APPLICATION NOTE 65727

Sampling the human population – ultra-high-throughput plasma protein profiling (uHTPPP) sample preparation for translational proteomics

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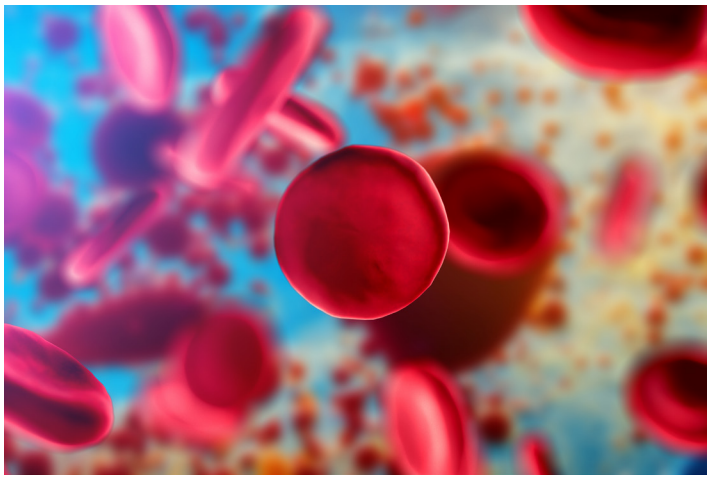


Goal

Present a robust, precise, and reproducible high-throughput sample preparation workflow for mass spectrometry-based large population cohort studies.

Introduction

Large human cohort studies require a robust and reproducible sample preparation workflow that achieves maximum efficiency with minimal sample handling. Manual sample preparation is a time-consuming and overwhelming task that can be a bottleneck for handling hundreds and thousands of samples and can introduce technical variability. To facilitate the efficient and precise processing of precious human blood samples, we developed an automated and high-throughput solution (uHTPPP) to enable large-scale proteomics analysis of plasma proteins. The uHTPPP workflow is a commercially available solution that automates the protocol of the Thermo Scientific™ EasyPep™ 96 MS Sample Prep Kit using a Hamilton® liquid handling robotic platform. We have developed quality assurance/quality control (QA/QC) operating procedures and demonstrated that we can produce precise and reproducible data with the uHTPPP workflow.

Experimental material and methods

Source of chemicals and reagents

The EasyPep 96 MS Sample Prep Kit (P/N A45733), Thermo Scientific™ EasyPep™ Mini MS Sample Prep Kit (P/N A40006), Invitrogen™ Fluorescein NIST-Traceable Standard (P/N F36915), and Thermo Scientific™ Pierce™ Quantitative Fluorometric Peptide Assay (P/N23290) were from Thermo Fisher Scientific. Glycerol was molecular biology grade from Fisher BioReagents. Acetonitrile and water were Fisher Chemical™ Optima™ LC/MS grade.

Sample preparation and peptide quantitation

Pooled normal human serum and non-small cell lung cancer adenocarcinoma plasma were purchased from BioIVT. Serum and plasma peptides were prepared with the EasyPep MS sample preparation kit using the automated uHTPPP workflow (EasyPep 96 MS kit) or spin column format (EasyPep Mini MS kit, manual) according to the manufacturer's protocol. For the uHTPPP workflow, serum and plasma samples were prepared with the EasyPep 96 MS Sample Prep Kit using the Hamilton® Microlab® STARLet liquid handling robotic platform equipped with the [MPE]^2 unit. A dialog was set up to allow the user to select specific protocol options, such as the number of samples and digestion time, or to allow the user to run additional protocols as needed. Then, another dialog was used to guide the user through loading of the required consumables and reagents onto the deck. The automated protocol began by diluting 5 µL of pre-aliquoted serum or plasma from a 96-well plate placed on the 4 °C chiller unit by the user into the Thermo Scientific™ EasyPep™ lysis solution and then transferring 10 µL of diluted serum or plasma into a second plate for in-solution digestion located on the Hamilton heater/shaker. Samples were then reduced and alkylated to break up the cysteine disulfide bonds and prevent reformation prior to trypsin digestion. During the incubation, trypsin/LysC were reconstituted and diluted on-deck to a pre-set concentration. After reduction and alkylation, the plate was cooled to 37 °C, and 2.5 µg of trypsin/LysC were added to each sample. Digestion occurred at 37 °C for 1.75 h. After incubation, the digestion reaction was stopped, and peptide clean-up began on the [MPE]^2 unit. The digested sample was first loaded into and passed through the EasyPep 96-well plate followed by wash buffer A and wash buffer B. Purified peptides were eluted with elution solution and dried using the [MPE]^2 Evaporator module. Finally, peptides were reconstituted in HPLC grade water with 0.1% formic acid and quantified using the Pierce Quantitative Fluorometric Peptide Assay and a Thermo Scientific™ Fluoroskan™ fluorescent microplate reader.

