Rapid and efficient sample preparation using EasyPep technology

Optimized sample preparation for MS-based proteomics applications

Introduction

Advances in mass spectrometry instrumentation has enabled routine analysis of complex protein samples. Sample preparation is a crucial step in the proteomics workflow, which greatly affects identification rates of proteins and peptides. However, the sample preparation methods are not standardized with many requiring long hours to process samples and potentially resulting in low peptide yield, poor digestion efficiency, and low reproducibility. To overcome these challenges, we developed an optimized and standardized workflow using the Thermo Scientific™ EasyPep™ sample preparation kits, which are simple and easy to use for high-quality sample preparation of proteins, cells, tissues, serum, and plasma for mass spec analysis. Our optimized workflow enables efficient and reproducible processing of samples with significantly reduced hands-on time for total sample processing time (under 4 hours) from intact cells to cleaned-up peptides (Figure 1).

Our EasyPep MS sample prep kits contain preformulated buffers, mass spectrometry (MS)-grade trypsin/Lys-C enzyme mix, peptide cleanup columns or plates, and an optimized protocol to generate high-quality peptide samples. The kit also includes a universal nuclease that eliminates the need for sonication to reduce viscosity in cells and tissue lysates. Addition of universal nuclease is not required for purified protein and plasma samples. After protein extraction, samples are heated at 95°C for 10 min for reduction and alkylation followed by trypsin/Lys-C digestion at 37°C for 1–3 hr (Figure 2, stage 1) using the preformulated kit buffers and enzyme mixture. Protein digests are then acidified by digestion stop solution before peptide cleanup on EasyPep peptide cleanup columns for detergent and contaminant removal (Figure 2, stage 2).
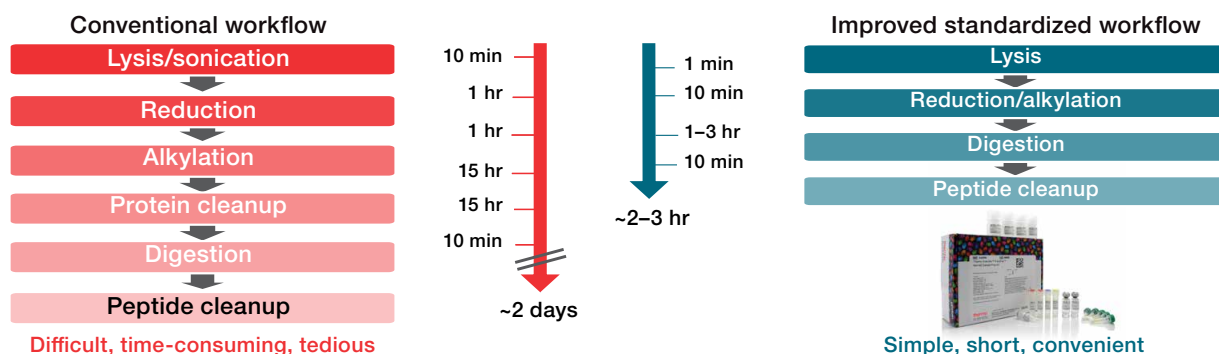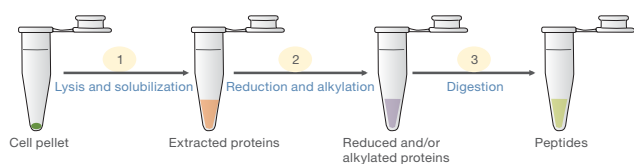

Figure 1. Comparison between conventional workflow and improved EasyPep standardized workflow.

Stage 2. Peptide sample cleanup
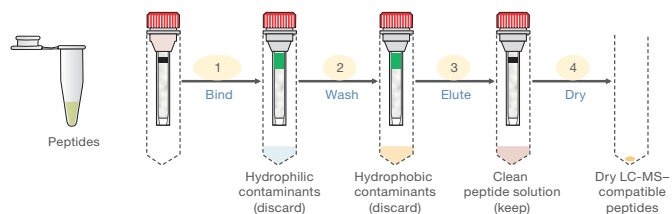
Figure 2. Schematic of the EasyPep MS Sample Prep Kit workflow.

Figure 3. EasyPep Mini, 96-well, and Maxi formats.

Our EasyPep MS sample preparation kits are available in three formats which support wide range of input amounts (Figure 3). Each kit uses the same chemistry and protocol to process biological samples to yield nearly same protein and peptide identifications with high reproducibility (coefficient of variation (CV) <5%) in less than 4 hours. The Thermo Scientific™ EasyPep™ Mini MS Sample Preparation Kit has been optimized for efficiently processing 10-100 μg of protein samples using a microcentrifuge. The Thermo Scientific™ EasyPep™ Maxi (large scale) Sample Preparation Kit has been optimized to process 0.5–2 mg of protein samples using either centrifugation or a vacuum manifold to facilitate peptide cleanup, helping to streamline analysis of posttranslational modifications. Lastly, the Thermo Scientific™ EasyPep™ 96 MS Sample Preparation Kt is optimized to process 10–100 μg of protein samples to enable high-throughput sample preparation and is compatible with automated cleanup by centrifugation, vacuum manifold, and positive pressure modes.

