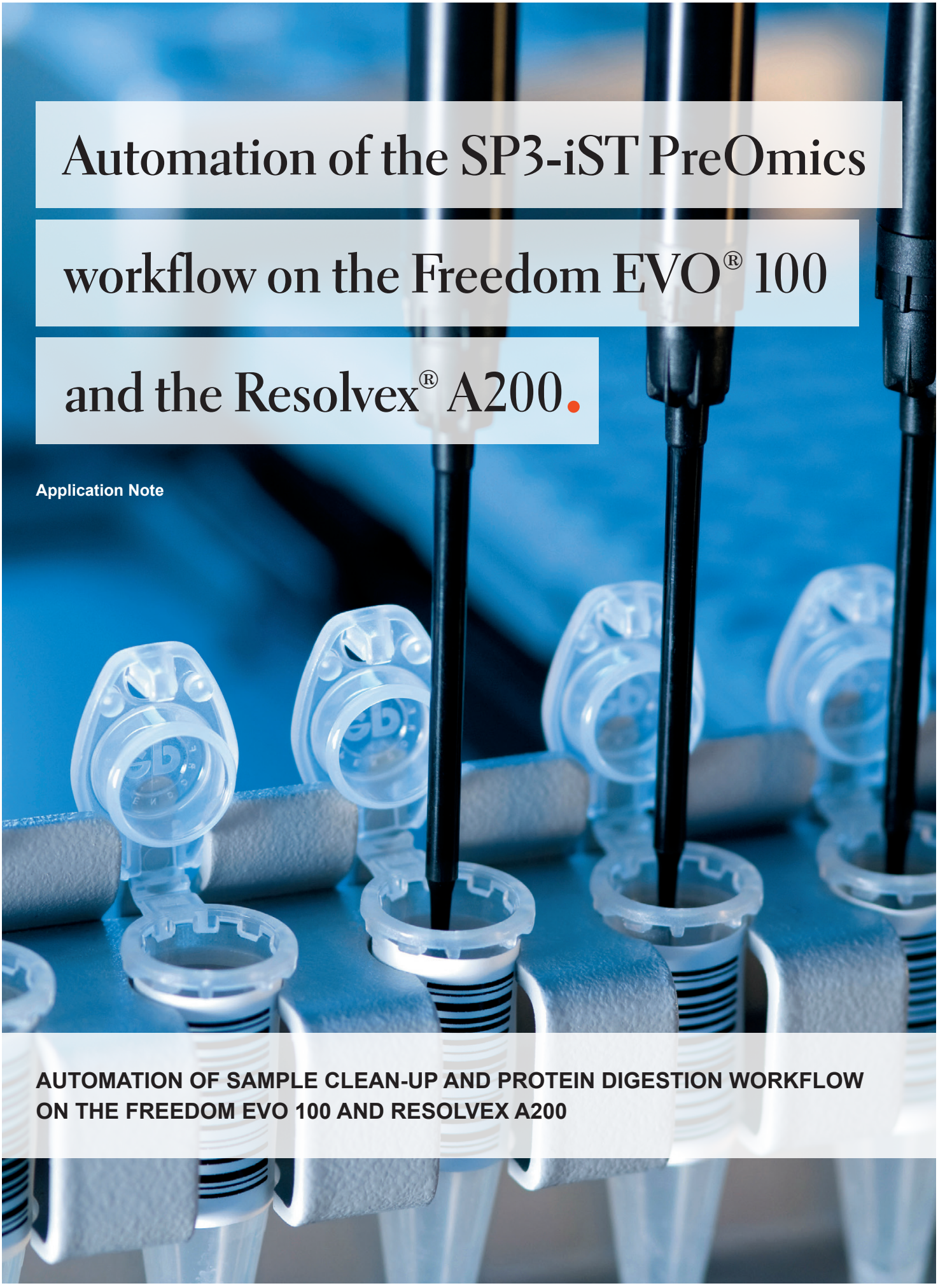
Automation of the SP3-iST PreOmics workflow on the Freedom EVO® 100 and the Resolvex® A200.

