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Automated gas-phase purification for accurate, multiplexed quantification on a stand-alone ion trap mass spectrometer

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Abstract

Isobaric tagging enables the acquisition of highly-multiplexed proteome quantification but is hindered by the pervasive problem of precursor interference. The elimination of co-isolated contaminants prior to reporter tag generation can be achieved through the use of gas-phase purification via proton transfer ion/ion reactions (QuantMode); however, the original QuantMode technique was implemented on the high resolution linear ion trap-Orbitrap hybrid mass spectrometer enabled with electron transfer dissociation (ETD). Here we extend this technology to stand-alone linear ion trap systems (trapQuantMode). Facilitated by the use of inlet beam-type activation (i.e., trapHCD) for production and observation of the low mass-to-charge reporter region, this scan sequence comprises three separate events to maximize peptide identifications, minimize duty cycle requirements, and increase quantitative accuracy, precision, and dynamic range. Significant improvements in quantitative accuracy were attained over standard methods when using trapQuantMode (trapQM) to analyze an interference model system comprising tryptic peptides of yeast that we contaminated with human peptides. Finally, we demonstrate practical benefits of this method by analysis of the proteomic changes that occur during mouse skeletal muscle myoblast differentiation. While trapQM's reduced duty cycle led to the identification of fewer proteins than conventional operation (4,050 vs. 2,964), trapQM identified more significant differences (>1.5 fold, 1,362 vs 1,132, respectively; $P < 0.05$) between the proteomes of undifferentiated myoblasts and differentiated myotubes and nearly ten-fold more differences with changes greater than 5-fold (96 vs. 12). We further show that our trapQM dataset is superior for identifying changes in protein abundance that are consistent with the metabolic and structural changes known to accompany myotube formation.

Introduction

Accurate determination of protein abundance is essential for the study of biological systems. Isobaric tag-based strategies^{1–2} are an attractive option because they enable a high level of multiplexing and are compatible with tissues and biofluids.^{3–4} The large-scale, multiplexed experiments these tagging chemistries enable are quickly becoming a requisite for practitioners of systems biology as they enable the measurement of time-course experiments, the collection of biological replicates, and the direct comparison of transcriptomic and proteomic data. Despite these advantages, the quality of quantitative data achievable with isobaric tags is degraded by the pervasive problem of precursor

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interference.^{5–8} This problem stems from the relatively low resolution of precursor isolation, typically 1–3 Th, and the dissociation of this entire mass-to-charge (m/z) region to produce reporter tags. As a result, the quantitative signal in the reporter region is a compilation of tags from both the intended target precursor and any co-isolated contaminants within the isolation window. For highly complex mixtures co-isolation of multiple species is commonplace. Recently we documented that, on average, only ~65% of the reporter ion signal originates from the target peptide, the remainder coming from low-level contaminants.⁶ This problem significantly erodes quantitative accuracy (~70% relative error) and truncates dynamic range, as measured ratios tend to be compressed toward the median ratio of 1:1.⁶

Several strategies have been suggested for the elimination of precursor interference. We initially investigated methods which narrow the precursor isolation window and/or reject precursors containing impurities above a certain threshold, but noted only modest quantitative improvement.⁶ More recent efforts have focused on the utilization of gas-phase purification.^{6,9} This strategy can be implemented using MS³ or by gas-phase ion-ion chemistry. For the latter, ion-ion reactions of the multiply protonated precursor and singly charged anions result in rapid (40 ms), predictable, and efficient charge-reduction of all species within the isolation window.^{10–11} The method, termed QuantMode, separates the target precursor from contaminants by dispersing them in m/z space followed by a second isolation of the first charge-reduced species. Figure 1 illustrates the QuantMode method, which was developed for a high-resolution quadrupole linear ion trap-orbitrap hybrid mass spectrometer. QuantMode offers certain advantages over the MS³ method. First, the products of proton transfer ion/ion reactions (PTR) are predictable from precursor charge state so the entire method can be accomplished in a single scan. Second, the method is compatible with peptides produced by any protease, including trypsin. Third, QuantMode is effective for peptides bearing labile PTMs such as phosphorylation. Finally, PTR is efficient with ~30–40% conversion efficiency of target to purified target as compared to ~<10% for the MS³ method. This benefit is particularly relevant as reporter ion signal-to-noise ratio is key to achieving high quantitative precision and accuracy.

