Reproducible and sensitive proteomic sample preparation workflow for µLC-MS/MS analysis

Authors: Chloé Moritz and Zuzana Demianova
PreOmics GmbH, Planegg/Martinsried, Germany

Introduction

Nanoflow liquid chromatography (nLC) has historically been the gold standard in proteome research. The low flow rates improve electrospray ionization of peptides prior to mass spectrometry analysis enabling a gain in sensitivity. However, this comes at the cost of less reproducible and less durable columns and difficulties in maintaining a stable chromatographic system over long periods.

These factors can limit the reproducibility of peptide identification, quantification, data completeness, reliability, and acquisition throughput[1]. Microflow liquid chromatography (µLC) is now being increasingly utilized to address some of these challenges.

This study evaluated the well-established iST kit[2] for global proteomics approaches when coupled to µLC-MS platforms (Figure 1). Protein standards of known quantities were selected to represent a wide range of protein properties (hydrophobicity/isoelectric points/molecular weight) and to evaluate workflow performance. The results demonstrate the suitability, reproducibility, and sensitivity of the iST kit in combination with µLC-MS/MS analyses. Additionally, it allows for the identification of several proteins (host cell proteins, HCPs) present in trace amounts and copurified with a biopharmaceutical drug product.

Keywords

Proteins, Host cell proteins, Proteomics, Sample preparation, Mass spectrometry, iST, Microflow LC-MS, Capillary-flow LC-MS, Monoclonal antibody

Key takeaways

- The iST kit is an efficient, fast, reproducible solution for proteomic sample preparation workflows.
- The iST kit can process the amounts of biological sample material required for downstream microflow proteomic analyses.

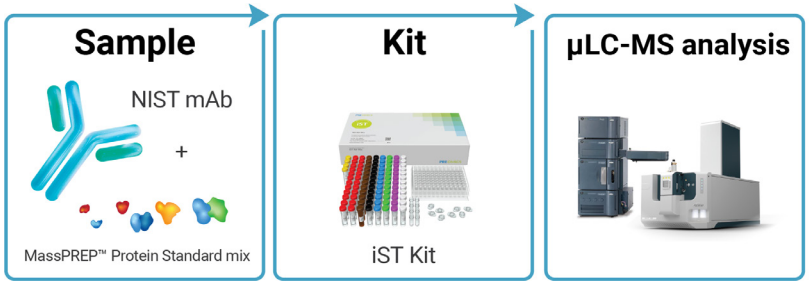

Figure 1 | A standardized workflow from protein sample preparation with the iST kit to purified peptides measured on a µLC-MS/MS platform.

Methods

A NIST monoclonal antibody (20µg) was purchased from the National Institute of Standards and Technology (NIST mAb, RM 8671) and resuspended in water to have a stock solution of 10 µg/µL. The MassPREP™ Protein Standard Mix[3] (186004900, Waters, Milford, MA, US) was solubilized with 50 µL of 1X Phosphate Buffer Saline (PBS) to prepare the stock solution. The MassPREP is a mixture of six proteins with varying physicochemical properties, present in different amounts and covering a range of molecular weights from about 12 to 97 kDa. The iST kit (PreOmics GmbH, Germany) was used for sample preparation. For the lysis step, 0.1 µL of NIST mAb and 14 µL of MassPREP stock solution were added to 50 µL of LYSE buffer. The resulting peptides were resuspended in 187.5 µL of LC-LOAD.

The ZenoTOF 7600 (AB Sciex, Framingham, MA, US) was coupled with a Waters ACQUITY UPLC M-Class microsystem (Waters), fitted with a Kinetex XB-C18 column (100Å, 2.6µm, 150x0.3 mm, Phenomenex, Torrance, CA, US). Solvent A was water containing 0.1% formic acid, and solvent B was acetonitrile containing 0.1% formic acid. 5 µL of resuspended peptides were injected into the µLC column and separated with a gradient from 2% to 40% of solvent B over 60 min at a 2 µL/min flow rate. A Data-dependent acquisition (Top30) strategy was used to acquire the data.

