Application Note

EVOSEP

# **Evotip Pure** simplifies workflows with excellent reproducibility, storage and recovery

## 1. Introduction

The Evosep One enables clinical proteomics and facilitates the processing of large cohorts in a robust, reproducible and rapid manner. The Evotip is a key feature of the technology as it seamlessly integrates up-front sample preparation through desalting, utilized in typical bottom-up proteomics workflows, with sample loading and partial elution on the Evosep One. Here we evaluate the reproducibility of sample loading and assess the linear range for Evotip loading across the standard 60 SPD method as well as the Whisper 40 SPD method, coupled to a targeted mass spectrometry assay. The aim is to guide applications where Evosep One is utilized in routine testing environments such as targeted bottom-up proteomics workflows.

Further, storage of samples prior to mass spectrometry analysis is often necessary and typically performed in tubes, plates or HPLC vials. This, however, will lead to various degrees of sample losses, through the additional transfer steps and peptide adherence to plastics, in particular when working with low amounts of material. In order to further streamline the transition from sensitive sample preparation to mass spectrometry analysis, we evaluate the utility of the Evotip as a storage device for low input digested sample with peptides bound to the Evotips and analyzed across fourteen to twenty eight days post sample loading.

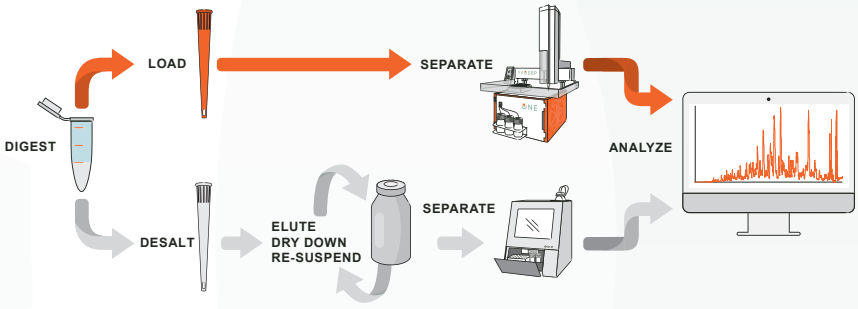


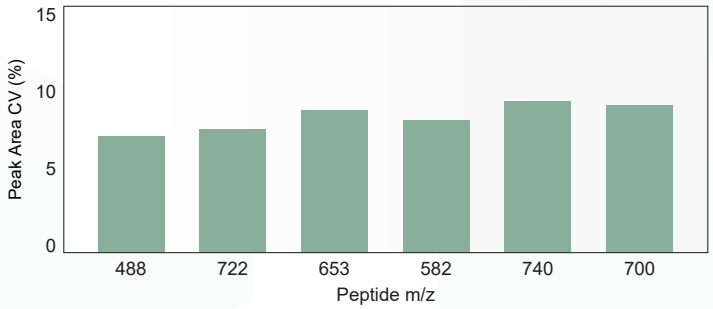

*Figure 1:* Evotip Pure streamlines the proteomics workflow by integrating peptide purification with sample loading and elution.