Application Note



AUTOMATION OF SAMPLE CLEAN-UP AND PROTEIN DIGESTION WORKFLOW ON THE FREEDOM EVO 100 AND RESOLVEX A200

INTRODUCTION

Proteins are active cellular components that provide fundamental functions in living organisms, and their accurate measurement can yield essential knowledge to help understand and describe biological systems. Liquid chromatography-mass spectrometry (LC-MS) has emerged as the gold standard technique for high throughput identification and quantification of proteins in a large variety of samples.

Typically, proteomics sample preparation involves protein extraction from cells or tissues, followed by reduction and alkylation of disulfide bonds, then enzymatic digestion to shorter peptides that can be separated and detected by LC-MS analysis. Unfortunately, many of the most efficient extraction buffers considerably reduce proteolytic activity and/or MS ionization efficiency, limiting overall proteome coverage. This means that they must be removed prior to protein digestion.

Single-pot, solid phase-enhanced sample preparation (SP3) technology was recently introduced as a rapid and efficient method for intact protein clean-up [1]. The SP3 protocol employs paramagnetic beads that are functionalized with hydrophilic groups for protein binding. This allows washing and digestion steps to be completed while proteins are bound to the beads. The workflow is compatible with a large variety of extraction buffers, leading to unbiased protein recovery with minimal sample processing. For improved sample quality with optimal digestion efficiency, the SP3-based protein clean-up can be readily combined with PreOmics' robust iST technology for efficient protein alkylation, digestion and subsequent peptide clean-up. This novel SP3-iST workflow is highly versatile, and suitable for a wide variety of sample types.

This application note describes high throughput automation of the SP3-iST protocol on a Freedom EVO workstation, with automated peptide clean-up using the Resolvex A200 positive pressure processor. Starting from lysed and denatured samples, this automated workflow enables the processing of up to 96 samples (ie. cell lysates) in approximately five hours, while reducing overall hands-on time to less than one hour.

MATERIALS AND METHODS

Sample lysis

HEK293 cells were grown in Dulbecco's modified Eagle 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 5 minutes at 95 °C in lysis buffer (50 µl/2e^6 cells) containing 5 % sodium dodecyl sulfate (SDS) detergent and 5 mM TCEP in 100 mM ammonium bicarbonate. Cell lysis was followed by sonication (10 cycles, 30 seconds on/off) using a Bioruptor® (Diagenode, Belgium) to shear DNA. Finally, proteins were alkylated with 10 mM chloroacetamide for 30 min at 37 °C (the PreOmics iST-REG-PSI 96HT (192rxn) kit does not generally require a pre-alkylation step, as alkylation is performed with the iST Lyse). Lysates were cleared by centrifugation (10 min, 17,000 × g, 25 °C) and protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Lysed samples were then submitted to the automated SP3-iST workflow on a Freedom EVO 100 workstation, followed by iST-based clean-up on a Resolvex A200.

Equipment and consumables for automation

The Freedom EVO workstation is 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, a BioShake 3000-T elm thermoshaker (QInstruments) and a Magnum™ EX Universal Magnet Plate (Alpaqua) (Figure 1).

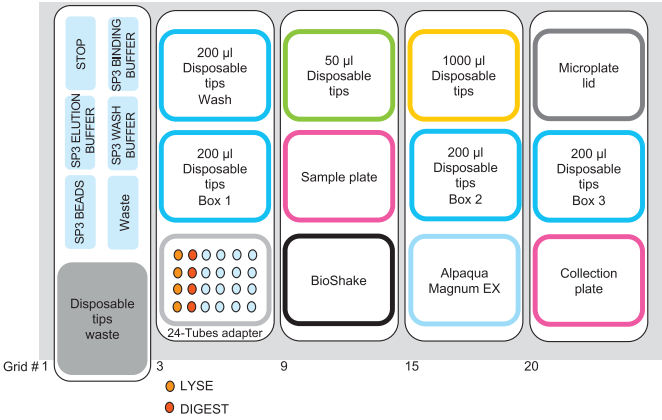


Figure 1: Freedom EVO workdeck layout for the PreOmics SP3-iST application.

