High-Throughput Automation of the PreOmics iST Technology for Proteomics LC-MS Sample Preparation

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Introduction

Proteomics workflows are becoming increasingly important in the clinical diagnostics and biotech industries, such as in therapeutic drug monitoring or biomarker detections. Liquid Chromatography Mass Spectrometry (LC-MS)-based assays in particular offer the great advantage of measuring multiple analytes at once (and is quantitative).

Proteomics workflows have been traditionally limited by LC-MS measurement time and sample preparation throughput. While advances in LC-MS instrument technologies and workflows have now significantly increased the number of samples that can be processed on a weekly basis, the bottleneck has shifted to efficient, robust, and reliable high-throughput sample preparation.

In this Application Note we demonstrate for the first time a completely automated high-throughput LC-MS sample preparation workflow, combining the Hamilton liquid handling technology with the PreOmics iST workflow.

- **Save time and costs with a maximized walk away time for 96-Well LC-MS sample preparation**
- **Full flexibility to process 1 to 96 samples with minimal tip usage**
- **Standardized workflow with high data reproducibility and process safety**

Figure 1: The Hamilton Microlab® VANTAGE Liquid Handling System® 1.3m

Workflow Description

Automated iST workflow

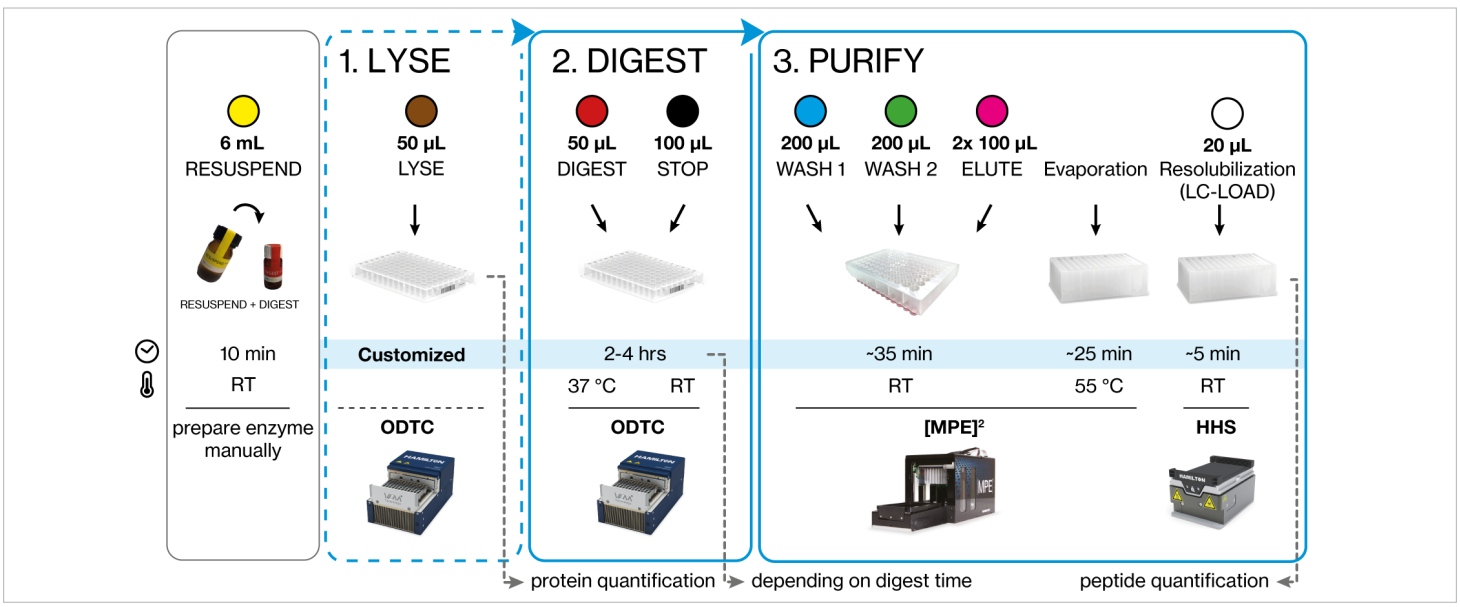

Figure 2: Automated iST workflow – In this figure, the reagents, labware and modules are described for processing 96 samples on a Hamilton liquid handling platform. The temperature and run time on the robot are displayed for each step. The conditions for lysis may vary, according to the sample material. After resolubilization of the digested peptides, the 96-well plate can be directly loaded onto a LC-MS autosampler.

