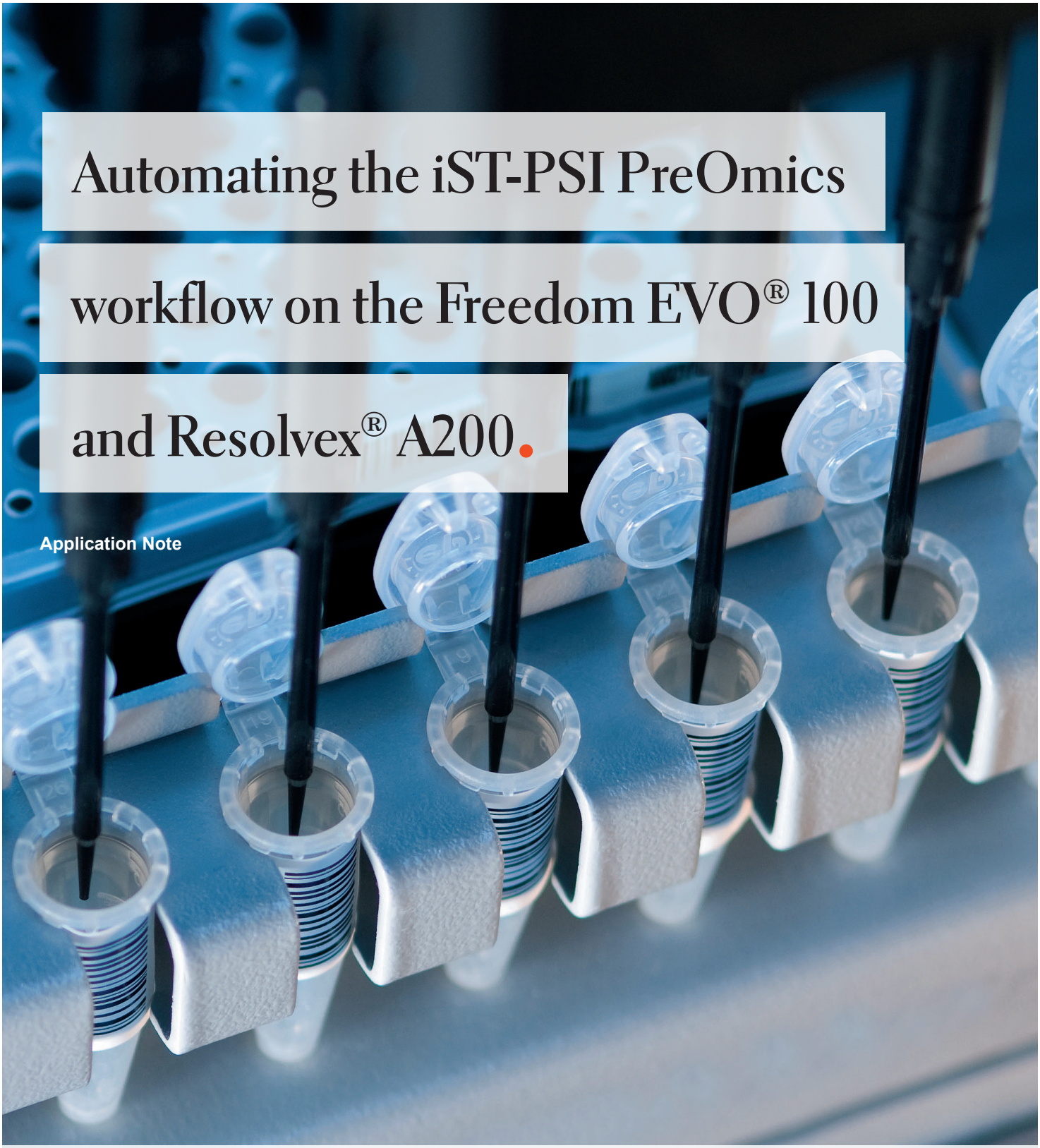
Automating the iST-PSI PreOmics workflow on the Freedom EVO® 100 and Resolvex® A200.

Application Note



RAPID PROTEOMIC SAMPLE PREPARATION INCLUDING PROTEIN DIGESTION AND PEPTIDE CLEAN-UP

TECAN.

INTRODUCTION

Proteins are essential effectors of cellular mechanisms, providing vital functions in living organisms. Proteomics studies – defined as large-scale characterization and quantification of proteins – can reveal the mechanisms underlying healthy and diseased cellular processes. This approach offers an appealing opportunity for translational research, including elucidation of various molecular mechanisms, identification of disease biomarkers, therapeutic drugs monitoring and patient stratification.