LC-MS and data analysis

Peptides from digested samples (500 ng) were loaded onto disposable EvoTip trap columns (Evosep, Odense, Denmark) by centrifugation following the recommended protocol. Peptides were eluted at high flow into a pre-formed gradient using the EvoSep One LC system and separated using an 8 cm Evosep column (Evosep, Odense, Denmark). All samples were analyzed using the "60 sample per day" method, which consists of a 21-minute gradient with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The column was connected to a Thermo Scientific™ EASY-Spray™ ion source through a Thermo Scientific™ EASY-Spray™ adapter (EV-1072) with a stainless-steel emitter (EV-1086). The chromatographic system was coupled to a Thermo Scientific™ Q Exactive™ HF-X hybrid quadrupole-Orbitrap™ mass spectrometer.

Data processing and consensus templates from Thermo Scientific™ Proteome Discoverer™ 2.4 software were used to search the acquired MS^2 spectra against the human protein database (UniProt reviewed, December 2018). A 1% FDR was set as the filtering threshold for protein and peptide identification.

Results and discussion

Automation of a streamlined and optimized protocol from the EasyPep sample preparation kit

The EasyPep MS Sample Prep Kit provides pre-formulated reagents and a robust method for the preparation of high-quality samples for mass spectrometry (MS) analysis. A streamlined protocol and reagents minimize the number of steps and time needed to process samples within 3 hours (Figure 1).

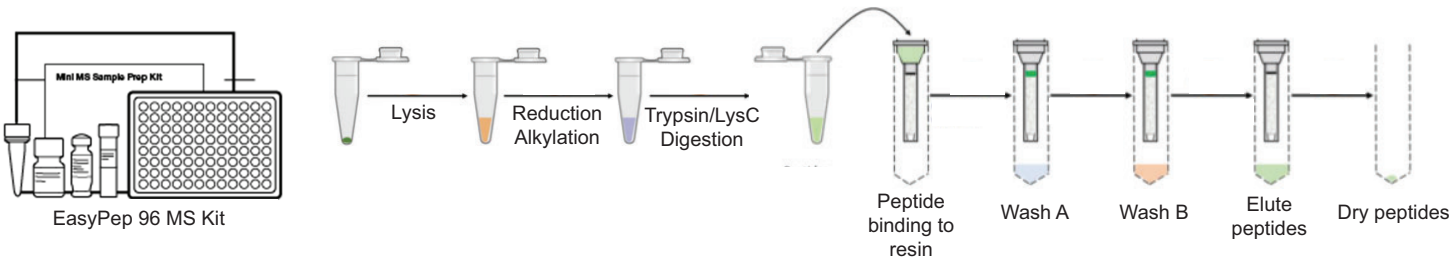
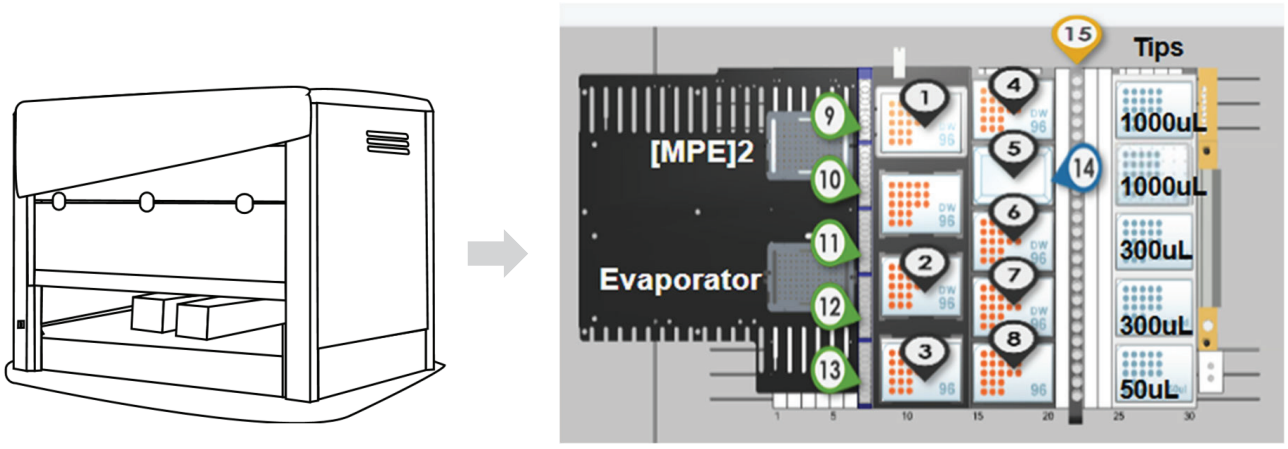