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Deck Layout Description

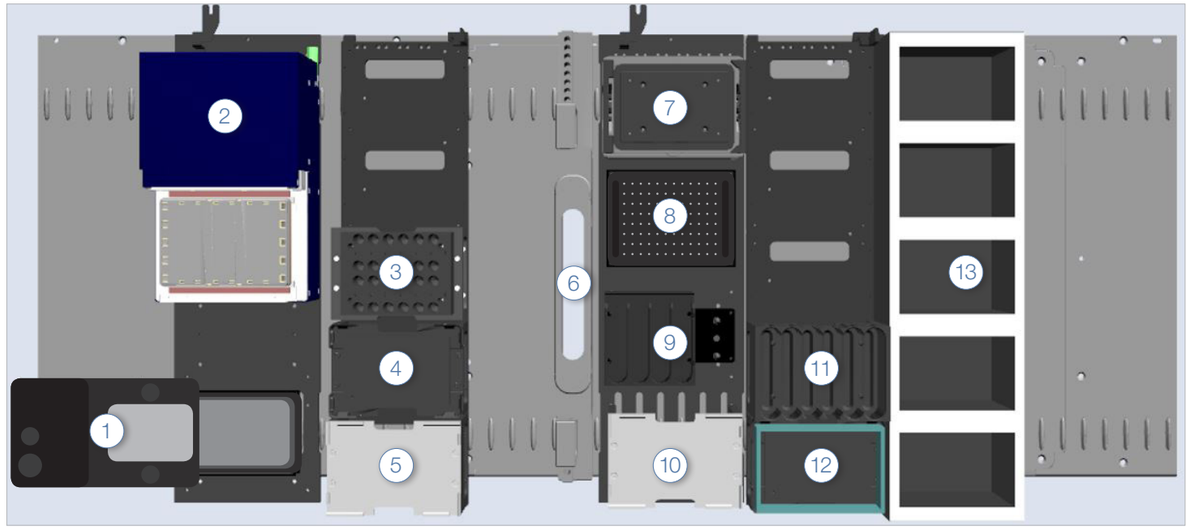


Figure 3: Deck layout

1. [MPE]² (Monitored Multi-Flow Positive Pressure Evaporative Extraction module)
2. ODTC (On-Deck Thermo Cycler)
3. Tube module (reagents)
4. DWP (Deep-Well Plate) module (collection plate)
5. MTP (Micro-Titer Plate) module (sample plate)
6. Tip Waste (2 Tracks)
7. HHS (Hamilton Heater Shaker)
8. [MPE]² Evaporator module parking position
9. QCG (Quad CO-RE Gripper) & 60 ml trough module (buffers)
10. MTP module (PCR Comfort lid)
11. 60 ml trough module (buffers)
12. DWP module (frame for filter plate)
13. Tip carrier (50 µl and 300 µl Tips)

Application Software

Via a Graphical User Interface, an operator can define the parameters of each run, such as the digest time or the volume for resolubilization, prior to LC-MS.

Kit Description

The PreOmics iST kit contains proprietary reagents to denature, reduce and alkylate proteins in one step, as well as the enzymes to perform a tryptic digestion. The final peptide clean-up includes two positive-pressure 96-well plates (iST-REG-PSI 96HT (192 samples): P.O. 00108; iST-REG-PSI 96HT (384 samples): P.O. 00112).

Technology

LC-MS sample preparation assays, such as the PreOmics iST kit, often use organic and volatile liquids in their workflows. A distinct pressure system in the Hamilton channels allows for the reliable monitoring and control of such liquids with, for example, Anti-Droplet Control (ADC).

The CO-RE (Compressed O-Ring Expansion) technology integrated in the channels permits the transport to-and-from each module on-deck without the need for an additional transport tool.

The ODTC, in combination with the proprietary Hamilton PCR Comfort lid, controls the temperature during protein digestion with high-precision and uniformly, without the loss of liquid, due to evaporation. The [MPE]² provides the positive-pressure functionality to process filter plates and the evaporator module to rapidly dry down samples, eliminating the need for a centrifuge.

Results

Four experiments were performed to assess the reproducibility and the robustness of the automated workflow **(Fig. 2)**:

(I) A cross-contamination test was performed with *Saccharomyces cerevisiae* protein extracts, demonstrating that there is **no cross-contamination** occurring during the [MPE]² procedure or any other step in the workflow **(Fig. 4)**.

