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# Mass Spectrometry-based Proteomics for Microscale Sample Amounts: From Cells to Tissues

Amarjeet Flora, Anastasia Klenke, Bhavin Patel, Ryan Bomgarden  Thermo Fisher Scientific, Rockford, IL, USA, 61101


## ABSTRACT

Advances in mass spectrometry (MS) instrumentation have enabled routine analysis of complex protein samples. However, protein sample preparation for mass spectrometry from microscale sample amounts is still a big challenge and largely lacks cohesive standardization, which generally leads to substantial sample loss, inconsistent and irreproducible analyses. Recently, we developed Thermo Scientific™ EasyPep™ sample preparation technology for efficient and rapid sample preparation for MS-based proteomics for 10 µg to 100 µg sample amounts. Currently, we are focused on adapting our chemistry to minute sample amounts to support higher throughputs. Here, we describe an optimized workflow for high-throughput proteomic analysis of 1 µg to 10 µg sample amounts from cells and tissues with high peptide recovery and reproducibility in less than 3 hours in a 96-well format.


## INTRODUCTION

Proteomic analysis of microscale sample amounts is challenging due to inefficient sample preparation at smaller scales and sample loss during multiple sample processing and handling steps. Recently, we developed the EasyPep sample preparation technology for efficient and rapid sample preparation for MS-based proteomics for 10-100 µg sample amounts. Currently, we are expanding our EasyPep technology by introducing EasyPep 96 Micro MS sample preparation kit for processing 1-10 µg of protein samples from cells and tissues, etc. in a 96-well format in less than 3 hours to enable high throughput. Three operation modes including centrifugation, vacuum, and positive pressure were evaluated and optimized for the 96-well EasyPep sample preparation plate.

Our optimized chemistry and clean-up are fully compatible with Thermo Scientific™ TMT™ or TMTpro™ reagent multiplexing for processing and quantitative analysis of 1-10 µg sample amounts in a 96-well format, and high pH reversed-phase fractionation with TMT/TMTpro-pooled samples. Overall, our EasyPep sample preparation technology has been shown to enable rapid and efficient processing of different samples and throughput for mass spectrometry-based proteomics.

## MATERIALS AND METHODS

### Sample Preparation

HeLa S3 cells were grown in sMEM media supplemented with 10% FBS, 1X Glutamax, and 1% Pen/Strep. Protein lysates were prepared from several cell and tissue types, including FFPE samples, as well as limited cells from tissue, using our standardized EasyPep sample preparation procedures in replicates. Protein concentration was measured using Pierce™ Rapid Gold BCA Assay kits. The samples were labeled with TMTpro reagents according to the manufacturer's protocol before sample cleanup. Pierce High pH Reversed-Phase Peptide Fractionation kit was used to quantify differentially expressed proteins for the samples.

### LC-MS Analysis

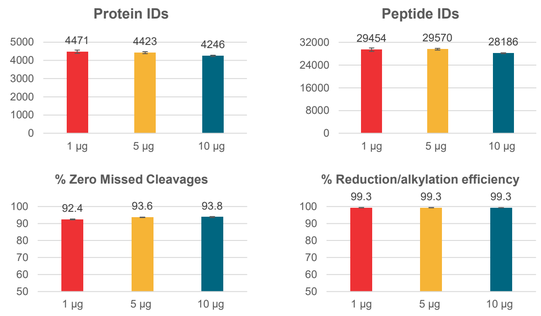
Triplicate protein digest samples were separated using a Thermo Scientific™ Dionex™ UltiMate™ 3000 Nano LC system using a 50 cm C18 Thermo Scientific™ EASY-Spray™ column with an acetonitrile gradient from 3% to 28% over 85 min, 28% to 45% over 30 min, at a flow rate of 300nL/min on a Thermo Scientific™ Orbitrap™ Eclipse™ Tribrid™ mass spectrometer.

### Data Analysis

LC-MS data were analyzed using the SEQUEST® HT search engine in Thermo Scientific™ Proteome Discoverer™ 3.0 software using static carbamidomethyl (C), dynamic oxidation (M), TMTpro 18plex (K, N-term), and deamidation (N, Q) modifications. Data were searched against the UniProt human protein database and results were filtered using a 1% protein FDR threshold.

