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## Peptide Quantification Using 8-Plex Isobaric Tags and Electron Transfer Dissociation Tandem Mass Spectrometry

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### Abstract

Isobaric tags for absolute and relative quantitation (iTRAQ) allow for simultaneous relative quantification of peptides from up to eight different samples. Typically peptides labeled with 8-plex iTRAQ tags are pooled and fragmented using beam-type collision activated dissociation (CAD) which, in addition to cleaving the peptide backbone bonds, cleaves the tag to produce reporter ions. The relative intensities of the reporters are directly proportional to the relative abundances of each peptide in the solution phase. Recently, studies using the 4-plex iTRAQ tagging reagent demonstrated that electron transfer dissociation (ETD) of 4-plex iTRAQ labeled peptides cleaves at the N-C $\alpha$  bond in the tag and allows for up to three channels of quantification. In this paper we investigate the ETD fragmentation patterns of peptides labeled with 8-plex iTRAQ tags. We demonstrate that upon ETD, peptides labeled with 8-plex iTRAQ tags fragment to produce unique reporter ions that allow for five channels of quantification. ETD-MS/MS of these labeled peptides also produces a peak at 322  $m/z$  which, upon resonant excitation (CAD), gives rise to all eight iTRAQ reporter ions and allows for eight channels of quantification. Comparison of this method to beam-type CAD quantification shows a good correlation ( $y$ ) = 0.91x + 0.01,  $R^2$  = 0.9383).

Protein quantification has become an important and, in many cases, critical component of modern mass spectrometry-based proteomic research.<sup>1-9</sup> Because of its ability to quantify peptides and proteins from multiple samples (up to eight) in a single experiment, the use of isobaric tagging reagents has garnered significant attention.<sup>10-13</sup> In these approaches isobaric amine-reactive tags are attached to peptides from as many as eight separate peptide pools. Independent of which tags are used to label the respective groups, peptides present in multiple pools will have the same nominal mass, chromatographic properties, and ionization efficiencies. Once labeled, the samples are combined and the peptides are interrogated via tandem mass spectrometry (MS/MS) with activation by beam-type collision activated dissociation (CAD). Cleavage of peptide backbone bonds provides sequence information while cleavage of the tag gives rise to unique reporter ions. The relative abundance of these reporter ions is directly proportional to the relative solution phase abundance of these molecules. Because these tags were specifically designed for use with beam-type CAD, it is unknown whether these tags are compatible with alternative dissociation methods.

Electron transfer dissociation (ETD) is one such alternative fragmentation method that has gained popularity, in part, for its ability to provide complementary information to CAD and

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its utility in sequencing peptides with post translational modifications.<sup>14-18</sup> We described the fragmentation behavior of peptides labeled with 4-plex isobaric tags for absolute and relative quantitation (iTRAQ).<sup>19</sup> We demonstrated that ETD results in a set of reporter ions that allows for three channels of quantification. When reporter ions were present at or above a certain intensity the quality of quantitative information was similar to that produced by CAD. However, since ETD does not preferentially cleave the intra-tag bonds, reporter ions of sufficient intensity were not always produced. In fact, often an intense peak at  $m/z$  162 was observed resulting from cleavage of the entire tag, balance and reporter regions, from the peptide. This peak does not provide quantitative information but McLuckey et al. showed that activation of this peak via resonant excitation CAD gives rise to the CAD-generated reporter ion and suggested such an approach may allow for four channels of quantification.<sup>20</sup>

Recently, Applied Biosystems has released a second generation iTRAQ tagging reagent (iTRAQ 8Plex) that allows for up to eight channels of relative quantification.<sup>12</sup> The complete structure is not publicly available but the molecular weight is roughly twice as large as the 4-plex reagent, and it produces chemically very similar reporter ions. Here we describe the ETD fragmentation patterns of peptides labeled with these tags and assess their quantitative utility using an ETD-enabled hybrid linear ion trap-orbitrap mass spectrometer.

## MATERIALS AND METHODS

*Saccharomyces cerevisiae* (strain BY4741) was grown in YPD at 30 °C to midlog phase. Cells were harvested by centrifugation at 4 °C for 5 min at 8000g and washed twice with sterile water before storage of the cell pellets at -80 °C. Frozen pellets were thawed and washed three times prior to lysis with Y-per (Pierce, Rockford, IL), 0.1 M DTT, complete mini ETDA-free protease inhibitor (Roche Diagnostics, Indianapolis, IN), and phosSTOP phosphatase inhibitor (Roche Diagnostics, Indianapolis, IN). Samples were pelleted, and supernatant was collected. Proteins were precipitated by addition of chilled acetone and resuspended in 50 mM HEPES pH7.5/4 M Urea. To extract nuclear proteins 8 M Urea/0.4 N H<sub>2</sub>SO<sub>4</sub> was added to the pellet. Extracted proteins from both fractions were mixed and subsequently reduced and alkylated prior to overnight digestion with endoproteinase Lys-C (Princeton Separations, Adelphia, NJ). After digestion peptides were desalted using SepPak cartridges (Waters, Milford, MA), split into eight tubes, and labeled with iTRAQ reagents (Applied Biosystems, Foster City, CA) according to the manufacturer supplied protocol. Samples were then mixed, dried to completion, and desalted again. The mixture was resuspended in 0.2% formic acid and separated online by nanoflow reversed-phase high performance liquid chromatography using a nanoAcquity UPLC (Waters, Milford, MA).