(II) Proteins from *Pichia pastoris* and commercial human plasma were digested on two different days and the data was acquired in a single experiment. A **100% inter-day overlap of identified proteins was achieved** for both *P. pastoris* and human plasma **(Fig. 5)**.

(III) Aliquots of *P. pastoris* samples were also digested, following the standard manual protocol. **98% of the identified proteins were detected in both the manual and inter-day runs (Fig. 5A)**.

(IV) To test a full 96-well plate automation run, aliquots from 48 *P. pastoris* and 48 commercial human plasma protein extracts were processed. The normalized protein intensities obtained demonstrated a mean **correlation of 0.97 and 0.98** for *P. pastoris* and human plasma, respectively **(Fig. 6)**.

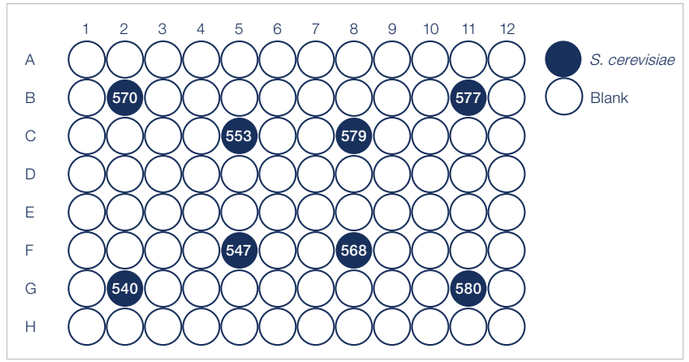


Figure 4: Cross-contamination – 8 aliquots of *S. cerevisiae* protein extracts (manually prepared, 5 µg) and 88 blank samples (only LYSE buffer) were arranged as shown and processed according to a customized procedure: 100 µl of protein extract or buffer, respectively, and 10 µl of DIGEST were incubated for 1.5 h in the ODTC.

The dried samples were dissolved in 20 µl LC-LOAD and 2 µl was injected on a UPLC, coupled with an Orbitrap Fusion Lumos (Thermo Scientific). A 20 min gradient of 5-35% acetonitrile was applied to separate the peptides. Protein identification and visualization were performed using Mascot (Matrix Science) and Scaffold (Proteome Software).

After filtering for 0.1% peptide/1% protein False Discovery Rate (FDR) and 3 peptides/protein, the result showed 540 to 580 proteins identified in the *S. cerevisiae* samples and no proteins detected in the blanks.

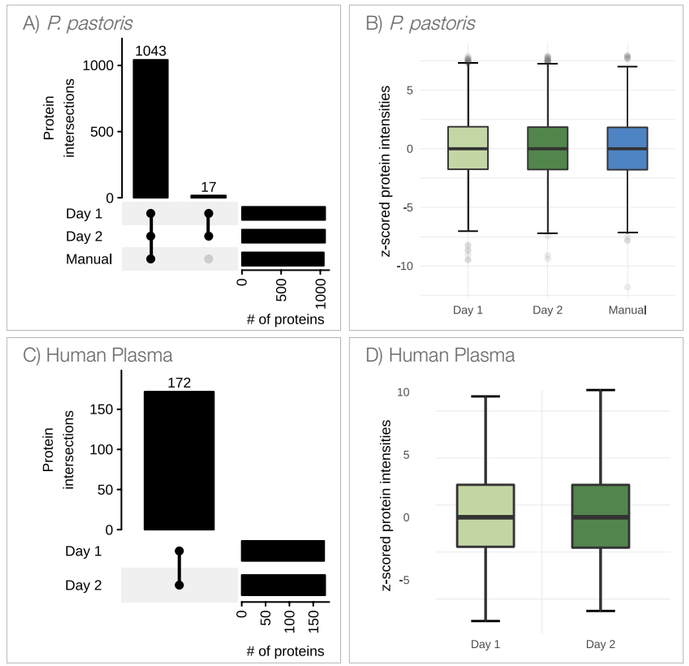
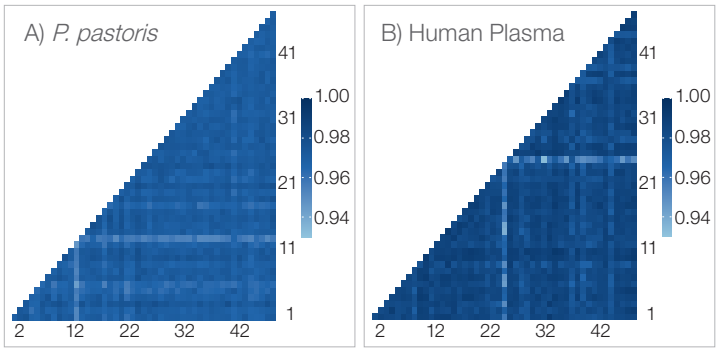


Figure 5: Inter-day reproducibility and comparison to manual processing – (A and B) 8 aliquots of manually prepared *P. pastoris* protein extracts (50 µg each) were processed on two different days, and 4 additional samples were digested with the manual procedure. The automation was customized as follows: 50 µl of proteins, 50 µl of DIGEST, and 10 µl of additional RESUSPEND were incubated for 1.5 h in the ODTC.