In this study we compared the three EasyPep formats (Mini, Maxi, and 96-well) for processing different sample types, including human cell lines, human serum/plasma, yeast, *E. coli*, fresh tissues, and formalin-fixed, paraffin-embedded (FFPE) tissues. In addition, we assessed the compatibility of EasyPep MS sample preparation chemistry with downstream applications, including phosphopeptide enrichment using Fe-NTA resins and Thermo Scientific™ Tandem Mass Tag™ (TMT™) reagent labeling before or after digestion. Overall, our EasyPep sample preparation technology enables rapid and efficient processing of different sample types, amounts, and throughput for various mass spectrometry–based proteomic applications.
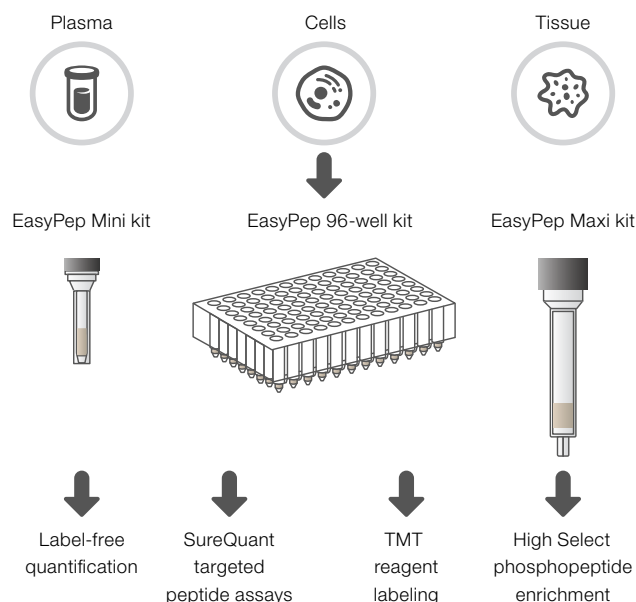
Methods

Cell culture

HeLa S3 cells were grown in Gibco™ MEM media supplemented with 10% FBS, 1X GlutaMAX™ reagent and 1% penicillin/streptomycin (pen/strep). HEK293 and A549 cells were grown in MEM media supplemented with 10% FBS and 1% pen/strep. Human plasma was obtained from BioIVT. Invitrogen™ Normal Human Serum was obtained from Thermo Fisher Scientific (Cat. No. 31876). Mouse tissues (brain, liver, and heart) were obtained from Pelfreeze. BL21-Gold (DE3) *E. coli* competent cells obtained from Agilent Technologies were grown in lysogeny broth (LB). Yeast BY4741 WT Parental strain, MAT A was obtained from GE Healthcare and was grown in YPD broth. Fresh-frozen tissues samples were obtained from BioIVT. Formalin-fixed, paraffin-embedded (FFPE) blocks from fresh-frozen tissues (colon, lung, breast) were prepared at Mercy Health Rockford. The tissue sections from FFPE blocks were generated using a microtome, and 2–3 sections were collected in a 1.5 mL low–protein-binding tube.

For phosphopeptide enrichment, HeLa cells were grown in MEM media supplemented with 10% FBS and 1% pen/strep. The cells were grown until they were 90–95% confluent and treated with 0.1 μg/mL of nocodazole for 22 hours. The cells were harvested and the cell pellets were rinsed with ice-cold PBS 3 times before storing them at –80°C.

Sample preparation

Cell types (HeLa S3, HEK293, A549): Protein lysates were prepared from several cell types (HeLa S3, HEK293, or A549) using our standardized EasyPep sample preparation procedure in replicates.

Bacteria: For cell pellets from bacterial cultures at OD_{600} = 1, 1–2 µL of 50 mg/mL lysozyme (Cat. No. 90082) was added to 200 µL of Thermo Scientific™ EasyPep™ Lysis Buffer provided in the kit. The cells were lysed by pipetting the sample repeatedly and centrifuged at 16,000 x g for 5 min. The supernatant was transferred to a fresh tube, and protein concentration was measured using the Thermo Scientific™ Pierce™ Rapid Gold BCA Protein Assay Kit. (Alternatively, *E. coli* cells can be lysed in EasyPep Lysis Buffer using bead beating or sonication instead of lysozyme.) After lysis, sample preparation was done per the EasyPep protocol for reduction, alkylation, digestion, and cleanup as described in the product manual.

Yeast: For cell pellets from yeast cultures at OD_{600} = 1, 100 µL of EasyPep Lysis Buffer was added to the yeast cell pellet and vortexed for 10–20 sec. The glass beads were added to the lysate (1.3 times more to the total volume of the lysate) and vortexed at high speed for 1 minute. The tube was placed in the rack for a minute followed by vigorous vortexing for 8 min continuously. The lysate was centrifuged at 4,000 x g for 2 min. The supernatant was transferred to a fresh tube and centrifuged at 16,000 x g for 5 min. The supernatant was again transferred to a fresh tube and protein concentration was measured using the Pierce Rapid Gold BCA assay. After lysis, sample preparation was done per the EasyPep protocol for reduction, alkylation, digestion, and cleanup as described in the product manual.

Human plasma and serum: Undepleted human plasma and serum samples (100 µg protein) were processed using our optimized EasyPep workflow in replicates. 10 µL of human plasma was depleted using Thermo Scientific™ Pierce™ High Select™ Top 14 Abundant Protein Depletion Mini Spin Columns. After depletion, the samples were dried in a speed vacuum concentrator, reconstituted in 100 µL of EasyPep Lysis Buffer, and prepared per the EasyPep protocol as described in the manual.

Mouse tissues: Approximately 20 mg of mouse tissue samples (brain, heart, and liver; N = 3) were weighed and rinsed in ice-cold PBS a few times; 500 µL of EasyPep Lysis Buffer was added and the sample was disrupted with a tissue homogenizer until the sample was homogenized. The tissue lysates were centrifuged at 16,000 x g for 10 min at 4°C. The supernatant was transferred to a fresh tube and protein concentration was measured using the Pierce Rapid Gold BCA assay. After lysis, sample preparation was done per the EasyPep protocol for reduction, alkylation, digestion and cleanup as described in the product manual.