Glu-fibrinopeptide peptide, EGVNDNEEFFSAR, was acquired from Sigma-Aldrich (St. Louis, MO). EGVNDNEEFFSAR was labeled with 8-plex iTRAQ according to the manufacturer supplied protocol. After labeling, samples were dried to completion, resuspended in 0.1% TFA, and desalted using SepPak cartridges (Waters, Milford, MA). Desalted samples were again dried to completion and resuspended in 30% acetonitrile/100 mM acetic acid. Labeled EGVNDNEEFFSAR was mixed in known ratios ranging from 1:1 to 1:20 and infused using a nanospray robot (Nanomate Triversa, Advion BioSciences, Ithaca, NY) into the mass spectrometer.

Tandem mass spectrometry was performed on a hybrid linear ion trap-orbitrap mass spectrometer (Orbitrap, Thermo Fisher Scientific, Bremen, Germany) that was modified to perform ETD as previously described.<sup>21,22</sup> Since iTRAQ quantification has already been validated for beam type CAD, those ratios were considered correct in the infusion experiments and used as a control to which ETD results were compared.<sup>12,23,24</sup> Beam-type CAD experiments were performed with a normalized collision energy setting of 35, a cation AGC

target value of 300,000, and orbitrap mass analysis. The normalized collision energy and AGC target values for PQD were 31 and 40,000 respectively. ETD reactions were carried out for 60-100 ms with cation AGC target values of 40,000 (for ion trap mass analysis) and 300,000 (for orbitrap mass analysis) and an anion AGC target value of 300,000. Resonant excitation of the peak at  $m/z$  322 was performed with an activation window of 10  $m/z$  at a normalized collision energy of 35 for 30 ms. There was no isolation step prior to CAD activation of the peak at an  $m/z$  of 322 LC-MS/MS experiments composed of 11 scan events; an MS<sup>1</sup> scan followed by PQD and ETD (with CAD of  $m/z$  322) of the top five most abundant precursors.

MS<sup>2</sup> spectra were searched using open mass spectrometry search algorithm (OMSSA) against a concatenated forward and reversed *Saccharomyces Genome Database* (SGD) database. Results were filtered by precursor mass error and e-value until less than 1% of the hits were from reversed sequences. Removal of these sequences resulted in a list of peptides identified with a 1% false discovery rate (FDR). Quantification was performed by software written in-house. For infusion experiments, scans were averaged for approximately 30 s. Peak areas for each reporter ion were extracted and summed using an  $m/z$  width of 1 Th for ion trap scans and 0.1 Th for orbitrap scans. For LC-MS/MS experiments only proteins that were identified by at least two spectra each containing a sum reporter ion intensity of 1000 or more were used. Protein ratios were determined by averaging the ratios of each of the peptides.

## RESULTS AND DISCUSSION

To determine the fragmentation patterns of peptides labeled with 8-plex iTRAQ reagents, eight aliquots of the synthetic peptide EGVNDNEEFFSAR were labeled separately with each of the eight iTRAQ tags. Triply charged precursor cations were isolated, fragmented by ETD, and mass analyzed. Figure 1A shows single scan ion trap mass analysis of the ETD-generated fragment ions of EGVNDNEEFFSAR labeled with the 113 iTRAQ tag. ETD leads to formation of *c*- and *z*<sup>•</sup>-type ions, as well as peaks at 322 and 101  $m/z$ . To determine the chemical composition of these peaks we analyzed the same fragments in the high mass accuracy orbitrap mass analyzer. By recalibrating using a peak of known chemical composition ( $z_1^+$ ) we typically witnessed subpart per million mass accuracy, allowing for unambiguous assignment of reporter ion chemical. The peak at 101.10725  $m/z$  (C<sub>5</sub>N<sub>2</sub>H<sub>13</sub>) results from cleavage of the N-C<sub>α</sub> bond of the iTRAQ tag whereas the peak at 322.23912  $m/z$  corresponds to cleavage of N-C<sub>α</sub> bond of the N-terminal amino acid. Further analysis of the remaining seven tags, shown in Figure 1C, reveals that ETD produces reporter peaks at 101.10732  $m/z$  (113 and 114 tag), 102.10436  $m/z$  (115 tag), 104.11107  $m/z$  (116 and 117 tag), 106.11146  $m/z$  (118 and 119 tags), and 108.11817  $m/z$  (121 tag). All eight iTRAQ tags produce a peak with a nominal mass of 322. The exact mass of this peak was different for some of the tags indicating the incorporation of different heavy isotopes. Resonant excitation of this peak gives rise to the same reporter ions that are generated by beam-type CAD. Besides producing these reporter ions CAD of the ion at 322  $m/z$  has very little affect on the MS/MS spectra (Figure 1B).