The primary drawback of the QuantMode method is availability. At present the only commercial MS systems capable of performing the technique are those outfitted with electron transfer dissociation (ETD)^{12–14} capability, since ETD-enabled systems can generate suitable PTR reagents. Further, our original QuantMode implementation was designed for use on high-resolution QLT-Orbitrap hybrid systems that can perform both beam-type collisional activation (for reporter tag generation) and high resolution MS analysis (for precursor charge state assignment). Here we describe development of a low-resolution compatible QuantMode methodology that extends the QuantMode method to encompass a broader subset of MS instrumentation – namely low resolution devices such as stand-alone ion traps. Termed ‘trapQuantMode,’ this method introduces rapid charge state determination scanning to facilitate PTR-based purification. Once precursor cations have been purified and re-isolated we employ AP inlet beam-type CAD (trapHCD)¹⁵ to produce abundant reporter ion signals. We evaluate the utility of trapQuantMode (trapQM) to boost dynamic range and quantitative accuracy with a two-species quantitative accuracy model. Finally, we apply the approach to the analysis of mouse-derived C2C12 myoblasts to elucidate the dynamic protein changes that occur during myogenic differentiation. We show that trapQM is a powerful method for identifying changes in important biological pathways that accompany myotube formation, including the electron transport chain. Additionally, our trapQM dataset serves as a rich resource for investigating proteins or pathways that might be important for myoblast differentiation.

Methods

Sample Preparation. Interference Sample

Yeast peptides were split into six equal mass aliquots; each aliquot was labeled with one of six TMT 6-plex reagents (m/z 126 – 131), as described previously,¹⁶ and mixed in the mass ratios 1:5:10:10:5:1, respectively. Human peptides were split into three equal mass aliquots; each aliquot was labeled with one of three TMT 6-plex reagents (m/z 129 – 131), as described previously,¹⁶ and mixed in the mass ratios 1:1:1, respectively. A small aliquot was obtained from each individual sample to provide material for control experiments. Yeast and human samples were then combined in a 2:1 mass ratio, respectively. All samples were dried to completion and re-suspended in 0.2% formic acid for LC-MS analysis. *Mouse Myoblast Differentiation Sample*: ‘Day 0’ mouse myoblast and ‘day 6’ mouse myotube samples were split into three equal mass aliquots; each of the ‘day 0’ aliquots was labeled with one of the lower three TMT 6-plex reagents (m/z 126 – 128) while each of the ‘day 6’ aliquots was labeled with one of the upper three TMT 6-plex reagents (m/z 129 – 131), as described previously.¹⁶ The six aliquots were desalted using C18 solid-phase extraction columns, dried to completion, and combined in equal masses. The labeled mouse peptide mixture was fractionated using SCX, as described previously.¹⁷ Each fraction was lyophilized, desalted, dried to completion, and resuspended in 0.2% formic acid for LC-MS analysis.

Liquid chromatography-mass spectrometry

All experiments were performed using a NanoAcquity UPLC system (Waters, Milford, MA) coupled to an ETD-enabled LTQ Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA).

Non-QuantMode instrument methods consisted of an MS¹ scan (300 – 1600 m/z) followed by ten data-dependent trapHCD MS² scans, all analyzed in the ion trap at a normal scan speed. MS² scans employed a precursor isolation window of 3Th and a trapHCD normalized collision energy (NCE) setting of 60 for 2 ms.