Liquid chromatography-mass spectrometry (LC-MS) is the gold standard technique for high throughput identification and quantification of proteins in a large variety of complex sample matrices. However, the main challenge is establishing a robust, reliable, reproducible and high throughput sample preparation workflow that allows for efficient protein extraction, enzymatic digestion and sample clean-up.

This application note describes a high throughput iST-PSI PreOmics workflow using the Freedom EVO 100 workstation, including peptide clean-up with the Resolvex A200 positive pressure processor. This automated workflow enables the processing of up to 96 samples – cell lysates, plasma, purified proteins, etc. – in a single run, while reducing the overall hands-on time to a few minutes.

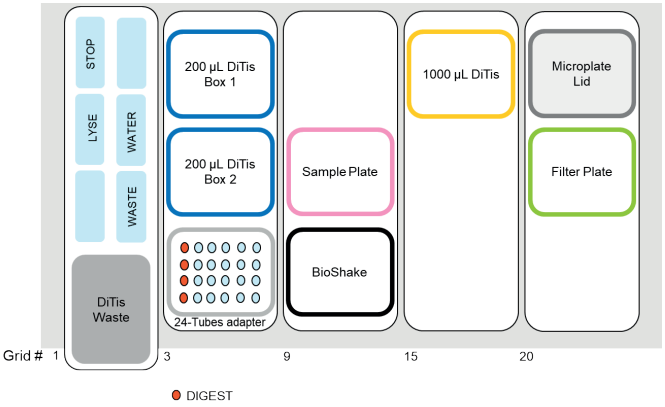
MATERIALS AND METHODS

Sample lysis

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 g/l glucose and 10 % heat-inactivated fetal bovine serum. Cells were harvested at 80 % confluency, and lysed by heating for 10 minutes at 95 °C in iST LYSE buffer (80 µl/1e^6 cells). Cell lysis was followed by sonication (10 cycles, 30 seconds on/off) using a Bioruptor® (Diagenode) to shear the DNA. Lysates were cleared by centrifugation (10 minutes, 17,000 rcf, 25 °C), and protein concentrations were determined using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Lysed samples then underwent the automated iST-PSI workflow on a Freedom EVO 100 workstation, followed by peptide clean-up on a Resolvex A200. As a control, the iST workflow was performed manually in parallel.

Equipment and consumables for automation

The Freedom EVO workstation was equipped with a four-channel Air Liquid Handling Arm™ (Air LiHa) with a disposable tip adapter, a Robotic Manipulator Arm™ (RoMa) – to transport plates from the microplate carriers – and a BioShake 3000-T elm thermoshaker (QInstruments) (Figure 1).



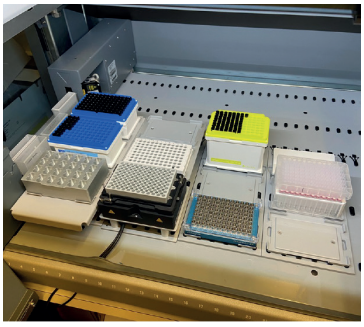


Figure 1: Freedom EVO workdeck layout for the automated PreOmics iST-PSI workflow.

All buffers, chemicals and enzymes required for the workflow were provided in the PreOmics iST-REG-PSI 96HT (192rxn, P.O.00108) kit. For the manual iST peptide clean-up, iST cartridges from the iST 8x kit (P.O.00001) were used.

Consumables
Freedom EVO workstation
Disposable tips: conductive, SLAS-format, non-filtered, non-sterile, in 200 and 1,000 µl volumes (Tecan)
Disposable troughs: 25 and 100 ml (Tecan)
4titude® Auto-Sealing PCR Plate Lid: Black (Azenta Life Sciences)
Hard-Shell® 96-Well PCR Plates: low profile, thin-wall, skirted, white/clear (Bio-Rad)
Screw cap micro tubes: 2 ml (Sarstedt)
Resolvex A200
Clean-up plate: provided in the PreOmics iST-REG-PSI 96HT (192rxn) kit
Deep-well plate: Protein LoBind® 96/1,000 µl (Eppendorf)

Table 1: Overview of the consumables required to run the application.

PreOmics automated iST-PSI workflow and experimental design

Five replicates of HEK293 cell lysate containing 100 µg of total protein per sample were diluted to a volume of 50 µl and loaded into a 96-well PCR plate. 3 blank samples (LYSE buffer only) were placed on the microplate next to the samples, to evaluate potential cross-contamination. Peptide clean-up was performed on the Resolvex A200 for both HEK293 cell and blank samples.

