Development of a targeted plasma quantitation assay using dia-PASEF

Blood plasma is the key sample of interest in clinical proteomics research applications. Comprehensive proteome analysis still remains challenging due to the large dynamic range of concentration.

Abstract

Here, we developed a large-scale targeted assay for quantitation of peptides in non-depleted human plasma samples using dia-PASEF® [1]. As dia-PASEF is a data-independent acquisition (DIA) technique it can be integrated in a platform for large-scale biomarker studies without the need for tedious setup of methods and in-depth method optimization as is required for typical targeted approaches.

Analysis of the PQ500™ kit (Biognosys) spiked in non-depleted human plasma using the timsTOF HT in combination with dia-PASEF allowed all 804 SIS peptides to be analyzed in a 30-min gradient. To validate the developed assay, we applied it to a sample set consisting of plasma from 20 different patients (10 healthy and 10 confirmed lung-cancer donors).

Keywords: PQ500, dia-PASEF, targeted quantitation, plasma, timsTOF HT, Spectronaut

Authors: Stephanie Kaspar-Schoenefeld[1], Sebastian Mueller[2], Tejas Gandhi[2], Sira Echevarria[2], Nagarjuna Nagaraj[1], Gary Kruppa[1]; [1]Bruker Daltonics GmbH & Co. KG, Bremen, Germany; [2]Biognosys, Schlieren, CH.

Introduction

DIA approaches are very attractive when the aim is to analyze a large sample cohort, e.g. in clinical studies, due to the very high reproducibility and data completeness. dia-PASEF has already been proven to deliver reproducible identification and quantitation information in discovery proteomics studies [1].

Here, we developed an easy-to-use targeted quantitation assay for human plasma proteins by combining:

- PreOmics' iST kit for fast, robust, and reproducible sample preparation.
- Biognosys' PQ500™ kit offering absolute quantitation of more than 500 human plasma proteins using stable isotope-labeled standard peptides.
- dia-PASEF on the timsTOF HT providing unmatched data completeness and analytical depth for high-throughput quantitative proteomics on the latest timsTOF platform.
- Spectronaut software (Biognosys) enabling data processing from raw data to biological interpretation.

The major advantage of the presented approach is that there is no need for tedious targeted method development and optimization as is typically required for targeted approaches like SRM and MRM. In addition, the dia-PASEF workflow preserves information about peptides not part of the targeted assay, thereby enabling a combination of targeted and discovery workflows.

The newly developed timsTOF HT instrument enhances the current benefits of the existing platform with improved ion storage capacity. This results in an increase in dynamic range for the eluting peptides providing additional analytical depth for quantitative proteomics and retaining the additional specificity