All QuantMode instrument methods consisted of an MS¹ scan (300 – 1600 m/z) followed by three data-dependent QuantMode scan cycles, all analyzed in the ion trap. Precursor isolation windows of 3Th were used. The QuantMode scan cycle utilizes proton transfer reactions (PTR) to achieve gas-phase purification. For all experiments, the nitrogen adduct of fluoranthene (m/z 216) was used as the PTR reagent ion. Reagent anions were generated by an integrated chemical ionization source (commercial ETD module; Thermo Fisher Scientific, San Jose, CA); source conditions and all associated ion optics were optimized for this reagent prior to each set of experiments.

Data Analysis

Data was processed using the in-house software suite COMPASS.¹⁸ Interference data was independently trimmed to 1% FDR and subsequently filtered to remove all human-derived peptides, enabling only yeast-derived peptides to be considered for analysis. Myoblast differentiation data fractions were collectively filtered to 1% FDR for each set of experiments. Statistical and GO/KEGG-term analyses of myoblast differentiation data were conducted using Persius¹⁹ For all statistical comparisons, a Students *t*-test was utilized with $P < 0.05$.

Results and Discussion

Low-Res QuantMode Scan Sequence

Two obstacles prevent QuantMode from being directly implemented on low-resolution instrumentation: 1) with no dedicated beam-type collision cell, traditional beam-type CAD (HCD^{20–21}) fragmentation for reporter tag generation is unavailable and 2) precursor charge state knowledge, which is necessary for determining the m/z of the purified precursor ion for secondary isolation, cannot readily be determined from MS¹ spectra when using a QLT mass analyzer at typical scan speeds.²² This first impediment, lack of beam-type fragmentation, may be overcome by use of the AP inlet region for collisional activation (trapHCD).¹⁵ Although resonant excitation CAD can be used to generate reporter ions, the low-mass cutoff associated with this type of dissociation prevents their retention and subsequent mass analysis. With a fragmentation mechanism identical to HCD, trapHCD allows for the effective activation of isobaric-labeled peptides at RF amplitudes low enough to retain the low m/z reporter ions. The precursor-to-product conversion efficiency of trapHCD is comparable to HCD (~40%); both fragmentation methods generate similar amounts of iTRAQ reporter tag signal.¹⁵ In the absence of trapHCD, pulsed Q dissociation^{23–24} (PQD) may be employed for peptide identification and tag generation on a stand-alone ion trap system; however, compared to PQD, trapHCD yields a 2-fold improvement in peptide identifications and a 10-fold improvement in reporter ion intensities.¹⁵ Precursor charge state knowledge, the second barrier, can theoretically be obtained by the incorporation of a dedicated scan event. Substantial improvements in spectral resolution are achievable on QLT mass analyzers by slowing the speed of resonance ejection scanning while maintaining a suitably low auxiliary RF amplitude in the ion trap.²⁵ A three-fold reduction in scan speed (~10,000 amu/sec, Velos ion trap) is attainable through the use of ‘enhanced’ scan rate analysis.²² When precursor ions are isolated in a 3Th window and subjected to m/z analysis using this slower scan rate, baseline resolution is improved such that subsequent charge state determination for doubly and triply protonated precursor peptides becomes possible.

Figure 2 presents an overview of the trapQuantMode (trapQM) scan cycle. Unlike the original version of QuantMode, which benefited from high-resolution MS¹ analysis, all events cannot be completed in a single MS scan in the low-resolution implementation. The method instead utilizes three disparate scan events: 1) precursor charge state determination, 2) quantification, and 3) identification (Supplemental Figure 1). The rapid scan speed of the QLT system (which is approximately twice as fast as that of an Orbitrap for MS² analysis) allows for rapid completion of the cycle.^{22, 26} Additionally, we find the separation of steps can offer certain advantages. Knowledge of precursor charge state, for example, not only allows for the purification step, but permits tailored collision energy scaling to maximize precursor-to-product conversion efficiencies. Another advantage provided by the segregated scan events is the decoupling of sequence and reporter ion generation, a tactic previously shown to improve quantitation with isobaric tags.²⁷ Decoupling allows independent optimization of collision energies for both identification and reporter ion generation. Figure 3 illustrates this advantage by plotting reporter ion signal (Fig. 3A) and the number of identifications (Fig. 3B) generated as a function of trapHCD collision energy. Higher collision energies maximize reporter tag signal-to-noise, but at a cost of reduced sequence ions so that sequence identification is often hampered. Ideal sequence ion generation (and therefore peptide identification) is attained at lower collision energies, where reporter tag signal is not as large. By generating reporter tag and sequence ions in separate scan events, we can optimally produce both types of ions without having to settle for a collision energy which lies somewhere between the two different peak energies.

