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Developing the Research to Routine Workflows with FAIMS: Automating Large-scale SRM Method Creation for Routine HeLa Peptide Screening

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ABSTRACT

Purpose: To demonstrate a discovery to quantitation work flow for peptide panel using FAIMS.

Methods: Used high resolution data to pick peptides for proteins and converted them to an SRM table to be analyzed on a triple quadrupole mass spectrometer.

Results: Improvement of 95% of the transitions monitored was seen using the new FAIMS source which lead to lower limits of detection.

INTRODUCTION

Highly multiplexed protein panels are developed to enable routine sample screening while maintaining high throughput. The challenge to creating an analytically robust SRM method is determining which peptides to select per protein and creating the resulting SRM table for confident data acquisition. Each protein added to the target list increases total SRM count by 9 quickly causing acquisition challenges on triple quadrupole mass spectrometers as most proteotypic peptides cluster into small hydrophobicity groups. To increase the selectivity space, we have incorporated a novel source, field asymmetric waveform ion mobility spectrometry (FAIMS) interface for both profiling and screening to increase the selectivity metrics for an SRM method monitoring over 300 HeLa proteins in 60 minutes.

MATERIALS AND METHODS

Sample Preparation

A stock solution of Thermo Scientific™ Pierce™ HeLA Protein Digest was used for all experiments, injection 200 ng of HeLa. Pierce Retention Time Calibration (PRTC) mixture was spiked in at 5 fmol/uL.

LC/MS

HeLa proteome profiling was performed using an Thermo Scientific™ Easy-nLC 1200™, Thermo Scientific™ Orbitra tribrid™ mass spectrometer with a Thermo Scientific™ FAIMS™ Pro interface. A HeLa digest was injected and analyzed using a single compensation voltage (CV) setting by standard DDA methods and repeated for eight different CV settings. Each RAW file was processed to create a data matrix of proteins and peptides, retention time, CV, and precursor and product ion distribution profiles.

Data Analysis

A routine was created to construct a scheduled SRM table for the top 300 HeLa proteins using over 2500 SRM transitions. The SRM table was imported into a triple quadrupole mass spectrometer, Thermo Scientific™ TSQ Altis™ with the FAIMS Pro interface and evaluated for analytical performance.

Figure 1. **From left to right, the new FAIMS Pro interface, Easy Nano 1200, and TSQ Altis triple quadrupole mass spectrometer.**

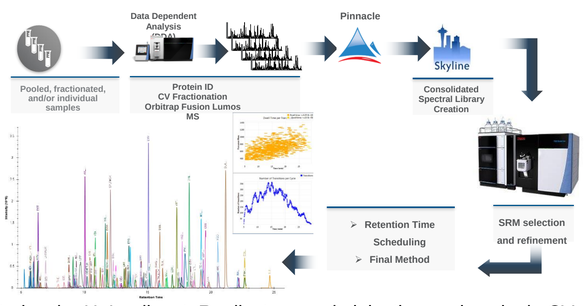


Figure 2. **Workflow of discovery to routine quantitation of a large peptide panel.**

RESULTS

Discovery HRAM Experiments

The discovery method was used to fully characterize the HeLa digest. Replicate sample injections using single CV settings significantly increases the protein coverage from 310 proteins without FAIMS to over 500 proteins with FAIMS. Example of the base peak is shown in Figure 3, demonstrating the improvement using FAIMS.

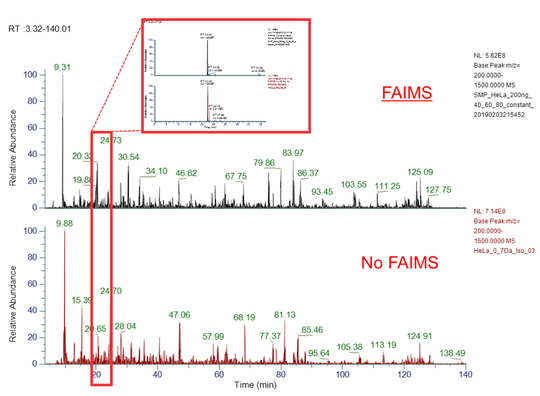


Figure 3. **HRAM spectra of the overall base peak chromatogram with and without FAIMS. The inset demonstrates a low level peptide gaining intensity with FAIMS**