All buffers, chemicals and enzymes required for SP3 protein clean-up, protein denaturation, alkylation and digestion, as well as peptide clean-up, were provided in the SP3-iST (96rxn) Add-on (P.O.00108) and iST-REG-PSI 96HT (192rxn) (#00108) kits.

Consumables
Freedom EVO workstation
Disposable tips, conductive, SLAS format, non-filtered, non-sterile, 50, 200 and 1,000 µl; Tecan
Disposable troughs, 25 and 100 ml; Tecan
4titude® Auto-Sealing PCR Plate Lid, Black; Brooks Life Science
PCR plate, 96-well, AB1300; Thermo Scientific
Resolvex A200
Clean-up plate; provided in the PreOmics iST-REG-PSI 96HT (192rxn) kit
Deep-well plate 96/1,000 µl (#0030 501.209); Eppendorf

Table 1: Overview of the consumables required to run the application on the Freedom EVO 100 and Resolvex A200.

PreOmics SP3-iST automated workflow and experimental design

The automated, highly reproducible SP3-iST protocol enables up to 96 samples to be processed in a single run. Sample preparation was performed with protein input amounts ranging from 15 to 50 µg in a total sample volume of 90 µl (up to a protein concentration of 0.6 µg/µl). Before starting the protocol, the digest and the SP3 beads (working concentration = 50 mg/ml) were prepared following the SP3-iST (96rxn) Add-on kit instructions.

For the experiment, replicates (n=6) of samples containing 15, 25 or 50 µg of total proteins from HEK293 cell lysates were diluted to a volume of 90 µl and loaded into a 96-well PCR plate. In order to evaluate the performance of the automated protocol on the Freedom EVO 100 workstation, the same cell lysates (50 µg of protein, n=3) were processed manually in parallel, using the same experimental conditions. The peptide clean-up was performed on the Resolvex A200 for both the automated and manual protocols.

The automation protocol was developed following the PreOmics SP3-iST (96rxn) Add-on kit instructions, as described below:

1. Load 90 µl of lysed sample onto a 96-well plate (manual step).
2. Add 10 µl of SP3 beads, shake (30 sec, 1,000 rpm).
3. Dilute 1:1 with 100 µl of SP3 Binding Buffer.
4. Transfer microplate to the BioShake using the RoMa.
5. Shake microplate briefly (30 sec, 500 rpm).
6. Sample incubation (10 min, room temperature, 600 rpm).
7. Transfer sample plate to the universal magnet plate.
8. Magnetization (3 min).
9. Wash magnetic beads with 3 x 150 µl of SP3 Washing Buffer.
10. Add 25 µl of iST Lyse.
11. Add 25 µl of iST Digest.
12. Cover microplate with a black auto-sealing lid using the RoMa.
13. Transfer microplate to the BioShake for protein digestion (3 hours, 37 °C, 1,000 rpm).
14. Add 50 µl 13 mM NaOH for elution (Not provided in iST-SP3 (96rxn) Add-on kit since not required for sufficient peptide elution from SP3 beads)
15. Shake (5 min, room temperature, 1,000 rpm).
16. Magnetization (3 min).
17. Collect samples in a new microplate.
18. Add 100 µl of iST Stop.
19. Shake (30 sec, room temperature, 500 rpm).
20. Place clean-up filter plate on the Freedom EVO worktable (manual step).
21. Transfer samples to the clean-up filter plate.

iST positive pressure peptide clean-up on the Resolvex A200.