## 2. Method details

Bovine Serum Albumin (BSA) digest was purchased from Bruker Daltonics LabScape (8217498) and diluted to a final concentration of 10 fmol/µl. HeLa digests were generated using Protein Aggregation Capture (PAC) based on-bead protein binding, clean-up and digestion with peptide amount estimated using a Nano-Drop (Thermo Scientific).
For linearity experiments, HeLa peptide digests were serially diluted from 1000 ng to 62 pg and loaded in triplicate for each amount. For storage experiments, HeLa digests were loaded in quadruplicates at 200 pg and 50 ng and stored at 4 °C submerged in solvent A for 14 days (200 pg load), 28 days (50 ng load) or analyzed immediately after sample load. All samples were analyzed on the same day.
For Evotip load reproducibility experiments, BSA digest samples were measured in a randomized order using the 100 SPD method with the EV1109 column (Evosep). For the Evotip linearity experiments, HeLa digests were processed using the Whisper 40 SPD method using the EV1112 column (Evosep) and the 60 SPD method, also using the EV1109 column. Both columns were operated at 40 °C. The Agilent 6495C QqQ mass spectrometer was operated in positive ion mode with a gas temperature at 200 °C and a drying gas flow of 11 L/min. The capillary voltage was 1750 V, the High/Low pressure RF voltage was 200/110 V and the Delta EMV was 200 V. Q1 and Q3 were set to Wide/Wide. Min and Max dwell time were set to 2.61 and 497.83 ms respectively. A cycle time of 500 ms was used for 183 MRMs. A minimum of three transitions were monitored per peptide. Skyline (21.2.0.425) was used to extract raw data and the top ranking transition was used to calculate peptide peak areas.
For storage experiments, 200 pg loads were analyzed using the Whisper 80 SPD method with the Aurora Rapid75 column (IonOpticks) at 50 °C. Samples were measured on a timsTOF Pro 2 mass spectrometer. For dia-PASEF acquisition, a window placement scheme consisting of 8 TIMS ramps with 3 mass ranges per ramp spanning from 400-1000 m/z and from 0.64-1.40 1/K0 with a cycle time of 0.95 seconds, including one MS1 frame, was utilized. 50 ng loads were measured using the 100 SPD method with the EV1109 column operated at 40 °C on a timsTOF Pro 2 mass spectrometer using the “dia-PASEF - short gradient method”. Data from each peptide load was processed independently using DIA-NN (version 1.8) in library-free mode against the reviewed human proteome (UniProt, Nov 2021, 20,360 entries without isoforms) with minor modifications from the standard settings of 2 missed cleavages and methionine oxidation as variable modifications. MBR (match-between-runs) was enabled across technical replicates and protein interference was set to ‘Genes’. The quantification strategy was set to ‘Robust LC (high precision). Protein numbers represent unique protein groups from the DIA-NN matrix report.

## 3. Reproducibility

First, the reproducibility of Evotip Pure sample loading across a set 96 samples was investigated by monitoring six peptides from a BSA digest. The peptides included in the assay spanned the full gradient elution of the 100 SPD method. High robustness was observed with coefficient of variation (CV) below 10% across 96 Evotips for each of the six monitored peptides (Figure 2).




**PEPTIDE**

*m/z 488* - TCVADESHAGCEK
*m/z 722* - YICDNQDTISSK
*m/z 653* - HLVDEPQNLIK
*m/z 582* - LVNELTEFAK
*m/z 740* - LGEYGFQNALIVR
*m/z 700* - TVMENFVAFVDK

*Figure 2:* Peptide CVs for all detected peptides (n=96).

## 4. Linearity

Next, the linear response of Evotip Pure, in combination with the Agilent 6495C mass spectrometer was evaluated by loading a Hela digest in the range of 62 pg to 100 ng. Two peptides representing the HeLa proteins Actin (ACTB) and Protein S100-A4 (S10A4) were monitored in a targeted manner using the standard 60 SPD or Whisper 40 SPD methods. Calibration curves were calculated and regression fit $R^2$ >0.99 were obtained in all cases (Figure 3). A good linearity above 100ng was also observed from standard curves generated using Evotip Pure loaded with HeLa digests in the range of 0.125 ng to 500 ng (Table 1).

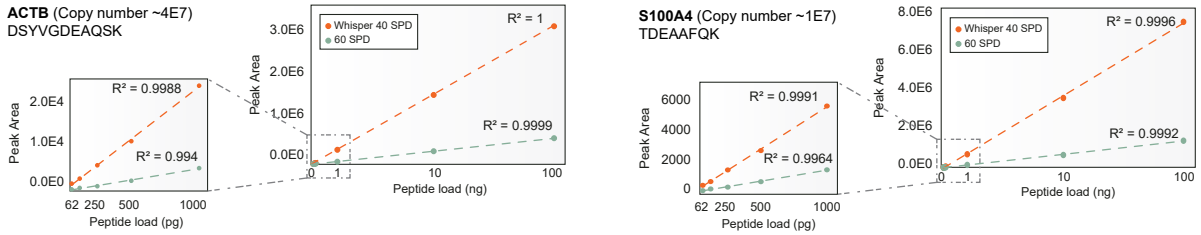



*Figure 3:* Calibration curves for HeLa dilution series for proteins ACTB and S100A4.

DSYVGDEAQSK

| Conc. range (ng) | n | 60 SPD | W 40 SPD |
|---|---|---|---|
| 0.125 - 100 | 7 | 0.9964 | 0.9992 |
| 0.125 - 200 | 8 | 0.9864 | 0.9880 |
| 0.125 - 500 | 9 | 0.9965 | 0.9964 |

TDEAAFQK

| Conc. range (ng) | n | 60 SPD | W 40 SPD |
|---|---|---|---|
| 0.125 - 100 | 7 | 0.999 | 0.9995 |
| 0.125 - 200 | 8 | 0.9957 | 0.9897 |
| 0.125 - 500 | 9 | 0.9968 | 0.9962 |

*Table 1:* $R^2$ values obtained from a linear least square regression fit across different ranges of the 0.125 – 500 ng standard curves.

