It is therefore not feasible to take advantage of this highly cost-effective isobaric labeling reagent to perform ETD experiments. To circumvent this problem, we employ a newly developed EThcD method to preserve HCD type of reporters.

EThcD is a dual fragmentation technique consisting of ETD and HCD, therefore optimization on both reaction time and NCE were performed respectively. We first optimized EThcD reaction times with various values tested for each individual charge state²⁹ while maintaining the same HCD NCE (Figure 2). Tryptic HEK293 peptides were used to investigate +2 and +3 precursor ions. Although regular ETD for a +2 ion required ~140 ms and +3 ion took ~60 ms (Table S1, Supplemental Information), our results suggested that 50 and 20 ms were sufficient for these two precursor ions respectively in EThcD, showing significantly reduced time and thus improved duty cycle. We compared not only peptide spectrum match (PSM) numbers but also their Sequest XCorr values. It appeared that 50 ms yielded the most PSMs and the best average quality for +2 ions (Figure 2A). For +3 ions, we found that the PSM number showed a slight increasing trend at 20 ms but the XCorr value started to drop (Figure 2B), therefore 20 ms was selected for a balance between the two parameters. Similarly, EThcD reaction times needed for +4 and +5 ions were optimized with Lys-C digested HEK293 cell lysate, in order to enrich the higher charge states. Although not as dramatic as +2 and +3 ions, EThcD also required at least 1/3 less reaction time compared to regular ETD for +4 (20 ms) and +5 ions (10ms). Part of our ultimate goal was to preserve original DiLeu reporters, so we compared median reporter intensity of each charge state based on each reaction time (Figure 2E, Figure S1, Supplemental Information). We inferred from our preliminary observation that increasing EThcD reaction time would cause decrease in reporter ion intensity as more ETD-type of reporters would be generated. Despite a decreasing trend in reporter ion intensity as reaction time increased, the reaction times used were able to generate $\sim 1e^6$ reporter intensity, which was sufficient for accurate quantitation.

Next, we determined the EThcD NCE value when fragmenting DiLeu-labeled peptides following reaction with electron anions. In a regular HCD experiment, NCE at 27 or 30 is usually used.^{26,27,30} However, we found that higher NCE value at 33 was required to break ion species generated after the defined reaction time. We reasoned this was due to abundant charge-reduced precursors generated in former step that would require higher NCE to fragment (Figure 3). NCE value at 33 with EThcD produced only slightly lower reporter intensity (median value = $2.13e^6$) than regular HCD with NCE at 30 (median value = $3.31e^6$)

(Figure S1D, E, Supplemental Information). We also noted that TMT produced similar reporter intensity with EThcD or HCD in the following discussion. Therefore, such slight decrease in reporter ion production would not impair quantitative performance.

3.2 Evaluation of EThcD method on DiLeu-labeled peptides

After a systematic optimization of EThcD parameters, we applied these values and compared them to traditional ETD and HCD approaches to validate its utility in the context of global proteomic studies. Lys-C digested HEK293 cell lysate was labeled with DiLeu⁰ and duplicate experiments were performed using ETD, EThcD or HCD respectively. In total, we acquired 49690 ETD, 60442 EThcD and 103476 HCD MS² spectra which resulted in 9465 ETD, 18050 EThcD, and 22509 HCD PSMs at 1% PSM and protein false discovery rate (FDR) level from each duplicate data set (Figure S2B, Supplemental Information). It was obvious that HCD acquired more MS² and PSMs due to the best instrument duty cycle among the three. But similar to previous studies with unlabeled peptides,^{14,16} EThcD of DiLeu-labeled peptides produced more complete peptide sequence coverage by supplying both b/y and c/z fragment ion series. As an example, ETD of triply charged ADLINNLGTIAK generated 21 c/z ions but lacked quantifiable reporter ions, whereas HCD generated 15 b/y ions (Figure 4). EThcD produced 49 product ions from both ion series. But more importantly, EThcD yielded original reporter ion for DiLeu labels at *m/z* 114. Therefore, as expected, EThcD identifications generally were scored higher by Sequest than the other two techniques (Figure S2D, Supplemental Information). As another direct reflection of better sequence coverage, EThcD identified 29.86% on average of all MS², higher than 19.05% of ETD and 21.76% of HCD (Figure S2C, Supplemental Information). One well-known obstacle for large scale ETD application is its difficulty to produce efficient fragmentation with +2 ions. In our dataset only 3.51% of all ETD identifications came from +2 ions. By taking advantage of additional collisional activation, EThcD greatly mitigated such bias and had 35.22% of all its identifications from +2 ions. On the other hand, we noticed EThcD yielded higher percentage of identifications with charge states equal to or larger than +3 compared to HCD, due to electron-based activation preferring higher charge states. Therefore, more uniformly distributed peptide charge states were obtained with EThcD compared to traditional ETD and HCD (Figure S2A, Supplemental Information).