Following automated processing, the clean-up filter plate was positioned over a deep-well microplate and manually transferred to the Resolvex A200 for the final peptide clean-up step. For comparison purposes, the manually processed samples were loaded into the clean-up plate alongside those processed on the Freedom EVO 100 workstation. The Resolvex A200 gas supply was set to 80 psi, and the automated peptide clean-up protocol was performed according to the iST-REG-PSI 96HT (192rxn) kit instructions. In brief, peptides were bound to the SPE membrane of the clean-up plate and samples were washed with 1 x 200 µl Wash 1, and 1 x 200 µl Wash 2. The clean-up plate was then manually transferred to a new deep-well microplate, and the peptides were eluted with 2 x 100 µl Elute. 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 0.1 % aqueous formic acid solution, and 0.125 µg of peptides (measured by NanoDrop™, Thermo Fisher Scientific) were analyzed by LC-MS/MS using a Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer fitted with an EASY-nLC™ 1000 (both Thermo Fisher Scientific) and a custom-made column heater set to 60 °C. Peptides were resolved on an RP-HPLC column (75 µm × 36 cm) packed in-house with ReproSil-Pur® C18–AQ, 1.9 µm resin (Dr. Maisch) using gradient separation with 0.1 % formic acid in water (A) and

80 % acetonitrile + 0.1 % formic acid in water (B), and a flow rate of 0.2 µl/min. The following gradient was used: from 5 % B to 10 % B over 5 min, to 35 % B over 45 min, to 50 % B over 10 min, to 95 % B over 2 min, followed by 18 min at 95 % B.

The mass spectrometer was operated in data dependent acquisition (DDA) mode, with a total cycle time of approximately 1 s. Each MS1 scan was followed by higher-energy collisional dissociation (HCD) of the 10 most abundant precursor ions, with dynamic exclusion set to 45 s. For MS1, 3e6 ions were accumulated in the Orbitrap over a maximum time of 100 ms, and scanned at a resolution of 70,000 FWHM (full width at half maximum) at 200 m/z. MS2 scans were acquired at a target setting of 1e5 ions, with a maximum accumulation time of 100 ms and a resolution of 35,000 FWHM at 200 m/z. Singly charged ions and ions with an unassigned charge state were excluded from triggering MS2 events. The normalized collision energy was set to 27 %, the mass isolation window was set to 1.4 m/z, and one microscan was acquired for each spectrum.

DATA ANALYSIS

The raw data was imported into Progenesis QI software (v2.0, Nonlinear Dynamics) to extract peptide precursor ion intensities across all samples, by applying the default parameters. The resulting MGF files were searched against a forward and reverse decoy database of the predicted proteome from *Homo sapiens* (download date: 18/3/19, total of 41,616 entries), using MASCOT (v2.4.1, Matrix Science Inc.) with the following search criteria:

- Full tryptic specificity required (cleavage after lysine or arginine residues, unless followed by proline)
- Three missed cleavages permitted
- Carbamidomethylation (C) was set as a fixed modification
- Oxidation (M) and acetylation (protein N-term) were applied as variable modifications
- Mass tolerance of 10 ppm (precursor) and 0.6 Da (fragments)

The database search results were imported into Progenesis QI and then filtered using the ion score to set the false discovery rate to 1 % on both the peptide and protein levels, based on the number of reverse protein sequence hits in the datasets. The relative quantitative data obtained was normalized and statistically analyzed using an in-house script (SafeQuant, PMID: 27345528).

RESULTS

To assess the efficiency of the automated PreOmics SP3-iST workflow, the chromatographic separations and full MS profiles of each sample corresponding to different input amounts were compared (Figure 2). As shown in Figure 2A, the LC-MS runs showed retention time shifts within the acceptable technical performance for nanoLC and good peak alignment. These results indicate a successful sample clean-up, with complete removal of contaminants and an efficient protein digestion. Moreover, the comparison between the manually processed samples and those processed using the automated workflow showed very high consistency between the two methods, confirming the success of the automated protocol (Figure 2B).

