High-throughput and high-sensitivity biomarker monitoring in body fluid by FAIMS-enhanced fast LC SureQuant™ IS targeted quantitation

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Running title: SureQuant™ IS targeted quantitation in body fluids
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ABBREVIATIONS

60SPD 60 samples per day
100SPD 100 samples per day
AGC automatic gain control
CAA 2-chloroacetamide
COL collagen
CV compensation voltage
DDA data dependent acquisition
ELNE neutrophil elastase
FAIMS high-field asymmetric waveform ion mobility spectrometry
FDR false discovery rate
FINC fibronectin
HCD high collision dissociation
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IL1B interleukin-1 beta
IS internal standard
MMP matrix metalloproteinase
PRM parallel reaction monitoring
PSM peptide-spectrum match
TCEP tris(2-carboxyethyl)phosphine
TIC total ion current
TNFA tumor necrosis factor-alpha
ABSTRACT

Targeted proteomics methods have been greatly improved and refined over the last decade and are becoming increasingly the method of choice in protein and peptide quantitative assays. Despite the tremendous progress, targeted proteomics assays still suffer from inadequate sensitivity for lower abundant proteins and throughput, especially in complex biological samples. These attributes are essential for establishing targeted proteomics methods at the forefront of clinical use. Here, we report an assay utilizing the SureQuant™ internal standard triggered targeted method on a newest generation mass spectrometer coupled with a FAIMS (high-field asymmetric waveform ion mobility spectrometry) interface ion mobility device and an EvoSep One liquid chromatography platform, which displays markedly enhanced sensitivity and a high throughput of 100 samples per day. We demonstrate the robustness of this method by quantifying proteins ranging six orders of magnitude in human wound fluid exudates, a biological fluid that exhibits sample complexity and composition similar to plasma. Among the targets quantified were low-abundance proteins such at TNFA and IL1B, highlighting the value of this method in the quantification of trace amounts of invaluable biomarkers that were until recently hardly accessible by targeted proteomics methods. Taken together, this method extends the toolkit of targeted proteomics assays and will help to drive forward mass spectrometry-based proteomics biomarker quantification.
INTRODUCTION

Analysis of disease-related protein biomarker panels in various tissues and body fluids is essential for reliable clinical diagnostics and evaluation of treatment regimens, as well as in pharmaceutical research and development. This calls for a reproducible, robust, and sensitive high-throughput workflow. Over the past decade, parallel reaction monitoring (PRM) has been developed into a significant and very reliable mass spectrometry (MS)-based proteomics method for simultaneous quantification of multiple proteins in complex biological samples (1). PRM relies on selective and sensitive quantification of endogenous peptides and mostly uses quadrupole-orbitrap mass spectrometers (2,3). However, one of the major challenges in traditional PRM analysis is the requirement to accommodate more peptides into a narrow retention time window with an increasing number of target peptides and a decrease in sensitivity with higher degrees of multiplexing. This limitation is particularly critical in high-throughput studies where large numbers of samples are to be analyzed. Although shorter chromatographic gradients in these experiments are intuitively highly desirable, they are difficult to implement, since more peptide precursors are concurrently eluted from the analytical column, impeding detection and limiting sensitivity due to cycle time imbalances. This affects the overall performance of the method in large-scale studies (4).

To overcome limitations of pre-defined retention time windows, the PRM method has been optimized by including stable isotopically labelled peptides as internal standards (IS) to trigger the dynamic acquisition of the endogenous peptides in real-time. In this method, the detection of an IS peptide triggers a switch from a watch mode to a quantitative mode where the IS and endogenous peptides are measured. The time window for acquisition in the quantitative mode is set to match the actual elution profile of the peptides, ensuring a more effective use of the instrument time (5).
Recently, the IS-PRM acquisition has been further refined to maximize efficacy of the method (6). This new method termed SureQuant™ takes advantage of enhanced capabilities of the latest generation of orbitrap instruments. In this refined acquisition, the watch mode interrogates information from both the MS1 and MS2 data to profile the elution of the internal standard. Instead of matching a spectral library, the identification of an IS triggers a low resolution MS2 and if the fragments match a defined number of transitions specified in the method, the instrument triggers a high resolution MS2 scan for the target peptide (7). This dynamic acquisition mode ensures a higher number of successful scans compared to traditional PRM, allowing analysis of more targets with consistently higher sensitivity and less total instrument time. As an example, the SureQuant™ acquisition approach has been successfully used to quantify more than 500 endogenous peptides in human non-depleted plasma (8).