To assess the quantitative utility of ETD with 8-plex iTRAQ reagents, EGVNDNEEFFSAR was labeled separately with five of the iTRAQ tags (113, 115, 116, 118, and 121). Four different mixtures of the five differentially labeled peptides were prepared, with the ratios between individual peptides in the mixture ranging from 1:1 to 1:20. The mixtures were infused into the MS and fragmented using either beam-type CAD or ETD. Note that current instrument configuration limits the detection of beam-type CAD fragments to the orbitrap. ETD-generated reporter ions had low signal-to-noise ratios when analyzed in the orbitrap so ion trap  $m/z$  analysis, which has higher sensitivity, was performed. Panels A and B of Figure 2 show single scan  $m/z$  analysis of the reporter regions after beam-type CAD and ETD, respectively. As expected ETD results in reporter ions at 101, 102, 104, 106, and 108, and the reporter ion ratios are similar to those observed via beam-type CAD (Figure 2A). ETD generated reporter ion

ratios were compared to the ratios provided by CAD and are shown in Figure 2C. The two fragmentation methods provide similar reporter ion ratios ( $y = 1.0165x - 0.00$ ,  $R^2 = 0.91$ ). Although ETD does produce quantitative reporter ions, this method is limited to only five channels of quantification.

Resonant excitation (ion trap CAD) of the more intense ETD peak at 322  $m/z$  provides an alternate strategy for quantification and allows for the use of all eight channels. After the ETD reaction but prior to mass analysis the peak at 322  $m/z$  was resonantly excited for 30 ms. For simplicity, ETD followed by ion trap CAD of the 322 peak will be referred to as ETD/CAD. Note there was no isolation step between ETD and CAD activation, so all the ions that were present in the ETD spectrum are also present in the ETD/CAD spectrum (with the exception of the peak at 322  $m/z$ ). Figure 2E shows the reporter ions generated by ion trap CAD of the 322 peak. Not only are all eight reporter ions present but the relative intensities correspond well with those produced via beam-type CAD (Figure 2D). EGVNDNEEFFSAR was labeled with each of the eight iTRAQ tags, and the differentially labeled peptides were combined into three mixtures with the ratios between individual peptides ranging from 1:1 to 1:20. The mixtures were infused and analyzed by both beam-type CAD and ETD with ion trap CAD of the 322 peak. Spectra were acquired for approximately 30 s. As shown in Figure 2F these two fragmentation methods exhibited very similar reporter ion ratios ( $y = 0.91x + 0.01$ ,  $R^2 = 0.94$ ).

Next, we assessed the compatibility of these approaches with LC-MS/MS analysis. Whole cell lysate from Yeast digested with Lys-C, labeled with each iTRAQ tag, and mixed in a 1:1:1:1:1:1:1:1 ratio. This sample was separated using reversed-phase chromatography and analyzed online using a hybrid linear ion trap-orbitrap mass spectrometer. Each of the five most abundant precursors were subjected to PQD and ETD/CAD. Since there is no isolation prior to CAD of  $m/z$  322 both sets of reporter ions (101 to 108 and 113 to 121) are present. Therefore, using one scan we evaluated two different quantification methods; one using the ETD-generated reporter ions ranging from  $m/z$  101 to 108 and the other using ETD/CAD-generated reporter ions ranging from 113 to 121. A total of 1283 unique peptides were identified (1083 by PQD and 1186 by ETD/CAD) corresponding to 389 proteins (331 by PQD and 329 by ETD/CAD). Protein quantification results are summarized in Figure 4. The percent contribution of each reporter ion was averaged for all proteins containing at least two spectra with a total reporter ion intensity of at least 1000 counts (163 proteins for PQD quantification, 177 for ETD quantification, and 153 for ETD/CAD quantification). This percent contribution was compared to the expected value. PQD, ETD, and ETD/CAD quantification had average  $\log_2$  values of 0.007, 0.006, and -0.052 respectively with average standard deviations of 0.227, 0.244, and 0.377. On average PQD generated the highest intensity reporter ions likely because iTRAQ tags were designed to be preferentially cleaved when using collision-based dissociation methods such as PQD. ETD does not preferentially cleave the iTRAQ tag and therefore does not necessarily produce high abundance reporter ions. Nevertheless, the ETD generated reporter ions between 101 and 108 provided the same quality of quantification as the PQD approach. ETD/CAD-generated ions between  $m/z$  of 113 and 121 were the lowest intensity reporter ions, and correspondingly the ETD/CAD approach provided less accurate protein quantification.