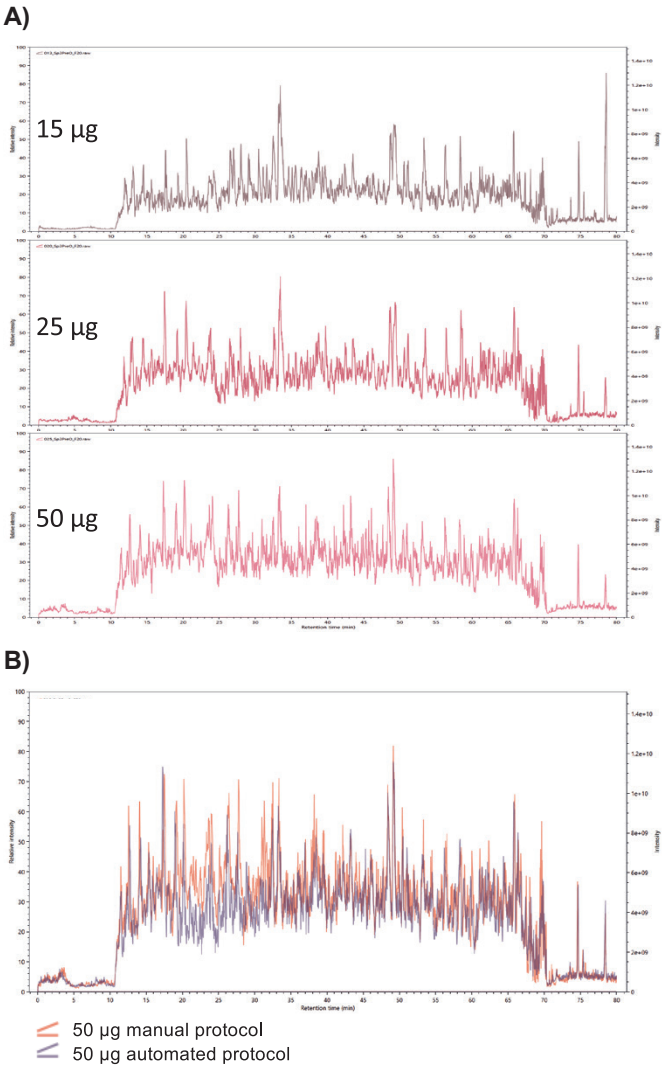


Figure 2: A) Example total ion current (TIC) chromatogram of the samples obtained for 15, 25 and 50 µg of protein input. B) TIC chromatograms of the 50 µg input samples processed with the manual (pink line) and automated (purple line) methods.

Starting protein amount	PSMs	Peptides (IDs)	Proteins (IDs)	Median peptide CV (%)	Median protein CV (%)	% no missing cleavages	% 1 missing cleavage	% 2 missing cleavages
Automation protocol								
15 µg	31,200	27,390	2,964	14	9	91.3	8.3	0.4
25 µg	31,361	27,395	2,963	21	12	91.3	8.3	0.4
50 µg	31,371	27,392	2,964	21	11	91.3	8.3	0.4
Manual protocol								
50 µg	31,363	27,389	2,961	15	8	91.3	8.3	0.4

Table 2: nanoLC-MS results for the SP3-iST automated and manual protocols.

The LC separation quality and sample clean-up efficiency, number of peptide-spectrum matches (PSMs) and identified peptides/proteins (IDs), peptide/protein coefficients of variation and percentage of missing cleavages were used to evaluate the performance of the automated SP3-iST protocol (Table 2).

The numbers of PSMs and IDs (both peptides and of proteins) were very consistent for all samples, regardless of protein input, as well as between the manual and the automated protocols. Approximately 27,370 peptides and 2,960 proteins were identified in all samples, in agreement with the expected performance of the nanoLC-MS measurements conducted. This demonstrates the high efficiency, quality and robustness of the SP3-iST workflow for the entire range of tested input amounts (15-50 µg). The low percentage of missing cleavages demonstrated the high efficiency of the protein digestion step. Moreover, the method reproducibility – calculated as % CV for peptide and protein quantification – was homogenous between the different tests (Figure 3A/B).

The method reproducibility and sample consistency between the manual and automated protocols is also demonstrated in the heat map in Figure 4, with all samples clustering together as expected.