High Resolution Scan for Charge state Elucidation—Knowledge of precursor charge state is an essential component of the trapQM scan sequence since the purified precursor must be re-isolated at its charge-reduced m/z value. For this reason we have dedicated the first scan event in the trapQM method to charge state elucidation. In this step we perform m/z analysis on the 3Th region surrounding the precursor at a slower, ‘enhanced’²² scan rate. The improved resolution afforded by the slower scan speed enables the charge state prediction of doubly and triply protonated precursors; the charge state information gleaned from this scan is then used to conduct subsequent events more effectively and efficiently.

We first evaluated the utility of the enhanced scan rate for charge state determination independently from the trapQM method. A complex mixture of peptides, generated upon digestion of mouse cell lysate with trypsin, was gradient-eluted into the mass spectrometer. Each peptide precursor selected for MS² analysis was subjected to two independent scan events: 1) the trapQM high-resolution scan and 2) trapCAD activation followed by m/z analysis. The latter scan event, following database analysis, served to verify the charge state determined by the enhanced scan; CAD spectra were interrogated using offline database searching such that the identity and charge state of each sampled precursor was obtained. From these data the enhanced scan permitted charge state assignment 42% of the time and, of this 42%, ~60% of these were from precursors amenable to PTR (i.e., +2 charge, Fig. 4A). Although the enhanced scan only predicted useful charge state information for about 25% of the sampled peptides, the doubly and triply charged precursor assignments it did make were correctly assigned ~88% of the time (Fig. 4B).

The enhanced scan correctly predicted charge state information for over half of the doubly charged precursors identified; however, it struggled to identify higher charge states, generating significantly fewer predictions for the triply charged precursors and no predictions for the quadruply charged precursors (Fig. 4C). Performing this higher resolution scan event using a slower ‘zoom’ scan speed resulted in no improvement in charge-state identification and negatively affected both duty cycle (increased the scan time for each high resolution event by approximately 11 ms) and total peptide identifications (2,489 vs. 2,891 for the zoom and enhanced scan speeds, respectively). Peptides labeled with isobaric tags tend to ionize to higher charge states than non-labeled peptides (Fig. 4D), which means that precursors with three or more charges make up a significant portion of the sample. The lack of charge state predictions for triply and quadruply charged species was too prevalent to completely ignore. Therefore, the enhanced scan was mainly utilized as a screening tool; if no charge state was determined, the peptide was evaluated in two separate, consecutive quantitation scans which perform the analysis assuming both a triply and quadruply protonated precursor. Charge state was later confirmed during data processing using typical database searching algorithms to interrogate each identification scan.

Without the inclusion of the enhanced scan event for charge state determination, trapQM would require the incorporation of three separate quantitation scans, such that each precursor could be evaluated as a doubly, triply, and quadruply charged species. Integration of the enhanced scan into the trapQM scan sequence therefore greatly improves duty cycle by eliminating the need to quantitatively evaluate each possible precursor charge state. The benefit of this improved duty cycle is illustrated in Figure 4E, which presents a comparison of our trapQM method to a modified trapQM scan sequence composed of three quantitation scans (interrogated for +2, +3, and +4 precursors, respectively) and an identification scan. These data demonstrate that incorporation of the charge state determination scan into the trapQM method increases sampling depth, translating to an increased number of identifications (1,506 vs. 1,179, respectively).