Figure 1. EasyPep MS sample preparation kit. Schematic of the EasyPep kit chemistry workflow. Samples are lysed, reduced, alkylated, and digested with Trypsin/LysC before peptides are purified by a mixed bed resin. There are two versions of the EasyPep sample preparation kit: 96-well filter-plate based kit (left) and spin column-based kit (right).

We automated the protocol and integrated the 96-well filter plate-based version of the reagent kit on a Hamilton liquid handling robotic platform to enable digestion and peptide purification from 48 or 96 samples (plasma and serum) in 4 hours. The fully automated protocol design includes an on-deck sample dilution process that allows for precise volume-based sample normalization. The deck layout required to run the automated script is shown in Figure 2. A stepwise illustration of the workflow and time required to complete each step is shown in Figure 3A. Also, a screenshot of the protocol section user dialog is shown in Figure 3B.



Hamilton Microlab STARLet

Deck Layout

Figure 2. The deck layout to run the uHTPPP workflow on the Hamilton Microlab STARLet platform. A schematic of the deck layout is shown. Deck layout key: 1) heater/shaker (sample digestion); 2) blank plate space; 3) 4 °C chiller (starting sample plate); 4) EasyPep 96 filter plate; 5) 12 column trough; 6) filtrate collection plate; 7) elution collection plate; 8) sample dilution plate; 9) wash buffer A; 10) wash buffer B (1); 11) wash buffer B (2); 12) elution solution; 13) lysis solution; 14) digestion reagents in trough; 15) Trypsin/LysC vials.

