Application Note
iST Sample Preparation for Mammalian Tissue

PREOMICS

AUTHORS

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OBJECTIVE

Efficient and simplified processing of various mammalian tissues using the iST technology for reproducible and sensitive proteomic analyses.

KEYWORDS

Sample preparation; proteomics; protein analysis; mass spectrometry (MS); mammalian tissue; fresh frozen; brain; heart; liver

INTRODUCTION

Proteins are the chief actors in regulating physiological activity of mammalian tissues. Mass spectrometry (MS)-based analyses of tissues have revealed striking dynamics of the proteome, including substantial differences in protein abundances, cell type- and time-dependent protein expression changes, as well as significant regulation by a large number of post-translational modifications (1,2).

In order to perform global and quantitative proteomic tissue analyses, sample preparation is a key step and workflows must be fast, robust and sensitive to ensure deep proteome coverage and minimize sample loss during the preparation. Since chemical lysis alone requires dilution and/or precipitation to achieve compatibility with downstream protein digestion and MS analysis, mechanical disruption has become a standard for efficient protein extraction of tissue.

In this application note, we present a streamlined preparation of three different fresh frozen tissue samples for MS-based proteomic analysis utilizing the PreOmics iST Kit in combination with protein extraction beads and ultrasonication (**Figure 1**). The whole workflow takes less than 4.5 hours from tissue lysis to ready-to-measure peptides, providing significant time savings compared to common tissue preparation protocols. The optimized and patented peptide washing steps eliminate both hydrophobic and hydrophilic contaminants. Thus, the resulting clean samples help to decrease MS downtime by enabling longer cleaning cycles and to generate more reproducible and reliable results.

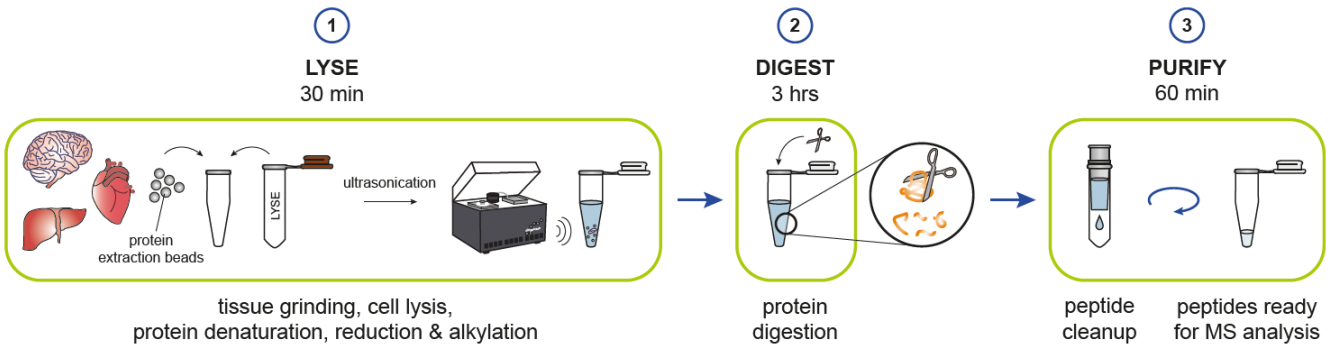

Figure 1 - iST workflow for mammalian tissues

RESULTS & DISCUSSION

In order to determine the best protein extraction method employing mechanical force in the presence of the PreOmics LYSE buffer, we compared traditional bead milling coupled with high speed shaking against ultrasonication bead lysis. We assessed both lysis methods for three distinct tissue types (brain, heart, liver) and performed LC-MS/MS analyses on a LTQ-Orbitrap XL system. We found that ultrasonication bead lysis outperformed high speed shaking in terms of both peptide and protein identifications, although the differences between both methods became smaller with increasing tissue rigidity (**Fig. 2A+B**).

To further increase the depth of protein identifications, we performed peptide fractionation downstream of the iST Kits as previously reported (3) and analyzed the fractionated peptides on a QExactive HF-X instrument that generates much higher numbers of peptide and protein identifications (**Fig. 2C+D**). We identified ~10,500 protein groups in all three tissue types combined, ~9,000 protein groups in brain, ~7,500 protein groups in liver and ~5,800 protein groups in heart. The differences in protein identifications can be explained by increased tissue rigidity and higher dynamic range of the respective proteomes (brain<liver<heart). Tissues with a higher content of fibers (e.g. heart, muscle, lignified plant tissue) may require even stronger mechanical shearing forces to sufficiently extract proteins. In such cases, liquid nitrogen grinding has proven to be the method of choice (4). The resulting powdered material can be directly processed by adding the PreOmics LYSE buffer before continuing with the regular iST protocol. Furthermore, the detection of low abundant regulatory proteins is often limited by highly abundant proteins. To overcome the high dynamic range of tissues such as the heart, additional LC-MS/MS analyses of defined anatomical sub-regions and in combination with cell-type resolved proteomes are beneficial (4).

