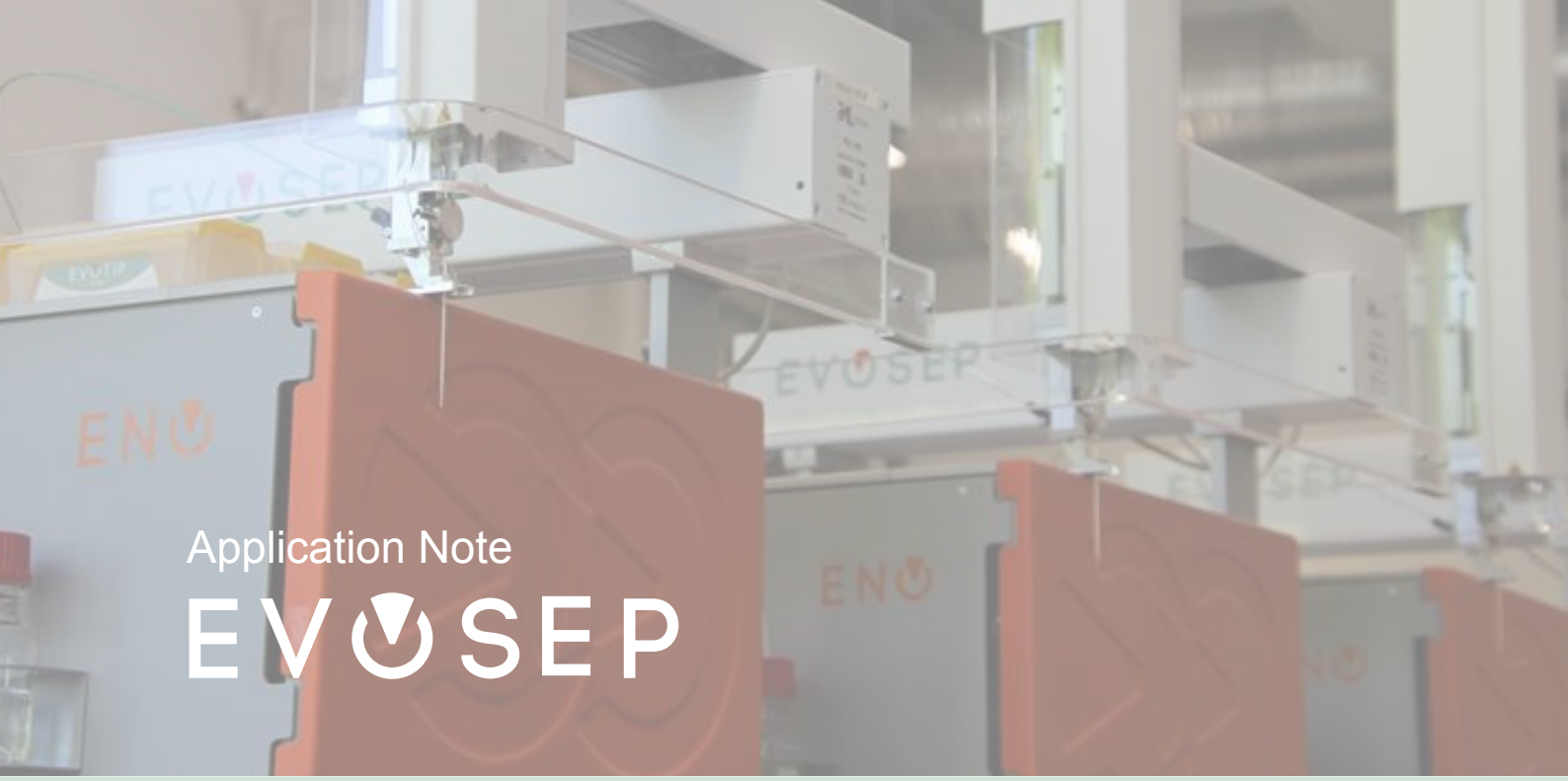

Application Note

EVOSEP

Reproducible, inter-laboratory, scalable and routine LC-MS based proteomics - **with the Evosep Eno**

Highlights

- Excellent inter-lab reproducibility of complete workflow from sample to MS.
- Consistent chromatographic performance across three different laboratories.