PreOmics manual iST workflow

Triplicates of 100 µg cell lysate were processed manually in parallel to assess the performance of the automated protocol. The manual digestion step was performed under the same experimental conditions as the automated workflow – including identical buffers and enzymes. For the manual peptide clean-up, the same buffers and volumes were applied as for the automated workflow, however iST cartridges were used and centrifugation steps – 1 minute at 3,800 rcf – replaced the positive pressure sample handling steps.

Automated iST digestion on the Freedom EVO

The automation protocol was developed following the PreOmics iST-REG-PSI 96HT (192rxn) kit instructions, as described below:

1. Add 50 µl LYSE buffer to 100 µg protein sample. *Take the plate out of the Freedom EVO, seal it, and perform offline lysis on a heating block (95 °C, 1,000 rpm, 10 min). After a brief centrifugation, remove the sealing, place the plate back on the Freedom EVO.** Alternatively, the on-board lysis step can be performed by using the integrated BioShake heating block.
2. Add 50 µl DIGEST reagent.
3. Transfer microplate to the BioShake using the RoMa.
4. Cover microplate with a black auto-sealing lid using the RoMa.
5. On-board protein digestion with the BioShake (3 hours, 37 °C, 500 rpm).
6. Add 100 µl iST STOP reagent.
7. Shake (30 sec, room temperature, 500 rpm).
8. Transfer the sample from the microplate to the 96 WELL SPE-PLATE included in the iST-REG-PSI kit.
9. *Move the filter plate to the Resolvex A200.*

**Manual steps are described in italics*

Automated iST peptide clean-up on the Resolvex A200

Following automated processing, the 96 WELL SPE-PLATE was manually transferred to the Resolvex A200 for the final peptide clean-up step. Gas supply was set at 5.5 bar (80psi), and the protocol was performed according to the iST-REG-PSI 96HT (192rxn) kit instructions. In brief, the 96 WELL SPE-PLATE containing the loaded peptide samples was washed twice – 1x 200 µl WASH 1 and 1x 200 µl WASH 2 – and then eluted with 2x 100 µl ELUTE in an 96-well, deep-well plate 96/500 µl. Finally, the plate containing the eluates was removed from the Resolvex A200, the samples were dried, and the peptides were stored at -20 °C until LC-MS/MS analysis.

LC-MS/MS analysis

Peptides were resuspended in LC-LOAD, and 300 ng peptide loads were analyzed on an EASY-nLC™ 1200 system (Thermo Fisher Scientific) coupled with a timsTOF Pro mass spectrometer (Bruker). Peptides were separated on a reverse phase column (self-pack column from ESI Source Solutions; column length: 50 cm; I.D: 75 µm) packed with C18 particles (ReproSil-Pur C18 particles, 1.9 µm diameter, Dr. Maisch). A 45-minute LC-MS gradient was run at a constant flow rate of 450 nl/min using 0.1 % formic acid in H_20 as mobile phase A, and 0.1 % formic acid in 80 % acetonitrile as mobile phase B (Table 2).

Time (min:sec)	Duration (min:sec)	%B (%)
00:00	00:00	5
25:00	25:00	35
40:00	15:00	60
42:30	02:30	95
45:00	02:30	95

Table 2: 45-minute LC gradient to analyze the samples.

Peptides were ionized with an electrospray voltage of 1.5 kV, and the capillary temperature was set to 180 ºC. The timsTOF Pro was operated in DDA-PASEF mode over an MS and MS/MS range of 100 to 1,700 m/z, using an accumulation and ramp time of 100 ms . Ion mobility range was set to 0.8-1.4 1/k0, and MS/MS spectra were acquired using stepped collision energy.

Data analysis

Raw files were analyzed using the MaxQuant software (v.2.0.1.0), and searched against the human Uniprot FASTA database (reference proteome with isoforms; version December 2021). The false discovery rate was set to 0.01 on both protein and peptide level, and was determined by searching a reverse database. The search was performed with a minimum peptide length of seven amino acids. Tryptic specificity – cleavage after arginine and lysine – with a maximum of two missed cleavages was applied. Cysteine carbamidomethylation was set as a fixed modification, while N-terminal acetylation and methionine oxidation were set as variable. Label-free quantification (LFQ) was performed with a minimum ratio count of 2, and statistical analysis was performed using the Perseus software platform (v.1.6.0.9).

RESULTS

There was no evidence of cross-contamination, with blank samples situated across the microplate lacking a specific signal.

