Standardization of Sample Preparation for Proteomics Applications

Aaron M. Robitaille[1], Michael Krawitzky[1], Greg Foster[1], Gina Tan[1], Ryan Bomgarden[2], Sergei Snovida[2], Daniel Lopez-Ferrer[1]

[1]Thermo Fisher Scientific, 355 River Oaks Pkwy, San Jose, CA 95134; [2]Thermo Fisher Scientific, 3747 N Meridian Rd, Rockford, IL 61101

ABSTRACT

Mass spectrometry-based proteomics is the preferred method for in-depth characterization of the protein components of biological systems. However, there is no standardized method for proteomic sample preparation, contributing to a lack of reproducible results. Therefore, we developed a standardized workflow that overcomes the aforementioned disadvantages and/or limitations.

INTRODUCTION

Current proteomic sample preparation methods may consist of some or all of the following steps: cell lysis, protein quantification, reduction, alkylation, digestion, desalting, and peptide quantification. These steps are performed sequentially, requiring significant user involvement and long incubation times, which translates into a major inconvenience for proteomic scientists. In fact, sample preparation is a significant bottleneck in high-throughput proteomic studies. To increase sample throughput while also improving reproducibility, we identified key steps in the traditional workflow that could be modified to create a standardized method for proteomic sample preparation.

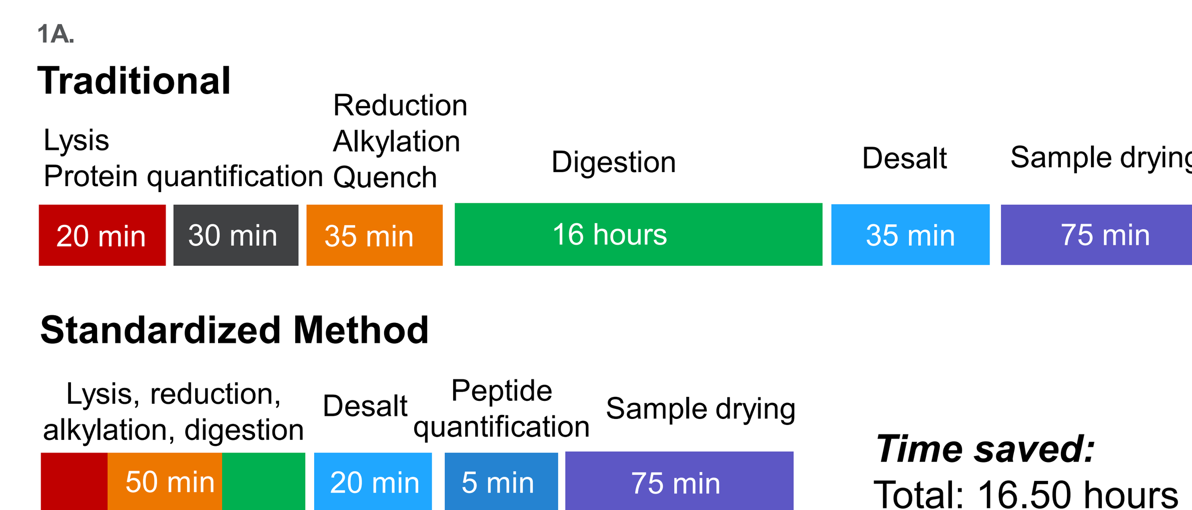
MATERIALS AND METHODS

Here, we show the scalability, versatility, and reproducibility of a single-step protocol that combines the cell lysis, protein denaturation, reduction, alkylation, and digestion while reducing the sample preparation time to approximately one hour. Samples are directly added to a reagent mixture that includes pre-measured quantities of a buffering salt, a detergent, a reducing agent, an alkylating agent, and an immobilized proteolytic enzyme. Next, the rapid digestion of proteins is facilitated by agitated heating. Peptides are then desalted and detergent is removed through a mixed mode clean-up procedure. The resulting peptides are quantified by UV absorbance at 280 nm on a Thermo Scientific™ NanoDrop™ spectrophotometer and normalized prior to LC-MS analysis. All experiments were analyzed using a Thermo Scientific™ EASY-Spray™ C18 50 cm column with a Thermo Scientific™ EASY-nLC™ 1200 HPLC running a 2–32% acetonitrile (vol/vol) gradient with 0.1% (vol/vol) formic acid in either 120 min or 180 min. The Thermo Scientific™ Q Exactive™ HF hybrid quadrupole-Orbitrap™ mass spectrometer was operated in data-dependent mode with 120,000 MS1/ 30,000 MS2 resolution, 375–1500 *m/z*, Top22, and a 1.4 *m/z* isolation window. Data analysis was completed in PMI-Preview™ or Thermo Scientific™ Proteome Discoverer ™ 2.2 software using a 10 ppm mass tolerance and 1% false discovery rate. All reagents were purchased from Thermo Fisher Scientific except for the reducing agent (Sigma-Aldrich) and IgG1 antibody (NIST).