Quantitation Scan(s)—Peptide quantification is achieved in the second trapQM scan event through the utilization of gas-phase purification via PTR. Precursor ions are subjected to PTR such that the production of species incurring a single proton loss is maximized and these first-generation charge-reduced products are isolated using the charge state information determined in the previous enhanced scan. The ‘purified’ precursor population is then fragmented with trapHCD activation at a collision energy optimal for tag generation. As mentioned above, in cases where charge state information was unavailable, two quantitation scans were performed: one which assumes a precursor charge state of +3 and one which assumes a precursor charge state of +4. Note these quantitation scans are performed over a narrow m/z range corresponding to the reporter tag region (m/z 110 – 140). The intent is to improve the duty cycle (the m/z scanning took ~ 1 ms on average).

Identification Scan—Peptide identification is achieved by use of a third scan event using trapHCD activation with collision energies that maximize the formation of sequence ions. In all cases, activation energy was scaled according to precursor charge state and m/z . When charge state information was unavailable, activation energy was scaled based on the user-defined default charge state of +3. We note that by decoupling reporter and sequence ion generation alternative dissociation methods such as trap CAD or ETD could be applied.

Evaluation of trapQuantMode using an Interference Model

To evaluate the ability of trapQM to remedy the problem of interference, we first tested the method using a mixed organism quantitative accuracy model designed to mimic a ‘worst-case’ interference scenario. A complex mixture of peptides generated upon digestion of yeast cell lysate with trypsin was labeled with TMT tags 126, 127, 128, 129, 130, and 131 and mixed in a 1:5:10:10:5:1 ratio, respectively. A complex mixture of peptides produced by digestion of human embryonic stem cell lysate with trypsin was labeled with TMT tags 129, 130, or 131 and mixed in a 1:1:1 ratio, respectively. To generate the mixed organism sample, the labeled yeast peptide sample was spiked with the labeled human peptide sample in a 2:1 stoichiometric ratio (this assured an equal amount of both species in each of the upper reporter channels, i.e., m/z 129–131). nanoLC-MS/MS analysis of the interference sample highlights the breakdown of quantitative accuracy which occurs in the reporter tag region when multiple species are co-isolated; any yeast-identified peptide containing human interference will contain skewed 5:1 and 10:1 ratios in the upper reporter channels while maintaining the correct 5:1 and 10:1 ratios in the lower reporter channels (i.e., m/z 126–128). For the purpose of our analysis, the lower reporter channels will be referred to as ‘control channels’; control channel ratios are determined by comparing tags 127:126 and 128:126 (5:1 and 10:1 ratios, respectively). Similarly, the upper reporter channels will be referred to as ‘interference channels’; interference channel ratios are determined by comparing tags 130:131 and 129:131 (5:1 and 10:1 ratios, respectively). Baseline quantitation was established through trapHCD analysis (data-dependent top 10, ddTop10) of our yeast control (i.e., our interference sample prior to the addition of human peptides). With no interfering species present, we observed 4.8:1 and 8.9:1 ratios in the control channels and 4.5:1 and 8.2:1 ratios in the interference channels. We attribute the deviation from the expected 5:1 and 10:1 ratios to sample preparation inconsistencies.

The interference sample, composed of both yeast and human peptides in the amounts specified above, was analyzed twice: once using only trapHCD (ddTop10, NCE60) and once using trapQM (as described). Data was filtered to provide quantitative results for only the yeast peptides identified in each experiment; outcomes are shown in Figure 5. The severity of the precursor interference problem is demonstrated by the truncated ratios observed in the trapHCD-only analysis. While control channels retained ratios of 4.9:1 and 9.2:1, interference channels displayed highly compressed ratios of 1.8:1 and 2.6:1. This translates

to a 3-fold underestimation of the 129:131 ratio and a 2.5-fold underestimation of the 130:131 ratio. Implementation of trapQM, however, recovered these diminished proportions. Again, control channels retained ratios of 4.9:1 and 9.2:1, but interference channels now displayed ratios of 3.0:1 and 4.9:1, numbers which are substantially closer to the respective 4.1:1 and 8.2:1 ratios observed in the yeast control. This marks a 28% and 27% improvement in quantitative accuracy for the 129:131 and 130:131 reporter tag ratios (32% to 60% and 40% to 67%), respectively, when comparing quantitative analyses conducted with and without trapQM. These improvements in quantitative accuracy, though substantial, are not as great as we routinely achieve using QM on a high-resolution system.⁶ We attribute this discrepancy to differences in the detection modes of the two mass analyzers – ion trap vs. orbitrap – and have observed, in general, a somewhat reduced dynamic range with trap detection (data not shown). This phenomenon is the subject of current investigation in our laboratory; that said, quantitative accuracy is significantly improved by use of gas-phase purification.