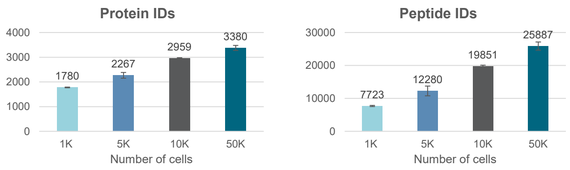
## RESULTS

### Figure 1. Efficient sample preparation in less time with higher identification rates



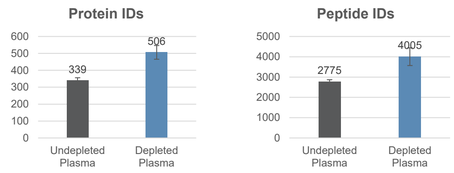

HeLa S3 cell pellets were lysed with lysis buffer. 1 µg, 5 µg, and 10 µg were reduced, alkylated, and digested with Trypsin/Lys-C followed by the clean-up on a 96-well Peptide Clean-Up Plate. Protein digest (300 ng) was analyzed by LC-MS and processed as described in the methods. The results in the figure show that our standardized workflow enables efficient sample preparation for 1-10 µg sample amounts in less than 3 hours yielding higher protein/peptide IDs with lower missed cleavages compared to conventional workflows.

### Figure 2. Sample preparation with a limited number of cells



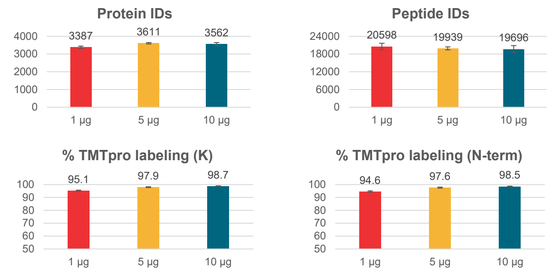

Freshly-dissected mouse liver tissue was dissociated using mechanical microfluidic force along with enzymes at 37˚ C. Dissociated cells were counted and different numbers of cells were aliquoted into the tubes. 1K, 5K, 10K and 50K cells were lysed with lysis buffer, reduced, alkylated, and enzymatically digested using EasyPep 96 micro protocol. Following digestion, the samples were cleaned-up on a 96-well Peptide Clean-Up Plate. 80% of each peptide sample was analyzed by LC-MS and processed as described in the methods. The results in the figure show that our standardized workflow can process a limited number of cells.

### Figure 3. Sample preparation with human plasma (Undepleted and depleted)



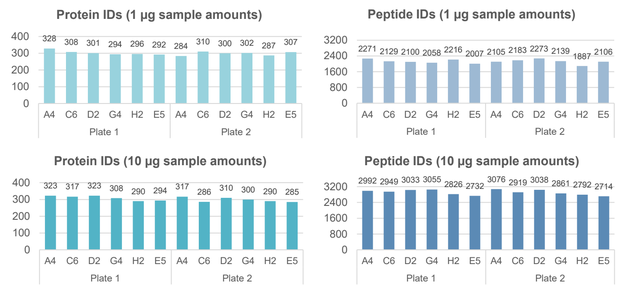

Human Plasma Digest was prepared using EasyPep workflow. Human Plasma was depleted using High Select™ Top14 Abundant protein depletion mini spin columns before processing with EasyPep technology. Protein digest (1 µg) was analyzed by LC-MS and processed as described in the methods. The results demonstrate that our workflow is compatible with both non-depleted and depleted human plasma.

### Figure 4. Compatibility with isobaric tags



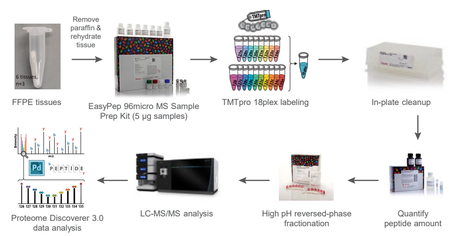

HeLa S3 cell pellets were lysed with lysis buffer. 1 µg, 5 µg, and 10 µg samples were reduced, alkylated, enzymatically digested, labeled with TMTpro zero reagents, and cleaned up on a 96-well Peptide Clean-Up Plate. Labeled samples (400 ng) were analyzed by LC-MS and processed as described in the methods. The results in the figure show that our standardized workflow is compatible with isobaric labeling reagents with a labeling efficiency of ≥ 95%.