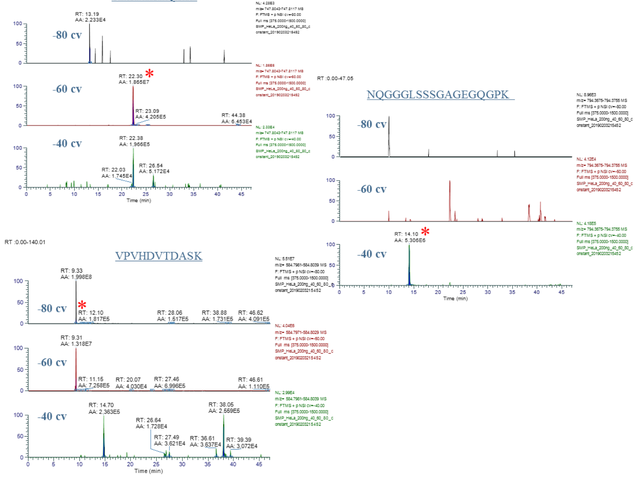


Figure 4. **Showing the importance of selecting the right compensation voltage for three different peptides. (*) representing the optimal CV.**

Optimization of compensation voltages is demonstrated in Figure 4 for three different peptides, showing the importance of using the correct CV for individual peptides.

Figure 5 is an example of the improvement of peptide fragmentation when using FAIMS.

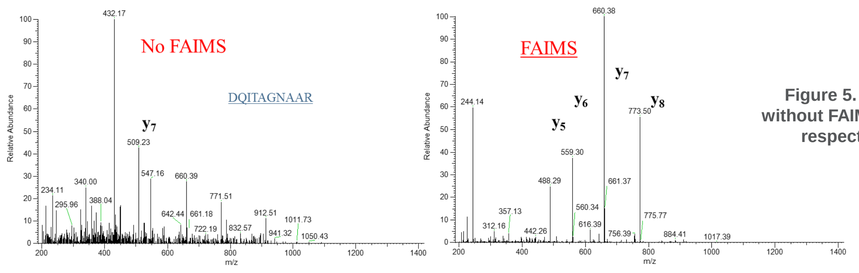


Figure 5. **Spectra of peptide DQITAGNAAR without FAIMS and with FAIMS, and the peptides respective –y ion fragmentation series.**

Peptide/Protein Selection – Pinnacle Software

Pinnacle software (Optys Tech Corporation) offers capabilities of searching routing (either spectral or sequence matching), new library creation, facilitating user-defined protein selections, establishment of peptide selection rules, and building the PRM/SRM assay. Example of the peptide selection is shown in Figure 6.

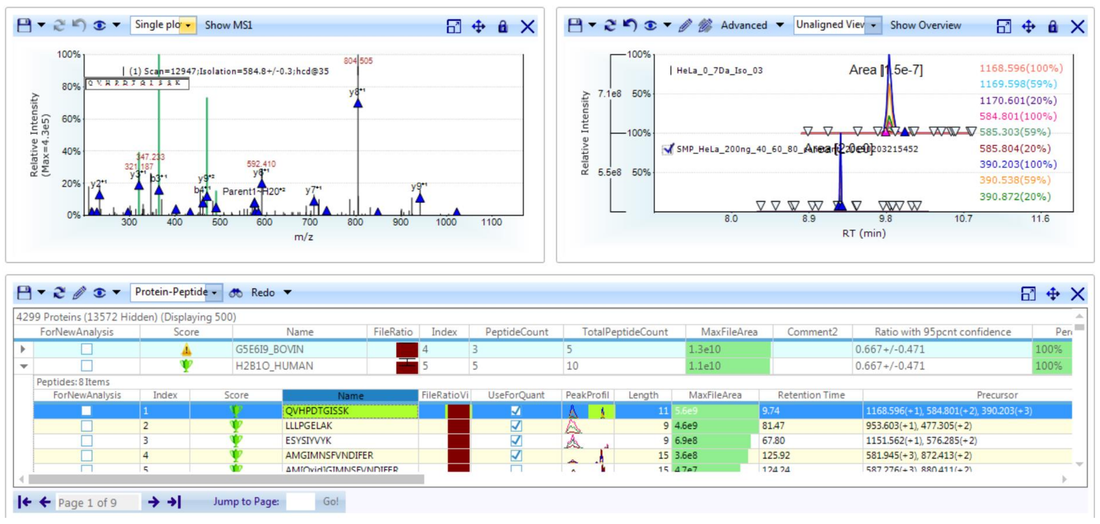


Figure 6. **Screenshot of workflow of the selection of proteins/peptides from Pinnacle.**

In addition, the resulting data are used to create a four-dimensional library that consists of the protein and corresponding peptides, and for each peptide, the measured retention time, CV setting, and optimal precursor m/z value and product ion distribution.

Targeted Experiments – Triple Quadrupole Mass Spectrometry

The addition of FAIMS enhances the selectivity and sensitivity of peptides, increasing the number of available peptides per targeted protein and resulting in more options to be considered in creating the scheduled SRM table.