Another experiment was conducted to test whether EThcD could also facilitate phosphopeptide characterization with DiLeu-labeled peptides.^{14,16} HEK293 cell Lys-C digest was labeled with DiLeu⁰ and phosphopeptides were enriched with IMAC.³¹ Enriched phosphopeptides were analyzed directly or fractionated by a high-pH reversed phase chromatography and recombined into five fractions.³² Samples were analyzed with ETD, EThcD and HCD, respectively. Strikingly, 2988 unique phosphopeptides were observed with EThcD, more than either 2392 with ETD or 2789 with HCD from five fractions (Figure 5A) and same trend was observed with unfractionated sample (Figure S3, Supplemental Information). Furthermore, as expected EThcD delivered higher MS² identification success rate (Figure 5B) and overall better XCorr values representing better spectral quality (Figure 5C, Figure S3, Supplemental Information). ETD had a median XCorr of 2.26 and HCD had 3.09, whereas EThcD had 4.03. Apart from identifying phosphopeptides, localization of phosphorylation sites is another important goal and is always complicated by additional

factors, such as multiple potential sites and insufficient sequence information. We used PhosphoRS probability score to evaluate the influence of different fragmentation techniques on phosphorylation site localization.³³ Among all 7019 phosphopeptide PSMs obtained by EThcD, 4799 (68.37%) had a phosphoRS probability larger than 0.9, showing great confidence localizing the exact site. In contrast to EThcD, ETD only had 46.63% of all phosphopeptide PSMs with such high confidence and HCD had 56.00% (Figure 5D). Overall, EThcD showed great potential to be coupled with DiLeu isobaric tagging and applied in quantitative phosphoproteomics.

3.3 Evaluation of EThcD method on TMT-labeled peptides

As a well-established isobaric tag, TMT has been widely used in a great variety of MS-based quantitative proteomic studies. However, few studies use ETD as its primary fragmentation approach even though its fragmentation behavior has been characterized.^{24,28} As EThcD is able to preserve original reporter ion, improve data quality and duty cycle, we reason that it could be beneficial for TMT-labeled peptides. In order to support our hypothesis, TMT⁰ was used to label HEK293 cell lysate tryptic peptides following manufacturer's protocol and analyzed by ETD, EThcD or HCD in duplicates. Upon ETD, TMT⁰ cleaves at a bond adjacent to the CID fragmentation site, which results in the proximal carbon relocating from the reporter ion to the balancer region, producing reporter ion at m/z 114 instead of 126 upon HCD.^{24,28} NCE ramp revealed that TMT⁰-labeled peptides required an EThcD NCE at 38 (Figure S4A, Supplemental Information). Head-to-head comparison between ETD, EThcD and HCD was then conducted. In contrast to EThcD and HCD where reporters were always present, ETD did not always produce reporter ions, represented by a +3 ion for tryptic peptide ADLINNLGTIAK (Figure 6). Reporter ion at m/z 126 was clearly present in EThcD and HCD spectra but neither m/z 114 nor 126 peak was visible in its ETD spectrum. When exploring entire duplicate ETD data sets, only 68.5% of all PSMs were found to have such 114 peak, leaving over a third of spectra not producing quantitative information, which presented a major obstacle towards reliable quantitation. More importantly, this absence of reporter ions in ETD spectra was universal in all charge states. As TMT tag was able to enhance peptide charge states,³⁴ +3 ions constituted the majority of the peptide precursors as well as the major part of the PSMs not having reporters (Figure S5A). Due to this ineffectiveness in reporter production, the median reporter ion intensity generated by ETD was only 1.3×10^4 , whereas EThcD and HCD had 3.6×10^5 and 5.1×10^5 respectively (Figure S5B). When calculating the relative frequency of PSMs without reporters according to each charge state, we did notice a clear trend showing that the lower charge state was more inclined to have PSMs without reporter ions (Figure S5A). Since there were still ~12% of +4 and ~8% of +5 precursor ions that did not produce measurable reporter ions, it would be difficult to circumvent this reporter loss by choosing different enzymatic digestion. Therefore, if ETD-generated reporter is desired for a particular reason, larger peptide pieces or even intact protein analysis is preferred to regular tryptic digest. Additionally, comparison among these three MS² modes using TMT suggested the same trend as we described for DiLeu tags. Better data quality and higher MS² identification success rate were observed with EThcD (30.8%) in comparison with ETD (15.23%) or HCD (28.29%) (Figure S4B, C).