FFPE: For FFPE samples, paraffin removal was carried out by heating samples at 56°C for 3 min in xylene followed by sequential ethanol washes (100%, 95%, 80%, and 50%). The samples were then homogenized in EasyPep Lysis Buffer using micropestle grinding for at least 30 sec followed by probe sonication for ten 70 amp pulses each lasting for 1 sec. Samples were heated at 95°C for 2 hr followed by centrifugation at 14,000 x g for 10 min to pellet the debris. The supernatant was transferred to a fresh tube, and protein concentration was measured using the Pierce Rapid Gold BCA assay. After lysis, sample preparation was done per the EasyPep protocol for reduction, alkylation, digestion, and cleanup as described in the product manual.

Protein, cells, tissues, plasma, and serum samples were processed using the Thermo Scientific™ Pierce™ Mass Spec Sample Kit for Cultured Cells (i.e., conventional workflow) or Thermo Scientific™ EasyPep™ MS Sample Prep Kit (improved standardized workflow). Protein concentration was measured using the Pierce Rapid Gold BCA assay. Peptide concentration was determined using the Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay Kit prior to LC-MS analysis.

For label-free quantification of *E. coli* peptides in a mixed proteome (HeLa and *E. coli* digest), HeLa and *E. coli* cell pellets were processed using the EasyPep workflow. The HeLa cell digest was used as a background proteome at fixed concentrations, and *E. coli* digest was spiked into HeLa digest at different ratios (0, 1, 2, 4, and 8) as shown in Table 1. Peptide concentration was determined using the Pierce Quantitative Colorimetric Peptide Assay.

Table 1. Sample information for label-free quantification of *E. coli* peptides.

	Total amount (ng)	HeLa (ng)	*E. coli* (ng)	Final *E. coli* ratio
Sample 1	800	800	0	0
Sample 2	824	800	24	1
Sample 3	848	800	48	2
Sample 4	896	800	96	4
Sample 5	992	800	192	8

TMT labeling

HeLa cell pellets were lysed, reduced, alkylated, and digested using the EasyPep workflow. After digestion, the samples were labeled with TMT reagents according to the manufacturer's protocol. The samples were cleaned up using EasyPep peptide cleanup columns. For conventional workflow, HeLa cell pellets were processed using the Thermo Scientific™ Pierce™ Mass Spec Sample Kit for Cultured Cells. The samples were labeled with TMT reagents according to the manufacturer's protocol followed by cleanup using peptide-desalting spin columns. Peptide concentration was determined using the Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay prior to LC-MS analysis.

Phosphopeptide enrichment

For phosphopeptide enrichment, 1 mg of nocodazole-treated HeLa digest was prepared using the EasyPep workflow and Pierce Mass Spec Sample Kit for Cultured Cells (i.e., the conventional workflow). The protein digest was enriched for phosphopeptides using the Thermo Scientific™ Pierce™ Hi-Select™ Fe-NTA Phosphopeptide Enrichment Kit according to the manufacturer's protocol.

LC-MS and data analysis

Triplicate protein digest samples (1 µg per injection) were separated using a Thermo Scientific™ Dionex™ Ultimate™ 3000 Nano LC system with a 50 cm Thermo Scientific™ EASY-Spray™ C18 column using an acetonitrile (ACN) gradient from 3% to 28% over 85 min, 28% to 45% over 30 min, at a flow rate of 300 nL/min on a

Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer. A Thermo Scientific™ Orbitrap Fusion™ mass spectrometer was used to analyze TMT reagent–labeled samples.

LC-MS data were analyzed using the SEQUEST™ HT search engine in Thermo Scientific™ Proteome Discoverer™ software 2.4 using static carbamidomethyl (C), dynamic oxidation (M), Thermo Scientific™ TMT6plex™ labeling (K, N-terminal), phosphorylation (S, T, Y), and deamidation (N, Q) modifications. Data were searched against the UniProt human, *E. coli*, or yeast protein databases; and results were filtered using a 1% protein false discovery rate (FDR) threshold.

For the unlabeled HeLa and *E. coli* samples, 2 µL, which corresponds to 800–996 ng of protein depending on the sample, was injected directly onto a 75 µm x 25 cm, 1.6 µm C18 Aurora analytical column (IonOpticks, Australia). The column was kept at 50°C using a Sonation column oven (Sonation GmbH, Germany). Peptides were separated over 120 min at 300 nL/min using a Thermo Scientific™ Easy-nLC™ 1200 System (Thermo Fisher Scientific, Cat. No. ES803A) over a gradient from 3% B (A: 99.9% H_2O, 0.1% formic acid (FA); B: 80% ACN, 0.1% FA) to 24% B in 100 min followed by a ramp to 40% B in 20 min. The column was washed at 98% B for 9 min.

Data were acquired on the Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer equipped with the Thermo Scientific™ FAIMS™ Pro interface. The instrument was operated at "top speed", data-dependent, MS/MS mode using FAIMS compensation voltage (CV) values of –50 and –70. First, a full scan was acquired from m/z 375–1,575 at 240,000 resolving power with the FAIMS interface set to –50 CV using an ion funnel radio frequency (RF) lens value of 30% and an automatic gain control (AGC) target of 1 x 10^6. Data-dependent MS/MS spectra were acquired with a 1 sec cycle using the following filters: monoisotopic precursor selection mode set to "peptide"; charge state filter set to 2–8; 45 sec dynamic exclusion with isotopes excluded; intensity threshold set to 5 x 10^3. The quadrupole mass analyzer was set to 0.7 Da for precursor isolation. The MS/MS spectra were generated by high-energy collision dissociation (HCD) with a normalized collision energy of 30%. MS/MS spectra were acquired in the ion trap using "turbo" scan rate, an AGC target of 1 x 10^4 ions, and a maximum inject time of 10 ms. The FAIMS device was then switched to –70 CV and the entire cycle was repeated. Samples were analyzed in triplicate.