Doubtless the trapQM scan cycle provides substantial improvements in quantitative accuracy; however, it does so at the slight expense of peptide and protein identifications. The original QM can be executed in a single scan event, but relies on high-resolution MS¹ scanning. Adaptation of this approach to lower resolution systems requires multiple scan events, as detailed above, resulting in a significantly reduced duty cycle (compared to conventional scanning); this translates to a 53% loss in peptide identifications and a 37% loss in protein identifications. In contrast, QM execution on high-resolution systems only incurred a 21% loss in peptide identifications when compared to control (HCD, ddtop10) experiments.⁶ We conclude that the most viable option for the compensation of this protein and peptide identification loss is performing trapQM analysis on a single sample multiple times to increase sampling depth. The trapQM scan sequence does not affect identification sensitivity as that scan is disparate from both the charge state determination and purification events. By increasing the number of experiments performed on a single sample, we can achieve similar depth to control experiments while drastically improving quantification. To examine whether the boost in quantitative accuracy that is achieved with trapQM is worth the added analysis time we evaluated the ability of both trapQM and trapHCD (control) to detect differences in a real biological sample (see below).

Evaluation of trapQuantMode using C2C12 myogenesis sample

The mixed organism model experiments document improvements in quantitative accuracy when using trapQM in a ‘worst case’ interference scenario; however, we next sought to determine if trapQM could improve analysis of large-scale, complex biological samples.

The differentiation of mouse-derived C2C12 myoblasts has been extensively studied as a model system for the development and interaction of skeletal muscle myocytes.^{28–32} Over the course of six days, C2C12 myoblasts undergo myogenic differentiation to form myotubes, and this development process is accompanied by dynamic changes in protein expression (Fig. 6A). In recent years, quantitative mass spectrometry methods, such as spectral counting²⁸ and SILAC³², have been utilized to investigate these myogenic protein dynamics. All studies find significant changes in the presence of metabolic and structural proteins during various stages of the differentiation process.

To validate the improved quantification obtained by combining isobaric tagging with trapQM, we employed trapQM to evaluate relative protein levels present in the myogenic cells at day 0 and day 6 of the differentiation process. Myoblast (day 0) and myotube (day 6) cells were separately harvested, lysed, digested, and split into three equal mass aliquots. Each myoblast aliquot was separately labeled with TMT 6-plex tags 126 to 128 *m/z* while each myotube aliquot was separately labeled with TMT 6-plex tags 129 – 131 *m/z*. All

aliquots were combined in equal mass ratios; the resulting sample was fractionated by strong cation exchange chromatography (SCX) and analyzed using both trapHCD and trapQM methods. Figure 6 presents the results of this analysis. As was observed in the interference experiments, trapHCD analyses identified a greater overall number of proteins than trapQM analyses (4,050 vs. 2,964, respectively). Both data sets contained a similar percentage of quantifiable identifications (94.4% and 91.9%, respectively). We note this data provides evidence of the high efficiency of the PTR reaction as the primary determinant of “quantifiable” is sufficient reporter ion signal-to-noise. The main goal of the study was to profile differences in the proteomes between the myoblast (Day 0) to the myotube (Day 6) stages in C2C12 differentiation. Using trapQM, we identified 15% more proteins associated with significant changes greater than 1.5-fold than we did using trapHCD (1,362 vs 1,132, respectively; $P < 0.05$, Student's *t*-test) (Fig. 6B). Improvements in dynamic range only become more substantial as trapQM and trapHCD identifications were compared at higher fold-changes; overall, trapQM identified significantly more proteins associated with changes greater than 2-fold (766 vs. 438) and changes greater than 5-fold (96 vs. 12) (Fig. 6B). Given the 1,332 differentially expressed proteins detected in both sets of experiments, greater significant fold-changes were discovered, on average, when proteins were analyzed using trapQM (Fig. 6C).