3.4 Analysis of differentially treated MOVAS cell line

Beyond demonstrating the technical capabilities of the EThcD method with the HEK293 cell digest, we sought to demonstrate the practicality of the method using a large-scale proteomics experiment. To this end, we prepared a DiLeu 4-plex sample that consisted of MOVAS cell line with four different treatments: control, PDGF, serum and rapamycin. PDGF and serum are known to stimulate SMC proliferation while rapamycin is a macrolide product inhibiting this process. We grew cell in triplicate, applied treatments and after 48 h harvested proteins in order to explore their long term effects on phosphoproteomes. Proteins were digested with trypsin, labeled and phosphopeptides were enriched with IMAC. Offline high pH reversed phase fractionation kit from Pierce was used to collect 5 fractions, each of which underwent subsequent MS analysis using a 2h EThcD method.

Collectively, 1949 phosphopeptides from 738 phosphoproteins were quantified in at least one of the replicates and 1049 phosphopeptides from 445 phosphoproteins in all three (Table S2, Supplemental Information). In order for a protein/peptide to be quantifiable, all four DiLeu channels had to be measurable. Most of the identified phosphopeptides (1878) had more than one potential phosphosites. From all 738 phosphoproteins, 3075 phosphosites were mapped, including 2673 serine, 386 threonine and 16 tyrosine (Figure 7B). Out of all these phosphosites, 2826 were localized with at least 90% confidence, most of which (2442) were even with 100% certainty (Figure 7C). In quantitative proteomics, as each protein can generate many peptides after digestion, the changes of protein expression level are usually determined by averaging the ratios of several corresponding isobaric-labeled peptides derived from the same protein to eliminate any variations.^{35–37} However, this strategy is not suitable for phosphoproteomics since the same protein can have multiple phosphosites that could potentially have various stoichiometry due to regulation from different kinases/phosphatases. It is necessary to quantify each peptide individually. Therefore, stringent criteria were required to control the accuracy.^{38–41} All phosphopeptides included in following quantitation had at least 3 PSMs to ensure any potential biases were minimized, such as precursor co-isolation.^{35,42,43} The coefficient of variations (CV) of phosphopeptide ratios among biological triplicates were then evaluated. Average CVs for phosphopeptides quantified in all three replicates were 25.5%, 25.4%, and 24.8% for PDGF/control, serum/control, and rapamycin/control, which were comparable to previous phosphopeptide studies.^{38–40,44} To ensure reproducibility, improve accuracy and avoid potential false positives, a CV cutoff at 30% was implemented. All criteria implementations guaranteed a good correlation between replicates, as represented in Figure 8A and yielded 798, 746 and 809 unique phosphopeptides in each pair of comparison respectively for statistical test. The correlation between other biological replicates 2 and 3 as well as 1 and 3 can be found in Figure S6A and Figure S6B, respectively (Figure S6, Supplemental Information).