Since only few proteins exhibit truly tissue-specific expression (1,2), we focused on gene set enrichment analyses of each tissue type (**Fig. 3A-C**). While the brain samples were found to be enriched in GeneOntology (GO) terms such as myelin sheath, neuron projection or synapse part, we found that the liver samples were conversely highly enriched in multiple GO terms associated with metabolic processes. Finally, the heart samples revealed GO terms such as cell substrate and anchoring junctions or focal adhesions as the most enriched annotations, highlighting the importance of structural integrity for a highly dynamic mechanical tissue.

CONCLUSION

Here, we demonstrate deep proteome coverage of three distinct tissue types using the PreOmics iST technology. With the adaption of adding the protein extraction beads during the recommended sonication step, the workflow ensures efficient lysis and shearing of nucleic acids at the same time without introducing exogenous DNase enzymes that can contaminate the sample. The described workflow is also compatible with chemical labeling and can thus be performed with the PreOmics iST-NHS kits as well. We believe that this fast and convenient workflow enables reproducible and sensitive tissue proteomics in a high-throughput manner.

MATERIAL AND METHODS

Protein content varies considerably across different tissue types and thus requires proper quantification beforehand. As a rule of thumb, 1 mm^3 of tissue corresponds to roughly 100 µg protein. Here, we dissected ~1 mm^3 of brain, liver (*M.musculus*) and heart (*R.norvegicus*) tissue and placed it into a clean Eppendorf LoBind tube (cat. #0030108116). 50 mg of protein extraction beads and 100 µL of LYSE (PreOmics GmbH) were added and proteins were subsequently extracted by means of ultrasonication using the Diagenode Bioruptor® Pico (10 cycles, 30 sec ON/OFF). For comparison of the protein extraction procedure, dissected tissue samples were processed using the FastPrep® instrument and Lysing Matrix D (MP Biomedicals) in 100 µL LYSE buffer. Next, samples were boiled at 95°C for 10 min. After the heat and sonication treatment, 50 µL of DIGEST was added and incubated at 37°C for three hours. Subsequently, 100 µL STOP buffer was added and the samples were then centrifuged for one minute at 13,000 rpm to clear tissue debris and pellet the protein extraction beads. The supernatant was then transferred to the CARTRIDGES, before continuing with the peptide cleanup according to the iST Sample Preparation Kit`s instructions. Resulting peptide samples were fractionated into 8 fractions according to Kulak et al. (3).
Samples were measured using LC-MS instrumentation consisting of an EASY-nLC 1000 coupled to a LTQ-Orbitrap XL or a EASY-nLC 1200 coupled to a QExactive HF-X mass spectrometer (Thermo Fisher Scientific) with standard settings. Peptides were eluted with a linear 100 min gradient. Column temperature was kept at 60°C. Raw files were analyzed using MaxQuant v.1.6.0.16 (5), the false discovery rate was set to 0.01 for both proteins and peptides with a minimum length of seven amino acids, and was determined by searching a reverse database. Enzyme specificity was set as C-terminal to arginine and lysine, using trypsin as the protease, and a maximum of two missed cleavages were allowed in the database search. Label-free quantitation (LFQ) was performed with a minimum ratio count of 1. Statistical analysis was performed using Perseus (6).