RESULTS

We demonstrate that the one-step protocol reduces hands-on time and total sample processing time from intact cells to cleaned-up peptides in 75 min. We determined this workflow scales from 10 µg to 100 µg of protein input, and the procedure is versatile and applicable to purified proteins, lysates, and intact mammalian cells. Finally, we show this procedure is compatible with isobaric labeling reagents such as Tandem Mass Tags® (TMT®) and Label-Free Quantitative (LFQ) methods to reproducibly quantify protein abundances.

Figure 1. Schematic representation of a traditional proteomics sample preparation workflow (Top) and the standardized method (Bottom). Compared to a traditional approach, the standardized workflow reduces the total protocol length by 16.5 hours (1A).



SCALABILITY AND STABILITY

Figure 2. The standardized method scales from 25 µg to 100 µg protein input, with a small decrease in IDs at 10 µg. We monitored performance of the standardized method across a range of protein inputs for chemical modifications (2A), Protein IDs (2B), and Peptide IDs (2C). HeLa cell pellets were lysed using the standardized method and quantified by UV absorbance at 280 nm on a NanoDrop instrument prior to digestion and peptide clean-up to measure input. Afterwards, 1 µg of peptides were then analyzed on a 3 h gradient using an EASY nLC-1200 system and Q Exactive HF MS. Searches were done in PMI-Preview or Proteome Discoverer 2.2 software.