## CONCLUSIONS

We demonstrate here that 8-plex iTRAQ reagents are compatible with ETD. ETD of labeled peptides generates  $c$ - and  $z$ -type ions that allow for peptide identification. Quantitative information can be obtained from ETD MS/MS analysis of peptides labeled with 8-plex iTRAQ reagents in two ways (Figure 3). In the first method, ETD of iTRAQ 8-plex labeled peptides generates five unique reporter ions, ranging from 101 to 108  $m/z$ , which allows for comparison of peptide abundances across five samples. In the second method, ETD is followed by resonant

excitation CAD of the ETD-generated ion at 322  $m/z$  giving rise to reporter ions ranging from 113 to 121  $m/z$  and allowing for eight sample comparisons. Since there is no isolation step between the ETD activation step and the CAD activation step both the ETD-generated reporter ions located between 101 and 108  $m/z$  and the ETD/CAD-generated ions located between 113 and 121  $m/z$  are available for quantification.

Because of its utility in sequencing post-translationally modified peptides we envision that ETD in combination with 8-plex ITRAQ reagents may be especially beneficial for, among other things, time course studies of these modifications. ETD has been used to identify such molecular events as phosphorylation and glycosylation, but to our knowledge ETD has never been combined with a multiplexed quantification strategy for the analysis of PTMs.<sup>25-29</sup> Taking advantage of ETD-generated reporter ions or performing supplemental activation of the ETD-generated 322 peak opens the door for these types of experiments and may help probe the temporal dynamics of these post-translational events.