In targeted proteomics, it is a significant advantage if the complexity of the sample can be reduced without affecting the targeted peptides. The high-field asymmetric waveform ion mobility spectrometry (FAIMS) interface effectively removes interfering background ions, reducing the complexity of the MS spectra and improving the analyte signal-to-noise ratio (9,10). FAIMS can be used in combination with the latest generations of orbitrap instruments and adds a new dimension of selectivity to the MS analysis pipeline (11). In a previous study, more than 5000 proteins have been successfully quantified using a short LC gradient and data independent acquisition on a quadrupole-orbitrap instrument coupled with a FAIMS device. This demonstrates how FAIMS can improve the MS results both qualitatively and quantitatively (11).

The current depth of proteome coverage in proteomics studies is not only based on technological advances of mass spectrometers but also on optimization of sample preparation and fractionation steps prior to MS analysis (12). Systems integrating multiple pre-analysis steps are continuously developed and refined. One example is the EvoSep One system, which combines sample clean-
up with a fast and efficient chromatographic separation without compromising the sensitivity and robustness known from nano-LC systems. This system uses a low-pressure pump to elute the analyte along with the elution gradient from the clean-up cartridge into a capillary loop and then a single high-pressure pump to apply the sample to the analytical column. This abolishes the need to form a gradient at high pressure and the gain is a reduced idle time between injections. The EvoSep One concept has proved very promising for high-throughput proteomics (13).

In this study, we combined the sensitivity of SureQuant™ acquisition on an ThermoFisher Scientific Orbitrap Exploris 480 quadrupole-orbitrap instrument with the speed of EvoSep chromatography to devise a workflow for high-throughput and high-sensitivity biomarker monitoring in complex biological matrices that can be further enhanced by FAIMS ion filtering. We showcase the power of the method by time-resolved monitoring of wound biomarkers spanning a concentration range of six orders of magnitude in non-depleted clinical wound exudates.

**EXPERIMENTAL PROCEDURES**

**Wound exudate preparation**

**Wound dressings.** The clinical study was planned and managed by Paul Hartmann AG (Heidenheim, Germany). The study was approved by the Bioethics Committee at the Silesian Medical Chamber, Katowice, Poland (Opinion No. 35/2018), and registered with the German Clinical Trials Register (DRKS00015832). It was conducted in accordance with the Declaration of Helsinki, and laws and regulations in Poland. All patients provided written informed consent prior to entry into the study. The wound fluid was extracted from wound dressings from a cohort of six patients with venous leg ulcers (Supplemental Table S1). The patients were enrolled in the study for a total of seven visits with approximately 14-day intervals in between. At each visit the patient and wound was examined, and the ulcer dressing was changed. At the first visit the wound was treated with Medicomp® for 2 hours. Subsequently, a two-layer bandage compression therapy
(PütterPro2) along with a Hydroclean® wound dressing was applied. The Hydroclean® wound dressing was removed during the second visit, serving as the sample containing the wound exudate for visit 2. A new Hydroclean® dressing was used to cover the wound, and this procedure was repeated until visit 7, resulting in 1 Medicomp® and 6 Hydroclean® dressings containing wound exudates from each chronic wound patient. Dressings were stored at the clinical site at -20 °C and shipped on dry ice. Upon arrival, dressings were stored at -80 °C until further processing.

**Extraction of wound fluids from wound dressings.** A buffer solution consisting of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM sodium chloride (NaCl), 10 mM EDTA was used to incubate the dressings for protein extraction. A protease inhibitor cocktail (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, 04693159001, Roche) was added to the buffer solution according to the manufacturer instructions. For Hydroclean® wound dressings, the inner polyacrylate-based layer was removed from the outer layers, using scissors to cut around the edge of the dressing. The inner layer was placed on a petri dish, buffer solution was applied on the polyacrylate, at a ratio of 500 µl/cm². For Medicomp® dressings, the dressing was transferred to a 15 ml falcon tube, and buffer solution was added until the dressing was fully immersed. The dressings were incubated on ice for a minimum of 2 hours on a rocking platform. Following incubation, Medicomp® dressings were placed in a 20/50 mL syringe, and the absorbed liquid was squished through. Hydroclean® dressings polymer material is prone to disruption, so the material was directly placed in a cell strainer and drained using a mortar. The liquid phases extracted for both dressing types were passed through a 40 µm cell strainer (Fisherbrand™ Sterile Cell Strainers, 22-363-547, Fischer Scientific) in order to separate polymer residues from the solution. Cell debris and remaining particles were removed by centrifugation (3 x 15 min, 4000 g, 4 °C). The samples were desalted and concentrated by ultrafiltration using 3 kDa molecular weight cutoff concentrators (Amicon® Ultra-15 Centrifugal Filter Unit, UFC9003, Millipore, Merck), and
the final protein concentration was determined with a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, Waltham Massachusetts, USA). Samples were stored at -80 °C until further processing.