Restenosis, the re-narrowing of blood vessel, is a common adverse event following angioplasty in artery, featuring intimal hyperplasia which also refers to abnormal proliferation and migration of vascular SMCs.^{45,46} Rapamycin has applications in immunosuppression, preventing rejection in organ transplantation.⁴⁷ It is one of the most-widely used drugs to prevent restenosis after angioplasty.⁴⁸ The progression of restenosis involves enhanced phosphorylation of several key proteins, which can be inhibited by

rapamycin. Even though a few phosphoprotein studies have been reported, the coverage of phosphoproteome changes induced by rapamycin is still limited.^{45,49–51} We applied our EThcD strategy to explore the phosphoproteome landscape after rapamycin treatment. Student t-tests were performed with biological triplicate and a q-value at 0.05 after correction for multiple testing with Storey method was implemented.⁵² Evaluation of differentially regulated phosphopeptides between control and rapamycin treatment revealed 58 of them that were significantly altered in abundance. Gene ontology analysis revealed that a great portion of the proteins these phosphopeptides belonged to were nucleus proteins (Figure 8B) and related to mRNA processing and transcription regulation (Figure S7, Supplemental Information) as expected.⁵³ As examples, histone deacetylase 1 and 2 (HDAC 1 and 2) are responsible for the deacetylation of lysine residues on the N-terminal part of the core histones and important epigenetic controllers in transcriptional regulation whose dysregulation has been implicated in a variety of pathological situations, including hyperplasia and restenosis.^{54–59} HDAC inhibitors are emerging treatments for a variety of cellular proliferation disorders.^{54,59–61} Increase in class I HDACs induces abnormal histone deacetylation, transcriptional repression and a phenotypic switch from a quiescent contractile state to a proliferative-migratory state, which are characteristics of hyperplasia.^{62,63} Phosphorylation is another critical factor controlling HDAC activities post-translationally. Hyperphosphorylation of HDAC1 and HDAC2 leads to a significant increase in deacetylase activity and therefore subsequent transcriptional silencing.^{64,65} Rapamycin is able to prevent or mitigate restenosis and is expected to lower the phosphorylation level of HDAC1 and HDAC2.^{66–68} Quantitative phosphorylation result of HDAC2 was revealed based on an average of 37 PSMs. HDAC1 had two phosphosites identified in two peptide sequences that differed by only a miscleaved lysine. They were quantified based on 19 and 16 PSMs respectively in biological triplicates. A decrease in phosphorylation of HDAC1 (S421 and S423) and HDAC2 (S422 and S424) were observed (Figure 8C).

Although the majority of the identified phosphosites from the same protein were undergoing same trend of being up- or down-regulated, it was interesting to find those that did not. In Figure 8D, we highlighted the phosphorylation level of a protein, serine/arginine repetitive matrix protein 2 (SRRM2). SRRM2 is a core member of the spliceosome, regulates transcriptional machinery^{69,70} and cell migration⁷¹ and has also been implicated in diseases such as AIDS⁷² and Parkinson disease.⁷³ 14 of its phosphosites were identified and 5 of them showed decrease in phosphorylation whereas another 6 indicated enhanced phosphorylation.

PDGF stimulates SMC proliferation, de-differentiation, whereas the picture of serum was more complex due to contradictory reports suggesting that serum can promote a differentiated phenotype^{74,75} as well as stimulate SMC migration, proliferation and matrix synthesis which are characteristics of de-differentiation.⁷⁵ Interestingly, no significant change was observed in PDGF-treated sample after 48 hours and only 7 phosphopeptides exhibited differential regulations after serum treatment. This may be partly attributed to the difference between half-life of the exogenously added PDGF and rapamycin.⁷⁶ Additionally, phosphorylation is a fast and reversible response towards environmental stimulation. Extended exposure also induces feedback regulations to adjust phosphorylation level and further complicates the whole picture.^{77,78}