1. Introduction

As proteomics continues its rapid evolution from exploratory research into applied science, the demand for analytical platforms that deliver robustness and reproducibility at scale has never been greater. The Evosep Eno, designed with applied proteomics in mind, offers exceptional system stability, standardization, unmatched performance and ease-of-use. All of which are crucial requirements to accelerate the adoption of LC-MS technologies and pave the way to applied proteomics. Additionally, there is a need for scalable and standardized workflow solutions for continuous and robust operation.
To evaluate the inter-lab end-to-end performance, we conducted a comprehensive, multi-laboratory study assessing workflow sensitivity and reproducibility. Each lab used its own Opentrons® OT-2 liquid handler (Opentrons Labworks, Inc), Evosep Eno system, and Orbitrap Astral mass spectrometer (Thermo Scientific) with a locked-in MS method. This setup enabled us to capture the variability arising from differences in sample preparation protocols and individual LC-MS platforms.
The results demonstrated remarkably consistent sensitivity and reproducibility, with minimal variability between laboratories. Nonetheless, the study also revealed the potential for further standardization, as minor performance differences primarily attributed to different reagents and cell lysis conditions was used across the laboratories.

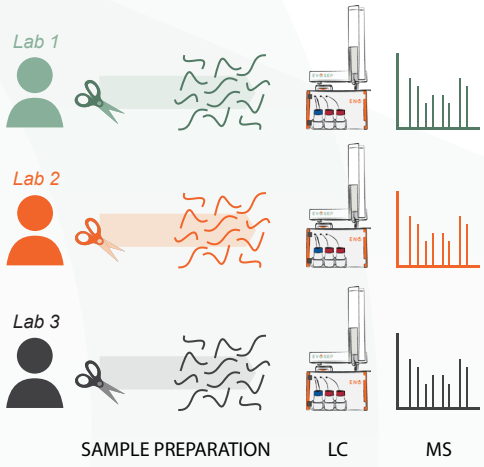

Figure 1: Standardized end-to-end workflows are key to unlocking applied proteomics.

2. Method details

The chromatographic reproducibility was assessed across three sites using the 300 SPD method by analyzing 200 ng of commercially available tryptic HeLa digest (Pierce). The 4 cm Performance column (EV1182, Evosep) was used at 40 °C using an Evosep Pod column oven (EV1187, Evosep), coupled to an EasyS-pray source. Samples were loaded from individual HeLa vials at the different sites.
The workflow studies were performed on Opentrons OT-2 liquid handlers located at the three sites. For information about how to set up the protocol, please find detailed step-by-step guides and scripts online at; www.evosep.com/-support/automation. Laboratory 1 and 2 used the same HeLa lysate, prepared from commercially available HeLa cell pellets (Ipracell, #CC-01-10-10), harvested in boiling 5% sodium dodecyl sylfate (SDS). Laboratory 3 used their own HeLa cells, harvested with the same protocol. Protein aggregation capture (PAC) assisted digestion was prepared from 1 µg HeLa lysate and 5 µl MagReSyn Hydroxyl (Resyn Biosciences). Digestion was carried out at ambient temperature for 4 hours using 10 ng Lys-C (Fujifilm) and 40 ng trypsin (Sigma Aldrich). 50% of the resulting digest was loaded on Evotips and analyzed with the 300 SPD method using the 4 cm Performance column (EV1182, Evosep), operated at 40 °C. All samples were analyzed on individual Orbitrap Astral mass spectrometers (Thermo Scientific). For all three instruments, spray voltage was set to 1900 V, and capillary temperature was heated to 275 °C. The mass spectrometers were operated using identical settings using full MS resolution of 240,000 with a full scan range of 380 − 980 m/z. The full MS AGC was set to 500%. MS/MS scans were recorded with 2 Th isolation window, 3 ms maximum ion injection time. Precursor mass range was from 380-980 m/z. Data was analyzed with DIA-NN (version 1.9.2) in library-free mode against the human proteome database (Uniprot, Oct 2020, 20,600 entries) with trypsin/P as protease allowing 2 missed cleavages.

3. LC-MS robustness across sites

To evaluate reproducibility of the Evosep Eno, we analyzed 6 replicate injections of 200 ng commercially available HeLa digest using the 300 SPD method across three independent sites. Each site used its own Orbitrap Astral mass spectrometer, running the same locked-in MS method. The Evosep Eno demonstrated highly robust chomatographic performance, with reproducible retention time and full width half maximum (FWHM) for five diagnostic peptides with low standard deviation across the three sites. Comparison of the summed precursor intensities revealed highly consistent results between the three sites with inter-site variation below 10% underscoring the robustness and reliability of the Evosep Eno across locations.