Various peptide and protein identification and quantification parameters were evaluated to assess the performance of the automated PreOmics iST-PSI protocol. Figure 2 compares the chromatographic separation for the automated (red line) and manual (green line) workflows with 100 µg input samples, using all MSn total ion current (TIC) profiles. The LC-MS runs showed high consistency between the two methods, with retention time shifts within the acceptable technical performance for nanoLC separation and good peak alignment. Furthermore, these results indicate successful sample clean-up and efficient protein digestion, highlighting the performance of the automated protocol.

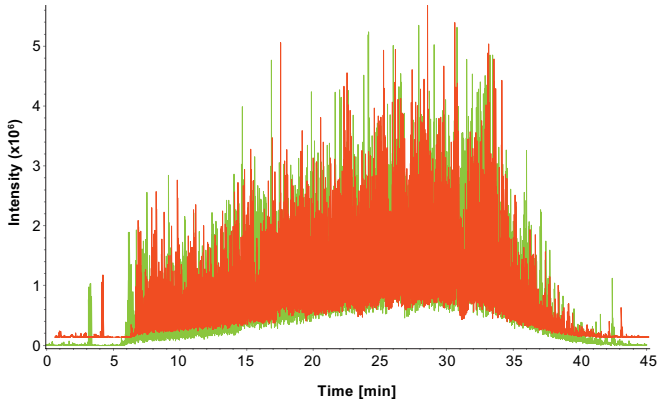


Figure 2: Stacked chromatograms showing exemplary TIC of all MSn for automated (red) and manual sample preparation (green).

The manual and automated protocols showed consistent results when considering the number of peptide and protein group IDs. Approximately 5,000 protein groups were identified for both workflows, and the number of peptides was also comparable, with a slightly higher number identified in the automatically processed samples (Figure 3).

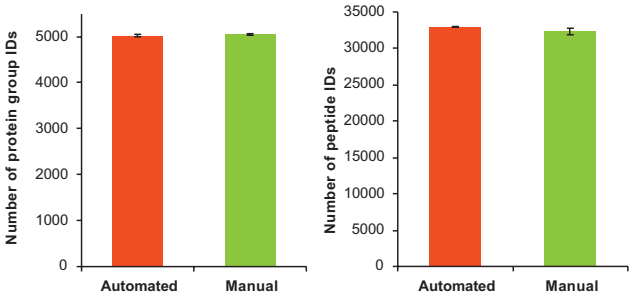


Figure 3: Average number of protein group and peptide IDs for the automatically (n=5) and manually (n=3) processed samples. The error bars depict the standard deviation.

Quantitative reproducibility was slightly better with the automated workflow, as demonstrated in Figure 4. The median coefficient of variation (CV) for protein LFQ intensities was 5.4 % for the automated workflow and 5.9 % for the manual workflow, indicating only minor sample-to-sample variation in the automated iST workflow.

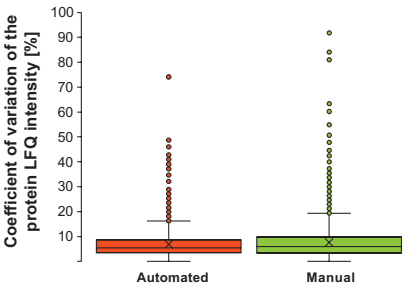


Figure 4: CV for protein LFQ intensities comparing the automated and manual iST workflows. The cross indicates the mean CV per group, and the line indicates the median CV per group.

The excellent reproducibility of the automated workflow was further supported by data from the multiscatter plot (Figure 5A). Protein LFQ intensities showed a median coefficient of determination (R^2) of 0.991 within replicates, versus a value of 0.983 for manual processing. The heatmap in Figure 5B confirms this, as well as displaying the high comparability between automated and manual sample preparation.

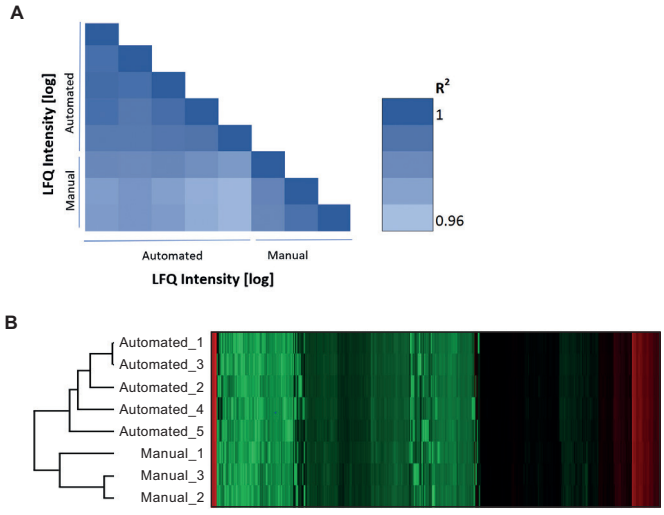


Figure 5: Comparison of HEK293 samples processed with the automated (n=5) or manual (n=3) iST workflow. (A) Multiscatter plot and R^2 of protein LFQ intensities. Protein LFQ intensities are plotted as log^{10} values. (B) Heatmap and hierarchical clustering of protein LFQ intensities. Protein LFQ intensities are plotted as log^2 values using the Euclidean distance (average linkage) method.