Data were processed in Proteome Discoverer software 2.4 for label-free quantitation using a two-stage search. The spectra were first analyzed by the MSPepSearch algorithm against the built-in NIST_Human_Orbitrap_HCD_20160923 version 1.0 and ProteomeTools HCD28_PD version 1.0 libraries as well as a Prosit-derived *E. coli* library (+2 and +3 charge states). The resulting peptide-spectrum matches (PSMs) were scored by the Percolator tool. Those PSMs that did not pass the strict FDR threshold with a q-value of 0.01 were automatically analyzed by the SEQUEST™ HT engine, searched against human and *E. coli* SwissProt databases with respect to dynamic methionine oxidation, N-terminal carbamylation, and N-terminal acetylation with and without methionine loss, and static cysteine carbamidomethylation. All PSMs were filtered to 1% FDR. MS1 feature extraction was performed using the Minora Feature Detector Algorithm, and precursor abundance was quantified based on intensity. Normalization was performed against a FASTA file comprising the 100 most abundant human proteins. Protein abundances were calculated from the summed unique and razor peptide abundances, and protein ratios were calculated as the median of all possible pairwise ratios between replicates. *P* values were calculated using a background-based *t*-test.

Results and discussion

Efficient sample preparation with EasyPep technology for larger sample amounts and throughput

Our optimized and standardized EasyPep workflow enables rapid adoption by both novice and expert users. HeLa S3 cell pellets were lysed in EasyPep Lysis Buffer as described in the product manual. All three EasyPep formats—Maxi, 96-well, and Mini—yielded identical protein and peptide IDs with high reproducibility (CV <5%) (Figure 4). The overall peptide quality was also virtually the same among the formats with >99% reduction/alkylation efficiency, >90% zero missed cleavages, <3% methionine oxidation, and 6% deamidation for identified peptides (Figure 4).

Compatibility with different sample types

Protein lysates from several sample types were processed using the EasyPep workflow as described in the "Methods" section (see "Sample preparation"). The results below demonstrate that our EasyPep standardized workflow generates high-quality data and is compatible with several sample types, including cell lines (Figure 5A), yeast and bacteria (Figure 5B), human plasma and serum (Figure 5C), mouse tissues (Figure 5D), and FFPE samples (Figure 5E), with high reproducibility (CV <5%) and lower missed cleavages (<10%).
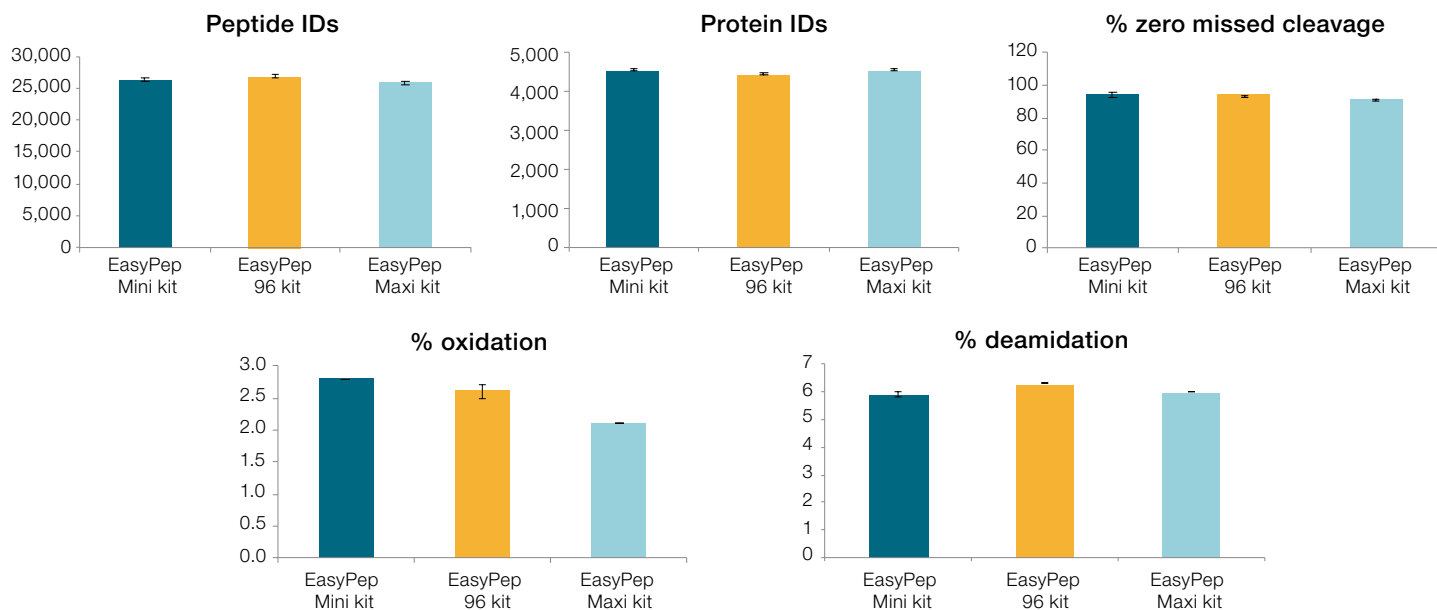