**Sample preparation for MS analysis.** One hundred micrograms of protein were diluted in 2.5 M guanidine hydrochloride (GuHCl), 100 mM HEPES pH 7.8 solution to a final concentration of 500 ng/µl. Cysteines were reduced and alkylated by addition of tris(2-carboxyethyl)phosphine (TCEP) to a final concentration of 1.5 mM, incubated for 60 min at 65 °C, and chloroacetamide (CAA) to a final concentration of 5 mM followed by incubation at 65 °C for 30 min. Afterwards, samples were diluted with 100 mM HEPES pH 7.8, in order to reach a final concentration of 0.5 M GuHCl. Trypsin was added to the samples in a final ratio of 1:50 (protease:protein w/w), and incubated for 16 hours at 37 °C, 600 rpm in an Eppendorf ThermoMixer® C. Reaction was stopped with 10% trifluoroacetic acid (TFA) (v/v), for a final concentration of 1% TFA v/v. The peptide concentration was measured with a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, Waltham Massachusetts, USA).

**SureQuant™ assay design**

**Selection of peptides for targeted analysis.** We included a total of nine proteins central in the wound healing pathophysiology covering a broad dynamic range of concentrations in wound exudates: matrix metalloproteinase (MMP) 2, MMP9, neutrophil elastase (ELNE), interleukin-1 beta (IL1B), tumor necrosis factor-alpha (TNFA), S100A8, S100A9, collagen 1 (COL1A1), and fibronectin (FINC). Based on data dependent acquisition (DDA) shotgun analysis survey runs and information from ProteomicsDB (14,15) and PeptideAtlas (16,17), 3 to 7 peptides per protein were selected for assay suitability and validation. For peptides selected from databases, the most frequently observed peptides that were identified in MS experiments with high confidence and satisfying a number of predetermined criteria (no methionine, length between 8 and 21 amino
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acid residues, C-terminal arginine or lysine, no missed cleavages) wherever possible were chosen for subsequent experiments. In proteins undergoing maturation due to proteolytic processing, preference was given to peptides matching the processed mature protein. The heavy isotopic arginine or lysine labelled peptides for IS-PRM analysis were ordered from JPT Peptide Technologies (Berlin, Germany). The quantotypic properties of the selected peptides were tested, and the two peptides with best profiles for each protein were selected for the final targeted analysis (Supplemental Table S2).

**LC-MS/MS analysis**

**EvoTip sample clean-up and loading.** EvoTip columns were conditioned with 100% isopropanol for 1 min, washed once with 50 µl buffer B (80% acetonitrile (ACN), 0.1% TFA) by centrifugation for 30 s, 1400 g. Activation with 100% isopropanol was repeated followed by equilibration with 50 µl buffer A (0.1% formic acid (FA)) by centrifugation for 10 s, 1400 g, leaving a considerable volume of buffer A on the EvoTip. The sample was loaded onto the EvoTip, followed by centrifugation for 40 s at 1400 g. The loaded peptides were washed two times with 200 µl buffer A using a centrifugation step for 40 s, 1400 g each. The washed peptides were kept wet by applying 250 µl of buffer A on top of the EvoTip and centrifugation for 10 s at 1400 g.

**Reverse phase liquid chromatography.** The peptides on the EvoTips were separated on an Evosep One chromatography system using a 2 cm x 75 µm, PepMap™ RSLC analytical column, packed with 2 µm C18 beads. Columns were washed and equilibrated before each run and peptides separated from the stationary phase over 11.5 min or 22 min according to the manufacturer standard method “100 samples per day” (100SPD) or “60 samples per day” (60SPD), respectively.
**Shotgun mass spectrometry analysis for synthetic peptide library.** For MS analysis an Orbitrap Exploris 480 mass spectrometer (ThermoFisher Scientific) equipped with a FAIMS Pro device was operated in DDA mode containing an inclusion list of heavy peptide masses (Supplemental Table S3) for 11.5 min (EvoSep One 100SPD gradient) or 22 min (EvoSep One 60SPD gradient), respectively. A sample volume corresponding to 1000 ng of sample peptides and 0.8 pmol of heavy peptides was injected. MS1 resolution was set to 120,000, automatic gain control (AGC) target set to 300%, maximum injection time set to 50 ms, scan range 340 to 1160 m/z, FAIMS voltage on (compensation voltage (CV) set to -50) or off, selecting the top 35 MS1 ions for MS2 analysis. MS2 scans used 7,500 resolution, AGC target of 1000%, maximum injection time 10 ms, and isolation window of 0.8 m/z at normalized collision energy of 28.