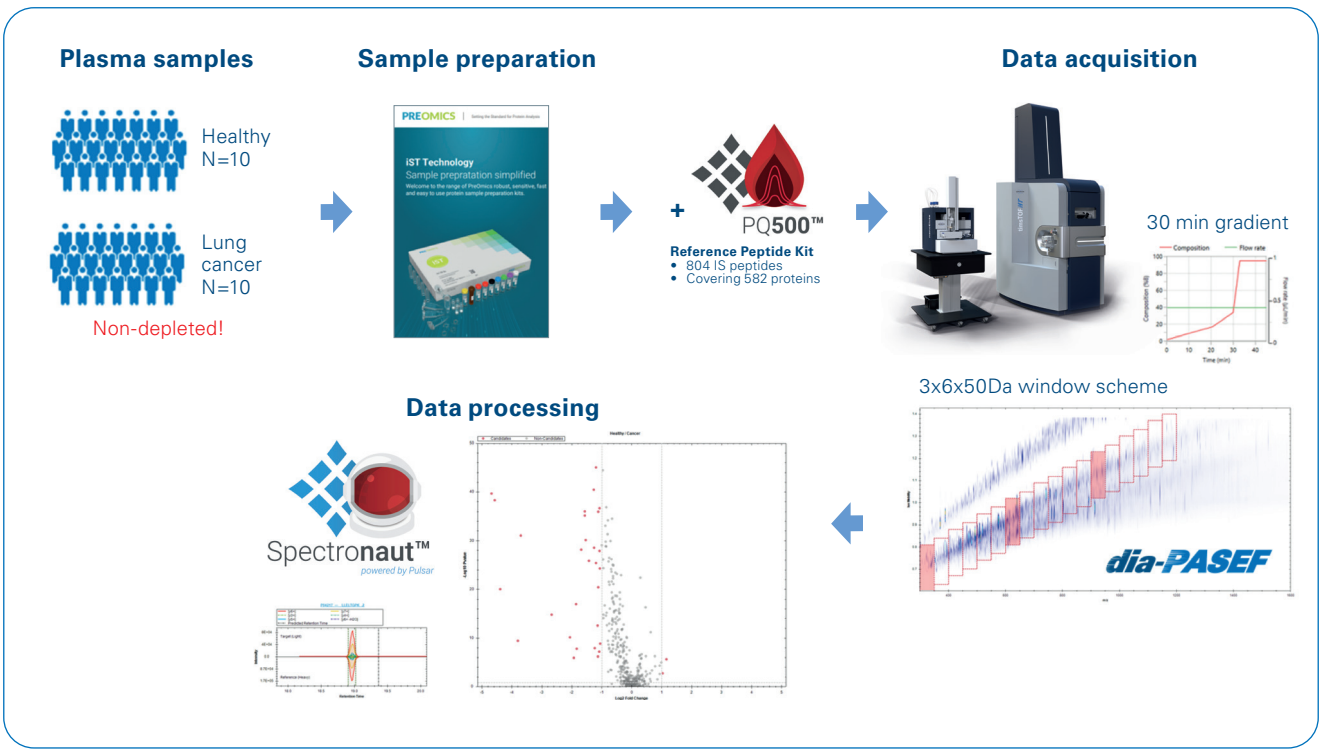

Figure 1: Schematic of the developed workflow for targeted quantitation of human plasma samples using dia-PASEF. Non-depleted human plasma originating from healthy (N=10) and confirmed lung cancer (N=10) source samples were prepared using iST kit (PreOmics). To enable absolute quantitation of human plasma proteins the PQ500™ kit (Biognosys) was spiked into the samples. Peptides were separated using a 30-min gradient on a nanoElute system and analyzed using an optimized dia-PASEF scheme. Data processing was done using Spectronaut (Biognosys).

provided by the TIMS separation. PRM or MRM methods used for targeted analysis on instruments without a high resolution on mobility separation typically use very small *m/z* selection windows (<2 Da) to eliminate interferences and improve specificity and quantitative performance, while DIA methods typically fragment everything in a wide *m/z* window (typically 5-25 Da). We hypothesized that the additional storage capacity when combined with the added selectivity of the TIMS separation could result in an easy-to-use dia-PASEF method providing results on targeted peptides with specificity, speed and quantitative performance similar to the best MRM and PRM performance obtained on other types of mass spectrometers. We report here the targeted proteomics performance of dia-PASEF acquisition on the timsTOF HT for the analysis of different non-depleted human plasma samples.