The LC-MS measurement was performed as described for the cross-contamination assay with 1 µl injected on the LC-MS system. Protein identification and quantification was performed using MaxQuant [1] (5% protein FDR; 2 peptides/protein). 1060 proteins were identified for the automated runs and 1043 for the manually processed samples (A) with a mean value of 0.12 for day 1, 0.10 for day 2 and 0.07 for manual when considering the protein intensities (median-normalized scaled) (B). Day 1 and 2 revealed an intersection of 100% and 98%, as compared to the manual run (A).

(C and D) 8 samples of commercially available human plasma (each ~70 µg; Sigma-Aldrich, P9523) were processed on day 1 and 2. The LC-MS measurement and analysis were executed as described for the *P. pastoris* proteins, with a gradient of 35 min. 172 proteins were identified for days 1 and 2, with an intersection of 100% (C) and a mean value of 0.22 for day 1 and of 0.25 for day 2 with regard to the protein intensities (median-normalized scaled) (D). All plots were generated in R [2].

Figure 6: 96 sample run – (A and B) 48 aliquots of manually extracted *P. pastoris* proteins were processed together with 48 samples of commercially available human plasma. For the automation run and LC-MS measurement, the same parameters were used as for the inter-day experiments, except for a 1 to 10 dilution of the plasma samples prior to the LC-MS measurement. The normalized protein intensities exhibited Pearson correlation values between 0.94 and 0.98 for the *P. pastoris* samples (A) and between 0.93 and 0.99 for the plasma samples (B). All plots were generated in R.



Summary

The qualification experiments and results demonstrate that the PreOmics iST technology effectively runs on a Hamilton liquid handling platform. The user is supported with a fully automated standardized and reproducible workflow, ultimately resulting in LC-MS grade peptides in less than 4 hours of total sample processing time. Successful processing of yeast, human plasma and cerebrospinal fluid (CSF) samples (not shown), demonstrates the applicability in clinical, biotech and research settings.

System Requirements	Part Number
Microlab VANTAGE 1.3m, INSTINCT V Software v1.9 (the power cord must be ordered for the specific country)	818050A13
Arm Channel/IPG	818009
8x Standard Pipetting Channels	196005
2T waste block	818047
Ejector plate for 2T waste	10088368

System Dimensions
Width: 1448 mm (including left extension [MPE]^2)
Height: 1360 mm (door open)
Depth: 1010 mm

Consumables	Part Number/ Provider
iST-REG-PSI 96HT (192 samples)	00108 / PreOmics
iST-REG-PSI 96HT (384 samples)	00112 / PreOmics
50 µl Conductive Tips without Filter	235966
300 µl Conductive Tips without Filter	235902
96 Well PCR FramePlate	814302
60 ml Reagent Container	194051
PCR Comfort Lid	814300
1.5 ml Eppendorf Tubes	0030123328 / Eppendorf
DWP (Collection plate)	186005837 / Waters

Labware Requirements	Part Number
[MPE]^2 with evaporator module (mounted on base plate with ODTC)	96160-04
ODTC 96 kit left VANTAGE (mounted on base plate with [MPE]^2)	10067561
Integration Kit [MPE]^2 and ODTC left VANTAGE (This kit is based on 95952-01 and 10066706. Panel for cosmetic is included)	10113023
2x MultiFlex Carrier Base Plate	188039
MultiFlex Tube / Cup Module	188048
2x MultiFlex DWP Module	188042
2x MultiFlex MTP Module	188228
Shaker Carrier Base	187001
HHS 3.0 mm orbit flat bottom	10068482
MFX Trough Module QCG Pos	10113303
QCG on MFX position	96006-01
MultiFlex Module Bracket 7T	188133
MultiFlex Reagent Trough Module	188404
Frame for filter plate	182712
Tip Carrier	182085

Citations:
1) Cox, J. and Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol, 2008, 26, pp 1367-72.
2) R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

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