**SureQuant™ analysis.** SureQuant™ analysis was performed on an Orbitrap Exploris 480 mass spectrometer (ThermoFisher Scientific) equipped with a FAIMS Pro device and operated in SureQuant™ mode for 11.5 min or 22 min (EvoSep One 60SPD gradient), respectively. A sample volume corresponding to 1000 ng of sample peptides and 0.8 pmol of heavy peptides was injected. MS1 resolution of 120,000, AGC target of 300%, FAIMS voltage on (CV at -50V) or off, 50ms injection time, and cycle time of 2 s. This was followed by heavy peptide precursors and fragment recognition from the list (Supplemental Table S4 and S5), at a resolution of 7,500, high collision dissociation (HCD) energy at 28, AGC target of 1000%, and a 10 ms injection time. On the fly product ion trigger had to equal at least 3 product ions to initiate an offset scan (Supplemental Table S6) at a resolution of 60,000, HCD collision energy at 28, AGC target of 1000%, and 250 ms injection time in centroided mode.

**MS Data analysis**

**Shotgun MS data analysis.** Data analysis was performed using Proteome Discoverer 2.2 (ThermoFisher Scientific). MS spectra were extracted from raw data files and searched against a
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complete Homo Sapiens database from the UniProt Database (TaxID 9606, 42,252 SwissProt entries, November 25, 2017). The database was concatenated with a list of all protein sequences in reversed order. Searches were run with 10 ppm precursor ion tolerance for total protein level identification and 0.02 Da for fragment ion tolerance using SEQUEST search algorithm and Percolator for false discovery rate (FDR) filtering. Carbamidomethylation of cysteine residues (+57.021 Da), heavy lysine and arginine amino acids (+8.014 and +10.008 Da) were set as static modifications, while oxidation (+15.995 Da) was set as a variable modification. Peptide-spectrum matches (PSMs) were set to not exceed 1% FDR. Filtered PSMs were further filtered for peptide and protein-level FDR of 1%.

**SureQuant™ data analysis.** To analyze SureQuant™ data we used Skyline 20.1 from MacCoss lab (18). Target peptides were imported after setting filter parameters at MS1 orbitrap to centroided, a mass accuracy of 10 ppm and MS/MS DIA centroided filtering at 20 ppm, with fixed isolation scheme of 0.007 m/z. All precursor masses had at least 3 transitions. Endogenous group of peptides had a fixed modification of carbamidomethylation of cysteine residues (+57.021 Da), synthetic peptides had additional static modifications of heavy lysine and arginine amino acids (+8.014 and +10.008 Da). The peptide transition peaks and their integration boundaries were manually revised and adjusted based on the heavy peptide retention times. Peak areas for the peptide fragments ions were exported from Skyline and the total area fragment column of the report for each precursor was used for quantification. Further processing and analysis were performed with custom made Python scripts (Python version 3.6), available upon request from the corresponding author. The peptide fragment areas were normalized based on the total ion current (TIC) chromatogram area, extracted from the raw data using the RawTools package ([https://github.com/kevinkovalchik/RawTools](https://github.com/kevinkovalchik/RawTools)). Peptide values from each protein were averaged to get protein quantification values. The protein values were rescaled on a 0-100 scale separately for each protein.
Immuoassay experiments

Multiplex immunoassay analysis. For the multiplex bead-based immunoassay analysis, we used a custom made Human Magnetic Luminex Assay LXSAM (R&D systems, Minneapolis, MN) according to manufacturer’s instructions (https://www.rndsystems.com/products/human-magnetic-luminex-assay_lxsahm). Reference standards for all analytes except ELNE were provided in the assay kit, and the assay was thoroughly validated by the manufacturer. An additional specificity validation was performed by eight different exclusion tests, where seven out of the eight recombinant biomarkers were mixed and analyzed. Final target concentrations for the recombinant biomarkers in the test were aimed at the middle of the 7-point standard curves (Table 1). The sample concentration was adjusted to 50 µg/µl using 50 mM HEPES. Initial dilution tests were performed on three patient samples at 1:2, 1:25, 1:50, 1:200, 1:4000 and 1:6000 dilutions with kit provided dilution buffer. After evaluation, each of the patient samples were diluted 1:50, 1:500 and 1:10,000 and analyzed. All standards were analyzed in triplicates and all samples were analyzed in duplicates. The plates were analyzed on a Luminex MAGPIX (Luminex corporation, Austin, TX).