Figure 4. Efficient sample processing of HeLa cell lysates using the EasyPep workflow. 100 µg of HeLa cell lysate was processed using the EasyPep workflow for the 96-well and Mini formats. 500 µg of HeLa cell lysate was processed using the EasyPep Maxi format. A Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer was used for LC-MS analysis of 1 µg peptide sample from each format.
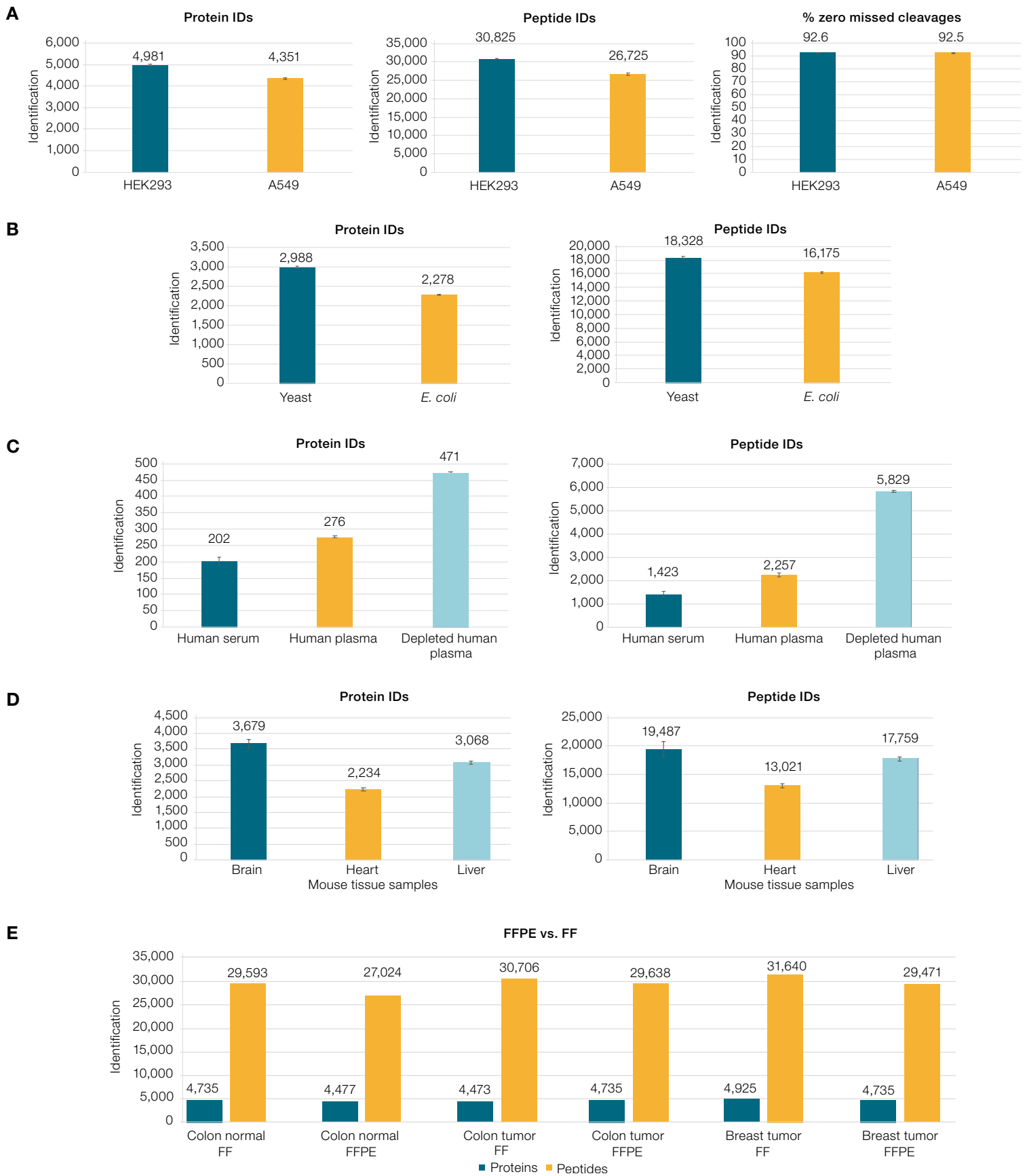
Figure 5. Compatibility of EasyPep workflow with different sample types. (A) Cell lines (A549 and HEK293), **(B)** yeast and *E. coli*, **(C)** human plasma (depleted and undepleted) and serum, **(D)** mouse tissues (mouse brain, heart, and liver), and **(E)** FFPE and fresh-frozen (FF) samples.

Compatibility with downstream applications and removal of excess TMT label during cleanup

