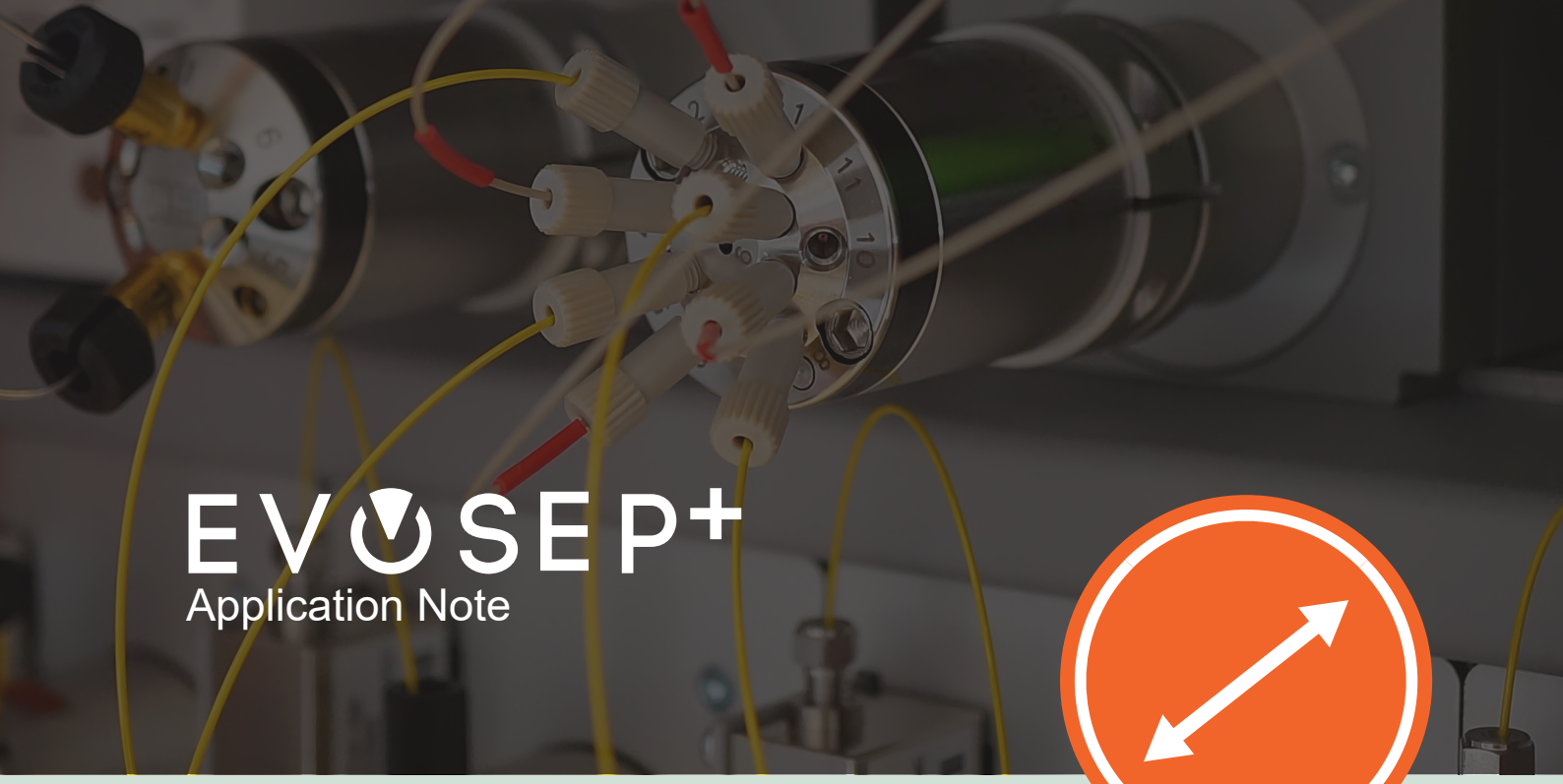

EVOSEP+

Application Note

# More proteome coverage in a single experiment with the **Extended method**

## 1. Introduction

We designed the Evosep One with the purpose of making clinical proteomics more robust and faster. The system uses four low-pressure syringe pumps in parallel to elute samples from a disposable and single use trap (Evotip) with a chromatographic gradient. The resulting gradient with the pre-separated sample is moved into a storage loop, that is then switched in-line with a single high-pressure pump and an analytical separation column for focusing. With this simplified workflow on pre-set gradients we offer a throughput from 30 to 300 samples per day. We have developed the Extended method to address the need for deeper proteome coverage in a single LC-MS experiment. The method has a 88 min gradient, equivalent to a throughput of a little more than 15 samples per day. This is possible due to the short 4 min overhead time between injections as the system in parallel with the LC-MS run is prepared for the next sample by ejecting the disposable trap column, washing the mixing cross and the ceramic needle, refilling the low-pressure pumps and aligning the solvents (Figure 1). The Extended method is the ideal choice for maximum proteome coverage in a single experiment, which is preferred, especially when the amount of starting material is limited. Reproducibility is a key aspect in clinical proteomics both in terms of limiting the technical variation between replica injections, but also the inevitable variation between instruments. In order