## 5. Capacity

Lastly, the Evotip Pure capacity was probed by loading 62.5 to 1000 ng digest on tip with subsequent collection and re-injection of the flow-through material. The calculated summed intensity of all identified precursors from the flowthrough and the carry-over from the Evotip (discarded with the tip) were used to estimate the amount of unbound material detected in the flow-through relative to both 1, 5 and 50 ng loaded Evotips. It was measured that the recovery was 99.6% from a 1000 ng loaded Evotip and 100% from a 125 ng loaded Evotip (Figure 4). These results indicate that the total summed losses are below 0.45%, which for the low loads will be below limit of detection.

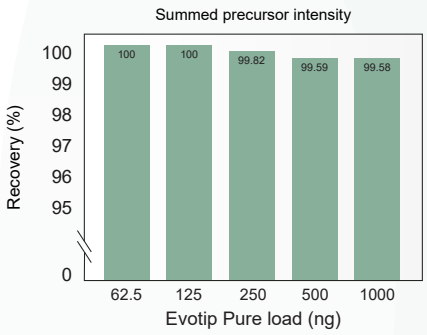



*Figure 4:* Percentage recovery measured from Evotip Pure tips loaded with 62.5 - 1000 ng, calculated as the sum of all identified precursors compared to the 50 ng sample load.

## 6. Storage

In order to evaluate the utility of the Evotip as a storage device of digests prior to mass spectrometry, an extended stability study was performed. Evotip Pure were loaded with 200 pg or 50 ng HeLa derived digests. Analyses were performed immediately, 14 (200 pg load) or 28 (50 ng load) days post Evotip loading. In order to minimize mass spectrometer bias, all the samples were analyzed on the same day. No statistical difference was observed from the measured precursor ion intensities across days 0, 14 and 28 for the respective loads. The number of identified precursors and proteins, identified with a CV below 20% were also similar for freshly loaded Evotips compared to Evotips stored for 14 or 28 days (Figure 5).

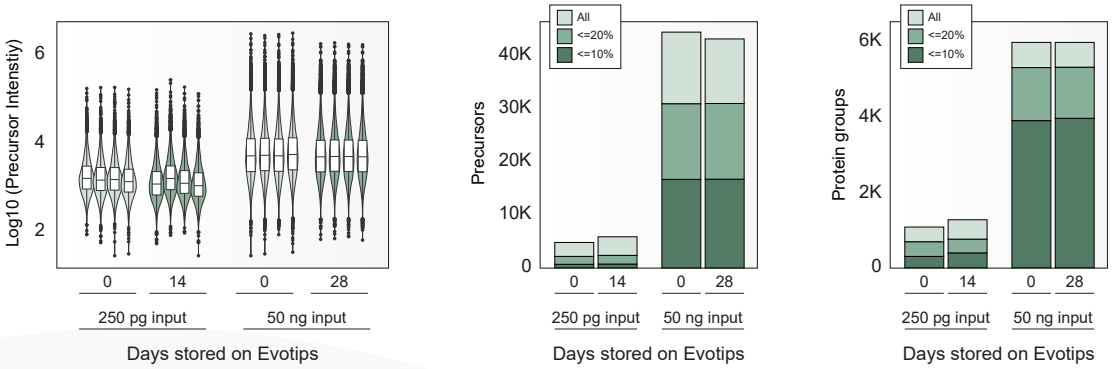



*Figure 5:* Precursor ion intensity and number of precursors and protein groups quantified in samples stored 14 and 28 days on Evotips.

## 7. Conclusion

The Evotip is a critical component of the Evosep One workflow, coupling front-end sample preparation with liquid chromatography and thus mass spectrometry analysis.
The experiments performed in this work highlight that Evotip loading can be accomplished in a highly reproducible manner. Evotip Pure can be utilized for sensitive targeted assays, here illustrated in combination with the Agilent 6495C, where a linear response is obtained for peptides covering a wide concentration range of 62 pg to 100 ng, which can be extended to 500 ng. Even at 1000 ng we measure that 99% of the loaded peptide digest is retained on the Evotip. In conclusion, the Evotip serves as an excellent sample introduction device with extremely high capture and recovery. Combined with sensitive and long-term storage of samples, the Evotip is a crucial component for integrated workflows.