

GRADIENT OFF-SET FOCUSING HPLC INSTRUMENT FOR ROBUST AND HIGH THROUGHPUT CLINICAL PROTEOMICS

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Introduction:

Mass spectrometry-based proteomics and metabolomics are fast growing and powerful technologies, with the potential to revolutionize health care and precision medicine. Human blood plasma and serum are already the most established samples for clinical analysis and are analyzed today with antibody-based assays. However, immunoassays have some inherent limitations that could be overcome by MS-based proteomics, which should be the optimal technology to investigate changes in the human plasma proteome in a specific and unbiased manner. Recently, we developed an automated, rapid and robust shotgun proteomics workflow

that allows us to analyse hundreds of plasma proteins. We call the workflow 'plasma proteome profiling' (Geyer et al., Cell Syst., 2016) and we have already applied it to several clinical studies, comprising up to 1,300 plasma proteomes (Geyer et al., Mol. Syst. Biol., 2016). However, until now, available separation technology has severely limited throughput and robustness and thereby prevented proteomics (and metabolomics) technologies from being fully integrated and routinely used in a clinical setting.

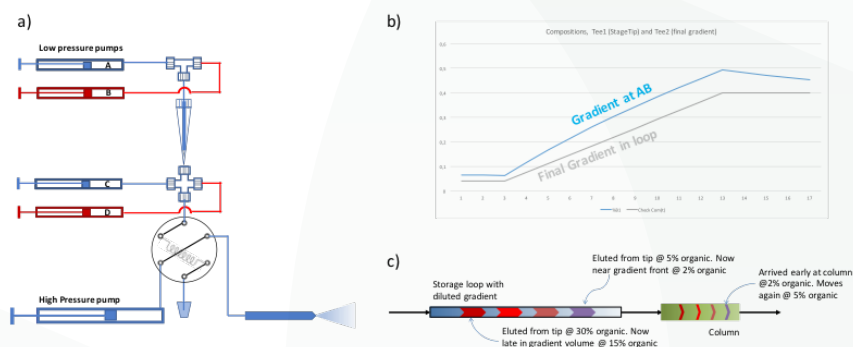


Figure 1: Evosep One a) Simplified plumbing diagram of the Evosep One. b) Illustration of the A+B gradient running through the Evotip and the following C+D modified gradient, resulting in an offset gradient for optimal focusing at the analytical column. c) Illustration of the preformed and offset gradient stored in the storage-loop containing the pre-separated analytes. The gradient offset helps to focus and significantly increase the capacity and chromatographic performance of the analytical column.

Evosep One is a conceptually novel chromatography system that dramatically increases robustness and sample throughput while maintaining the sensitivity of current nano-flow LC instruments. It uses four low-pressure pumps in parallel at relatively high flow to form a gradient that sequentially elutes analytes from a disposable Evotip. The initial gradient containing embedded analytes is created by pump A and B but pump C and D then modify that initial gradient just after the Evotip. This creates a gradient offset which ensures optimal focusing and maximal chromatographic performance of the analytical column, see figure 1. The preformed and offset gradient with the embedded and pre-separated peptides are first stored in a holding loop that subsequently is switched in-line with the analytical column. It is pumped out in an extremely robust way by a single high pressure pump with a constant flow rate (typically 1 uL/min). This configuration also ensures a very low overhead time of only

3 minutes per sample and virtual absence of cross contamination for crude biological samples such as body fluids, making it ideal for large cohort studies.

Method

Sample preparation was carried out as described in (Geyer et al., Cell Syst., 2016) with the automated plasma proteome profiling pipeline on an Agilent Bravo liquid handling platform. Non-depleted plasma samples were diluted 1:10 with ddH₂O and were prepared with the iST sample preparation kit (P.O. 00001) according to the manufacturer's instructions (PreOmics GmbH).

Peptides were loaded on C18 EvoTips and were separated using the Evosep One and analyzed in combination with a Q Exactive HF-X Orbitrap instrument (Thermo Fisher Scientific).

