**INTRODUCTION**

Recent developments in LC-MS instrumentation have paved the way for the single cell revolution. Improvements at different stages in the workflow have pushed sensitivity to at least one order of magnitude and have leveraged automated workflows with near-real time sample processing. These developments are key to moving towards standardized workflows and large-scale single cell MS studies. From a separation point of view, operation of capillary bore LC columns at ultra-low flow rates yields the highest absolute sensitivity, but these workflows are inherently laborious in terms of throughput, instrument productivity and workflow robustness. In the current contribution, we present a workflow capable of delivering robust low-flow separation with minimal instrument overhead and excellent coverage for low input samples.

**MATERIALS AND METHODS**

**Sample Preparation**

Thermo Scientific™ Pers™ HeLa-Protein Dimer Standard was dissolved in 1% Acetic acid, 0.1% DMSO with 0.1% TFA to a concentration of 25 ng/µL for direct injection experiments and to 1 ng/µL for trap and elute experiments. Figure 1. Experimental setup.

**LC-MS settings and data processing**

Active solvent gradients of 6.12 and 32 min were evaluated at flow rates of 125 and 65 nL/min. Injection volumes were set between 10 and 200 nL. Electrospray ionization voltage was set at 1.9 kV. FAIMS was operated with a single capture voltage at 500 V. Blank measurements were taken at each concentration before and after the samples. MS data were collected in a data-dependent acquisition mode (TopDown) with full scan data collected at 125,000 resolution and 50 microscans. Quadrupole isolation width for MS2 acquisition was set at 5 Th. Maximum injection time (MaxIT) was set at 118 ms. The scan range used was 375-1050 m/z. Dynamic exclusion was set to 10 min with a 90 second duration. Fragmentation was performed using HCD with a fixed collision energy of 30. The acquired raw data files were processed with Thermo Scientific™ Proteome Discoverer™ search software with the CHIMERE75 intelligent search algorithm.

**RESULTS**

**Increased ionization efficiency at ultra low flow rates**

High throughput and maximum sensitivity can be combined by using variable flow rate methods with high flow rates (170 nL/min) applied during the first 2 min of the injection, and slow flow rates (125 or 65 nL/min) applied as soon as analytes start eluting. Ionization efficiency is significantly increased when reducing the eluting flow rate down to 125 and even further down to 65 nL/min (Figure 2). For low input proteomics experiments where precursor ion concentrations tend to fall below the limit of detection, this can have a great impact as more ions can be collected for fragmentation and identification.

By reducing the eluting flow rate from 125 to 65 nL/min, we were able to increase peptide coverage by 47 and 22% for respectively 12 and 32 min gradient sample. For bulk HeLa cell extract amounts considered to be equivalent to the amount present in a single cell (200 pg) up to 1000 protein groups could be identified on a standard nontarget LC-MS setup (no FAIMS) with 80% instrument productivity (Figure 3).

As reducing the eluting flow rate inherently results in some loss of chromatographic performance (Figure 3C), increases in precursor coverage are predominantly attributed to the increased in ionization efficiency. Moreover, using target enrichment in quantification (50% in 75% below CV 20) could potentially be attributed to this observation (increase in datapoints per peak).

**SELECTED PROTEIN SEQUENCES**

**Peptide groups**

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<th>Protein group ID</th>
<th>Peptide group area (A)</th>
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**CONCLUSIONS**

- Low flow rates (125-65 nL/min) can be used with FAIMS as noise levels for low input samples.
- Trap placement of the trapping column allows obtaining 80% instrument productivity with 32 min of active scan/100 nL flow rate elution.
- Increased ionization and instrument productivity can be increased by using dedicated low-flow trapping column.
- Below 15 min active gradient, trap and elute has the highest impact on instrument productivity.
- FAIMS can successfully be implemented at these low flow rates and increases peptide coverage even further.

**TRADEMARKS/LICENSEING**

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**REFERENCES**

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**Figure 1. Experimental setup.**

**Figure 2. Comparison of the basepeak traces obtained for the separation of 250 pg HeLa digest using 32 min gradient methods with respectively 125 and 65 nL/min as eluting flow rate.**

**Figure 3. DIRECT INJECTION results.**

- (A) Protein and peptide group ID’s
- (B) Quantified proteins
- (C) FAIMS distribution of eluting peptides

**Figure 4. Direct injection versus (A) Trap and Elute (B) configuration on Yamapush Neo. 12 min active gradient profile for 65 nL/min elution.**

**Figure 5.**

**A** DIRECT INJECTION results. (A) Protein and peptide group ID’s. (B) Quantified proteins. (C) FAIMS distribution of eluting peptides. **B** TRAP AND ELUTE results. **C** Protein and peptide group ID’s. **D** Quantified proteins. **E** FAIMS.