Multiplex immunoassay data analysis. Raw data was analyzed using xponent software (version 4.2.1324.0, Luminex corporation, Austin, TX). Validation of data was performed as follows: If highest or lowest standards were < 10% higher or lower, respectively, from the nearest standard based on net MFI (median fluorescence intensity minus background) then it was removed due to flattening of the curve; If %recovery of a standard, based on the five parameter logistic regression made by xponent, were outside 80-120% then the standard was removed due to poor model fit; If the % coefficient of variance (%CV) of the net MFI was > 10% then the standard was removed due to poor replication. For the samples, net MFI values above or below the standard curve average net MFI was discarded. Also, sample measurements were removed
if %CV was >10%. Finally, the absolute concentration of the different biomarkers in each sample was calculated based on the standard curve and the dilution factor.

**Experimental design and statistical rationale**

Quantification relied on 5 transitions from two peptides for each protein in accordance with the HUPO guidelines for high confidence PRM/SRM assay protein quantitation (19). The absolute protein concentrations and dynamic range measurements were based on analysis of a total of 42 biological replicates.

**RESULTS AND DISCUSSION**

In this work, we introduce an optimized internal standard based SureQuant™ PRM workflow that we apply in a case study of biomarker quantification using a total of 42 human wound exudate samples. We demonstrate the robustness and sensitivity of the acquisition method by targeting peptides representing both highly and lowly abundant proteins in a highly complex matrix. This targeted proteomics workflow exhibited high sensitivity, reproducibility and robustness.

**Experimental workflow and design**

Our newly devised targeted proteomics workflow follows the general principle of SureQuant™ analysis with significant modifications by integration of the EvoSep One platform for fast LC and optionally FAIMS Pro ion filtering for enhanced signal to noise ratios on a ThermoFisher Scientific Orbitrap Exploris 480 instrument (Figure 1). In our case study, we used trypsin as digestion protease and analyzed nine biomarkers central in wound pathophysiology known to span a large dynamic range in concentrations in wound fluids (20,21). Thereby, we targeted two peptides per protein that had been selected either from DDA shotgun analyses or with help of ProteomicsDB (22) or PeptideAtlas (16), respectively, and following the HUPO guidelines for high confidence PRM/SRM assay protein quantitation (19) and criteria as outlined in Experimental Procedures.
**Dynamic range of selected wound fluid biomarkers**

To evaluate the power of our workflow, we first determined the actual concentration range of the selected wound biomarkers in the 42 patient samples included in this study. For this, we used a validated custom-made bead-based multiplexed immunoassay with the Luminex technology upon optimization of sample dilutions for each protein biomarker of interest (Supplemental Table S7). As anticipated from literature reports on protein amounts in similar samples (20,21), absolute concentrations of the selected proteins in extracted wound exudates spanned six orders of magnitude (Figure 2). As most extreme examples, fibronectin (FINC) had a median concentration value of 128 µg/ml, while the median concentration across all samples for TNFα was 1171 pg/ml (Supplemental Table S8). Thus, the samples and selected biomarker proteins used to test specificity and sensitivity of our targeted proteomics workflow presented a highly dynamic concentration range in a complex sample matrix.

**Adaptation of SureQuant™ to short LC gradients**

Since throughput is critical for biomarker studies with larger patient cohorts, we next sought to shorten LC gradients as major time-limiting step in sequential targeted proteomics analyses of many samples. This became possible with help of the EvoSep One system that provides optimized workflows for processing of either 60 or 100 samples per day, resulting in 22- and 11.5-minutes gradients, respectively. Despite inherent compression of total ion chromatograms (TICs) from shorter gradients, the 18 heavy spike-in peptides monitored to identify and relatively quantify the nine biomarkers in this study (Supplemental Tables S9 and S10), sufficiently resolved along the elution range both in 60 and 100 samples per day setups (Figure 3A). Moreover, shortening LC gradients did neither affect the number of reliably monitored transitions nor peak intensities but in many cases even resulted in sharper peak groups with respective smaller peak widths (Figure 3B). Applicability of fast LC has been demonstrated for standard PRM assays (23) but not yet reported in the literature for IS-PRM and SureQuant™. Hence, our workflow demonstrates the
potential to increase throughput of targeted analyses of proteins spanning a large dynamic range in a complex biological matrix.

**Increase of SureQuant™ sensitivity by FAIMS**

With the release of the FAIMS Pro interface, we next tested if the significant increase in intensity and signal-to-noise ratio that had been observed in discovery proteomics (24) also improves performance of targeted PRM assays. Therefore, the samples included in the study were analyzed utilizing the IS-PRM SureQuant™ MS method both with and without the FAIMS ion mobility device attached to the Thermo Fisher Scientific Orbitrap Exploris 480 instrument (Supplemental Tables S11 and S12). Different CVs applied to the FAIMS device determined a CV of -50 as the optimal value for our experimental setup, whereby at the time of the experimental runs of this study only a single CV could be applied per sample analysis in targeted runs. This CV value was in the middle of the recommended range of CV values (approx. -120 to -20) tested and can retain charge 2 and 3 peptides with positive polarity, while filtering out most neutral and less charged ions.