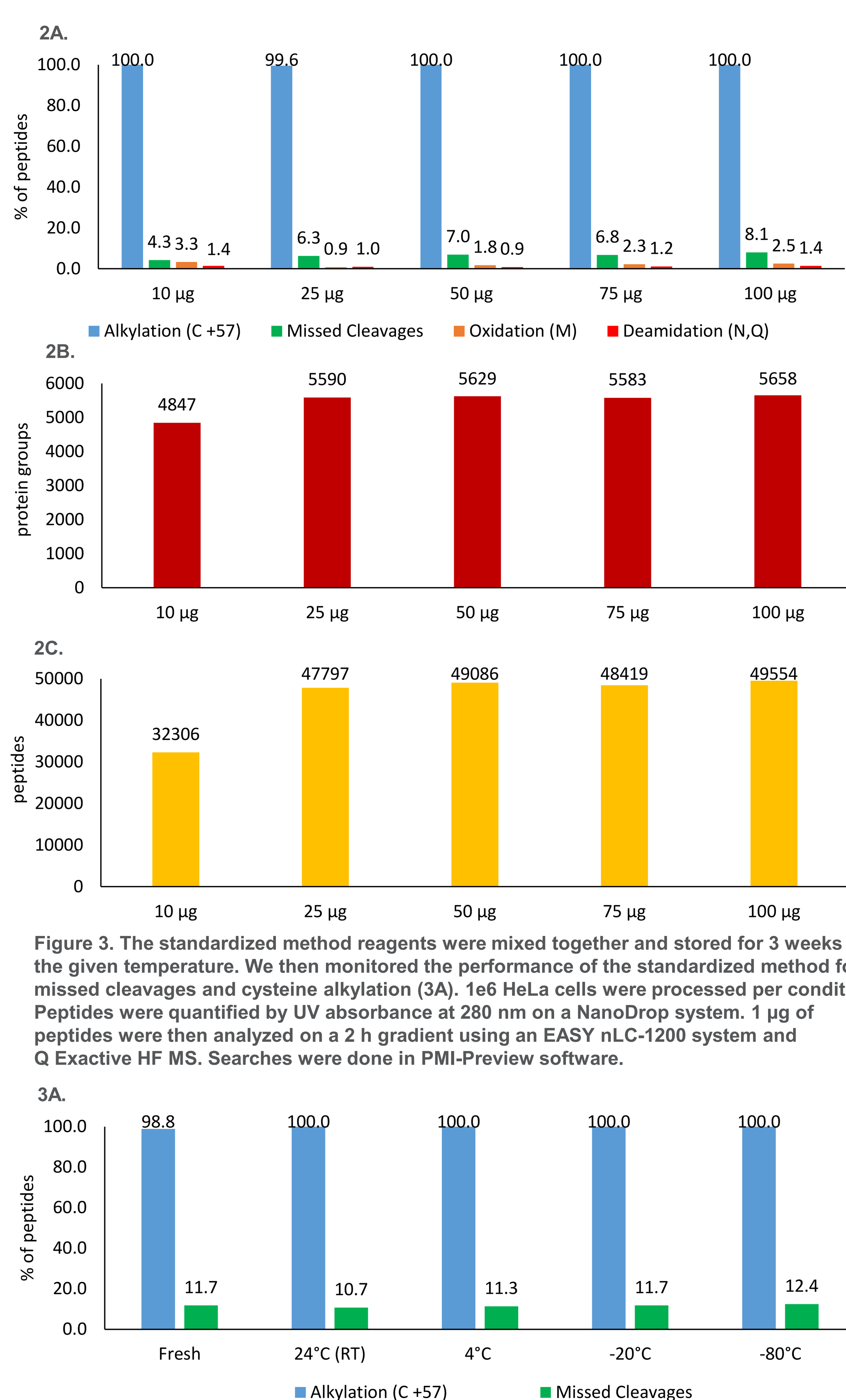
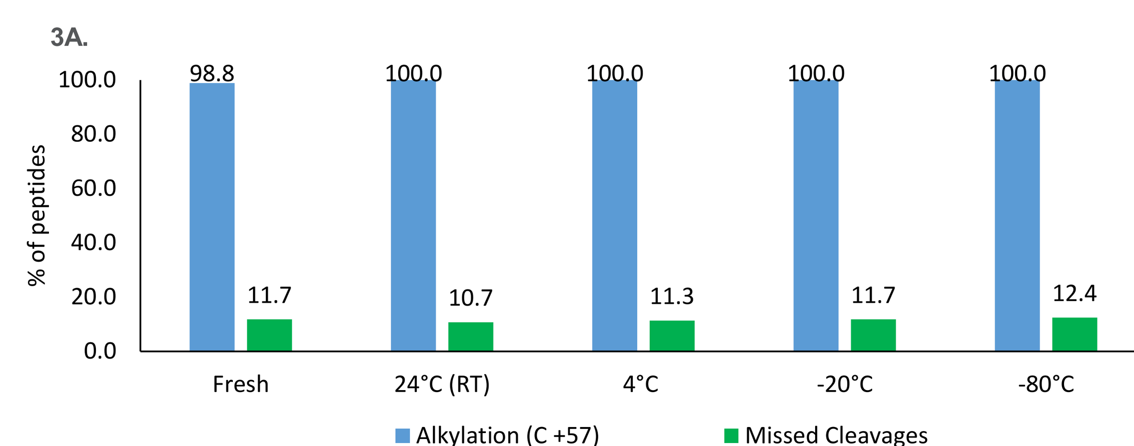