CONCLUSIONS

The PreOmics SP3-iST protocol has been successfully automated on the Freedom EVO liquid handling platform and Resolvex A200 workstation. The automated workflow shows efficient sample clean-up, good peptide recovery and high reproducibility comparable with the respective manual protocol. It demonstrates high throughput (up to 96 samples per run), reduces sample processing times and eliminates potential operator errors, enhancing the method's overall analytical performance.

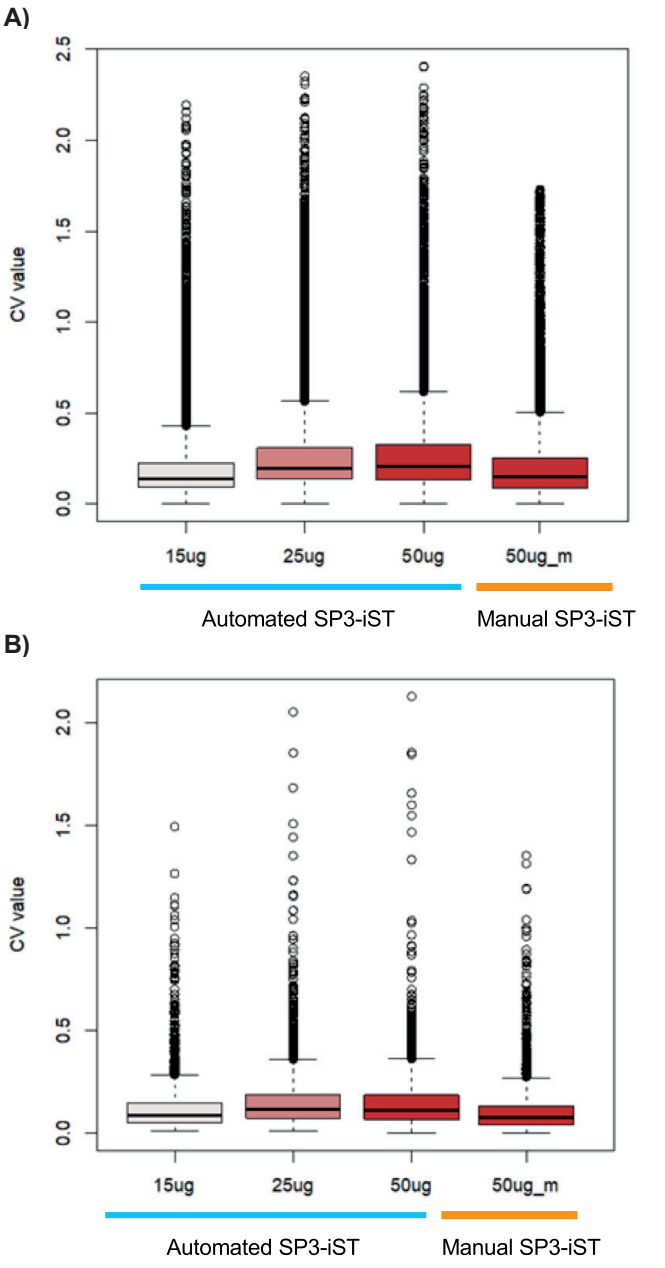


Figure 3: Peptide (A) and protein (B) CVs for the SP3-iST experiment.