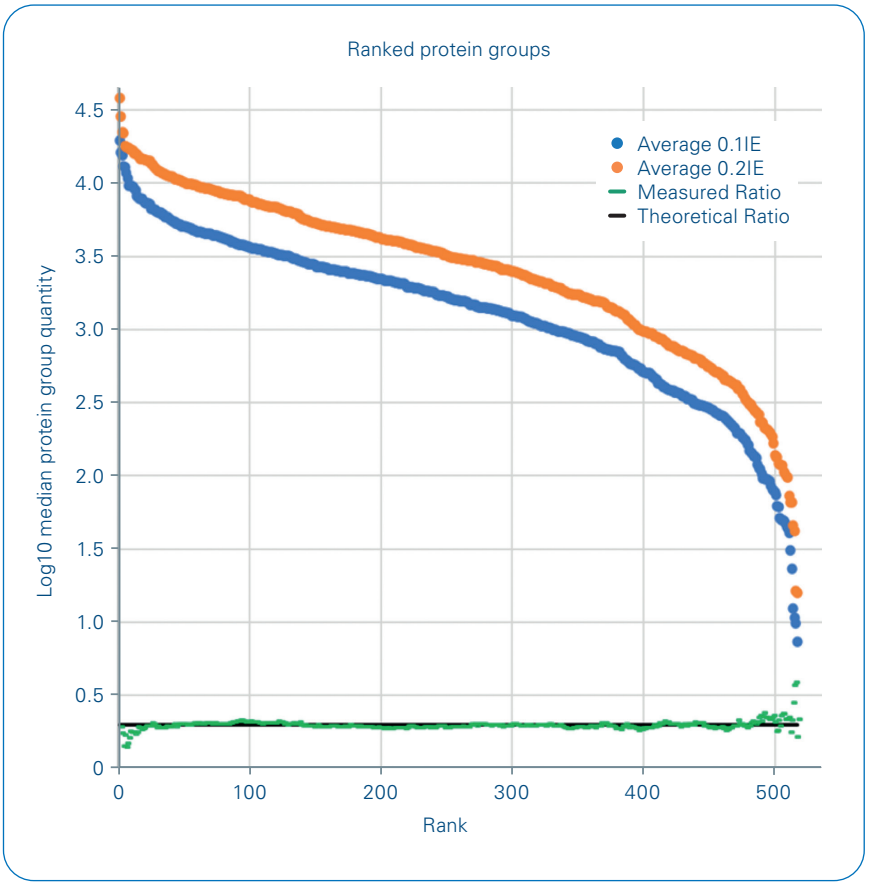

Figure 2: Investigation of the quantitation accuracy by spiking two different amounts of PQ500 (0.1 Injection Equivalents (IE) vs 0.2 IE) into non-depleted plasma background (theoretical ratio 1:2). Shown is the median protein group quantity (log10 transformed) for all quantified labelled PQ500 peptides as well as a comparison of the measured (green dots) and theoretical ratio (black line).

Methods

Pooled plasma used for method development was purchased from Sigma Aldrich (USA) and individual plasma samples were extracted from healthy and confirmed lung-cancer patients (N=10). Plasma proteins were digested using the iST sample digestion kit from PreOmics (Germany). The PQ500™ kit (Biognosys), containing 804 stable-isotope labeled peptides designed to allow absolute quantitation of 582 proteins, was prepared according to the manufacturer's instructions and spiked into the prepared digests.

The resulting tryptic peptides were separated on a 25 cm C18 column (75 μm inner diameter, 1.9 μm particle size, Aurora, IonOpticks) using a nanoElute coupled to a timsTOF HT mass spectrometer via a CaptiveSpray ionization source using a 30-min acetonitrile (ACN) gradient. For the dia-PASEF acquisition, a window placement scheme consisting of 6 TIMS ramps with 3 mass ranges per ramp spanning from 300–1200 *m/z* and from 0.6–1.40 $1/K_0$ with a cycle time of 0.9 seconds, including one MS1 frame, was utilized (Figure 1). Data was processed in Spectronaut (v16, Biognosys) using a developmental version of the default PQ500 library including ion mobility annotation for targeted data extraction. The library-free directDIA workflow was used for discovery-based proteomics of the non-targeted peptides and proteins.

Results and Discussion

The ideal method to perform large-cohort targeted proteomics analysis on plasma samples would include fast single-shot analysis without the need for tedious optimization for each targeted peptide. Especially with the recent developments of reference kits allowing absolute quantification of more than 500 proteins in plasma samples there is a strong need for easy-to-use methods which do not compromise the sensitivity and specificity known from classical targeted approaches like SRM and MRM. We developed an assay consisting of straightforward plasma sample preparation using PreOmics' iST kit, addition of the PQ500™ reference kit from Biognosys for absolute quantitation of target peptides, combined with dia-PASEF data acquisition on the timsTOF HT and processing using Spectronaut software (Biognosys, Figure 1). The PQ500™ kit covers more than 500 target proteins amongst them 130 FDA approved clinical markers. We used a 30-min gradient to enable high sample throughput without sacrificing analytical depth of the assay. The dia-PASEF method has been adapted to include the complete PQ500 panel by covering a mobility range from 0.6 to 1.4 $1/K_0$ and an *m/z* range from 300 to 1200. Application of an acquisition scheme with 6 TIMS ramps using 3 mass ranges per ramp resulted in a total cycle time of 0.9 s (including one MS1 frame per cycle) ensuring good coverage of the chromatographic peak.

To investigate the performance of the approach we spiked two different concentrations of the PQ500™ kit into a constant non-depleted human plasma background with a theoretical ratio of 1:2 (Figure 2). Targeted data processing in Spectronaut was done using an ion-mobility annotated PQ500 library.