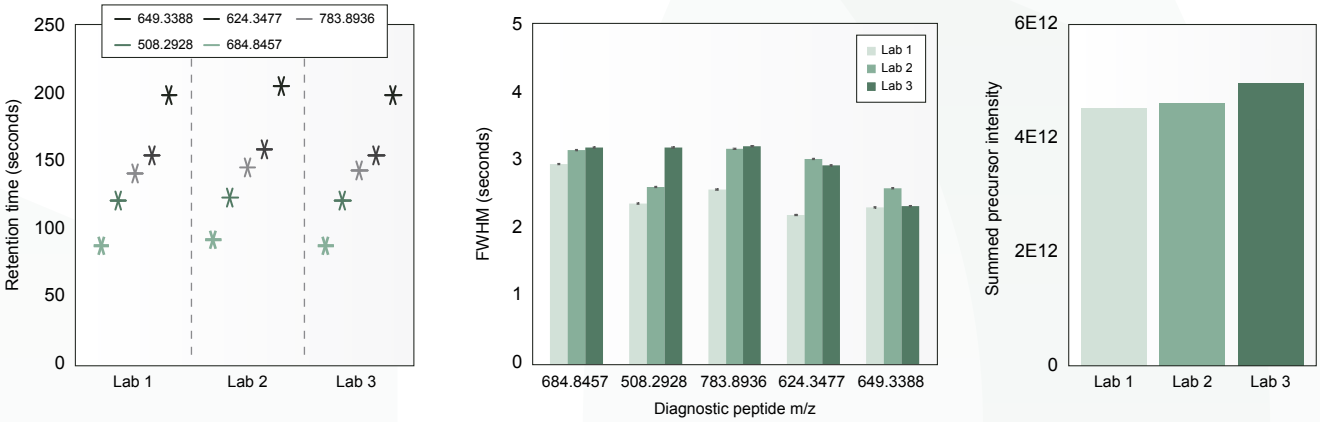

Figure 2: Average retention time and FWHM of five diagnostic peptides and summed precursor intensities from 200 ng HeLa Pierce samples analyzed with three different LC-MS setups.

4. Workflow reproducibility

In a controlled study, we assessed the effect of automated sample preparation using the PAC workflow and two different OT-2s. To evaluate LC-MS performance during the assessment, two sample plates were prepared simultaneously on a single OT-2 and analyzed at two independent laboratories (Figure 3). Additional variables such as input cell line, protein extraction conditions, operators, as well as source of proteolytic enzymes were kept constant at each site. Evotip blanks (EB), demonstrated less than 0.05% carryover calculated as relative precursor signal from parent sample, confirming that the Evotip Pure technology effectively protects the LC system and the analytical column. Plate blanks (PB) confirmed that the entire workflow was free from cross-well contamination (<0.20%) with minimal detectable signal in PBs attributed to trace amounts of enzymes and reagents introduced during the robotic processing steps.
The experiment also captured the variability introduced during the PAC workflow by different liquid handling robots, with coefficients of variation (CV) of 15.7% and 13.7%, respectively. Comparable CVs were obtained when identical samples were analyzed on two different LC-MS systems (13.1% and 14.1% respectively).

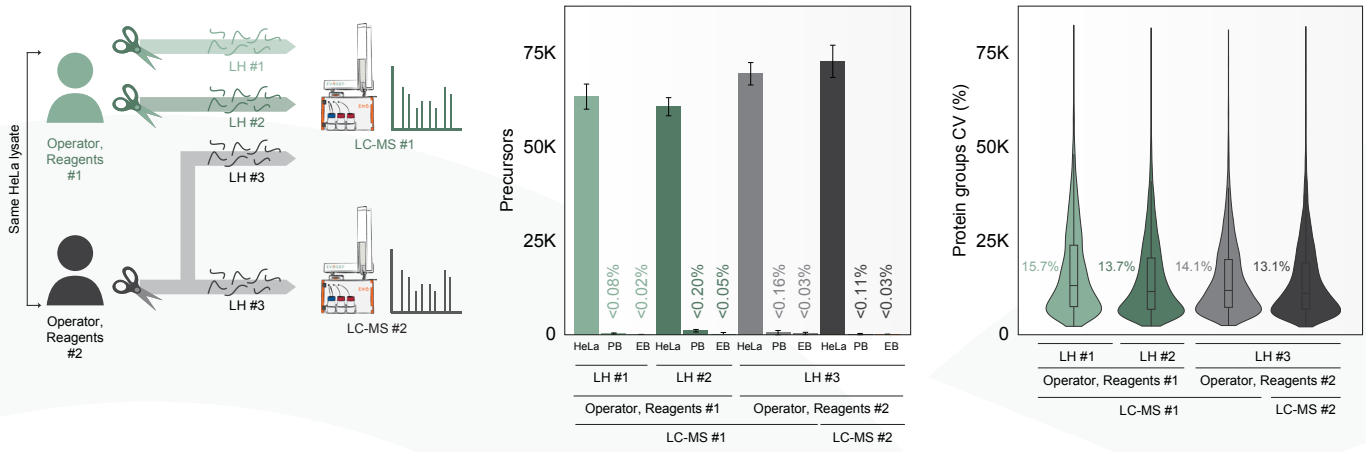

Figure 3: Complete workflow comparison with identified precursors, workflow QCs, and variance across identified proteins.