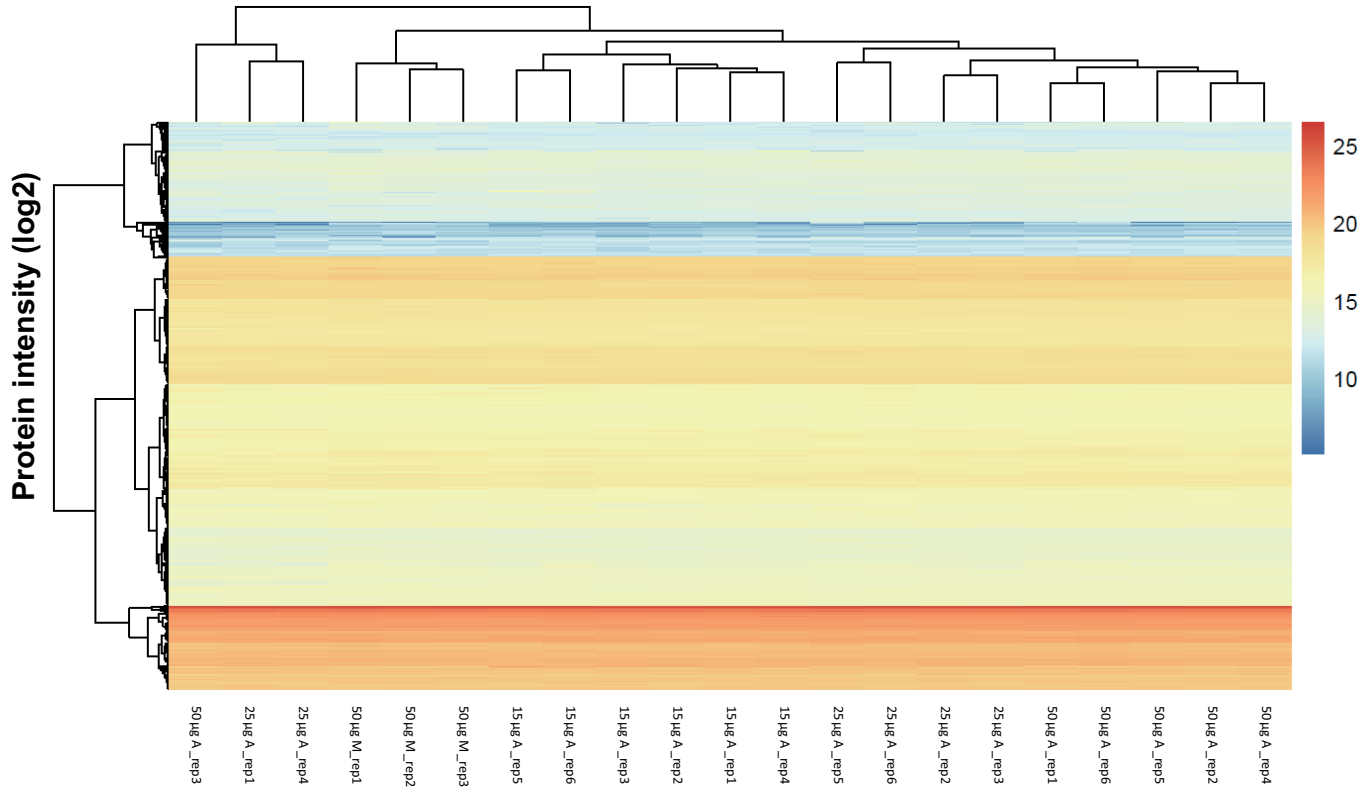


Figure 4: Heat map and hierarchical clustering of all samples processed with the automated and manual protocols. Protein MS intensities are plotted as log2 values.

REFERENCES

[1] Hughes C.S. *et al. Nat Protoc.* 2019, **14**(1), 68-85.

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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.

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Katrin Hartinger joined PreOmics in 2019, and works as a Senior Scientist in Research and Development. She studied biochemistry at the University of Regensburg and, after finishing her MSc in biochemistry, was introduced to LC-MS-based proteomics during an internship in the Mann lab at the Max-Planck Institute of Biochemistry, Martinsried. At PreOmics, Katrin focuses on the development of optimized and innovative LC-MS sample preparation workflows.

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Dr Sarah Lupton joined PreOmics in 2019 in Technical Sales, Northern Europe. She received her PhD from the University of Warwick, where she was introduced to mass spectrometry as part of her research on an extracellular enzyme produced by B. pumilis. Sarah worked in pharma and contract research for over 10 years, using LC-MS for a wide range of small molecules, lipids and proteins, before moving to technical and commercial support in sample preparation for major scientific suppliers.

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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 been CEO at PreOmics, where he has followed his passion of innovations, leading the R&D team.

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Dr Alexander Schmidt joined the Biozentrum, 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.

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