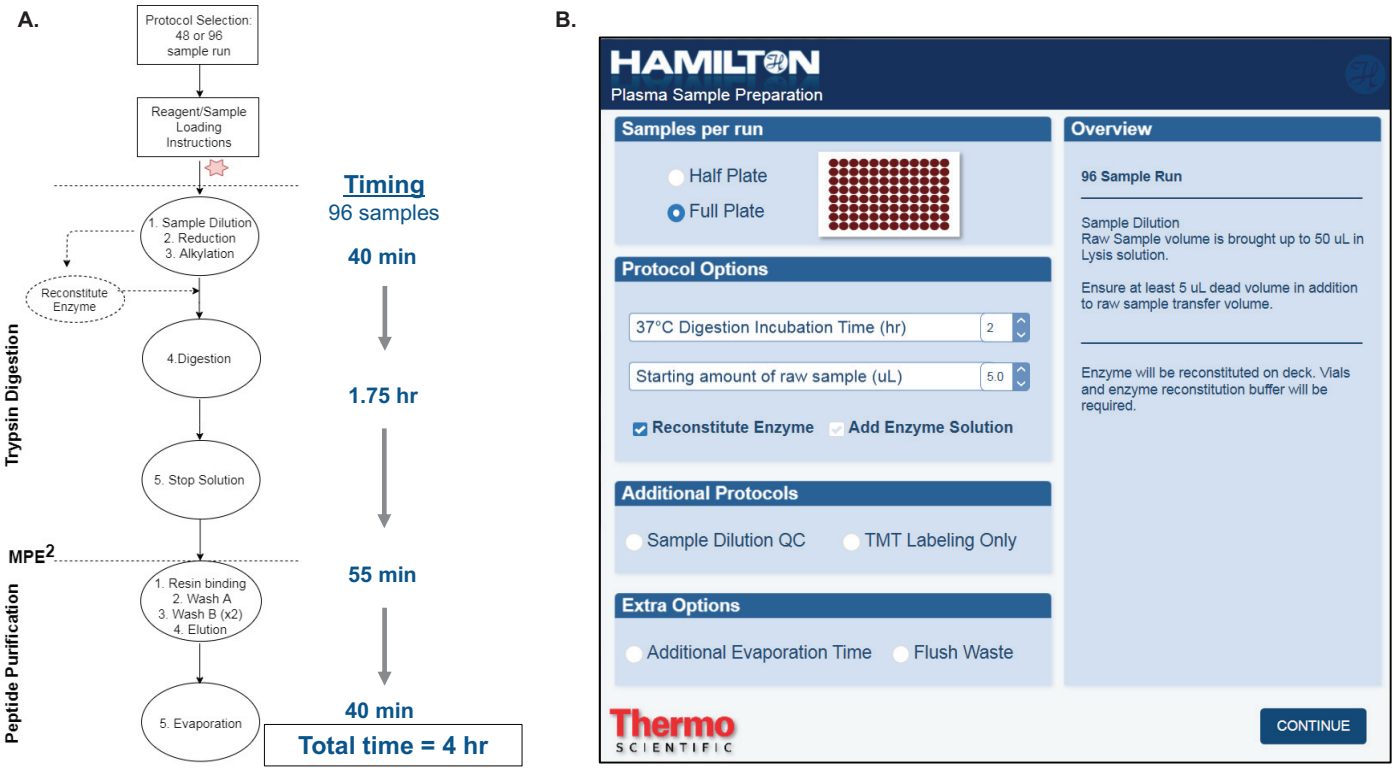


Figure 3. A stepwise illustration of the automated workflow and a screenshot of the dialog box. A). A schematic illustration of the uHTPPP workflow with the time required to complete each step. Red star indicates when the user would load the deck. B). A dialog box to start the automated script. Users are allowed to select various options or additional protocols such as the Sample Dilution QC to verify accurate pipetting and dilution of plasma samples.

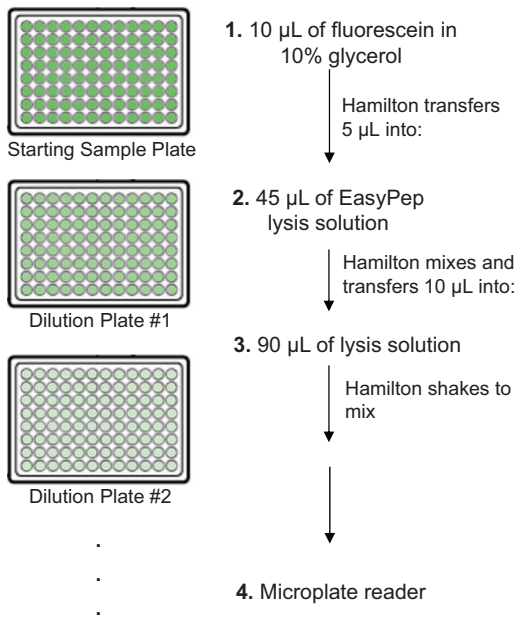
QA/QC operations of uHTPPP automation designed to assess accurate pipetting

Since small volume pipetting is needed for the sample dilution step, we included a sample dilution QC script in the workflow to allow the user to evaluate pipetting accuracy and consistency. To run the sample dilution QC script, fluorescein in 10% glycerol was used to mimic the viscosity of plasma and serum. The script will make a serial dilution of the fluorescent sample using the lysis buffer from the 96-well EasyPep MS sample prep kit. The plate can be read using a fluorescent microplate reader. A schematic of the Sample Dilution QC procedure is found in Figure 4A. We plotted a standard curve of data containing six replicate dilutions at fluorescein concentration from 31.25 to 500 nM. The sample dilution QC script resulted in very precise readings (coefficient of variations (CVs) <4%) as shown in Figure 4B. The protocol was expanded to a 48- or 96-well format with a single concentration to determine the pipetting consistency across the plate.

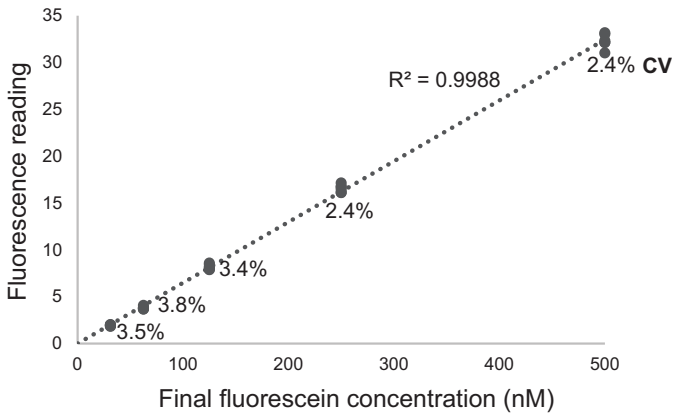
We observed less than 5% CV on average for the fluorescence reading for both the 48- and 96-dilution QC (Figure 3C). This QC script is included in the automated uHTPPP script as a stand-alone protocol and can be selected by the user anytime to test the pipetting consistency and accuracy (with a standard curve) of the Hamilton liquid handling robotic platform.