### Figure 5. Assessing workflow reproducibility



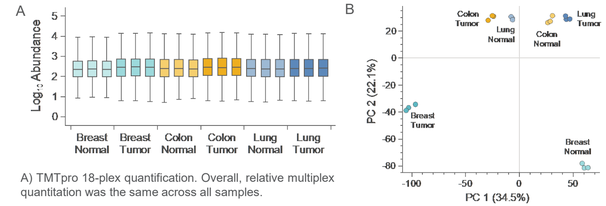

1 µg and 10 µg of Human Plasma samples were processed using EasyPep 96 micro protocol and cleaned up on two Peptide Clean-Up Plates. Six samples out of each plate from each sample amount were randomly selected and analyzed by LC-MS. ~400 ng and ~1 µg was injected for each 1 µg and 10 µg sample, respectively. The data was processed as described in the methods. The results show that our workflow is reproducible between the plates and yielded similar protein and peptide ID numbers with CVs < 5%.

### Figure 6. TMTpro multiplexing experiment with FFPE tissue samples




Paraffin removal was carried out using xylene and sequential ethanol washes. The optimized extraction protocol and EasyPep 96micro MS protocol were used to prepare digest samples from FFPE sections of normal and tumor breast, lung, and colon tissues. TMTpro 18plex reagents were used to label the samples followed by fractionation and nanoLC-MS/MS analysis.

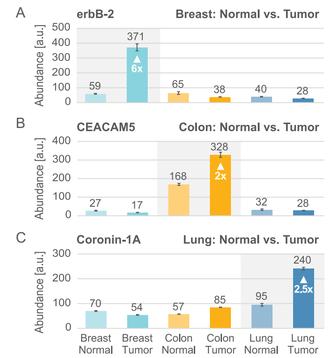
### Figure 7. Multiplex quantitation of different FFPE tissue samples




A) TMTpro 18-plex quantification. Overall, relative multiplex quantitation was the same across all samples.

B) Around 4000 unique proteins and 26000 unique peptides were identified after the fractionation with a labeling efficiency of ≥98%. Principal component analysis grouped the biological replicates based on similar protein/peptide abundance.

### Figure 8. TMTpro 18plex quantitative data analysis




A) Comparison of breast normal (n=3) vs. breast tumor (n=3) samples. Example of protein quantified using TMTpro 18plex after high pH reversed-phase fractionation, erbB2, Receptor tyrosine-protein kinase, showed a 6-fold increase in breast tumor compared to breast normal samples. This protein plays an important role in the development and progression of certain aggressive types of breast cancer.

B) Comparison of colon normal (n=3) vs. colon tumor (n=3) samples. CEACAM5 (CEA), Carcinoembryonic antigen-related cell adhesion molecule 5 showed a 2-fold increase in colon tumor compared to colon normal samples. CEACAM5 plays a significant role in the inhibition of apoptosis and differentiation in colon cells.

C) Comparison of lung normal (n=3) vs. lung tumor (n=3) samples. Coronin-1A (CORO1A) showed a 2.5-fold increase in lung tumor compared to lung normal samples.

## CONCLUSIONS

- Our EasyPep™ sample preparation technology enables rapid and efficient processing of 1-10 µg sample amounts to enable throughput for mass spectrometry-based proteomics.
- Our standardized workflow is compatible with several sample types including cell lines, plasma, tissues, FFPE, limited cell numbers with high reproducibility (CVs <5).
- Our sample preparation chemistry is directly compatible with isobaric labeling reagents such as TMT/TMTpro as well as high pH reversed-phase fractionation for relative protein quantification.

## TRADEMARKS/LICENSING



PO2023-59EN

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