4. Conclusion

In summary, we provide an improved and systematically optimized EThcD fragmentation strategy that enables preserving original HCD-generated reporter ions of isobaric tags (e.g., DiLeu, TMT) while producing informative spectra that possesses both ETD and HCD generated fragments. It is superior to conventional ETD in the context of isobaric tag-based quantitative proteomics and PTM studies due to significantly improved identification, data quality, minimal bias against lower charge states and preserved HCD-generated reporter ions. It is also better than HCD in the sense that PTM can be localized with increased confidence and higher MS² identification success rates can be achieved. We conclude that the EThcD method will be highly useful for simultaneous PTM characterization and quantitation with isobaric tagging strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported in part by the National Institutes of Health grants R01 DK071801, NIH R01 HL068673, and P41GM108538. The Orbitrap instruments were purchased through the support of an NIH shared instrument grant (NIH-NCRR S10RR029531) and Office of the Vice Chancellor for Research and Graduate Education at the University of Wisconsin-Madison. LL acknowledges an H. I. Romnes Faculty Research Fellowship and a Vilas Distinguished Achievement Professorship with funding provided by the Wisconsin Alumni Research Foundation and University of Wisconsin-Madison School of Pharmacy. The authors would like to thank Thermo Fisher for providing a complimentary high pH reversed-phase peptide fractionation kit. We appreciate Dr. Dustin Frost and Amanda Buchberger in the Li research group for assisting with the synthesis of 4-plex DiLeu reagents. The authors would also like to thank Dr. Matthew S. Glover in the Li/Kent laboratories for his critical reading of the manuscript draft and helpful discussions.

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Highlights

- EThcD was optimized for isobaric tag-labeled peptides for quantitative proteomics
- EThcD preserves HCD-type reporters of DiLeu and TMT tags and improves instrument duty cycle by lowering ETD reaction time
- With EThcD, traditional isobaric tags originally designed for HCD can be readily utilized with ETD
- EThcD is able to provide both b/y and c/z ion series for greatly improved data quality
- EThcD enables quantitative phosphoproteomics with enhanced phosphorylation localization

Figure 1. ETD spectrum of a DiLeu-labeled peptide GEFVTTVQQRGA AVI*K (**A**) and proposed ETD fragmentation pathway of DiLeu-labeled peptides (**B**). Starred peak represents the peak after loss of ETD-generated reporter ion.

Figure 2.

Charge-state specific EThcD reaction time optimization of DiLeu-labeled peptides. Peptide spectral matches (PSMs) are plotted (bar) against reaction times (+2 (A), +3 (B), +4 (C) and +5 (D)) with bar graphs representing the mean of duplicates $\pm$ S.D. Mean $\pm$ S.D. of the median XCorr values from duplicates for +2 and +3 are indicated in the same figures (blue solid line, right y axis). Median reporter ion intensity $\pm$ interquartile range (IQR) of +2 ions are calculated for each EThcD reaction time (E).

Figure 3. Number of PSMs identified from duplicates of DiLeu-labeled HEK293 cell lysate tryptic peptides according to HCD NCE (A) and EThcD NCE (B). Although most IDs using regular HCD are found at an NCE of 30, EThcD requires elevated NCE to generate sufficient fragmentation for identification.

Figure 4. MS² of the 3+ peptide ADLINNLGTIAK after fragmentation by ETD (A), EThcD (B) or HCD (C). * denotes DiLeu labeled site and the starred peak represents the HCD type reporter ion at m/z 114.

Figure 5. Number of unique phosphopeptides (A). MS² identification success rate of all PSMs and phosphopeptide PSMs (B). Median $\pm$ IQR of the XCorr value of phosphopeptide PSMs (C). PhosphoRS probability pie chart of phosphopeptide PSM (D).

Figure 6. MS² of the 3+ peptide ADLINNLGTIAK by ETD (A), ETHcD (B) or HCD (C). * denotes TMT labeled site and the starred peak represents the HCD type reporter ion at m/z 126.

Figure 7.

Four differentially treated MOVAS cell lines were grown in biological triplicate. Each replicate was digested with trypsin, labeled with DiLeu, fractionated, and analyzed using EThcD method (A). 2587 phosphosites were identified, including 2234 serine, 343 threonine and 10 tyrosine (B). 2373 phosphosites were localized with a PhosphoRS probability >0.9 (C).

Figure 8.

Correlation between the two replicated quantification results of control and rapamycin treatment after being filtered by the CV criterion to control the quantification accuracy (A). Gene ontology analysis of differentially regulated phosphoproteins in biological triplicates (B). Phosphopeptide quantitation of HDAC1 and HDAC2 (C) and SRRM2 (D) in biological triplicates. All peptides had q-values < 0.05.