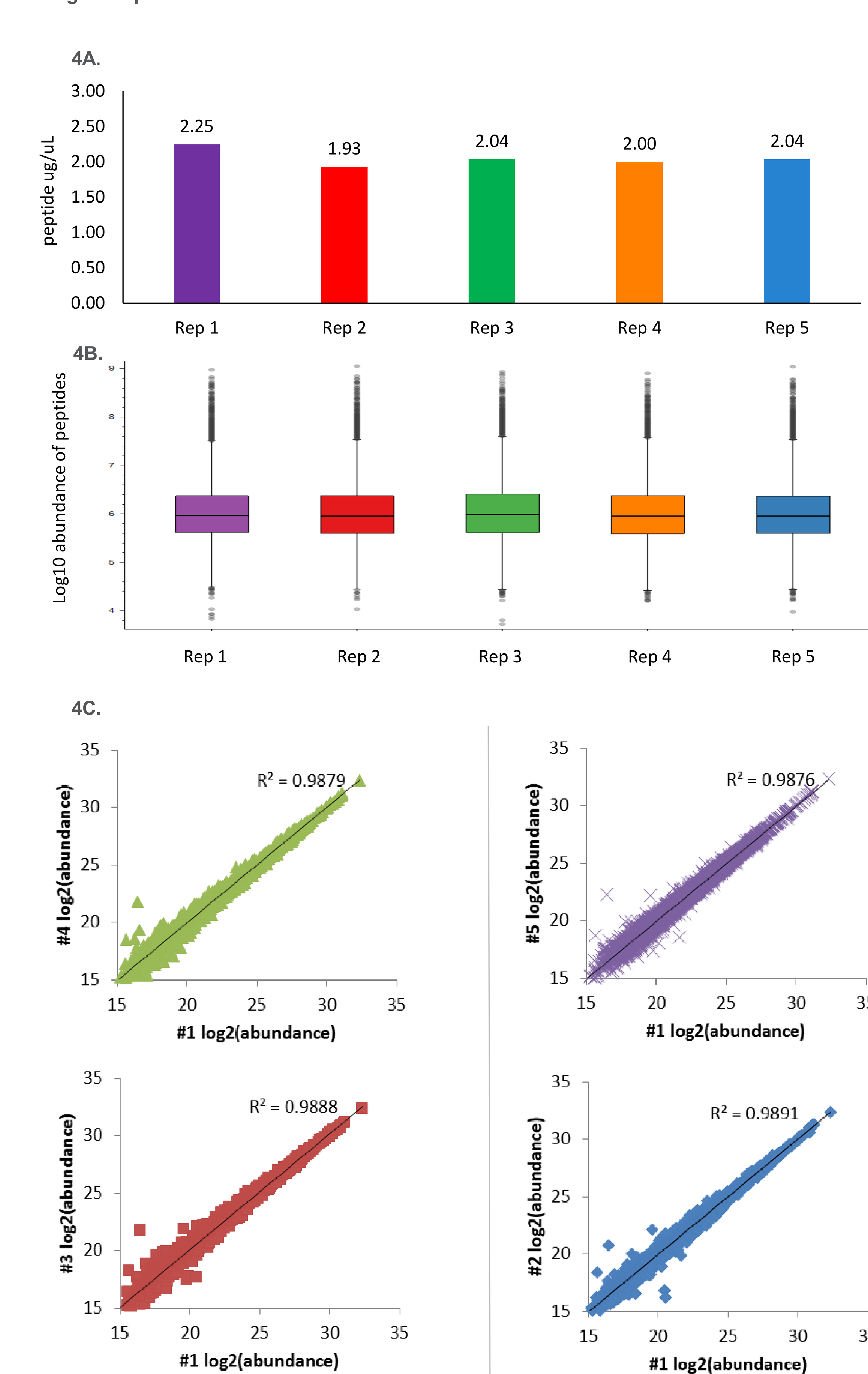