The set of experiments was performed on a high-end triple quadrupole mass spectrometer capable of acquiring robust data with less than 5 msec dwell times per SRM transition and variable dwell time settings per SRM transition. The SRM table is presented in Figure 7. For each peptide one to three transitions were selected to monitor; this resulted in many transitions to be monitored. In order to determine if there was enough dwell time, a visualization tool in the method editor software was used to schedule windows, and is shown in Figure 8.

	Compound	Retention Time (min)	RT Window (min)	Precursor (m/z)	Product (m/z)	Collision Energy (V)	Min Dwell Time (ms)	FAIMS CV (V)
458	LNQMDQDK(+2)	24.3	1.5	496.229	878.367	19.2	78.876	-80
459	NEISEMNR(+2)	24.3	1.5	496.726	878.404	19.2	78.876	-80
460	EAQSIC[+57.0214	24.37	1.5	496.726	577.276	19.2	78.876	-80
461	EAQSIC[+57.0214	24.37	1.5	496.726	664.308	19.2	78.876	-80
462	DSHGVAQVR(+2)	25.06	1.5	484.749	567.252	18.8	156.627	-50
463	DSHGVAQVR(+2)	25.06	1.5	484.749	572.351	18.8	156.627	-50
464	DSHGVAQVR(+2)	25.06	1.5	484.749	794.379	18.8	156.627	-50
465	TC[+57.021464]VA	25.27	1.5	732.298	1103.442	27.2	156.627	-50
466	IAEMETQK(+2)	25.44	1.5	475.236	505.262	18.5	156.627	-80
467	IAEMETQK(+2)	25.44	1.5	475.236	636.302	18.5	156.627	-80
468	IHNAENIQPGEQK(	26.08	1.5	493.251	460.733	16.1	242.036	-80
469	IHNAENIQPGEQK(	26.08	1.5	493.251	679.316	16.1	242.036	-80
470	EEEEGKEEDEIK(+2	28.59	1.5	732.322	1204.449	27.2	209.371	-55
471	EEEEGKEEDEIK(+2	28.59	1.5	732.322	1205.553	27.2	209.371	-55
472	HLTHAQSTLDAK(+	29	1.5	661.346	970.495	24.8	209.371	-50
473	VDC[+57.021464]T	29.25	1.5	699.292	1137.43	26.1	209.371	-60
474	VDC[+57.021464]T	29.25	1.5	699.292	1183.483	26.1	209.371	-60
475	NPQQQESLK(+2)	29.31	1.5	536.275	604.33	20.5	209.371	-80
476	NPQQQESLK(+2)	29.31	1.5	536.275	725.321	20.5	209.371	-80
477	AIQGGTSHHLGQN	29.44	1.5	561.285	793.42	18.1	209.371	-80
478	AIQGGTSHHLGQN	29.44	1.5	561.285	1067.538	18.1	209.371	-80

Figure 7. **SRM table of peptides targeted in HeLa analysis. Optimized CVs are represented in the far right column.**

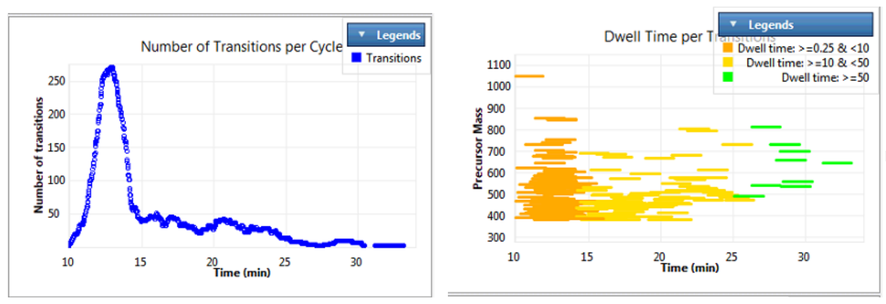


Figure 8. **Visualization tool with the method editor showing the number of transitions per time as well as the dwell times of different precursor masses over the chromatographic timescale.**

For the different protein groups targeted, the optimal peptides were selected based on relative response in the discovery method, but more importantly on the retention time and CV setting as the two values were used to create the final SRM table. Peptides were grouped into overlapping retention time and CV bins to maximize duty cycle while maintaining analytical performance. FIG. 7 depicts the variation of transmitted ion abundance with CV value for two peptides.

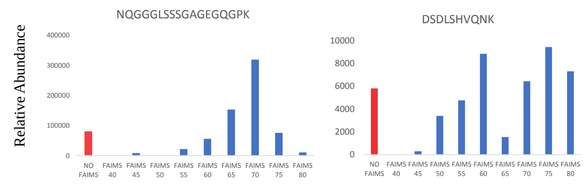


Figure 7. **Comparison of peak area of no FAIMS, red, to different compensation voltages highlighted in blue.**

PRTC, a well known heavy labeled peptide standard was used to show overall intensity improvement using FAIMS and the results can bee seen in Figure 8. The standard was than made into a dilution series to study if linearity or lower limit of detection can be obtained using FAIMS. Figure 8 illustrates that the linearity is not affected by FAIMS, and Figure 9 demonstrates the ability to reach a lower LLOQ because of diminishing interferences in the quadrupole's isolation window.