Additionally, we optimized the pipetting accuracy of the buffers from the EasyPep reagent kit required in the workflow. Results after liquid class optimization are shown in Table 1. The average transfer volume from 50 µL of different in-solution digestion buffers was accurate to within ±1 µL and consistent with less than 3% CV. The average transfer volume from 300 µL of different peptide purification buffers was accurate to within ±4 µL and consistent with less than 1% CV. The pipetting parameters obtained from the liquid class calibration are built into the automation script.

A.



B.



C.

	%CV
48 samples QC	3.5%
96 samples QC	4.7%

Figure 4. Sample dilution QC analysis. A). A schematic illustration of the Sample Dilution QC protocol. B). A serial dilution of fluorescein from 50 to 3.125 µM in 10% glycerol in the EasyPep lysis buffer (n=6 per concentration) was made by automated pipetting. %CV for the replicate dilutions were calculated and a scatter plot of the serial dilution with a best-fit line is shown. C). Using the Sample Dilution QC, the Hamilton performed 48 or 96 replicate serial dilutions of 25 µM fluorescein in the EasyPep lysis buffer. %CV of fluorescence readings from both formats were calculated and are shown in the table.

Table 1. Optimization of pipetting accuracy among different liquid classes. Liquid classes were developed using Hamilton's Liquid Class Verification kit (LVK) to accurately pipet 50 µL of EasyPep in-solution digestion buffers (lysis, reduction, alkylation, enzyme, and stop solutions) and 300 µL of EasyPep peptide purification buffers (wash buffer A, B, and elution buffer). The average volume pipetted and %CV after liquid class calibration are shown in the table with the number of replications for each solution in parentheses.

Hamilton pipetting	Lysis (n=48)	Reduction (n=96)	Alkylation (n=24)	Enzyme (n=96)	Stop solution (n=96)	Wash A (n=96)	Wash B (n=96)	Elution (n=36)
Average volume	50.1 µL	51.0 µL	50.4 µL	50.7 µL	50.9 µL	301.2 µL	303.4 µL	299.2 µL
%CV	1.0%	2.8%	0.8%	1.9%	2.8%	0.8%	0.9%	1.0%

uHTPPP workflow implements the Monitored Multi-Flow Positive Pressure Evaporative Extraction module, or [MPE]^2, which reduces processing time per sample and improves peptide recovery

The uHTPPP workflow utilizes the [MPE]^2 module instead of centrifugation for purifying peptides from the 96-well filter plate after the in-solution digestion. The [MPE]^2 unit is portable and integrated into the Microlab STARLet platform. The [MPE]^2 unit contains two modules:

(1) The Logistics Module uses a dual elevator and applies up to 100 psi of positive pressure to samples through a manifold connected to a control box. This eliminates inconsistency due to path of least resistance.

(2) The Evaporator Module provides sorbent drying, with variants for multiple microplate well densities.

We optimized the positive pressure air flow rate to maximize the peptide recovery efficiency from the 96-well filter plate. To calculate the peptide recovery efficiency, we measured the peptide amount from each well using the Pierce Quantitative Fluorometric Peptide Assay after peptide elution and compared it to the starting protein amount. To compare the reproducibility between the automated (uHTPPP) and manual (spin column) methods, we compared either the peptide purification only (half workflow) or in-solution digestion combined with peptide purification (full workflow) based on peptide recovery efficiency. From both the half and full workflows, we observed excellent %CVs from the automated method compared to the spin column method (Table 2).

Then, we compared the processing time per sample and peptide recovery efficiency between preparation of a full plate of serum using the automation script and preparation of 12 serum samples using the spin column format (EasyPep spin column MS kit). We included the entire workflow from aliquoted samples to dried down peptides. Not surprisingly, we found that the uHTPPP workflow processes each sample more efficiently (8 times faster) and more consistently than the spin column sample preparation procedure (Table 3).

Table 2. A comparison of peptide recovery efficiency between automated and manual methods. Positive air pressure was used in the automated method, and centrifugation was used in the manual spin column method for liquid collection. At least three replicates were performed per condition.

