Zeno MS/MS significantly improves quantification for iTRAQ reagent-labeled proteomic samples

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INTRODUCTION

Quantitative proteomics using isobaric labeling reagents, such as iTRAQ and TMT reagents, has been a workhorse for protein research since the commercialization of these reagents in 2003.[1] These labeling reagents consist of an amine labeling group to label peptides, a reporter group that fragments off to produce a low m/z ion and a balance group. Isotope labels are placed at strategic locations across the reporter and balance group, such that all reagents in the set have the same mass. As such, when peptides from different proteomic samples are labeled with each of the reagents, the same peptides will have the same precursor mass. During MS/MS analysis, the peptide will fragment to produce y- and b-ions that will enable identification of the peptide. In addition, the reporter and balance group fragment to create a set of low mass reporter ions, such that the areas of each of these ions will represent the relative abundance of that peptide across the proteomes studied.

The production of a rich MS/MS spectrum for peptide identification and the strong intensity of the reporter ions for quantification quality are key to the success of this technique. Zeno MS/MS on the ZenoTOF 7600 system delivers significant improvements in peptide and protein identifications due to the 5-6x increase in sensitivity of the peptide fragments.[2] The expected sensitivity gains in the reporter ion region is even higher, on the order of 10- to 20-fold.[3]

Here, the impact of Zeno MS/MS on the identification rates and quantification quality of an iTRAQ reagent proteomics experiment was assessed. Very high numbers of proteins were identified and 95% of proteins identified had quantitative information (Figure 1), confirming that Zeno MS/MS combined with iTRAQ reagent labeling is a robust approach for quantitative proteomics.

MATERIALS AND METHODS

Sample preparation: MCF-7 and A549 cell lysates were obtained from Rockland Immunochemicals. HeLa and K562 were obtained pre-digested from Thermo and SCIEX, respectively. Fifty µg of each were digested and labeled with 8plex iTRAQ reagents, according to standard manufacturing guidelines. K562 was labeled with reagents 113 and 117, HeLa with reagents 114 and 118, A549 with reagents 115 and 119 and MCF-7 with reagents 116 and 121. After mixing the labeled samples, the peptide mixtures were fractionated into 44 fractions using high-pH RP-HPLC, as previously described.[4]

Chromatography: Labeled peptide fractions (5% of each fraction) were separated using a Waters ACQUITY UPLC M-class system using a Phenomenex Kinetex XB-C18 (2.6 µm, 100 Å, 150 x 0.3 mm) LC column. A rapid linear gradient from 5 to 30% B over 21 minutes with a flow rate of 6 µL/min was used. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid.

Mass spectrometry: A ZenoTOF 7600 system equipped with the OptiFlow Turbo V ion source[5] using a low microflow probe and electrode was used for all data acquisition. Data-dependent acquisition (DDA) parameters included a TOF MS accumulation time of 250 ms and an MS/MS accumulation time of 10 ms. The maximum number of candidate ions per cycle was 45 and exclusion time was 6 seconds.

Data processing: Data were processed using an iTRAQ reagent application in OneOmics suite in the SCIEX cloud, which uses the ProteinPilot application multi-file search. Analysis of quantitative data was performed using the ProteinPilot software report.[6,7]

IMPROVEMENTS IN PROTEIN AND PEPTIDE IDENTIFICATIONS

After fractionation of the labeled samples, the fractions were analyzed using DDA on the ZenoTOF 7600 system, with or without the Zeno trap enabled. Data were processed using the ProteinPilot application in OneOmics suite.

When the Zeno trap was activated in the acquisition method, the MS/MS sensitivity increased, leading to the identification of 6,505 proteins and 88,411 peptides at a 1% global FDR rate. The activation of the Zeno trap resulted in the identification of approximately 24% more proteins and 94% more peptides than without the Zeno trap (Figure 2). The improved quality in the MS/MS is highlighted in Figure 3, showing an approximate 6x increase in fragment ion signal and a greater than 10x increase in reporter ion signal. Peak resolution on the reporter ions was typically between 27,000-30,000, while the peptide fragment ions were typically greater than 35,000-40,000. Note that the MS/MS accumulation time was 20 ms, therefore permitting MS/MS to be acquired at 50 MS/MS per second.

Much higher peptide identification numbers led to a significant improvement in the protein sequence coverage across the identified proteins (Figure 4). This provides both more confidence in the identification of the protein and more peptide measurements that can be used for quantification.

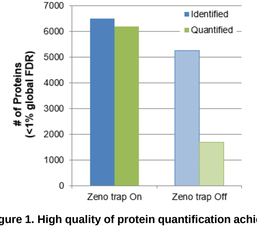

Figure 1. High quality of protein quantification achieved with Zeno MS/MS. Of the 6,505 proteins identified at <1% global FDR in the mixed proteome sample, 95% were quantified using the iTRAQ reagent reporter ions with <24 hours of data acquisition. Fewer proteins were identified and quantified without the Zeno trap enabled.

