**Introduction**

The data-independent acquisition (DIA) approach has been recently introduced as a novel mass spectrometric method that promises to combine the high content aspect of shotgun proteomics with the reproducibility and precision of selected reaction monitoring. Here we evaluate, the targeted identification of peptides with post translational modifications in SWATH-MS type DIA. Differences in success of identifying modified peptides from unmodified peptides could be reflected by differences in iRT retention time precision (Escher et al.) and coefficient of variation.

**Experimental Summary**

HeLa cells were prepared using Biognosys’ Sample Preparation Kit according to manufacturers’ specifications. The samples were desalted using C18 macrospin columns from The Nest Group. The Biognosys’ HRM Calibration Kit, was added to all of the samples according to manufacturer’s instructions (required for the DIA analysis using Biognosys Spectronaut).

**Mass spectrometry**

1 µg of the samples was analyzed on a self-made analytical column (75 µm x 30 cm) packed with 3 µm Magic C18AQ (Bruker) at 50°C using an Easy-nLC II connected to a Q Exactive mass spectrometer (Thermo Scientific). The peptides were separated by a 2 h linear gradient from 5 to 35 % ACN with 0.1 % formic acid at 300 nL/min. For DDA acquisition, the “fast” method from Kelstrup was used with the following alterations (Kelstrup et al.). The HRM DIA method consisted of a survey scan at 75,000 resolution from 400 to 1,220 m/z (AGC target of 5×10⁵ or 120 ms injection time). Then, 19 DIA windows were acquired at 35,000 resolution (AGC target 3×10⁵ and auto for injection time).

**Data Analysis**

The DIA data were analyzed with Spectronaut, a mass spectrometer vendor independent software from Biognosys. The default settings were used for the Spectronaut search. To generate a spectral library, DDA spectra were analyzed with the MaxQuant analysis software using default settings, using a searched a human UniProt fasta database. As variable modifications were used Oxidation of Methionine, protein-N terminal acetylation and deamidation of asparagine. The identifications were filtered to satisfy a FDR of 1 % on peptide and protein level. The spectral library was generated using Spectronaut. To select 1000 peptides with a intensity distribution, peptides were binned by intensity and then a selected range of 20 bins 50 peptides were sampled randomly. This was performed for unmodified and modified peptides separately for the comparisons.

**Figure 1: Comparison of elution of a peptide in its unmodified and modified form**

Extracted ion chromatograms are shown for two peptides in unmodified form and with post translational modification using Spectronaut. The upper graph shows the extracted ion current of the fragments. The lower graphs show the expected retention time stemming from the spectral library as determined with IRT. The green area indicates the integration area for the peaks. Note the coelution of the MS1 and the MS2 signals.

**Figure 2: Retention time analysis of peptides in modified and unmodified form**

To analyze the difference of retention time according to the expected retention time, a sample of 1000 unmodified and 1000 modified peptides were selected with a similar intensity distribution from HeLa HRM-DIA measurements. The difference was calculated in IRT (predicted from the spectral library - measured in DIA). One IRT unit represents about 0.5 min in the main linear part of the gradient. The mean and the standard deviation are shown. There is no significant difference between modified and unmodified peptides (p-value > 0.05).

**Figure 3: Coefficient on variation of unmodified and modified peptides**

The coefficients of variation of 1000 sample unmodified and 1000 modified peptides were analyzed from triplicate measurements of HeLa digests of HRM-DIA measurements. The graphs how the mean and the standard deviations. There is no significant difference.

**Figure 4: IRT difference of modified and unmodified peptides in relation to the HRM DIA IRT extraction window width**

Spectronaut performs a dynamic calculation of the required retention time extraction window in the HRM DIA measurements for optimal data analysis. For the spectral library used in this study, extraction window was below 4 min for a 2 h gradient. This is smaller than the retention time difference between the unmodified peptide and a Methionine oxidized peptide of about 7 min.

**Conclusions**

- The usage of high resolution MS1 full scans facilitates the targeted search for unmodified and modified peptides.
- Unmodified and modified peptides show no significant difference in IRT retention time of the spectral library compared to the retention time in IRT.
- Analysis of the coefficients of variation of modified and unmodified peptides shows no significant differences in quantification accuracy.
- This results strongly indicate, that the used DIA method containing high resolution MS1 and high resolution IRT is well suited for the analysis of post translational modifications of peptides in complex cellular digests.

**References**

- Escher C., Reiter L. et al. (2012) Using IRT, a normalized retention time formus for targeted measurement of peptides. Proteomics 12, 1111–1121
- Biognosys, Schlieren, Switzerland.