The FAIMS ion filtering resulted in a median increase of ~500% in ion intensities across the total ion chromatograms for all 42 samples analyzed. Figure 4A illustrates examples of increases in the ranges of ~200% (rep 26, 27) and ~600% (rep 61, 63) for samples with similar TIC profiles. Moreover, transitions recorded with FAIMS were significantly higher in intensity than their non-FAIMS counterparts, in many cases for both the endogenous and the heavy IS spike-in peptides (Figure 4B). This was particularly important for monitoring of the proteins with lowest abundances (IL1B and TNFA), for which FAIMS increased data completeness at the levels of transitions, peptides and proteins (Figure 5). Strikingly, also highly abundant proteins, such as FINC, strongly benefitted from FAIMS in intensity and completeness of quantitation across samples, allowing
reliable quantification of the nine biomarker proteins in almost all wound fluid samples. This setup provided us with higher intensity for all 18 peptides used for protein quantification (Figure 6).

The FAIMS ion mobility technology proved especially useful on the detection and quantification of lowly abundant proteins in the biomarker set of this study, namely the cytokines TNFA and IL1B. The amount of transitions identified with high confidence and their intensity increased in the runs where the FAIMS device was installed, corroborating the claim that FAIMS neutral ion filtering increases sensitivity and enables reliable quantification of peptides present in lower concentrations. Selected transition profile examples can be seen in Figure 4B.

To our knowledge, this is the first example of detection and quantification of the lowly abundant cytokines IL1B and TNFA by targeted proteomics in human samples with complexity and composition resembling blood plasma. However, it has to be noted that the samples used in this study likely showed elevated levels, in most cases more than 50-fold over baseline levels, of the two cytokines due to the chronic inflammatory wound environment. Near native concentrations of low-abundance biomarkers might not be reliably measurable yet but could potentially be realized in the near future with optimization of parameters and even more advanced targeted proteomics methods and MS instrumentation.

**High-throughput analysis**

With an increased sensitivity, we wanted to ensure the reproducibility and throughput of these assays. This was addressed by using the EvoSep One system together with SureQuant™ acquisition. Optimization trial runs determined the chromatographic gradient of 11.5 minutes to exhibit the highest benefits in terms of sensitivity, quantification reliability and high throughput analysis. The combination of the two systems retained the measurement window within 30 seconds and peak detection resulted in most cases in coverage of the entire area under the curve
of the endogenous peptides (Figure 7A). The retention time windows for all peptides showed very high consistency even after a stress test of non-stop analysis of 365 samples, with mean standard deviation of less than 7 seconds across the 18 quantified peptides and maximum standard deviation of 10 seconds (Figure 7B). By harnessing the high sensitivity of the ThermoFisher Scientific Orbitrap Exploris 480 mass spectrometer and the standardized 100SPD method from EvoSep system we were able to identify and quantify the target proteins.

CONCLUSIONS
This study demonstrates that the SureQuant™ IS-PRM method presents an excellent strategy for targeted, quantitative proteomics. The method provides the opportunity for high-throughput analysis of peptides in a wide dynamic range without sacrificing measurement sensitivity. The dependence on heavy peptides for the detection of the endogenous peptides bears some risks and requires cycle times to be optimized in order to preserve the initial peptide elution profile. However, the addition of heavy peptides in the matrix does not seem to affect sample measurement. The heavy peptides retain the same chromatographic properties with their native counterparts (5). In addition, it still seems to be a considerably better alternative to precursor monitoring scheduling windows, which is the strategy used in standard PRM experiments. This holds especially true in high-throughput experiments that span several days of continuous instrument running time, where retention time shifts are frequently observed due to multiple, continuous injections in the same analytical column (13,25). The combination of the EvoSep HPLC system and the SureQuant™ method present an excellent example of reliable high throughput targeted experiments monitoring multiple precursors, with the capability of running 100 samples per day without sacrificing sensitivity.
DATA AVAILABILITY
All mass spectrometry-based proteomics data discussed in this study have been deposited at PanoramaWeb (https://panoramaweb.org/faims_surequant.url) (e-mail: panorama+reviewer111@proteinms.net; password: dZEWxyvy) with the dataset identifier PXD032305 (http://proteomcentral.proteomexchange.org/cgi/GetDataset?ID=PXD032305).

CONFLICTS OF INTEREST
HS is a full-time employee of HARTMANN. The other authors declare to have no conflict of interests.