to assess this, we tested our retention time reproducibility between Evosep instruments and expanded it with a pilot study from two different laboratories to measure the general performance of the Extended method independent of sample preparation method.


*Figure 1:* Stepwise overview of all steps performed by the Evosep One in the Extended method

## 2. Method design

The Extended method is designed for maximum proteome coverage in a single-shot analysis with a throughput of approx. 15 samples per day. The analytical column is initially equilibrated at 1.5 µl/min, while the peptides in parallel are eluted from the Evotip with a gradient from pumps A and B. Directly after, a secondary gradient from pumps C and D modifies the initial gradient and thus, reduces the effective organic content. With this offset gradient strategy, peptides eluting from the loop are shortly retained at the head of the column and thereby focused. This results in sharp peaks with the highest possible peak capacity. The high-pressure pump then pushes the preformed and offset gradient with pre-separated peptides over the analytical column with a flow of 220 nl/min (Figure 2). The method is optimized for a 150 µm ID x 15 cm analytical column packed with 1.9 µm beads (EV-1106), which we recommend to use at ambient temperature in combination with a stainless steel emitter (EV-1086).

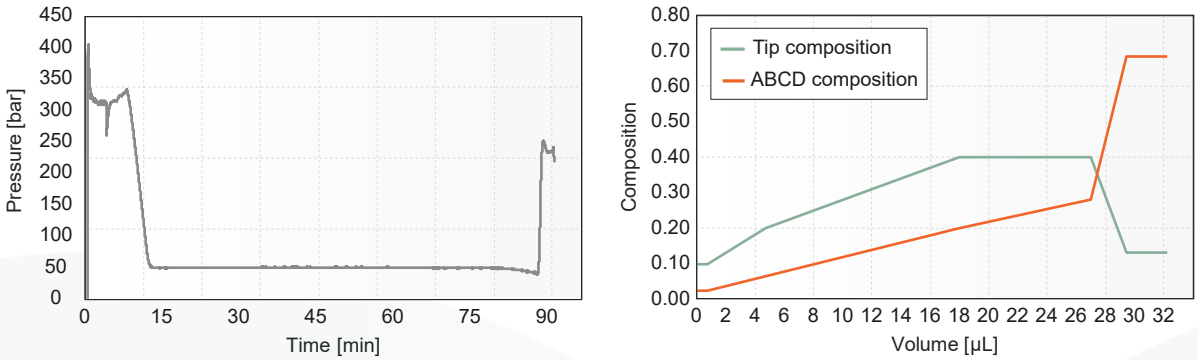


*Figure 2:* Pressure profile over the gradient and composition of the gradient. The x-axis designates the volume entering the storage loop and the proportion of acetonitrile is indicated on the y-axis.