The data were analyzed with MaxQuant (v.2.1.3.0) using a custom database containing the NIST mAb light and heavy chains, the MassPREP™ Protein Standard Mix sequences (Ribonuclease A (P61823), Cytochrome C (P00004), Albumin (P02769), Myoglobin (P68082), Enolase (P00924), Phosphorylase B (P00489)) and the Murinae SwissProt proteome (Taxon: 39107, 20th January 2023). Data were evaluated using a target-decoy strategy and a 1% FDR at both peptide and protein levels. Matched proteins contained at least one unique peptide. The minimum peptide size has been set at 6 amino acids.

Results and Discussion

To show the potential benefit of iST kits for standardization and reproducibility, different kit batches have been used to prepare samples for µLC-MS/MS analyses. Table 1 shows all identified protein standards, NIST mAb chains, and HCPs across all tested batches of the kit. Standard proteins and NIST mAb chains were identified across all batches. In addition to protein standards and NIST mAb, eighteen HCPs of NIST mAb were detected; from them, just two HCPs weren't identified in a single batch and two more were identified in only one batch. This highlights the reproducibility and robustness of the iST-µLC-MS/MS workflow, even when protein abundance levels differ by four orders of magnitude.

Along with the iST kit reproducibility, it is essential to be able to detect very low-abundance proteins in the presence of highly abundant ones by LC-MS. Table 1 illustrates the sensitivity of the iST workflow combined with µLC-MS/MS analysis by identifying eighteen HCPs present in trace amounts in the NIST mAb. These HCPs originated from the host organism and were co-purified with the NIST mAb during product manufacturing. Most HCPs are removed during purification processes, but trace amounts of them may still be present and could compromise the safety and efficacy of biopharmaceutical drugs. Therefore, monitoring co-purified HCPs is crucial and requires sensitive methods such as the iST-µLC-MS/MS workflow to detect them.

Quantitative reproducibility of the proteomics workflow is even more challenging to achieve than qualitative reproducibility. Figure 2 plotted the intensity of selected NIST mAb heavy chain peptides measured in 48 runs. These peptides were tracked in runs from 15 sample preparations from three iST kit batches. The CVs of the peptide peak intensities are 18%, 19%, 18% and 23% for peptides 1, 2, 3, and 4 respectively. Importantly, the average CV of peptide intensity for all four peptides considering only injection variability is 3.2%. These results illustrate the excellent standardization of the entire process, from iST sample preparation to peptide analysis by µLC-MS/MS.

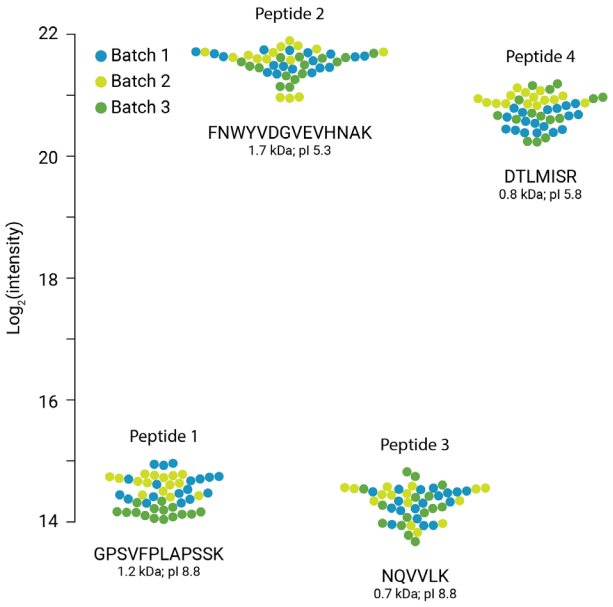

Figure 2 | The intensity of 4 peptides of NIST mAb heavy chain scatter across 48 injections from samples prepared using three iST batches.