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REFERENCES


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TABLES

**Table 1: Target concentrations for recombinant biomarkers in specificity test.**

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Target concentration (pg/ml)</th>
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<tr>
<td>Collagen I alpha 1 (6220-CL-020)*</td>
<td>260</td>
</tr>
<tr>
<td>Fibronectin (1918-FN-02M)*</td>
<td>59100</td>
</tr>
<tr>
<td>IL1B (201-LB005/CF)*</td>
<td>316</td>
</tr>
<tr>
<td>MMP-2 (in house, unknown origin)</td>
<td>5416</td>
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<tr>
<td>MMP-9 (in house, unknown origin)</td>
<td>2108</td>
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<tr>
<td>S100A8 (9876-S8)*</td>
<td>1205</td>
</tr>
<tr>
<td>S100A9 (9254-S9)*</td>
<td>426</td>
</tr>
<tr>
<td>TNFA (210-TA-005/CF)*</td>
<td>143</td>
</tr>
</tbody>
</table>

*(From Biotechne, Abingdon, UK)*

FIGURE LEGENDS

**Figure 1: Sample preparation and analysis workflow.** Wound exudates were extracted from dressings and prepared for analysis with no depletion of high-abundance proteins. Samples were digested on 96 well plates. After digestion, heavy peptides for wound biomarkers were spiked into the samples and the mixture loaded on EvoTip trap columns. Finally, columns were placed on an Evosep One platform and analyzed on an ThermoFisher Scientific Orbitrap Exploris 480 mass spectrometer with FAIMS or without FAIMS equipped.

**Figure 2: Absolute quantification of proteins of the biomarker panel in wound exudates.** Protein concentrations were measured using a custom-made Luminex assay in all 42 patient samples. The measurement distribution for each protein is given as boxplots displaying the median measurement and outlier measurements as whiskers (75th quantile). Measurements for all proteins are also given as a run plot indicating the full measurement range of the assay.
**Figure 3: Assessment of EvoSep One LC gradients.** A Selected example total ion current chromatograms for 11.5 (top) and 22 min gradients (bottom). Dashed lines indicate the peak of the elution window for peptide precursors and their transitions. B Selected example transition plots for endogenous peptides of neutrophil elastase and MMP9, using 11.5 (top) and 22 min gradients (bottom).

**Figure 4: Comparison of signal intensities without and with FAIMS.** A Chromatograms of analytical runs for selected samples, demonstrating the absolute total ion current signal increase during active chromatographic gradients when FAIMS was installed on the MS instrument. Percent increase of integrated area when samples were analyzed with the FAIMS device in comparison to no FAIMS is mentioned in the graph title. Blue line: Chromatogram with FAIMS, green filled line: chromatogram without FAIMS. B Selected example ion transitions. Left column corresponds to the result of a given sample being analyzed without FAIMS and right column to sample analyzed with FAIMS. For each replicate, endogenous peptides can be seen in the left side and heavy peptides in the right side of the transition plots panel. Inserts show ion transitions with a signal-adjusted y-axis.

**Figure 5: Analysis of data completeness and intensities.** Top heatmap: Log2 transformed values of integrated peaks of ion transitions for each peptide precursor. Bottom heatmap: Log2 transformed protein values of averaged integrated peaks for precursor peptides. Left side of heatmaps shows values for the SureQuant™ assays, right side shows values for the SureQuant™-FAIMSpro experiments.

**Figure 6: Relative quantification of wound biomarkers in wound exudates by SureQuant™ with and without FAIMS.** Log2 transformed raw intensity values of area under the curve for targeted experiments with and without FAIMS. Values are given as the averaged, normalized and...
rescaled intensity values deriving from peptide fragment integrated peak areas for each protein. Each boxplot includes data from six patients grouped for seven time points (summing up to 42 patient samples) and thus represent variance of biological replicates.

Figure 7: Retention time of peptides used in the targeted experiments. A Example of a sample chromatogram with the gradient used for all targeted experiments of the study and overview of elution windows for a set of six precursors. BRetention time of the selected precursors in a stress test analysis of 365 samples.
Fig. 1

Wound exudate is collected and digested. The resulting peptides are then spiked with heavy peptide standards and loaded onto an EvoTip device. The sample is then processed through SureQuant™ and FAIMS Pro™ for quantification and analysis of biomarkers.
**SureQuant™ without FAIMS**

- **y7**: 758,4771+ (10^3)
- **y6**: 644,4341+ (10^3)
- **y5**: 531,3501+ (10^3)
- **y4**: 418,2660+ (10^3)
- **b4**: 440,2867+ (10^3)