Results

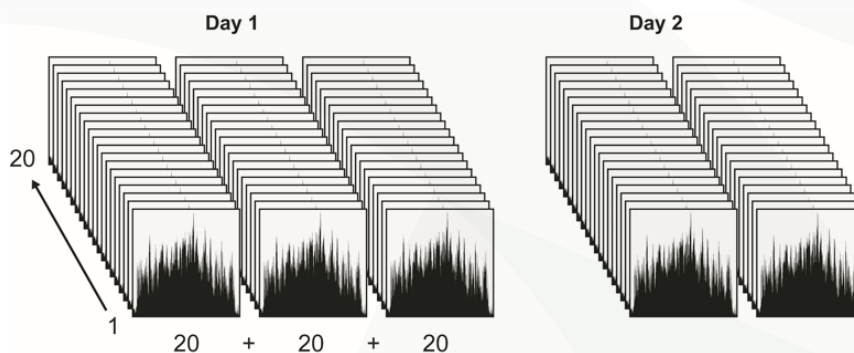


Figure 2: Measurement of 100 plasma proteomes with a throughput of 60 samples per day.

To show high throughput and to evaluate the performance of the Evosep One in a clinical context, we repeated the measurement of a single plasma sample 100 times (Figure 2). The applied 21 min gradient with 3 min overhead time allowed us to measure 60 samples per day. This resulted in a total analysis time of

just 40 h for 100 plasma samples. The system was able to measure over 1,500 HeLa runs without any technical issues and with a total analysis time of 25 days. This robustness and speed promises to finally enable routine, high throughput measurement of clinical samples.

For clinical decision making based on the concentration of a biomarker, it is crucial to ensure low carry-over from one analysis to the next. Therefore, we carried out a cross contamination experiment with alternate injections of plasma and a blank sample (Figure 3A). The average carry-over was just 0.1% and can be tracked back to 20 peptides which were responsible for 90%

of the carry-over. To evaluate the performance of the HPLC system for the 100 plasma runs, we randomly chose six peptides and monitored their retention time over the runs. The median, unadjusted standard deviation was of peak maxima was only 5.4 s.

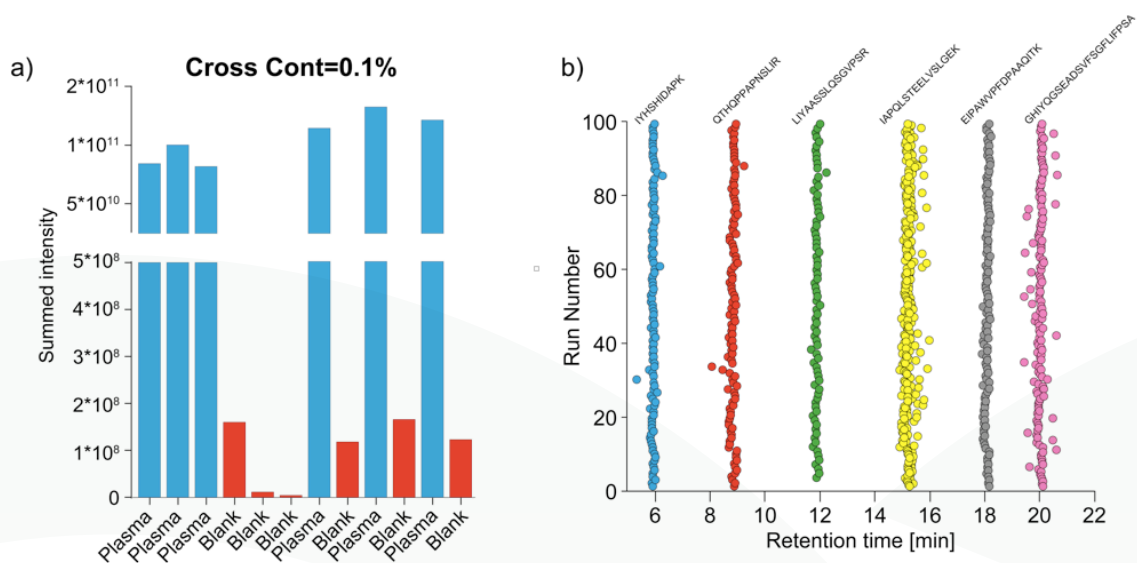


Figure 3: Robustness of the Evosep One for plasma samples. a) Evaluation of cross contamination in an experiment with alternate runs of plasma and blank samples. b) Retention times over 100 injections of the same plasma digest for six randomly chosen peptides over the gradient.