## 3. Chromatographic reproducibility

To show high reproducibility across Evosep systems, we analyzed ten replica injections of BSA on three different instruments, while keeping the analytical column and mass spectrometer constant. We evaluated the sample to sample retention time reproducibility within each set of measurements. We extracted peaks of five different BSA peptides throughout the gradient and calculated the standard deviation of the retention time for each set of ten measurements. With an overall average standard deviation of 8 seconds, we observed a minimal retention time shift. The error bars represent the standard deviation of the three sets of measurements (Figure 3).

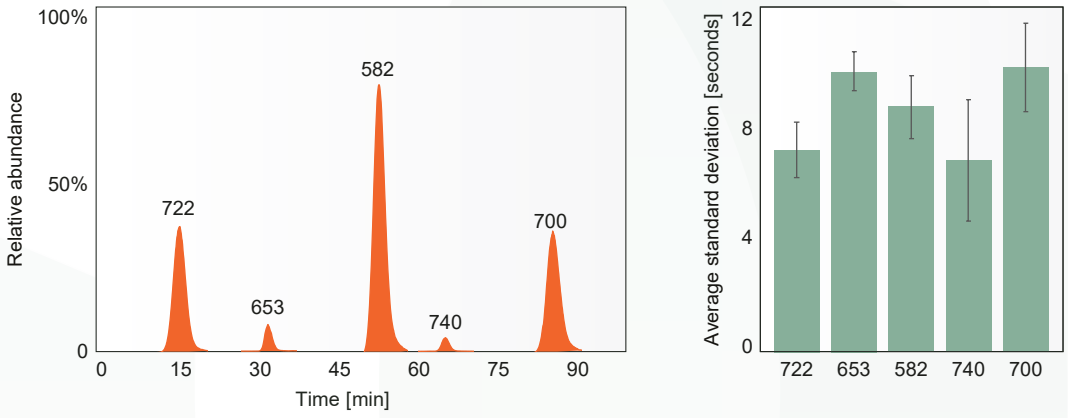


*Figure 3:* Extracted peaks from five different BSA peptides and average standard deviation of each set of measurements.

## 4. Multi-laboratory performance

In collaboration with two different laboratories, we evaluated the performance of the Extended method in terms of proteome coverage. With an aim to reflect everyday performance, each laboratory prepared a digested HeLa sample with their standard protocol. Peptides were diluted and loaded on Evotips and kept wet until analysis. To limit the comparison to the performance of the Extended separation method, we used an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific) in both laboratories. The MS instrument was operated in data-dependent acquisition mode using a Top12 method. Full MS resolution was set to 60,000 at m/z 200 and full MS AGC target value was 300% with an IT of 45 ms. Mass range was set to 350-1400. AGC target value for fragment spectra was set to 200% and the intensity threshold was kept at 2E5. Isolation width was set at 1.3 m/z and normalized collision energy was set at 30%. Peptide match was set to off, and isotope exclusion was on. Former target ions were dynamically excluded for selection for 30 sec. Higher-energy collision dissociation (HCD) fragment scans were acquired at 28 Hz speed with an injection time of 22 ms using an Orbitrap resolution of 15,000. The combined dataset was analyzed with the Spectromine software, version 2. The MS aquisition time was set to 88 minutes (Figure 4).