To further assess the advantages of trapQM over trapHCD, we used data from each method to evaluate abundance changes in electron transport chain (ETC) protein subunits following myoblast differentiation. The ETC is a series of four multi-subunit complexes (complexes I–IV) that harnesses energy from a series of redox reactions to establish an electrochemical proton gradient across the inner mitochondrial membrane. Consistent with a recent study that analyzed ETC subunit abundance during myoblast differentiation using immunoblotting³³, we observed an overall increase in complex I subunit levels with both trapHCD and trapQM (Fig. 6D). When quantified using trapQM, complex I subunits were consistently observed to increase by approximately 2-fold. We also observed a marked increase in complex III subunits when using trapQM. Interestingly, an increase in complex I, and a corresponding increase in complex I-derived reactive oxygen species (ROS), is critical for promoting myoblast differentiation³³. As complex III is also a major site of ROS generation in the mitochondria, our data suggests that complex III-derived ROS will likewise be an important player in signaling during muscle differentiation. Using Western blot, we confirmed the increased fold-changes of ETC proteins from all four subunit complexes (Fig. 6D). The trends discovered by our trapHCD analysis were not nearly as pronounced in our trapQM analyses, evincing that, if left unchecked, the problem of precursor co-isolation will mask important biological trends.

trapQM was also proficient at identifying important decreases in subunits of the COP9 signalosome complex, which were largely missed when using trapHCD (Fig. 6E). The COP9 signalosome is a complex of eight protein subunits and is an important regulator of ubiquitination, protein turnover, and cell proliferation.³⁴ We detected all eight subunits using both trapQM and trapHCD. With trapQM we detected statistically significant decreases in fold change for five of the subunits compared to only one such decrease when using trapHCD ($P < 0.05$, Student's *t*-test). This result demonstrates the power of trapQM to improve both quantitative accuracy and dynamic range, as the overall decrease in the subunits of the COP9 signalosome would not have been detected with trapHCD alone.

Conclusions

We have developed a method for stand-alone ion traps (termed trapQuantMode, trapQM) which markedly improves the quantitative accuracy and dynamic range achievable on low-resolution MS instrumentation for isobaric tag-based quantitative analyses. This is

accomplished through the use of gas-phase purification, which greatly mitigates the prevalent problem of precursor interference. We further demonstrate that segmenting the trapQM scan sequence enables the generation of both optimal reporter ions and optimal sequencing ions, producing the most informative data possible for the quantitation and identification of each peptide precursor sampled. Up to this point, methods enabling the elimination of precursor interference for the improvement of isobaric tag-based quantification have only been developed for high-resolution instrumentation. With the development of trapQM, this functionality has now been extended to low-resolution instruments, providing a more accessible solution to the problem of precursor interference.

The utility of trapQM in the quantitative analysis of biological samples is demonstrated by our mouse-derived C2C12 myogenesis study. Using trapQM we find many significant changes – changes that are not detected using standard methods – in the abundance of proteins involved in the metabolic and morphological changes that accompany myotube formation. More broadly, we demonstrate how our trapQM dataset can serve as a reliable resource for exploring key proteins and pathways important for muscle differentiation.

