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Leveraging the extended instrument capabilities of a Tribrid MS using Real-time PTM localization

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ABSTRACT

Purpose: To explore the utility of PTM localization confidence estimation during Real Time Search (RTS) as a means of guiding instrument method execution and the application of the extended capabilities of a modified Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ MS.

Methods: A custom build of the Tribrid™ Tune instrument control software was created to augment RTS scoring to include PTM localization confidence estimation. An enriched phosphopeptide sample was used to explore on-line decision-making using PTM localization confidence estimation, such as triggering orthogonal fragmentation chemistries.

Results: Predicating time-consuming fragmentation events on localization scoring allows us to maintain a high data quality while reducing instances of unnecessary supplemental fragmentation.

INTRODUCTION

Recent advances in mass spectrometry have demonstrated the utility of analyzing mass spectrometric data concurrently with data acquisition. As on-line method decision making becomes more sophisticated, we shift instrument activities away from deterministic or semi-stochastic towards more intelligent acquisition paradigms. The implementation of RTS has been largely focused on analyzing samples labeled with isobaric tag reagents, specifically for the selection of peptidic precursors and their associated fragment ions for SPS-MS3 quantitation. Herein, we explore the efficacy of augmenting the real-time identification of peptide analytes with PTM localization confidence estimation. During method acquisition, we use these site localization scores to guide acquisition logic, providing additional filters that control decision-making synchronously with peptide separation and generation of mass spectra.

MATERIALS AND METHODS

LC-MS Methods

For our initial testing, we used the ptmRS[1] algorithm for modification site localization. These localization scores were used in the method acquisition logic to regulate the triggering of additional fragmentation chemistries (EThcD vs. HCD).

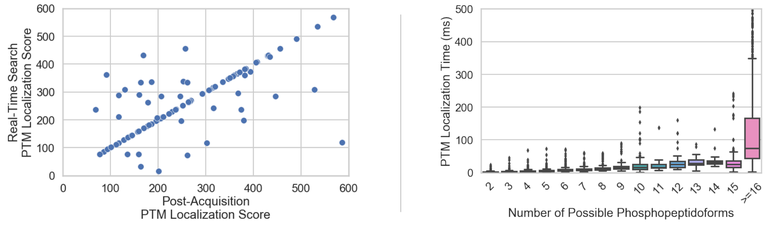
We compared a set of standard Orbitrap HCD MS2 acquisitions against runs utilizing real-time search and PTM localization confidence estimation. Orthogonal fragmentation by EThcD was executed when real-time search result identified a phosphopeptide in which the PTM localization score indicated a non-confidently localized phosphosite. Approximately 50ng of peptides were separated per injection on a two hour gradient with water with 0.1% formic acid as buffer A and 80% Acetonitrile with 0.1% formic acid as buffer B on a 75µM internal diameter, 15cm length IonOpticks Aurora column.

Data Analysis

Peptide spectrum matches were generated during RTS by the Comet search engine with a fragment mass tolerance of 0.02Da and allowing neutral losses for the variable modification phosphorylation. PSM false detection rates were assigned in real time (**MP 112, "Developments in Real-Time Search on an Orbitrap Tribrid mass spectrometer"**) PTM localization confidence estimation was carried out by the ptmRS algorithm. Peptides with an isoform confidence probability greater than 0.75 were considered as being confidently localized.

Post-acquisition analysis was performed in the Thermo Scientific™ Proteome Discoverer™ 2.4 software. Briefly, MS/MS spectra were identified by SequestHT database search. False detection rates were assessed by Percolator, and PTM localization confidence estimation performed by ptmRS utilizing the same threshold as during data acquisition.

Figure 1. Online PTM localization scoring produces similar scores as an offline analysis, with a median localization scoring time of 5ms.