Results show very accurate quantitation over the complete dynamic range with an average ratio of 1:1.98

Figure 3: Number of identified SIS peptides and protein groups from the PQ500 panel. An ion mobility annotated PQ500 library was used for data processing. Shown are the cumulative sparse and full profiles.

(±0.15). Results also show a very good reproducibility of the approach with median CV values of 7.3%. 90% of the PQ500 SIS peptides had %CV less than 20%.

The developed quantitation assay was applied to a proof-of-concept study of non-depleted plasma samples from patients diagnosed with lung cancer. All 804 SIS peptides and 578 protein groups from the PQ500 panel could be detected using the adapted dia-PASEF scheme (Figure 3). As constant PQ500 amounts have been spiked into the samples, the observed variation in the number of identifications on the protein group and peptide level is very low.

A higher variation was observed when looking at the endogenous peptides and protein groups identified in the plasma samples. This is due to the fact, that samples arise from 20 different individuals. In total, 663 peptides and 463 protein groups could be identified covering around 80% of the PQ500 panel (Figure 4).

In total, 55 proteins were found to be significantly regulated (p-value <0.05, fold change >2) between the two sample cohorts (Figure 5). Three of the proteins (Fibronectin, Immunoglobulin lambda-like polypeptide 1, Immunoglobulin lambda-like 1 light chain) were detected to be higher abundant in healthy donors, whereas the remaining proteins showed significant upregulation in donors diagnosed with lung cancer. Among them is the C-reactive protein (CRP), which is an inflammatory biomarker known to be elevated in diseased patients. Also the S100 protein family, whose members function as intracellular $Ca2^{+}$ sensor was detected to be significantly up-regulated. Members of the S100 family are involved in the pathogenesis of several malignancies including lung cancer [2]. Furthermore, elevated plasma levels of serum amyloid A (SAA) proteins (SAA1 and SAA2) have been detected in patients diagnosed with lung cancer. The detected regulation of the SAA

Figure 4: Number of identified endogenous peptides and protein groups from the PQ500 panel. An ion mobility annotated PQ500 library was used for data processing. Shown are the cumulative sparse and full profiles.