5. Inter-laboratory workflows

A comprehensive reproducibility study of the end-to-end workflows were conducted across the three different laboratories. To this end each lab prepared 84 replicate samples using automated digestion with 1 µg HeLa lysate for each sample following the OT-2 PAC protocol. Sites 1 and 2 used the same commercially available cell lysate, while site 3 utilized an internally cultured cell lysate. 50% of the resulting digest was automatically loaded onto Evotips at each site and analyzed using the 300 SPD method of Evosep Eno coupled to an Astral mass spectrometer. Comparison of digestion efficiency showed only minor differences despite the variation in lysate source and the multi laboratory setup. Across all three laboratories, 5795 protein groups were consistently identified, with approximately 75% of these quantified with a CV <20%, demonstrating strong inter-site reproducibility, even when using independently prepared HeLa digests.
The PAC workflow exhibited a dynamic range spanning 6 orders of magnitude for commonly identified proteins. High sensitivity and robustness were further demonstrated by consistent quantification (CV <20%) across a wide range of protein abundances.

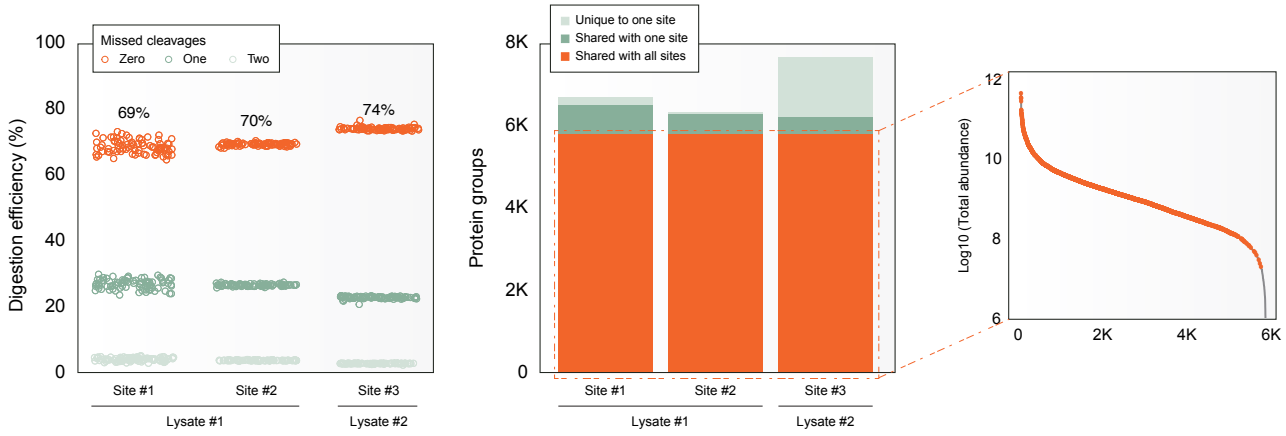

Figure 4: PAC workflow digestion efficiency, identification of unique and common proteins for each site and dynamic range plot of shared proteins annotated as proteins with CV below 20% in orange.

6. Conclusion

The complete, end-to-end workflow, multi-laboratory comparison highlights that high-throughput proteome profiling can be achieved with excellent reproducibility, enabled by the robustness of Evosep Eno and ease-of-use of the automated Evotip Pure loading.

Consistent retention times and FWHM for five diagnostic peptides across all three laboratories underscore the highly robust chromatographic performance of the Evosep Eno. The PAC protocol featuring short digestion time (4h) at ambient temperature combined with the high-throughput 300 SPD method allows for ultra-fast turnaround from sample to LC-MS readout. Despite differences in sample preparation and LC-MS systems across sites, the workflow delivered consistent protein identification rates with low CVs.

Overall, the success of the end-to-end workflow across three independent sites, despite independent sample preparations, emphasizes its reproducibility, robustness, and suitability for routine use.

The study confirms that standardized, high throughput applied proteomics - without compromising depth and sensitivity - is not only achievable, but also scalable and transferable across laboratories. This will only be improved as standardization is subjected to the complete workflow solution.