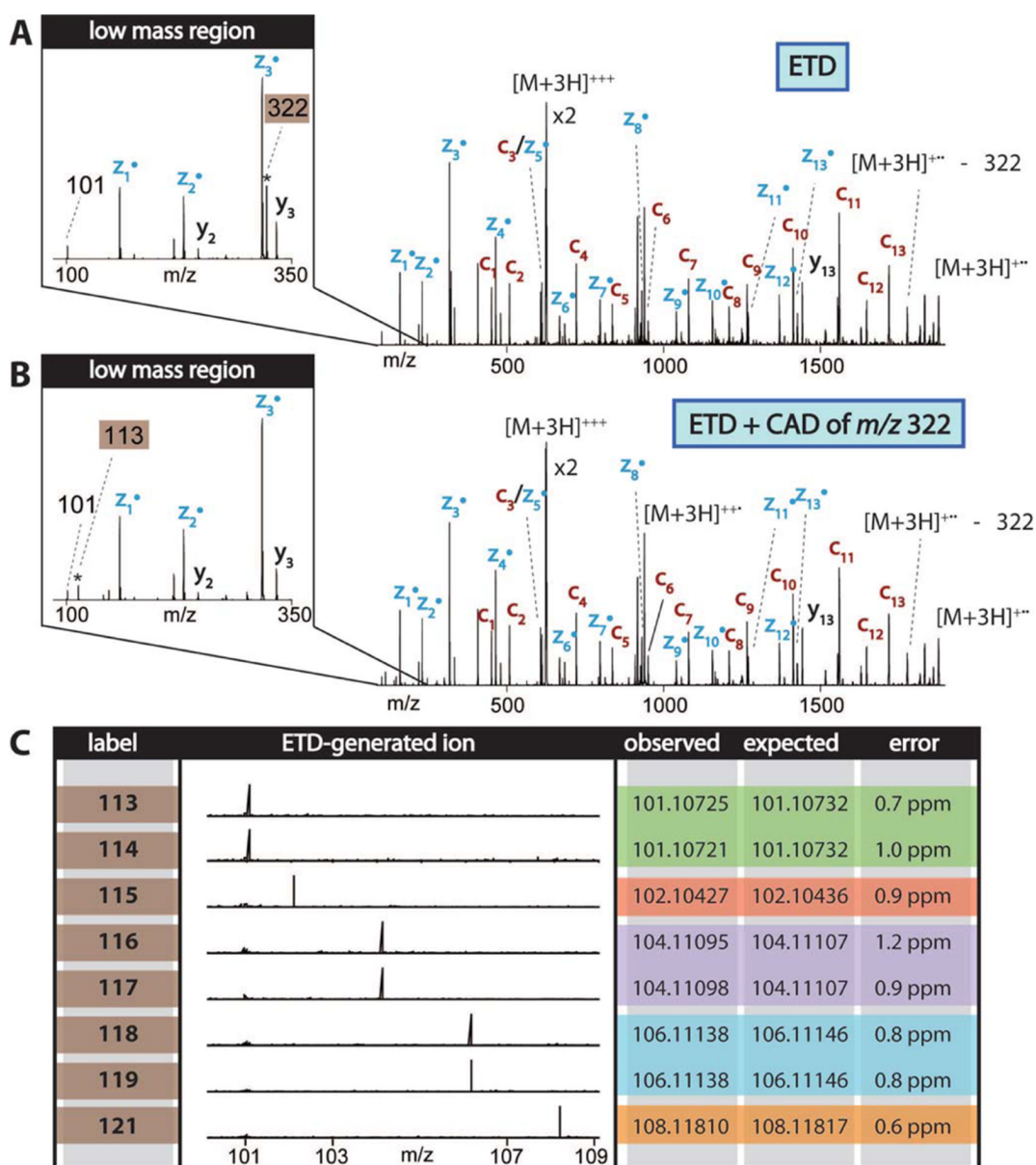
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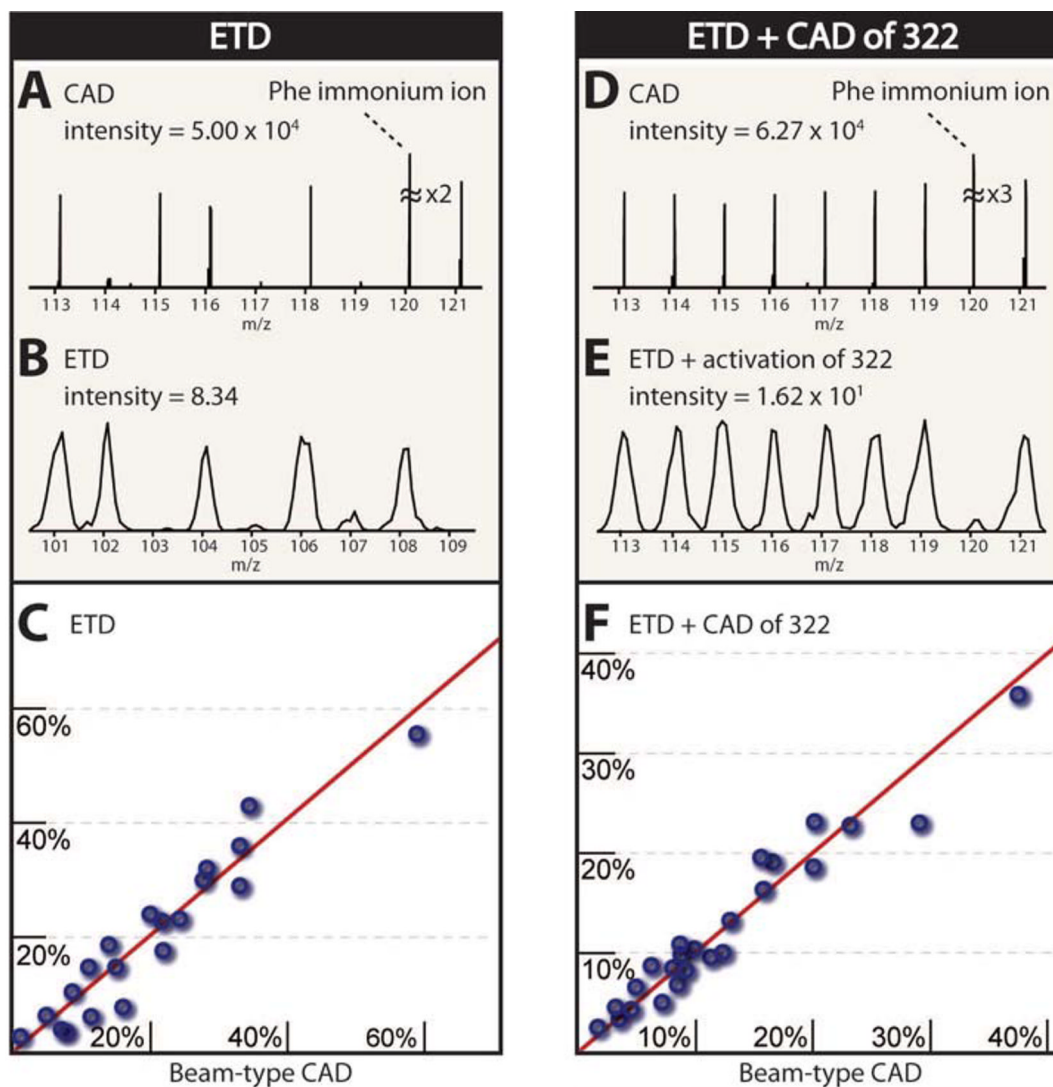
## References