**Retention Time**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Intensity (10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6,2</td>
</tr>
<tr>
<td>6.2</td>
<td>6,3</td>
</tr>
<tr>
<td>12</td>
<td>6,4</td>
</tr>
</tbody>
</table>

**SureQuant™ with FAIMS**

- **y7**: 766,4913+ (10^3) (heavy)
- **y6**: 652,4483+ (10^3) (heavy)
- **y5**: 539,3643+ (10^3) (heavy)
- **y4**: 426,2802+ (10^3) (heavy)
- **b4**: 440,2867+ (10^3) (heavy)

**Retention Time**

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<tr>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>5,4</td>
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<tr>
<td>5.4</td>
<td>5,5</td>
</tr>
<tr>
<td>12</td>
<td>5,6</td>
</tr>
</tbody>
</table>

**SureQuant™**

**TNFA, VNLLSAIK, replicate 27**

- **y7**: 758,4771+ (10^3)
- **y6**: 644,4341+ (10^3)
- **y5**: 531,3501+ (10^3)
- **y4**: 418,2660+ (10^3)
- **b4**: 440,2867+ (10^3)

**Retention Time**

<table>
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<tbody>
<tr>
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<td>5.8</td>
<td>5,9</td>
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<tr>
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<td>6,0</td>
</tr>
</tbody>
</table>

**SureQuant™ - FAIMS**

**TNFA, VNLLSAIK, replicate 27**

- **y7**: 766,4913+ (10^3) (heavy)
- **y6**: 652,4483+ (10^3) (heavy)
- **y5**: 539,3643+ (10^3) (heavy)
- **y4**: 426,2802+ (10^3) (heavy)
- **b4**: 440,2867+ (10^3) (heavy)

**Retention Time**

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<td>5.2</td>
<td>5.4</td>
</tr>
<tr>
<td>12</td>
<td>5.6</td>
</tr>
</tbody>
</table>

**IL1B, IPVALGLK, replicate 77**

- **y7**: 758,4749+ (10^3)
- **y6**: 608,4221+ (10^3)
- **y5**: 509,3537+ (10^3)
- **y4**: 438,3166+ (10^3)
- **y7**: 353,2411++ (10^3)
- **b6**: 551,3552+ (10^3)

**Retention Time**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Intensity (10^3)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>7.5</td>
</tr>
<tr>
<td>12</td>
<td>7.6</td>
</tr>
</tbody>
</table>

**MMP2, AFQVWSDVTPLR, replicate 62**

- **y7**: 758,4749+ (10^3)
- **y6**: 608,4221+ (10^3)
- **y5**: 509,3537+ (10^3)
- **y4**: 438,3166+ (10^3)
- **y7**: 353,2411++ (10^3)
- **b3**: 347,1714+ (10^3)

**Retention Time**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Intensity (10^3)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>7.6</td>
</tr>
<tr>
<td>12</td>
<td>7.7</td>
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</tbody>
</table>

**Intensity**

- **y9**: 1072,5786+ (10^3)
- **y8**: 973,5102+ (10^3)
- **y7**: 787,4308+ (10^3)
- **y6**: 700,3988+ (10^3)
- **y5**: 585,3719+ (10^3)
- **b3**: 347,1714+ (10^3)

**Retention Time**

<table>
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<tbody>
<tr>
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<td>7.5</td>
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<tr>
<td>12</td>
<td>7.6</td>
</tr>
</tbody>
</table>

**Intensity**

- **y9**: 1082,5868+ (10^3) (heavy)
- **y8**: 983,5184+ (10^3) (heavy)
- **y7**: 797,4308+ (10^3) (heavy)
- **y6**: 710,4071+ (10^3) (heavy)
- **y5**: 595,3801+ (10^3) (heavy)
- **b3**: 347,1714+ (10^3) (heavy)

**Retention Time**

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<td>7.6</td>
</tr>
</tbody>
</table>

**Intensity**

- **y9**: 1092,5988+ (10^3)
- **y8**: 983,5184+ (10^3)
- **y7**: 797,4308+ (10^3)
- **y6**: 710,4071+ (10^3)
- **y5**: 595,3801+ (10^3)
- **b3**: 347,1714+ (10^3)

**Retention Time**

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<td>7.6</td>
</tr>
</tbody>
</table>

**Intensity**

- **y9**: 1102,6008+ (10^3)
- **y8**: 973,5102+ (10^3)
- **y7**: 797,4308+ (10^3)
- **y6**: 710,4071+ (10^3)
- **y5**: 595,3801+ (10^3)
- **b3**: 347,1714+ (10^3)

**Retention Time**

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</table>
Fig. 5

Peptides

SureQuant™

SureQuant™ FAIMS

Proteins

SureQuant™

SureQuant™ FAIMS