EasyPep MS sample prep kits are compatible with various downstream applications, including phosphopeptide enrichment, fractionation, and TMT reagent labeling. As shown in Figure 6A, our EasyPep workflow is compatible with the TMT labels and yielded 10–20% higher protein and peptide IDs with lower missed cleavages (<10%) and labeling efficiency of >99%. EasyPep cleanup chemistry has been optimized for efficient removal of excess TMT reagents along with removal of detergents and interfering buffer salts from the samples, while maintaining high, unbiased peptide yields and improved peptide identifications as shown in Figure 6B.
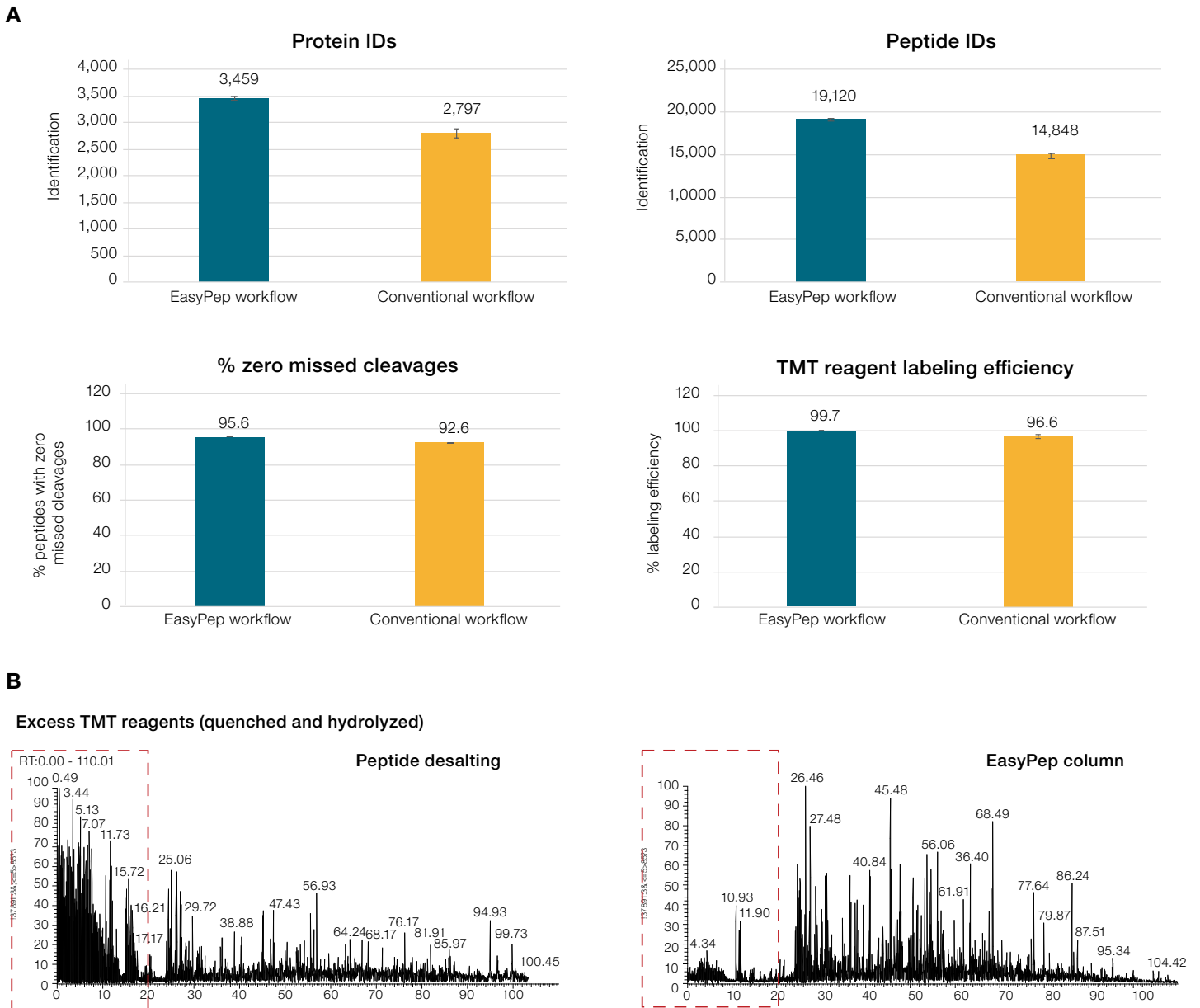
A

B

Figure 6. Improved peptide identifications using the EasyPep workflow. (A) HeLa S3 cell pellets were lysed, reduced, alkylated, and enzymatically digested. The samples were labeled with TMT reagents after digestion and cleaned up using the EasyPep peptide cleanup procedure. **(B)** Comparison of MS chromatograms of samples prepared using the EasyPep sample prep kits and conventional sample prep methods (peptide-desalting column cleanup).