CONCLUSIONS

The PreOmics iST-PSI protocol was successfully automated on the Freedom EVO liquid handling platform and Resolvex A200 workstation. The workflow showed efficient protein digestion and peptide purification, and reproducibility comparable with the manual protocol. The method described here allows robust processing of up to 96 samples per run, drastically reducing tedious manual processing times and the potential for operator errors.

About the authors

Claudia Martelli, Tecan Group

Dr Claudia Martelli joined Tecan Switzerland in 2019 as an Application Specialist for the company's automated MS sample preparation workflows. She studied analytical chemistry at the Sapienza University of Rome. During her PhD at the Catholic University of Rome, she focused on clinical proteomics using various MS-based proteomics approaches. Subsequently, she did a postdoc at ETH Zürich, where she developed and applied MS approaches for the comprehensive characterization of protein-protein interactions in clinical samples.

Katrin Hartinger, PreOmics GmbH

Katrin Hartinger is the Head of Applications at PreOmics. After finishing her master´s degree in biochemistry at the University of Regensburg, she discovered her passion for LC-MS-based proteomics during her time in the Mann lab at the Max-Planck-Institute of Biochemistry and joined the R&D team at PreOmics in 2019.
At PreOmics, Katrin and her team focus on developing optimized and innovative workflows and applications for LC-MS-based protein analysis.

Katarzyna Buczak, Biozentrum, University of Basel

Dr Katarzyna Buczak joined the Biozentrum of the University of Basel in 2019 as a Research Associate in the Proteomics Core Facility. She studied biotechnology at the Jagiellonian University in Krakow. During her PhD at the European Molecular Biology Laboratory in Heidelberg, she focused on the development of approaches allowing for the spatial analysis of proteomes derived from clinical samples.

Vincenzo Romaniello, PreOmics GmbH
Dr Vincenzo Romaniello joined PreOmics in 2021 as Product Manager for automation systems. He received his PhD in molecular physiology from the University of Göttingen, Germany. Over the last 5+ years, Vincenzo has worked in technical and application support roles in automated sample preparation workflows for major biotech and human diagnostics scientific suppliers. He is highly experienced with Tecan and Eppendorf automation systems.

Nils A Kulak, PreOmics GmbH
Dr Nils A Kulak is the founder and CEO of PreOmics. During his PhD at the Matthias Mann lab, he invented the iST technology and, together with Dr Garwin Pichler, started PreOmics based on this technology in 2016. Over the last couple of years, Nils has followed his passion of driving innovation by leading the R&D team.

Alexander Schmidt, Biozentrum, University of Basel
Dr Alexander Schmidt joined the Biozentrum of the University of Basel in 2009, and heads the Proteomics Core Facility. He studied analytical chemistry at the Friedrich-Alexander-University of Erlangen, and developed a quantitative proteomics isotope labeling approach during his PhD at the Max-Planck Institute of Biochemistry, Martinsried. In his postdoc at ETH Zürich, he developed sensitive, targeted MS approaches, and applied them to several biological projects including diabetes, cancer and systems biology research.

For Research Use Only. Not for use in diagnostic procedures.
Freedom EVO is a General Purpose product. Tecan makes no claims to the application performance.
The customer must validate the product for their defined application needs.

Australia +61 3 9647 4100 **Austria** +43 62 46 89 330 **Belgium** +32 15 42 13 19 **China** +86 21 220 63 206 **France** +33 4 72 76 04 80 **Germany** +49 79 51 94 170
Italy +39 02 92 44 790 **Japan** +81 44 556 73 11 **Netherlands** +31 18 34 48 17 4 **Nordic** +46 8 750 39 40 **Singapore** +65 644 41 886 **Spain** +34 93 595 25 31
Switzerland +41 44 922 89 22 **UK** +44 118 9300 300 **USA** +1 919 361 5200 **Other countries** +41 44 922 81 11

402374 V1.0, 2022-10

www.tecan.com

TECAN.