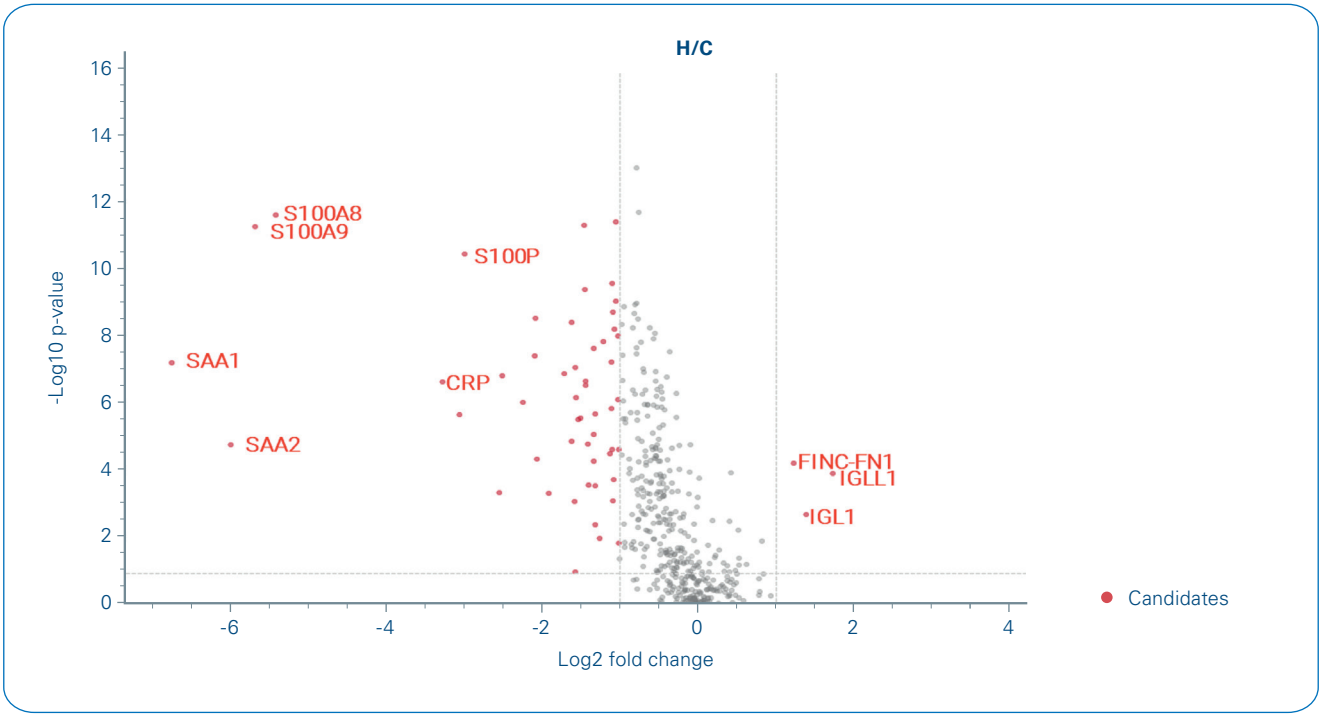

Figure 5: Volcano plot showing differentially regulated candidates from the PQ500 panel (p-value ≤0.05, fold change ≥2), marked in red. Most candidates were significantly enriched in subjects diagnosed with lung cancer, among them the S100 protein family.

proteins correlates to findings reported in previous studies [3].

By using dia-PASEF acquisition not only targeted peptides can be monitored, but quantitation information for all detectable peptides is preserved. Data processing using the directDIA approach supported in the Spectronaut software allows identification and quantitation of the non-targeted peptides without the need for library generation based on fractionated samples. In total 543 protein groups and 4540 peptides were identified during the experiment (Figure 6A). An additional 26 protein groups were found to be significantly regulated (p-value <0.05, fold change >2), which were not part of the targeted quantitation assay (Figure 6B). Among those, an extracellular superoxide dismutase (SOD3) was more highly expressed in lung cancer patients compared to healthy donors. SOD3 is known to be more highly expressed in tumor cells than in normal cells and its levels have been used to provide information about survival rates of diseased patients [4].

Our results show that the applied multiplexed approach has the potential to identify disease biomarkers in non-depleted plasma samples without in-depth expert knowledge by using a standard proteomics workflow supported on the timsTOF platform. A high correlation of the targeted dia-PASEF method with an advanced prm approach (prm-PASEF), which fully exploits the multiplexing capabilities of the timsTOF mass spectrometer, was already shown before [5]. In this study, the PQ500 kit was spiked in depleted human plasma samples originating from 20 patient plasma samples from a colorectal cancer cohort and analyzed using a 100-min gradient. The results clearly showed that both techniques could be used as a diagnostic tool for different diseases in future. In our study we further evaluated and optimized the targeted dia-PASEF approach as it is less complex to set up. Our results obtained from a 30-min gradient using non-depleted plasma show that an increase in sample throughput is possible without sacrificing analytical depth further extending the diagnostical potential of the developed assay.