Compatibility with phosphopeptide enrichment

Phosphorylation is a critical posttranslational modification that modulates the function of numerous proteins. For phosphoproteome analysis, 1 mg of nocodazole-treated HeLa digest was prepared using our Thermo Scientific™ EasyPep™ Maxi MS Sample Prep Kit or conventional workflow for subsequent phosphopeptide enrichment using immobilized metal affinity chromatography (IMAC). As shown in Figure 7, the number of protein and peptide identifications with the EasyPep workflow was similar to that of the conventional workflow. The results demonstrate that our standardized EasyPep workflow combined with IMAC phosphopeptide enrichment can generate samples with 95% phosphopeptide specificity in less than 5 hr.
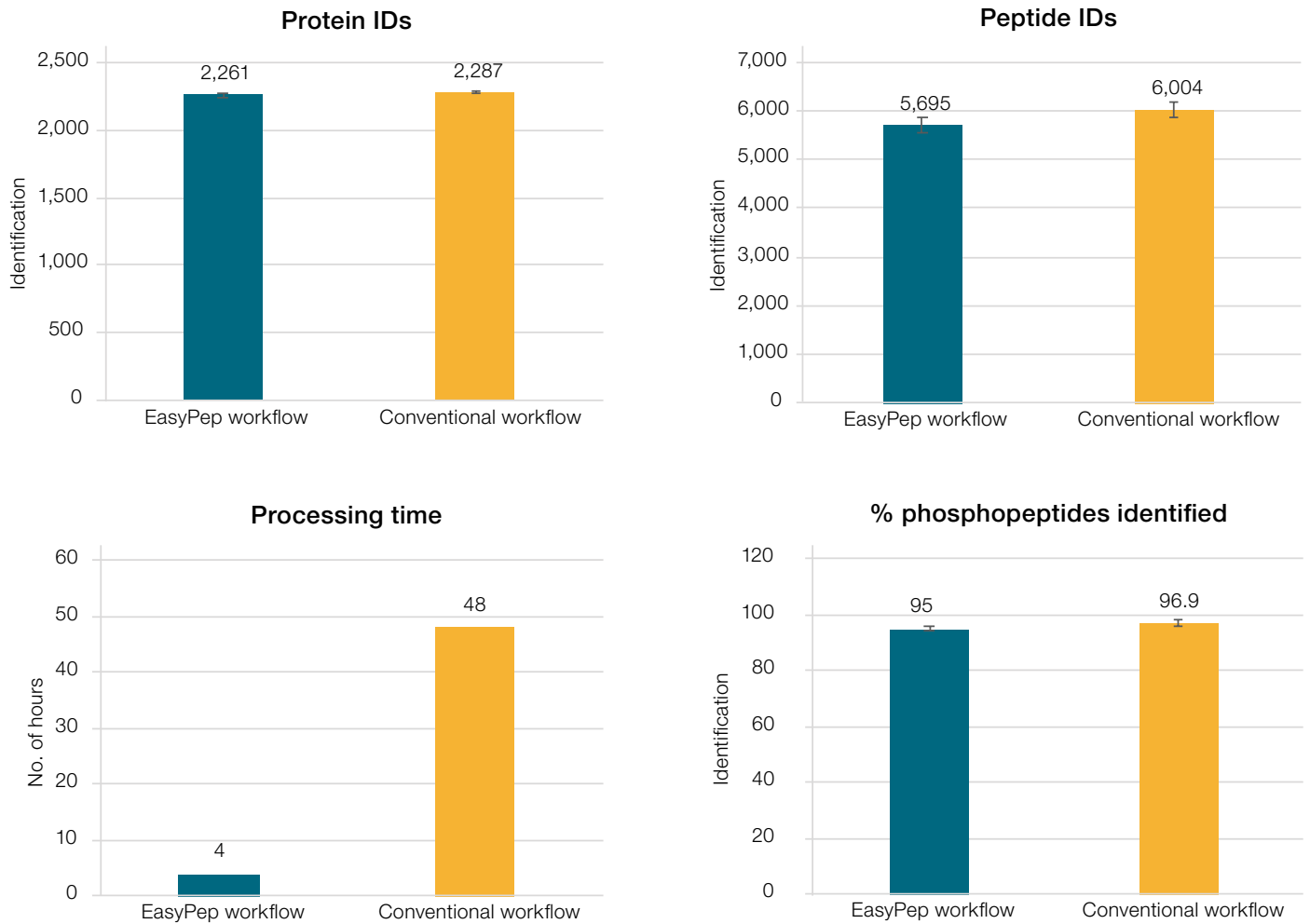

Figure 7. **Phosphopeptide enrichment using IMAC and the EasyPep workflow.** Nocodazole-treated HeLa cell pellets were processed using the EasyPep Maxi kit or conventional workflow followed by IMAC phosphopeptide enrichment using the Pierce Hi-Select Fe-NTA Phosphopeptide Enrichment Kit. A Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer was used for LC-MS analysis of 1 µg enriched peptide sample.

Label-free quantification of *E. coli* peptides using EasyPep sample prep kit

To demonstrate the quality, reproducibility, and depth of protein coverage achievable using EasyPep sample preparation upstream of our most advanced proteomic instruments, a set of mixed proteome standards were created using *E. coli* protein digests at different ratios. These standards were mixed with HeLa protein digests at a fixed concentration (Figure 8A). Samples were analyzed using the Orbitrap Eclipse Tribrid mass spectrometer with a FAIMS Pro interface that enables gas-phase fractionation of the proteomic samples for improved protein coverage.

Using the Orbitrap Eclipse Tribid mass spectrometer and FAIMS Pro interface, a total of 10,827 protein groups and 118,030 peptides were identified across all samples (Figure 8B). For HeLa protein digest, which was spiked in at a constant level across all five samples, an average of 7,789 protein groups and 74,477 peptides were identified