This measurements yielded on average 230 quantified proteins, which is on par with our recently published results (Geyer et al., Cell Syst, 2016). To analyze the quantitative reproducibility over the 100 plasma runs, we correlated all measurements to each other. This resulted in

an average **Pearson correlation coefficient of 0.99**. This dataset included in total 50 clinical applied biomarkers and the majority of these were quantified **with a very low coefficient of variation of below 20%** in these label-free measurements.

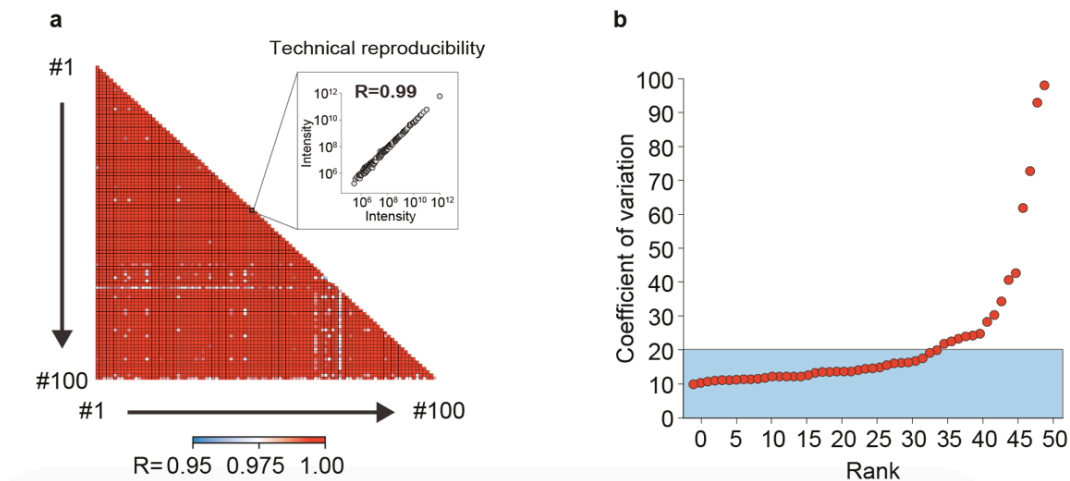


Figure 4: Quantitative plasma proteomes. a) Correlation of 100 repeated plasma runs. The Pearson correlation coefficients are color coded and one example for a comparison of two runs is highlighted. b) Coefficients of variation of 50 clinical biomarkers that were quantified with 21 min gradients.

Conclusion:

We have tested the Evosep One a 'gradient off-set focusing HPLC' instrument. We implemented this novel HPLC class in our recently published 'Plasma Proteome Profiling' pipeline for robust and high-throughput analysis of blood plasma samples. First, we evaluated the instrument and can confirm a low cross-contamination rate of less than

0.1% with plasma samples and a high reproducibility with Pearson correlations of 0.99. Next, we analysed 100 plasma samples in just 40h of measurement time, resulting in the quantification of on average 230 plasma proteins, including more than 50 approved clinical biomarkers.

References

1. Geyer, P.E., Kulak, N.A., Pichler, G., Holdt, L.M., Teupser, D., Mann, M. (2016) Plasma Proteome Profiling to Assess Human Health and Disease. *Cell Systems* 2, 185-195.
2. Geyer, P.E., Wewer Albrechtsen, N.J., Tyanova, S., Grassl, N., Iepsen, E.W., Lundgren J., Madsbad, S., Holst, J.J., Torekov, S.S., Mann, M. (2016) Proteomics reveals the effects of sustained weight loss on the human plasma proteome. *Molecular Systems Biology* 12: 901.