- (1). Aebersold R, Mann M. *Nature* 2003;422:198–207. [PubMed: 12634793]
- (2). Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B. *Anal. Bioanal. Chem* 2007;389:1017–1031. [PubMed: 17668192]
- (3). Conrads TP, Issaq HJ, Veenstra TD. *Biochem. Biophys. Res. Commun* 2002;290:885–890. [PubMed: 11798155]
- (4). Heck AJR, Krijgsveld J. *Expert Rev. Proteomics* 2004;1:317–326. [PubMed: 15966828]
- (5). Julka S, Regnier F. *J. Proteome Res* 2004;3:350–363. [PubMed: 15253416]
- (6). Leitner A, Lindner W. *Proteomics* 2006;6:5418–5434. [PubMed: 16972287]
- (7). Ong SE, Foster LJ, Mann M. *Methods* 2003;29:124–130. [PubMed: 12606218]
- (8). Ong SE, Mann M. *Nat. Chem. Biol* 2005;1:252–262. [PubMed: 16408053]
- (9). Righetti PG, Camprostrini N, Pascali J, Hamdan M, Astner H. *Eur. J. Mass Spectrom* 2004;10:335–348.
- (10). Thompson A, Schafer J, Kuhn K, Kienle S, Schwarz J, Schmidt G, Neumann T, Hamon C. *Anal. Chem* 2003;75:1895–1904. [PubMed: 12713048]
- (11). Ross PL, Huang YLN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ. *Mol. Cell. Proteomics* 2004;3:1154–1169. [PubMed: 15385600]
- (12). Choe L, D'Ascenzo M, Relkin NR, Pappin D, Ross P, Williamson B, Guertin S, Pribil P, Lee KH. *Proteomics* 2007;7:3651–3660. [PubMed: 17880003]
- (13). Zhang Y, Wolf-Yadlin A, Ross PL, Pappin DJ, Rush J, Lauffenburger DA, White FM. *Mol. Cell. Proteomics* 2005;4:1240–1250. [PubMed: 15951569]
- (14). Chi A, Huttenhower C, Geer LY, Coon JJ, Syka JEP, Bai DL, Shabanowitz J, Burke DJ, Troyanskaya OG, Hunt DF. *Proc. Natl. Acad. Sci. U.S.A* 2007;104:2193–2198. [PubMed: 17287358]
- (15). Good DM, Wirtala M, McAlister GC, Coon JJ. *Mol. Cell. Proteomics* 2007;6:1942–1951. [PubMed: 17673454]
- (16). Syka JEP, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF. *Proc. Natl. Acad. Sci. U.S.A* 2004;101:9528–9533. [PubMed: 15210983]

- (17). Molina H, Matthiesen R, Kandasamy K, Pandey A. *Anal. Chem* 2008;80:4825–4835. [PubMed: 18540640]
- (18). Molina H, Horn DM, Tang N, Mathivanan S, Pandey A. *Proc. Natl. Acad. Sci. U.S.A* 2007;104:2199–2204. [PubMed: 17287340]
- (19). Phanstiel D, Zhang Y, Marto JA, Coon JJ. *J. Am. Soc. Mass Spectrom* 2008;19:1255–1262. [PubMed: 18620867]
- (20). Han HL, Pappin DJ, Ross PL, McLuckey SA. *J. Proteome Res* 2008;7:3643–3648. [PubMed: 18646790]
- (21). McAlister GC, Berggren WT, Griep-Raming J, Horning S, Makarov A, Phanstiel D, Stafford G, Swaney DL, Syka JE, Zabrouskov V, Coon JJ. *J. Proteome Res* 2008;7:3127–3136. [PubMed: 18613715]
- (22). McAlister GC, Phanstiel D, Good DM, Berggren WT, Coon JJ. *Anal. Chem* 2007;79:3525–3534. [PubMed: 17441688]
- (23). Quaglia M, Pritchard C, Hall Z, O'Connor G. *Anal. Biochem* 2008;379:164–169. [PubMed: 18510936]
- (24). Pierce A, Unwin RD, Evans CA, Griffiths S, Carney L, Zhang L, Jaworska E, Lee CF, Blinco D, Okoniewski MJ, Miller CJ, Bitton DA, Spooncer E, Whetton AD. *Mol. Cell. Proteomics* 2008;7:853–863. [PubMed: 17951628]
- (25). Wu SL, Huehmer AFR, Hao ZQ, Karger BL. *J. Proteome Res* 2007;6:4230–4244. [PubMed: 17900180]
- (26). Mikesch LM, Ueberheide B, Chi A, Coon JJ, Syka JEP, Shabanowitz J, Hunt DF. *Biochim. Biophys. Acta, Proteins Proteomics* 2006;1764:1811–1822.
- (27). Khidekel N, Ficarro SB, Clark PM, Bryan MC, Swaney DL, Rexach JE, Sun YE, Coon JJ, Peters EC, Hsieh-Wilson LC. *Nat. Chem. Biol* 2007;3:339–348. [PubMed: 17496889]
- (28). Swaney DL, McAlister GC, Wirtala M, Schwartz JC, Syka JEP, Coon JJ. *Anal. Chem* 2007;79:477–485. [PubMed: 17222010]
- (29). Phanstiel D, Brumbaugh J, Berggren WT, Conard K, Feng X, Levenstein ME, McAlister GC, Thomson JA, Coon JJ. *Proceedings of the National Academy of Sciences of the United States of America* 2008;105:4093–4098. [PubMed: 18326628]