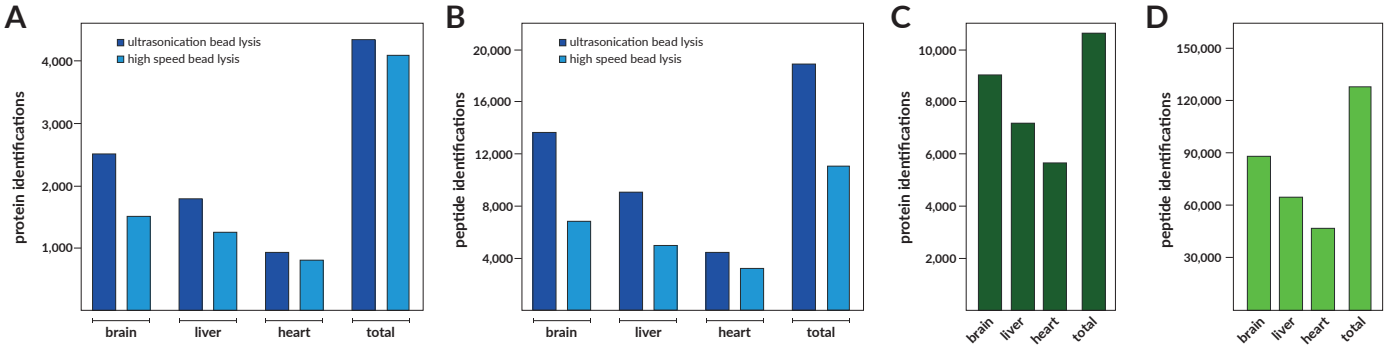

Figure 2
Protein (A) and peptide (B) identifications of tissue samples lysed by means of ultrasonication or high speed bead lysis. The data were acquired on a LTQ-Orbitrap XL system. Identified proteins and peptides (C) of tissue samples fractionated using high pH (8 fractions); The data were acquired on a QExactive HF-X system.

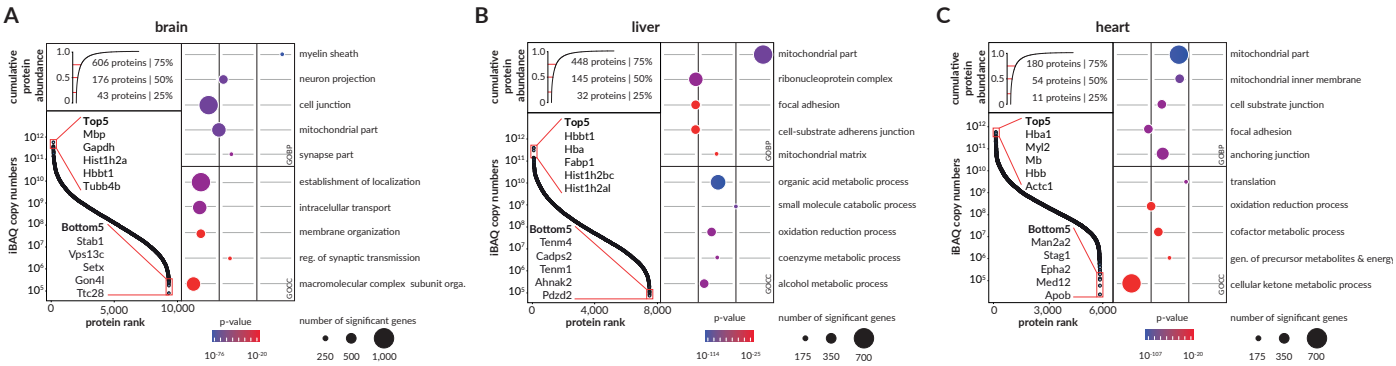

Figure 3
Plots for (A) brain, (B) liver and (C) heart tissue. Top left: Cumulative protein composition from the highest to the lowest abundant protein shows that only a few proteins make up most of the protein mass. Bottom left: Ranking of proteins by iBAQ (intensity-based absolute quantification) copy numbers from highest to lowest. Right: Top5 most significantly enriched Gene Ontology (GO) terms cellular component (GOCC) and biological process (GOBP). The x axis represents the relative enrichment of each GO term. Circle sizes represent the number of enriched genes in each GO term category; circle color represents the p-value.

PRODUCTS

http://www.preomics.com/products

Product	Quantity	Manufacturer	Product Code
iST Kit 8x	8 reactions	PreOmics GmbH	P.O.00001
iST Kit 96x	96 reactions	PreOmics GmbH	P.O.00027
iST-NHS Kit 12x	12 reactions	PreOmics GmbH	P.O.00026
iST-NHS Kit 96x	96 reactions	PreOmics GmbH	P.O.00030
iST-HT Kit 192x	192 reactions	PreOmics GmbH	P.O.00067
Bioruptor® Pico	1 unit	Diagenode SA	B01060010
Protein extraction beads	20 grams	Diagenode SA	C20000021

iST Kits compatible with label-free & metabolic labeling; iST-NHS Kits compatible with chemical labeling (iTRAQ, TMT)

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