Figure 3. The standardized method reagents were mixed together and stored for 3 weeks at the given temperature. We then monitored the performance of the standardized method for missed cleavages and cysteine alkylation (3A). 1e6 HeLa cells were processed per condition. Peptides were quantified by UV absorbance at 280 nm on a NanoDrop system. 1 µg of peptides were then analyzed on a 2 h gradient using an EASY nLC-1200 system and Q Exactive HF MS. Searches were done in PMI-Preview software.



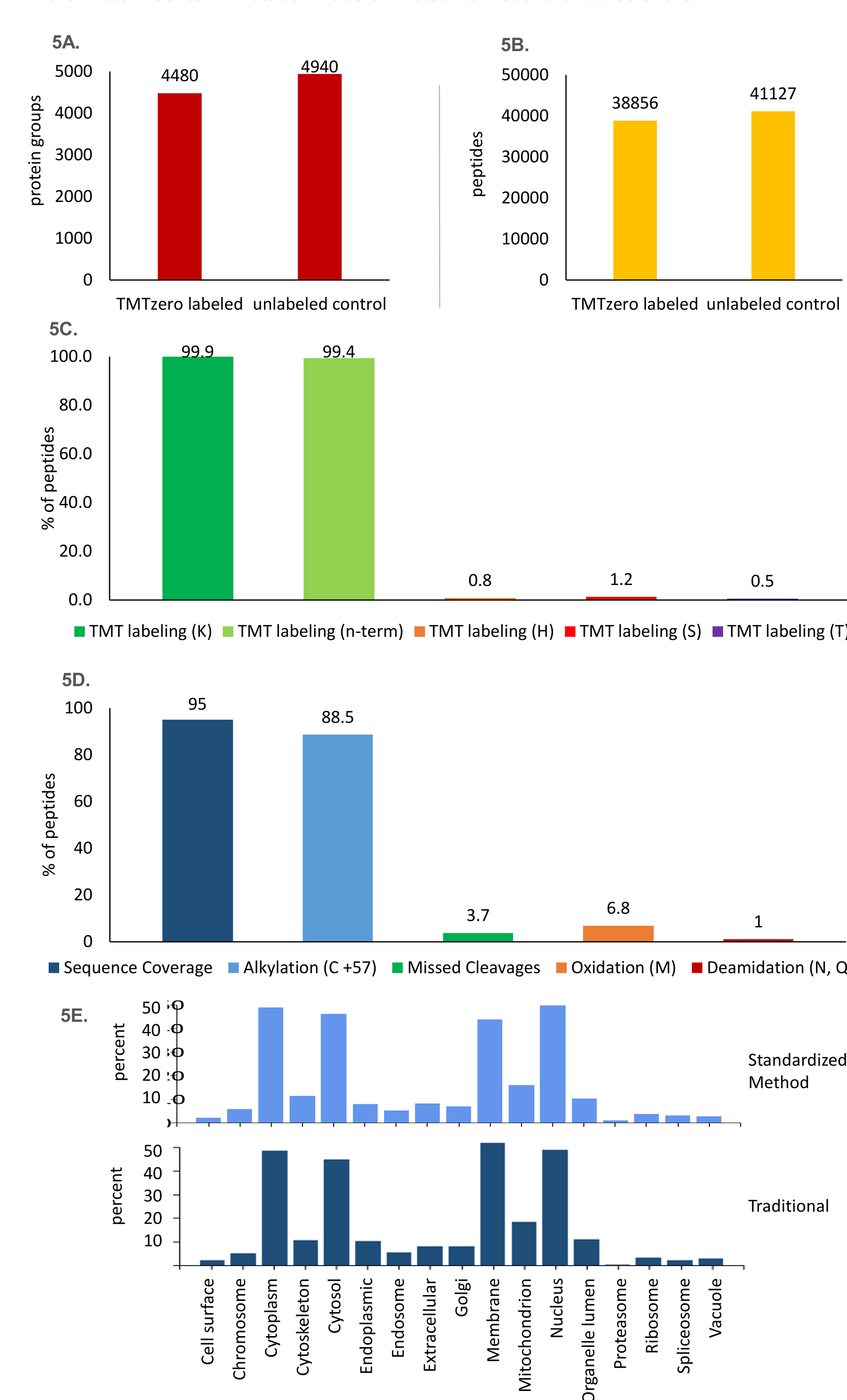
REPRODUCIBILITY

Figure 4. The standardized method is reproducible with a 6% CV for peptide yield after digestion, 14% CV for peptide quantification, and an average R^2 of 0.9885 for protein quantification. HeLa cell pellets were processed using the standardized method as described in Figure 3. We assessed the reproducibility of the standardized method for peptide yield after digestion (4A), peptide abundance (4B), and protein abundance between replicates (4C). Peptide yield was quantified by UV absorbance at 280 nm on a NanoDrop system. Peptide and protein abundances were measured using label-free quantification with the Minora Feature Detector node and Feature Mapper node in Proteome Discoverer 2.2 software with N=5 biological replicates.