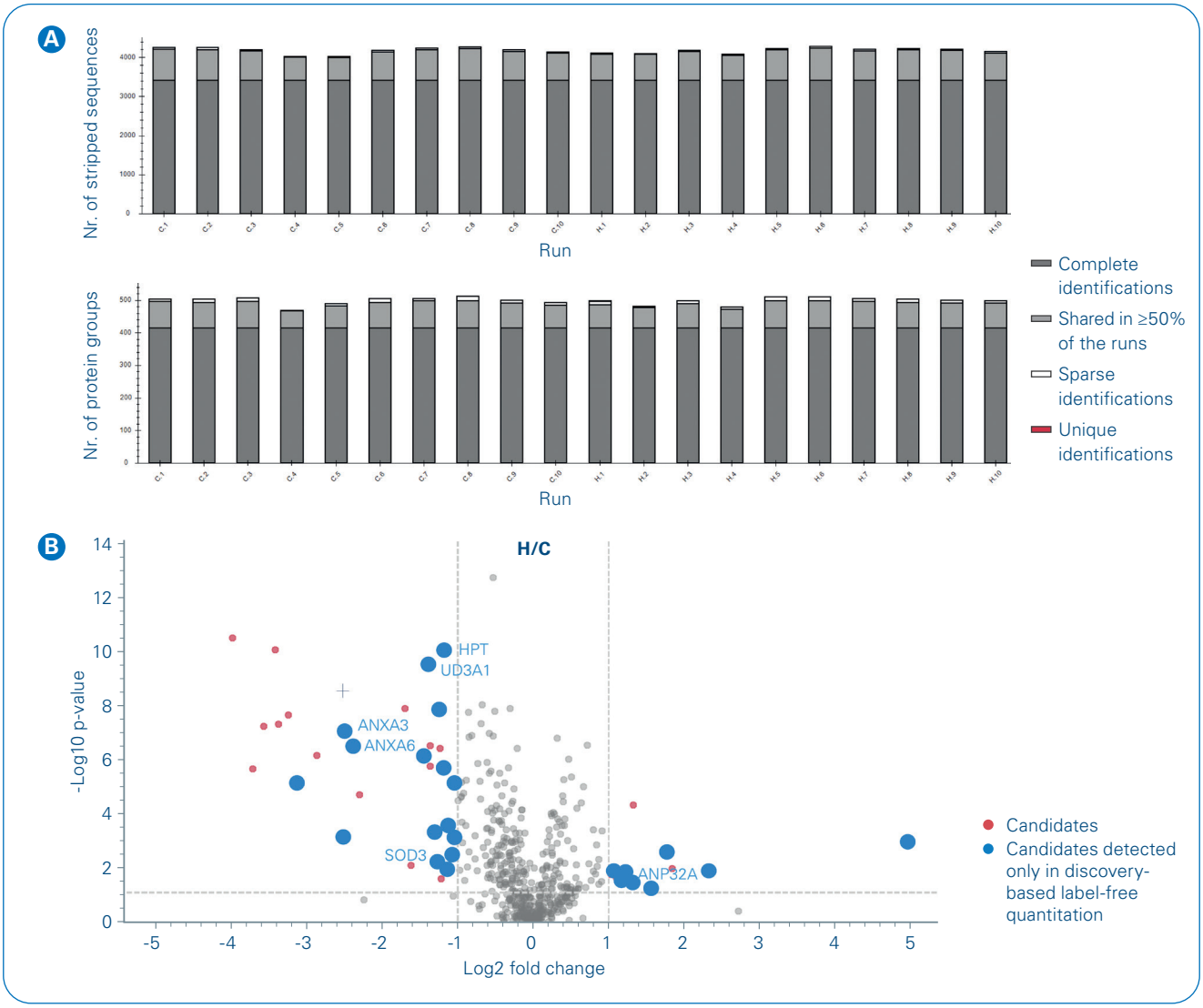

Figure 6: Discovery-based processing using the library-free directDIA approach (Spectronaut). (A) Number of identified proteins and peptides. (B) Volcano plot showing differentially regulated plasma peptides (p-value ≤0.05, fold change ≥2) from discovery based label-free quantitation (marked in blue). Most candidates were significantly enriched in subjects diagnosed with lung cancer.

Conclusions

A workflow for targeted peptide quantitation in non-depleted human plasma using dia-PASEF was evaluated. The PQ500 reference kit consisting of 804 heavy synthetic peptides enables reliable targeted quantitation of several hundreds of proteins in plasma.

- The presented approach eliminates tedious and time-consuming method development as known from standard targeted workflows and still yields excellent targeted quantitation results.
- The complete PQ500 panel can be covered in just 30-min run time using the dia-PASEF approach.
- By using a data-independent approach also proteins outside the target panel are measured and can be quantified resulting in a combination of targeted and discovery proteomics.
- The workflow has been applied to a biological relevant lung cancer study and shows regulation of peptides known to be associated with cancer. This confirms the potential of applying the presented approach for clinical research studies.

Learn More

You are looking for further Information?
Check out the link or scan the QR code.

www.bruker.com/timstof

References

[1] Meier et.al. (2020), Nat Methods, Dec;**17**(12):1229-1236

[2] Wang et al. (2021), Clin Chim Acta., Sep; **520**:67-70

[3] Kim et al. (2015), Proteomics, Sep; **15**(18):3116-312

[4] Zhang et al. (2022), Front Oncol.; **12**:722646.

[5] Lesur et al. (2022), bioRxiv preprint, https://doi.org/10.1101/2022.04.01.486663

For Research Use Only. Not for use in clinical diagnostic procedures.

Bruker Daltonics GmbH & Co. KG

Bremen · Germany
Phone +49 (0)421-2205-0

Bruker Scientific LLC

Billerica, MA · USA
Phone +1 (978) 663-3660

ms.sales.bdal@bruker.com – www.bruker.com