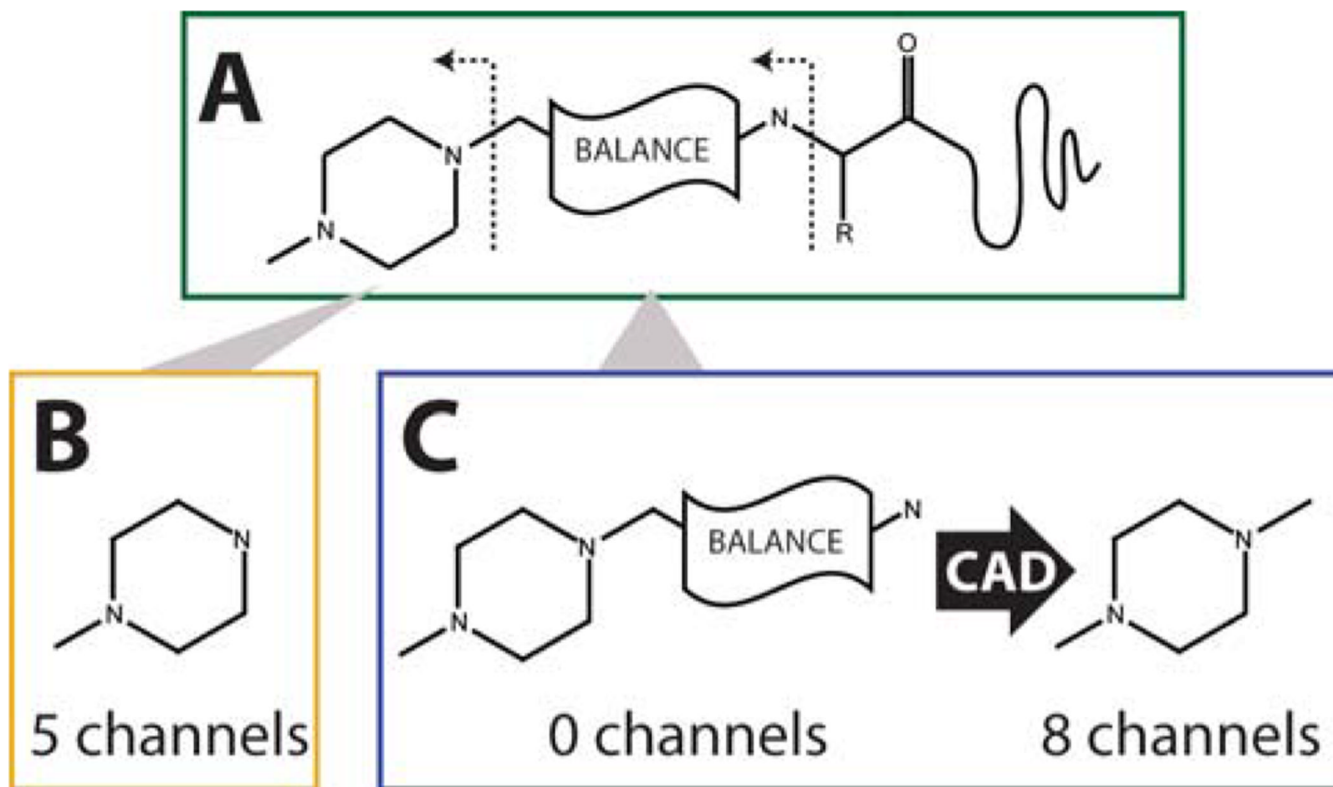


**Figure 1.** Fragmentation Patterns (a) Single scan ETD MS/MS analysis of EGVNDNEEGFFSAR labeled with the 113 iTRAQ tag with ion trap detection reveals all possible  $c$ - and  $z^*$ - type ions. The liftout on the left shows the ETD-generated reporter ion at an  $m/z$  of 101 as well as a peak at an  $m/z$  of 322 representing the entire iTRAQ tag. (b) Single scan mass analysis of the same peptide following ETD and resonant excitation of the peak at  $m/z$  322 results in a similar spectrum. The spectrum is not significantly different from the ETD spectrum except for the disappearance of the peak at 322 and the appearance of the peak at 113 seen in the liftout on the left. (c) The reporter regions of ETD MS/MS of EGVNDNEEGFFSAR labeled with each of the eight iTRAQ labels reveals five unique ETD generated reporter ions. The column on the left describes the iTRAQ tag that was interrogated. The spectra show orbitrap mass analysis of the ETD generated reporter ions. Detected masses, theoretical masses, and ppm error are shown on the right.