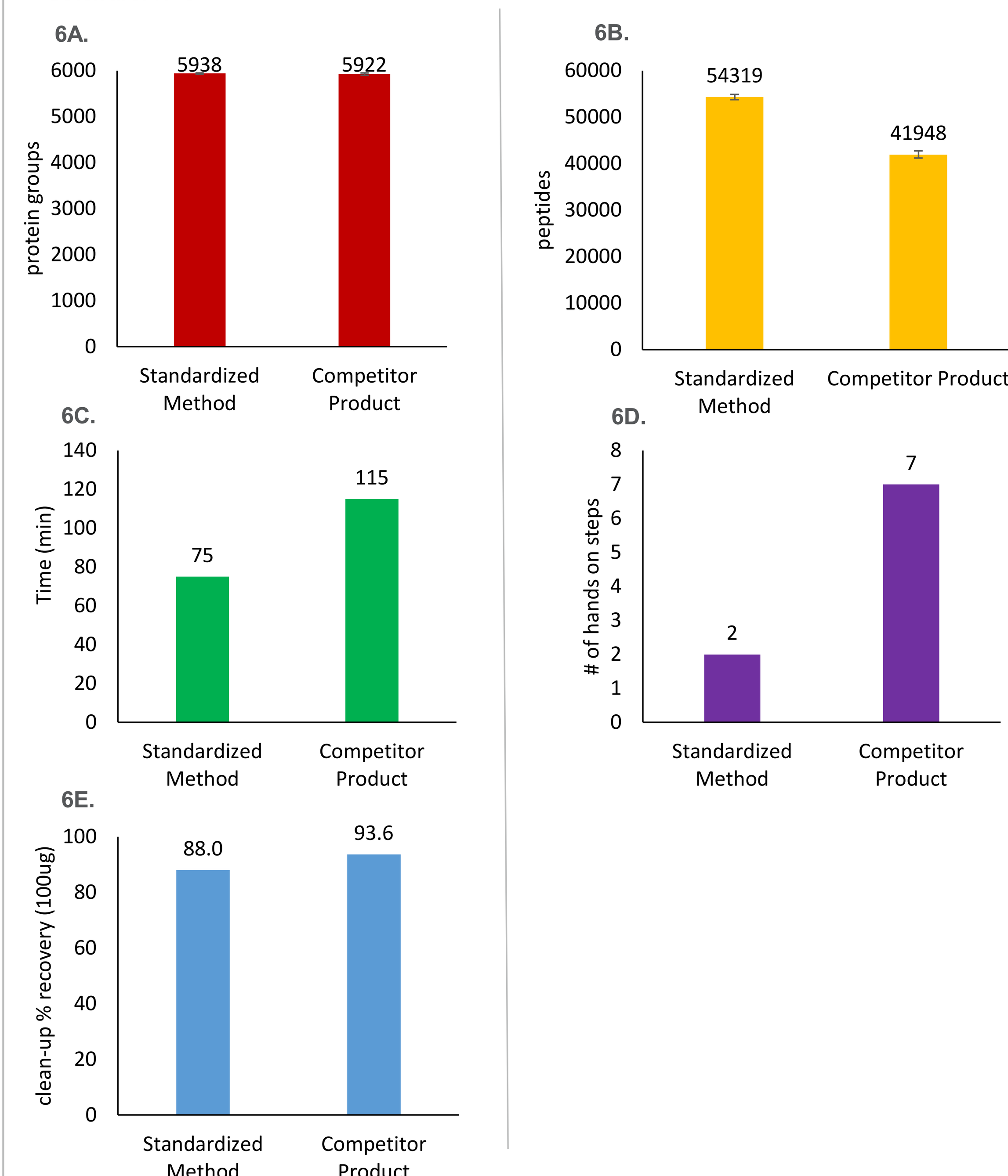
SAMPLE DIVERSITY

Figure 5. The standardized method is compatible with TMT labeling (5A-C). 5e5 HeLa cells were digested following the standardized method. Prior to clean-up, peptides were quantified by UV absorbance and 100 µg was labeled with 0.8 mg Thermo Scientific™ TMTzero™ label reagent for 1 hour. Searches were done in in Proteome Discoverer 2.2 software with TMT (224.152 Da) set as a dynamic modification. (5D) The standardized method can be carried out on purified proteins such as the NIST IgG1 antibody standard. (5E) The cellular components of proteins identified from HeLa cells exhibited the expected distribution, which was calculated in the Protein Center Annotation node of Proteome Discoverer 2.2 software.



SPEED AND COMPETITIVE PERFORMANCE

Figure 6. The standardized method is faster and performs better (higher peptide IDs) than a competitor product while also providing a standardized, reproducible process that is easy to use. 5e5 HeLa cells were digested using the standardized method or a competitor product according to the manufacturer's instructions. Peptides were quantified by UV absorbance at 280 nm on a NanoDrop system, and then 1 µg of peptides were analyzed on a 3h gradient using an EASY nLC-1200 system and Q Exactive HF MS. Searches were done in Proteome Discoverer 2.2 software. N=3



CONCLUSIONS

We present a standardized workflow for proteomic sample preparation that is quick, scalable, versatile, and reproducible, while being compatible with either isobaric mass tags such as TMT or LFQ. This method is applicable to purified proteins, lysates, and intact mammalian cells.

TRADEMARKS/LICENSING

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