Although duty cycle limitations reduce protein identification rate, this loss is compensated by significant improvements in quantitative accuracy, precision, and dynamic range, which enable the trapQM method to elucidate protein fold-changes that were undetectable without purification. Accurate determination of relative protein abundance is crucial to advance our understanding of complex biological systems. With these data we conclude (1) that the elimination of precursor interference is paramount to achieving quantitative accuracy with isobaric tagging and (2) that gas-phase purification is both particularly effective for this purpose and accessible on low and high-resolution MS systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PTR-based gas-phase purification de-convolves co-isolated precursors. Overview of QuantMode method

Inset depicts a selected precursor and the corresponding isolation window. In addition to the target precursor, and its isotopic cluster, a contaminant (denoted with black circle) is co-isolated. The contaminant, however, is separated in m/z space following the brief gas-phase ion/ion reaction (bottom). Once separated, the charge-reduced precursor is re-isolated and, hence, purified.

Figure 2. Workflow for QuantMode implementation on a stand-alone ion trap (trapQM)
The elapsed scan times reported for each scan event represent an average compiled from scans associated with a peptide spectral match; data was collected from four separate trapQM interference experiments.

Figure 3. Optimal collision energies for quantification and identification of doubly- and triply-charged peptides

A) Effect of normalized collision energy (NCE) on reporter tag signal generation. The maximum median total reporter tag intensity is attained at 90 NCE. B) Effect of NCE on identification rate. The optimal number of target peptide spectral matches (PSMs) identified from the generated sequence ions at 1% FDR is achieved at an NCE of 70 for doubly-charged precursors and an NCE of 40 for triply-charged precursors.

Figure 4. Evaluation of the trapQM high resolution ('enhanced') scan event

A) Distribution of enhanced scan charge-state predictions, B) amount of doubly- and triply-charged precursors correctly predicted by the enhanced scan, and C) enhanced scan charge-state predictions for all doubly- and triply-charged precursors identified in the analysis of a TMT-labeled tryptic mouse digest. D) Charge-state distribution of TMT-labeled tryptic yeast peptides identified in a data-dependent top 10, trapHCD MS/MS experiment. E) Duty-cycle comparison of trapQM with a version of QM which has a scan cycle composed of three quantitation scans (interrogated for +2, +3, and +4 precursors, respectively) and an identification scan (trapHCD, NCE 50). Assessment conducted by comparing the number of

precursors sampled and the number of target yeast PSMs identified during quantitative accuracy model experiments.

Figure 5. trapQM improves quantitative accuracy over trapHCD

Boxplots display results for the trapHCD MS/MS analysis of the yeast standard (left), trapHCD MS/MS analysis of yeast peptides in the yeast/human interference sample (middle), or trapQM analysis of yeast peptides in the yeast/human interference sample (right) at (i) 4.5:1, and (ii) 8.2:1 ratios. Boxplots indicate the median (stripe), 25th – 75th percentile (interquartile range, box), 1.5 times the interquartile range (whiskers), the 1st and 99th percentiles (o symbols), and the minimum and maximum values (lines, when applicable). The number of quantified yeast proteins and peptide spectral matches (below) obtained in the experiment are provided in boxplot (i). Median ratios are displayed below boxplots (i) and (ii) in bold.

Figure 6. trapQM achieves greater quantitative dynamic range than trapHCD and reveals insight into the metabolic and morphological changes that occur following myotube differentiation

(A) Light microscopy images of undifferentiated C2C12 mouse myoblasts at day 0 and fully differentiated myotubes at day 6. (B) Comparison of proteins significantly changing greater than (i) 1.5-fold, (ii) 2-fold, and (iii) 5-fold in trapQM and trapHCD analyses ($p < 0.05$). (C) Fold-changes ($\log_2$ scale) measured by trapHCD (y-axis) and trapQM (x-axis) for C2C12 mouse peptides identified in both trapQM and trapHCD analyses. Slope of best-fit line (0.58) reflects the ability of trapQM to identify greater fold-changes than trapHCD (D) Comparison of fold changes in (i) electron transport chain subunits as quantified by trapQM

and trapHCD using MS and (ii) specific electron transport chain subunits as quantified by trapQM/trapHCD using MS and immunoblotting (6 biological replicates per time-point). (E) Comparison of fold changes in COP9 signalosome complex subunits as quantified by trapQM and trap HCD ($p < 0.05$, Student's two-tailed t-test) using MS.