**Figure 2.**

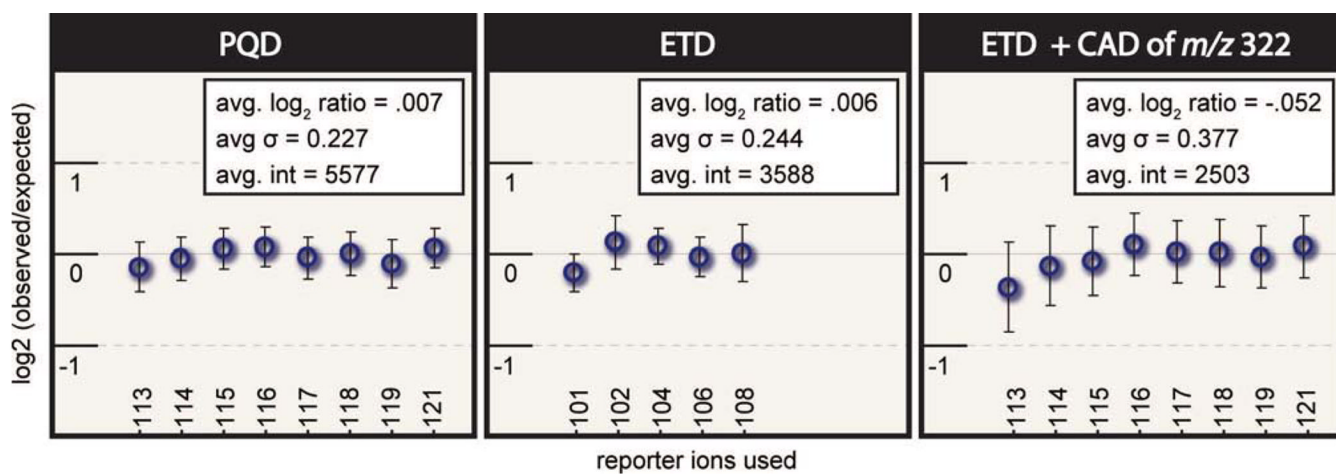
Evaluation of quantification (comparison to beam-type CAD) (Left) EGVNDNEEGFFSAR peptides were labeled with the 113, 115, 116, 118, and 121 iTRAQ tags, mixed in known ratios, infused into a hybrid linear ion trap-orbitrap mass spectrometer, and fragmented by beam-type CAD (a) and ETD (b). The reporter region from a single scan mass analysis of each is shown. (c) Four different mixes of the five labeled peptides were made. Ratios between samples ranged from 1:1 to 1:20. Plotted here are observed ratios via CAD on the *x*-axis and observed ratios via ETD on the *y*-axis. (Right) EGVNDNEEGFFSAR labeled with the all eight iTRAQ tags was mixed, infused into a hybrid linear ion trap-orbitrap mass spectrometer, and fragmented by beam-type CAD (d) and ETD with resonant excitation of 322 (e). Again, the reporter region from a single scan mass analysis of each is shown. (f) Three different mixes of the eight labeled peptides were made. Plotted here are observed ratios via CAD on the *x*-axis and observed ratios via ETD with resonant excitation of 322 on the *y*-axis.





**Figure 3.**

Two approaches for iTRAQ quantification using ETD. (a) The structure of an iTRAQ tag as attached to a peptide is shown. ETD cleaves the 8-plex iTRAQ tag as indicated by the dotted lines. (b) Cleavage of the N-C $\alpha$  bond of the tag results in reporter ions with the structure shown. Incorporation of different heavy isotopes allows for quantification of peptides from up to five different samples. (c) Cleavage of the N-C $\alpha$  bond of the first amino acid or corresponding bond on the lysine side chain produces a peak at an  $m/z$  of 322 which does not provide quantitative information since it retains both the reporter and balance regions. However, resonant excitation of the peak at an  $m/z$  of 322 produces all eight reporter ions allowing for eight sample comparisons.



**Figure 4.** Protein quantification results. Average  $\log_2$  reporter ion ratios (observed/expected) are shown for each quantification strategy. Error bars depict standard deviations.