The Beeswarm graph shows 48 injections of 15 samples from three different batches of the iST kit. Four peptides of different size (kDa) and isoelectric point (pI) were selected to display the CV of the peptide intensity which, taking into account the variability of the injection and sample preparation, is on average 19%. The average CV of the peptide intensity for the four peptides considering only the variability of the injection is 3.2%.

Table 1 | Table of the protein groups identified in the sample prepared using three different iST kit batches. Triplicate injections per batch were measured.

Protein group IDs		Batch 1	Batch 2	Batch 3
NIST mAb	Heavy chain	x	x	x
	Light chain	x	x	x
MassPREP™ Protein Standard Mix	P02769|ALBU_BOVIN	x	x	x
	P68082|MYG_HORSE	x	x	x
	P00489|PYGM_RABIT	x	x	x
	P00924|ENO1_YEAST	x	x	x
	P61823|RNAS1_BOVIN	x	x	x
	P00004|CYC_HORSE	x	x	x
HCPs	Q9WUB7|CLCKA_MOUSE	x	x	x
	P18421|PSB1_RAT	x	x	x
	Q7TNF8|RIMB1_MOUSE	x	x	x
	Q3UV17|K22O_MOUSE	x	x	x
	Q3UQU0|BRD9_MOUSE	x	x	x
	P08228|SODC_MOUSE	x	x	x
	P53534|PYGB_RAT	x	x	x
	P05064|ALDOA_MOUSE	x	x	x
	Q99LX0|PARK7_MOUSE	x	x	x
	P70623|FABP4_RAT	x	x	x
	P35427|RL13A_RAT	x	x	x
	P48500|TPIS_RAT	x	x	x
	Q922R8|PDIA6_MOUSE	x	x	x
	Q4FZC9|SYNE3_MOUSE	x	x	x
	O70362|PHLD_MOUSE	x		x
	O88767|PARK7_RAT	x		x
	Q9ERE9|JIP2_MOUSE			x
	P26043|RADI_MOUSE			x

Conclusions

In conclusion, the iST kit is capable of efficient, rapid, and reproducible protein sample preparation compatible with µLC-MS/MS analysis. This combination showed sensitive performance even for protein sample analysis with a wide dynamic range, such as HCPs in the presence of a highly abundant biopharmaceutical drug (e.g., NIST mAb). Moreover, outstanding reproducibility across three different iST kit batches was achieved. The average CV of peptide intensities for four selected peptides considering both injection and sample preparation replicates is 19% and 3.2% when only injection variability is considered.

Finally, iST coupled with µLC-MS/MS provides a robust workflow that reduces the analysis time and mitigates the challenges associated with maintaining nLC systems.

Products

Product	Manufacturer	Product Code
iST 8x	PreOmics GmbH	P.O.00001
iST 96x	PreOmics GmbH	P.O.00027
iST HT 192x	PreOmics GmbH	P.O.00067

Ordering information:

http://www.preomics.com/quote

order@preomics.com

Acknowledgements

We thank the Anaquant team, especially Xavier Homo and Tanguy Fortin, for their time and affords with project design and data acquisition.

References

1. Bian Y, Zheng R, Bayer FP, Wong C, Chang YC, Meng C, Zolg DP, Reinecke M, Zecha J, Wiechmann S, Heinzlmeir S. Robust, reproducible and quantitative analysis of thousands of proteomes by micro-flow LC–MS/MS. Nature communications (2020) 11(1): p1-2. DOI: 10.1038/s41467-019-13973-x

2. Kulak NA, Pichler G, Paron I, Nagaraj N, Mann M. Minimal, encapsulated proteomic-sample processing applied to copy-number estimation in eukaryotic cells. Nat Methods (2014) 11(3): p319-24. DOI: 10.1038/nmeth.2834

3. Waters MassPREP Protein Standard Mix (https://www.waters.com/webassets/cms/support/docs/720003229en.pdf)