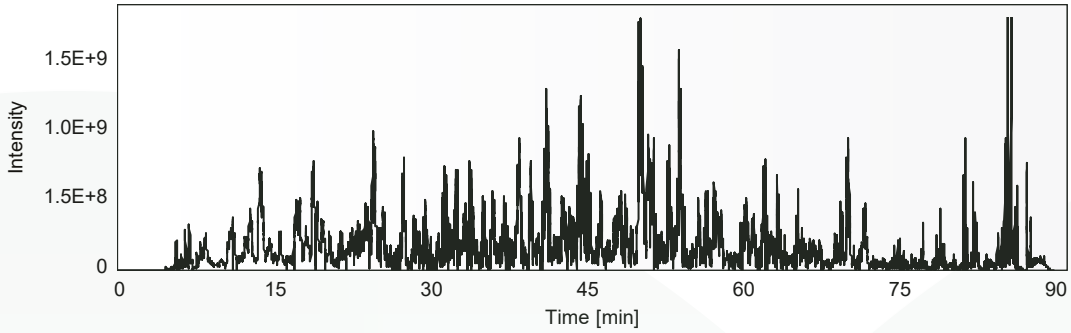


*Figure 4:* Base peak chromatogram from a sample loaded with 500 ng HeLa peptide.

To evaluate the performance and sensitivity of the Extended method, we analyzed three different loads ranging from 5 ng to 500 ng. The maximum load of 500 ng resulted in similar proteome coverage of ~35,000 unique peptides and close to 5000 proteins quantified in both laboratories, whereas the lower loads of 5 and 50 ng showed some performance difference, which is to be expected with different sample preparation methods for HeLa digestion. Importantly, the reproducibility between replica injections was excellent with a Pearson correlation of 0.95 (Figure 5) and the overall performance level was similar in both laboratories.

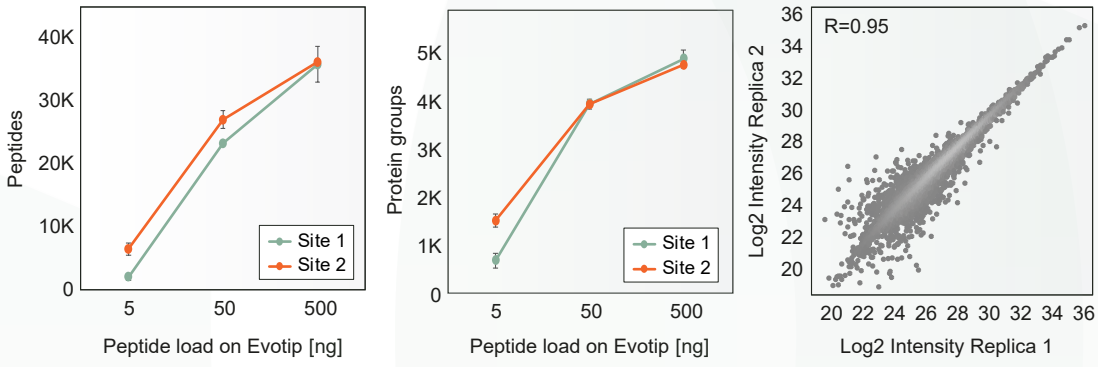


*Figure 5:* Identifications and correlation between replica injections of HeLa dilution series.

## 6. Conclusion

The Extended method is the latest addition to our toolbox. It is specifically designed for applications, where maximum proteome coverage is needed in a single-shot experiment, while maintaining the known Evosep advantages such as sensitivity, speed and robustness. We evaluated the sample to sample retention time reproducibility on different systems and found the overall standard deviation to be 8 seconds. We tested the performance of the method in two different laboratories and could routinely quantify around 5000 proteins from 500 ng of peptide load independent of the HeLa digestion method.

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### References


1. Bache N., Geyer PE., Bekker-Jensen DB., Hoerning O., Falkenby L., Treit PV., Doll S., Paron I., Müller JB., Meier F., Olsen JV., Vorm O., Mann M. (2018) A novel LC system embeds analytes in preformed gradients for rapid, ultra-robust proteomics. Mol Cell Proteomics., mcp.TIR118.000853


### Data courtesy


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### Adding the Extended method to the Evosep One

From the Windows Start menu, choose the Evosep One plugin.

Open the Change Evosep One applications and follow the installation procedure.

The Extended method is now available from the same menu as the standard methods.

EVOSEP