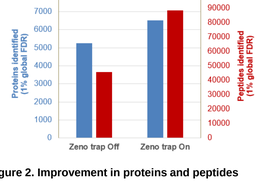

Figure 2. Improvement in proteins and peptides identified with Zeno MS/MS. The 44 fractions were run with DDA both with and without the Zeno trap enabled. Zeno trap activation provided approximately 24% more proteins (blue) and 94% more peptides (red) identified at <1% global FDR.

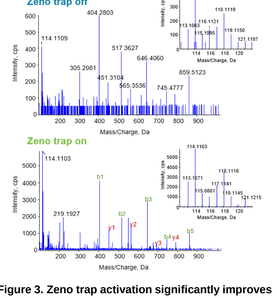

Figure 3. Zeno trap activation significantly improves MS/MS quality. MS/MS spectra for the VLEVNPQNK peptide from FKBP5 were compared without (top) and with (bottom) the Zeno trap enabled. The signals observed for the peptide fragment ions and the reporter ions were significantly improved using Zeno MS/MS.

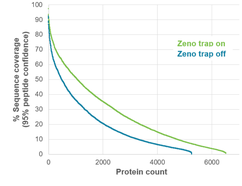

Figure 4. Protein sequence coverage. The large increase in peptides identified resulted in much higher sequence coverage across the measured proteins when the Zeno trap was enabled (green) vs. disabled (blue). Plot was generated from the peptides identified at 95% peptide confidence or greater but was not filtered based on quantitative annotations.

SIGNIFICANT INCREASE IN iTRAQ REAGENT REPORTER ION SIGNAL

To confirm the impact of Zeno MS/MS on the iTRAQ reagent reporter ions, the peak areas for all observed reporter ions across the acquired MS/MS spectra were measured and peak area histograms were plotted (Figure 5). This significant increase in reporter ion signal resulted in a large increase in the numbers of peptides that had good quantitative data and a very high percentage of identified proteins also having good quality quantitative data. Approximately 95% of proteins identified when the Zeno trap enabled were also quantified using the iTRAQ reagent reporter ions (Figure 1). Without the Zeno trap, however, only 32% of the proteins were quantified.

Here, K562 labeled with reagent 113 was set as the denominator and peptide ratios were measured by comparing the reporter ion area to the 113-peak area. K562 was also labeled with reagent 117 and the ratio of the peak areas for reagents 117/113 was approximately 1:1, as expected (0 on log scale, as shown in Figure 6).

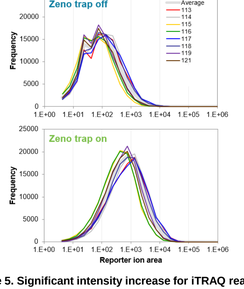

Figure 5. Significant intensity increase for iTRAQ reagent reporter ions with Zeno MS/MS. The intensity distributions for the reporter ions were plotted when the Zeno trap was disabled (top) and enabled (bottom). The median of the signal distribution increased approximately 10-fold for reporter ions when the Zeno trap was enabled.

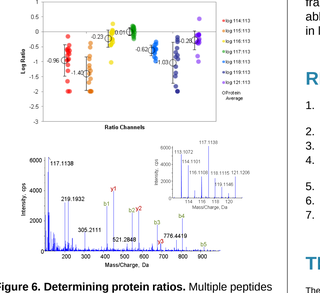

Figure 6. Determining protein ratios. Multiple peptides per protein were measured to determine protein quantification. Data for DNA topoisomerase 1 is shown (top). An example peptide [IT8]-LEEQLMK[IT8] from TOP1 is shown (bottom), highlighting the high-quality peptide fragments and reporter ions.

FINDING SIGNIFICANT CHANGES

Finally, the protein ratios were assessed between the 2 experiments to determine how many proteins had statistically significant protein changes. Plotting data as a volcano plot (Figure 7) highlights the distribution of the quantitative data by plotting log fold changes versus log p-value. Using a p-value filter of 0.001 (-3 on log scale), 814 proteins were found to be differentially expressed between the cell lines in the dataset collected using with the Zeno trap enabled. This is significantly higher than the 131 proteins that were significantly differentially expressed in dataset collected without the Zeno trap.

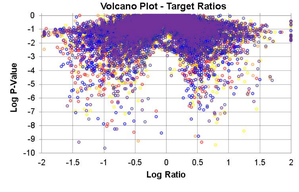

Figure 7. Differential proteins between cell lines. The volcano plot highlights the proteins measured with larger fold-change ratios and significant p-values from data collected with the Zeno trap enabled. Using a p-value filter of 0.001, 814 differential proteins were found with the Zeno trap enabled, while only 131 were found with the Zeno trap disabled. The different colors represent the different reporter ion ratios.

CONCLUSIONS

Differential expression analysis using the iTRAQ reagents requires the identification of large numbers of peptides and high sensitivity in the low m/z region of the MS/MS spectrum for high-quality quantification. Zeno MS/MS provides large improvements in MS/MS sensitivity, with 5-6x signal gains for peptide fragments and approximately 10x signal gains for the reporter ions. This increased sensitivity improved the number of proteins identified by approximately 24% and the number of peptides by 94%, when comparing a large-scale fractionation study collected with and without the Zeno trap enabled. Most of the identified proteins (95%) were able to be quantified, providing a rich protein expression dataset, consisting of 6,186 proteins, that was acquired in less than 24 hours.

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