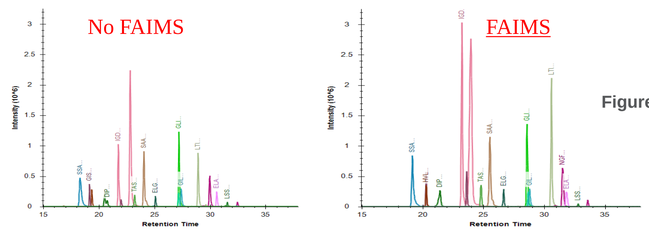


Figure 8. **PRTC total ion current of peak intensity with and without FAIMS.**

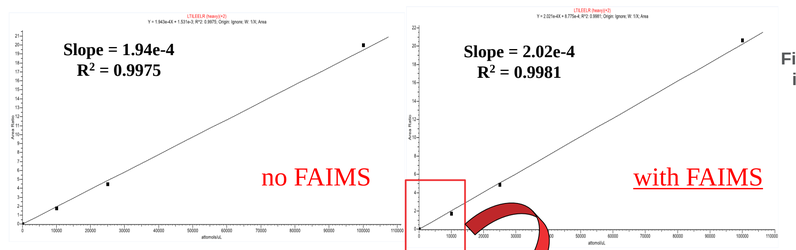


Figure 9. **Peptide LTILEELR (heavy labeled) spiked into HeLa in concentration range 1 attomol/uL – 100 fmol/uL linearity remains consistent with FAIMS across linear range.**

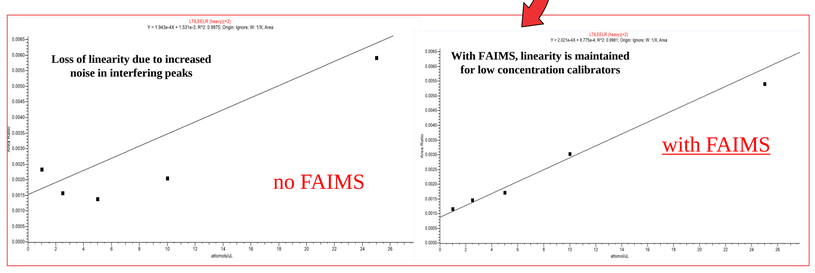
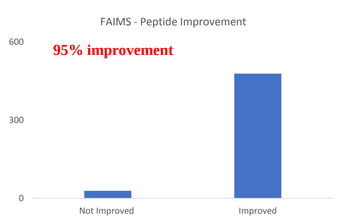


Figure 10. **Peptide LTILEELR (heavy labeled) spiked into HeLa in concentration range 1 attomol/uL – 1 fmol/uL at low concentration interferences are present and with FAIMS the linearity is improved at lower concentrations.**

Of the 150 proteins monitored there was 95% improvement of peptide signal, with varying results of improvement.



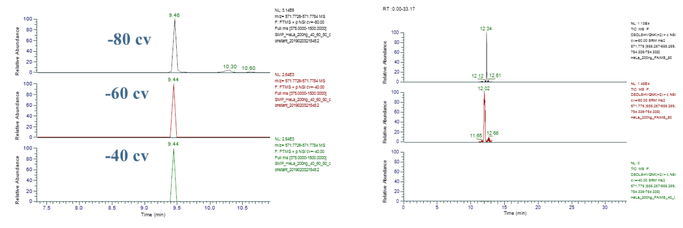


Figure 13. **Consistency of source and instrumentation. The figure to the left is HRAM data at different compensation voltage, whereas the right figure is compensation voltages on a triple quadrupole. The same CV is ideal for this peptide on both instruments**

CONCLUSIONS

- Field asymmetric waveform ion mobility spectrometry (FAIMS) can be used in a discovery environment to determine a peptide screening panel. These peptides can be put into a targeted panel to be monitored by a triple quadrupole mass spectrometer. The use of the FAIMS Pro interface increases signal-to-noise of these peptides.
- Different compensation voltages can be run early in the workflow process to determine the optimal voltage this is than translated to the targeted panel without additional optimization.
- Due to the improvement of signal-to-noise, linearity of calibration curves is improved and lower LLOQs can be obtained.
- Further analysis will be done to inquire on looking at the number of transitions per peptide and determining what protein expression levels can be monitored

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TRADEMARKS/LICENSING

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