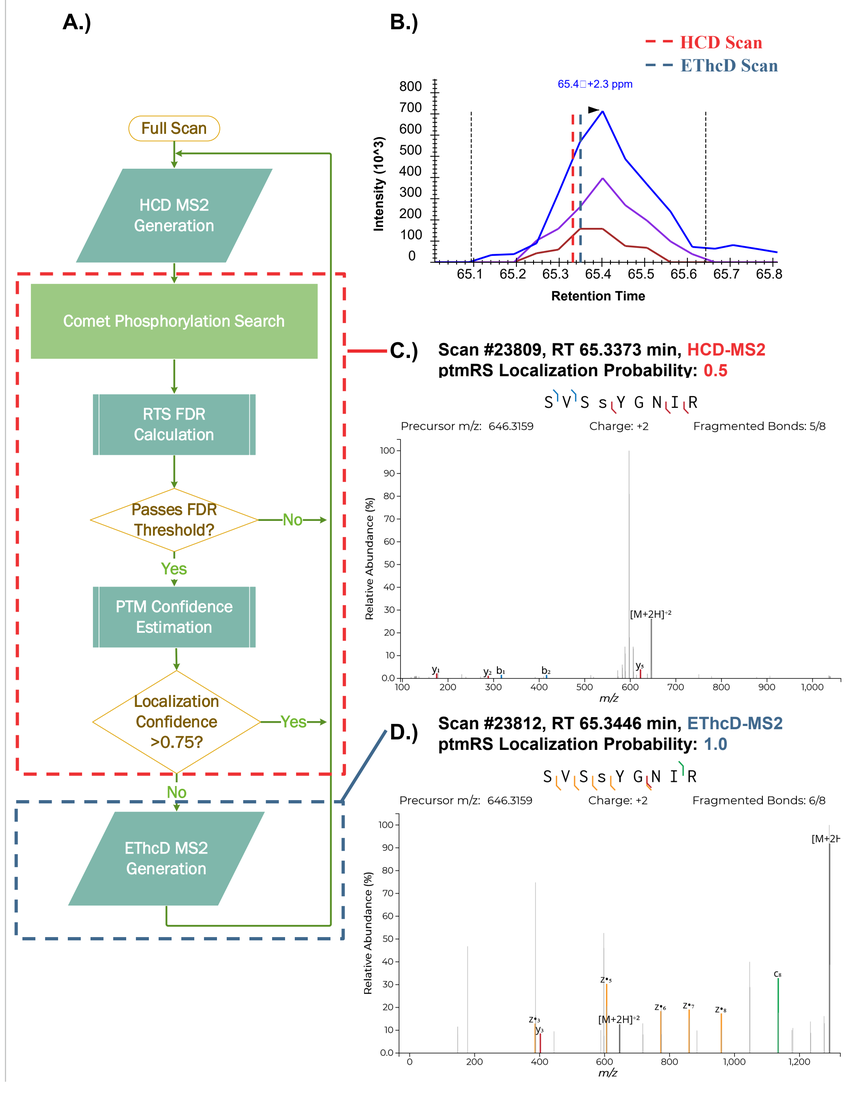


RESULTS

PTM Localization Acquisition Logic

Some modifications, like phosphorylation, are labile and can be difficult to localize to a specific residue based solely on collisional activation fragmentation spectra. Alternative fragmentation methods, like electron transfer dissociation (ETD), or a supplemental activation fragmentation like EThcD, are known to produce higher quality spectra that allow for easier site localization at the expense of a slower spectral acquisition rate. The triggering logic of our PTM localization method is explained in Figure 2.

Figure 2. Run logic during real-time PTM localization. A.) A simple decision tree displays the logic of the RTS-FDR-PTM Localization acquisition. B.) A chromatographic peak is detected and selected for fragmentation. C.) An example HCD-MS2 fails to confidently localize a phosphorylation event. D.) The triggered EThcD-MS2 confidently localizes the phospho-modification within the identified peptide sequence.



LC-MS Results

Figure 3. Identification of confidently localized phosphosites (left). EThcD PSMs account for the majority of the increase in confident phosphosite localization (right).

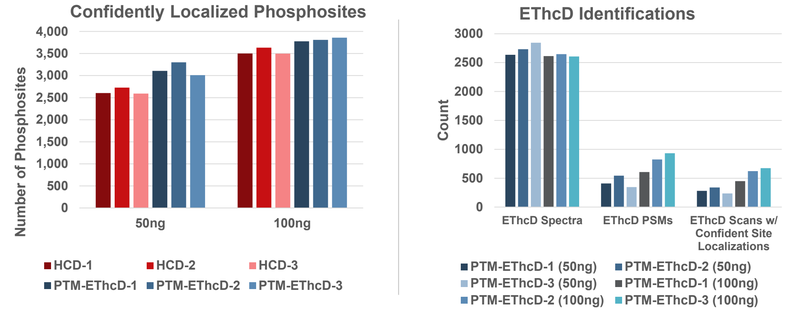


Table 1. Detailed acquisition and identification metrics from Proteome Discoverer analysis.

Run	Injection Mass	MS/MS	Total PSMs	Peptide Groups	Phospho-Sites
HCD-1	50ng	49,088	5,950	3,735	2,604
HCD-2	50ng	49,733	6,213	3,888	2,728
HCD-3	50ng	50,221	6,072	3,803	2,592
PTM-EThcD-1	50ng	43,128	6,875	4,207	3,107
PTM-EThcD-2	50ng	43,053	7,278	4,413	3,302
PTM-EThcD-3	50ng	42,100	6,624	4,065	3,010
HCD-1	100ng	55,198	8,347	4,854	3,502
HCD-2	100ng	55,690	8,371	4,986	3,634
HCD-3	100ng	55,351	8,384	4,815	3,499
PTM-EThcD-1	100ng	44,875	8,562	4,870	3,777
PTM-EThcD-2	100ng	45,294	8,619	4,871	3,810
PTM-EThcD-3	100ng	46,058	9,011	5,014	3,860

CONCLUSIONS

- Real-Time Search serves as an extensible base to facilitate a wide variety of decision-making processes during acquisition outside of SPS-MS3 fragment selection, like interrogation of PTMs.
- Estimating PTM localization confidence for PSMs synchronously with acquisition allows for triggering of alternative activation techniques to increase the number of localized phosphosites.
- At 50ng and 100ng loads, we observe an increase in confidently localized phosphosites when triggering EThcD on non-confidently localized, but confidently identified phosphopeptides in RTS.
- With increases of loaded peptide mass, triggering of EThcD may yield less advantage when compared to sampling alternate precursors by generating additional HCD scans (data not shown).

REFERENCES

1. Taus, T., et al. (2011). "Universal and confident phosphorylation site localization using phosphoRS." J Proteome Res 10(12): 5354-5362.
2. Brademan, D. R., et al. (2019). "Interactive Peptide Spectral Annotator: A Versatile Web-based Tool for Proteomic Applications." Mol Cell Proteomics 18(8 suppl 1): S193-S201.

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