per sample with no matching identification between runs. The number of identified *E. coli* protein groups and peptides ranged from 967 and 3,909, respectively, in sample 2, to 1,912 and 12,515 in samples 5. Overall, the label-free quantitation of these standards was excellent with coefficients of variation (CVs) ranging from 10 to 17.5%. Considering only *E. coli* protein groups identified with at least two *E. coli* peptides per protein, CVs ≤20% in each sample group, and with adjusted *P* values of <0.05 between samples, over 1,200 protein groups were determined to be significantly different in each sample group. Expected fold changes were also highly accurate, with a median $\log_2$-fold change of 1.13 (expected $\log_2$-fold change = 1) between samples 5 and 4; a median $\log_2$-fold change of 2.20 (expected $\log_2$-fold change = 2) between samples 5 and 3; and a median $\log_2$-fold change of 3.23 (expected $\log_2$-fold change = 3) between samples 5 and 2 (Figure 8C).
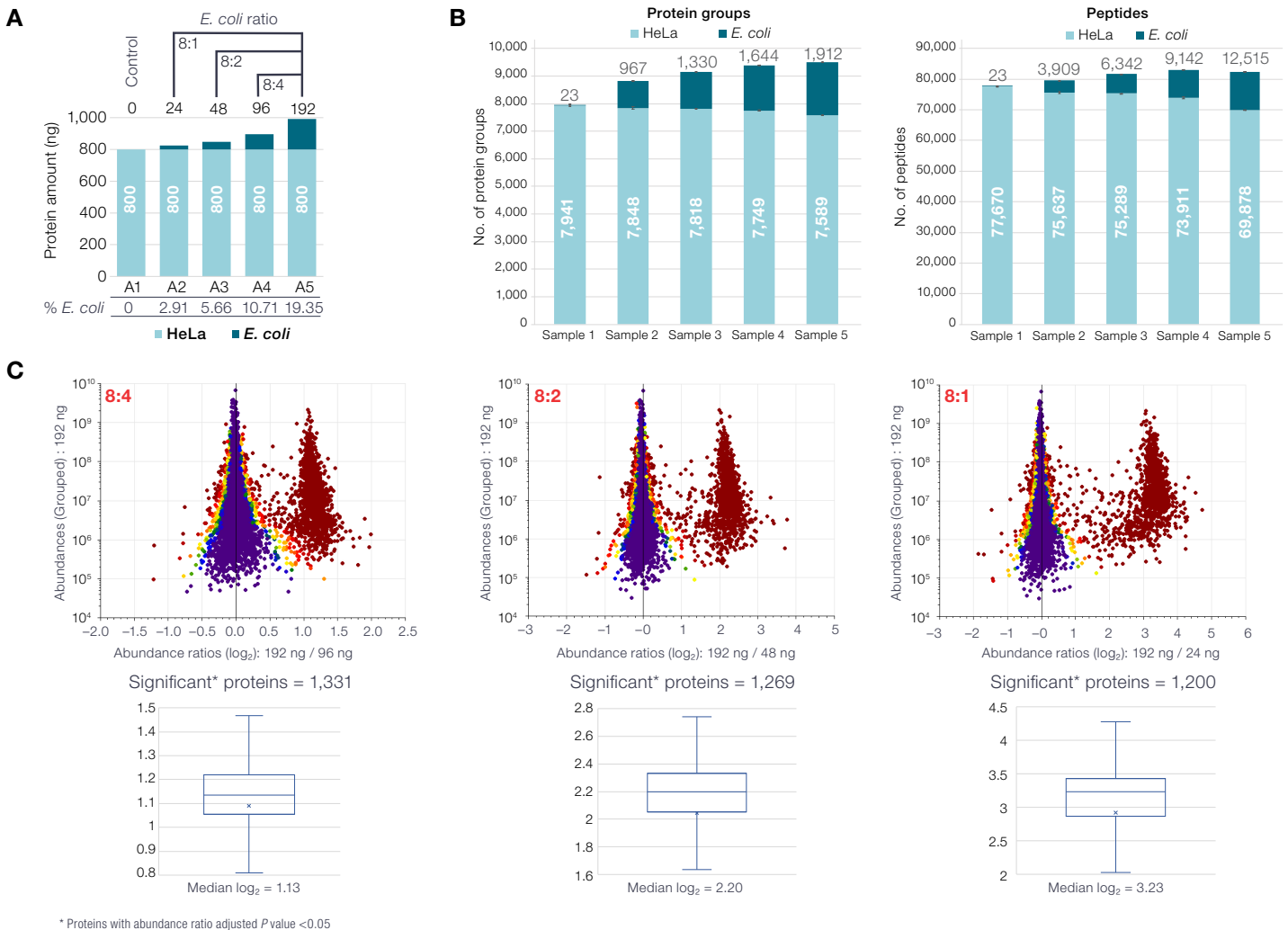

Figure 8. Quantitation of a set of two-proteome (HeLa and *E. coli*) standard digest mixtures. (A) HeLa/*E. coli* standard sample experimental design. **(B)** Protein and peptide identifications in HeLa and *E. coli* standards. **(C)** Label-free quantitation of *E. coli* peptides spiked at different ratios into the fixed HeLa digest background. Filters: >1 peptide/protein; abundance (grouped) CVs ≤20% in both sample groups.

Conclusions

EasyPep sample preparation technology enables rapid and efficient processing of different samples, scales, and throughput for mass spectrometry–based proteomics. Our standardized workflow shows identical peptide and protein identification rates, quality, and reproducibility for HeLa cell pellets and plasma samples processed using the EasyPep Mini, Maxi and 96-well filter-plate formats. The EasyPep sample prep kits are compatible with several sample types, including cells (mammalian, yeast, and *E. coli*), tissues (fresh and FFPE), and plasma, as well as downstream applications including TMT reagent labeling, high-pH reversed phase fractionation, and phosphopeptide enrichment. Combining EasyPep sample prep with the latest generation of Orbitrap Eclipse Tribrid mass spcetrometer equipped with the FAIMS Pro interface enables deep proteomic analysis of samples with the highest accuracy and precision for both TMT reagent–labeled and label-free protein quantitation.

Related products

- EasyPep Mini MS Sample Prep Kit, Cat. No. A40006

- EasyPep 96 MS Sample Prep Kit, Cat. No. A45733

- EasyPep Maxi MS Sample Prep Kit, Cat. No. A45734

- EasyPep Lysis Buffer, Cat. No. A45735

- Thermo Scientific™ Pierce™ Trypsin/Lys-C Protease Mix, MS Grade, Cat. No. A40007

- Pierce Rapid Gold BCA Protein Assay Kit, Cat. No. A53225

- Thermo Scientific™ Pierce™ BCA Protein Assay Kit, Cat. No. 23225

- Thermo Scientific™ Pierce™ Quantitative Fluorometric Peptide Assay, Cat. No. 23290

- Pierce Quantitative Colorimetric Peptide Assay Kit, Cat. No. 23275

- High-Select Fe-NTA Phosphopeptide Enrichment Kit, Cat. No. A32992

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