	Peptide clean-up only				Full workflow		
	Peptide input	Recovery	%CV		Protein input	Recovery	%CV
Manual spin column	20 µg	68.5%	5.7%	Manual spin column	45 µg	77.9%	10.7%
Automated 96-well plate on Hamilton	20 µg	72.1%	3.7%	Automated 96-well plate on Hamilton	45 µg	80.7%	7.2%

Table 3. A comparison of efficiency and reproducibility of processing samples between automated and spin column methods

	Processing time per sample	Serum		Plasma	
		% Recovery	%CV	% Recovery	%CV
Manual spin column	20 min	77.9%	10.7%	60.4%	6.2%
Automated 96-well plate	2.5 min	80.7%	7.2%	81.0%	8.9%

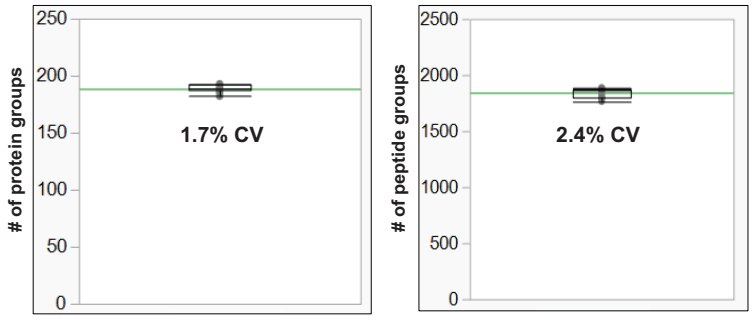
Reproducibility of the automated workflow

To demonstrate the reproducibility of the uHTPPP workflow, we examined both the well-to-well and plate-to-plate reproducibility. We processed 48 replicates of 50 µg pooled undepleted normal human serum. The efficiency of peptide recovery was determined using the peptide assay, and %CV are labeled in cells highlighted in green, summarizing row/column variations as well as the total variability (Figure 5A). On average, we observed over 70% peptide recovery efficiency across the plate with a total average %CV of 7.2%. Then, we selected 12 samples from different well locations across the plate (Figure 5A, yellow cells) to carry out LC-MS/MS analysis using a Q Exactive HF-X mass spectrometer coupled with an Evosep One LC system. The results are shown in Figure 5B. Overall, we observed very consistent protein and peptide identifications from the selected serum samples (Figure 5B). We also observed small variations (<5% CV) in other important parameters, such as the % of zero missed cleavage and the number of cysteine modified peptides from these samples (Figure 5C). Finally, we extended the study to process 96 samples and further optimized individual pipetting procedures throughout the automation script. As a result, we were able to increase the average peptide recovery to over 80% and maintain less than 10% total CV (Figure 5D).

A.

	1	2	3	4	5	6	%CV
A	74.0%	74.3%	72.6%	72.4%	83.7%	76.9%	5.6%
B	72.5%	73.8%	66.7%	68.3%	73.8%	82.5%	7.6%
C	68.9%	72.2%	69.7%	70.3%	77.3%	78.6%	5.7%
D	72.0%	71.7%	69.2%	100.0%	76.2%	74.4%	14.8%
E	74.3%	75.1%	72.2%	67.4%	73.1%	81.6%	6.2%
F	70.4%	75.3%	74.3%	67.9%	74.2%	73.8%	4.0%
G	75.6%	73.1%	72.6%	72.8%	77.5%	80.1%	4.1%
H	70.0%	72.5%	76.8%	71.7%	77.1%	77.1%	4.3%
%CV	3.2%	1.8%	4.4%	14.6%	4.4%	4.1%	**7.2%**

B.



C.

96-well plate location	Proteins	Peptides	PSMs	MS/MS Spectrum	% of zero missed cleavage	C mod
D1	193	1880	3622	17288	77.5%	636
H1	193	1880	3645	17640	77.5%	637
A2	190	1886	3641	16953	78.0%	635
F2	189	1888	3735	16379	77.0%	636
C3	185	1805	3618	17044	76.0%	619
G3	188	1872	3746	17163	77.5%	637
A4	188	1824	3605	16395	77.0%	627
F4	193	1857	3742	17466	76.5%	633
B5	187	1881	3737	17118	75.5%	637
H5	189	1797	3592	16592	78.0%	607
A6	187	1789	3495	15784	77.0%	619
D6	182	1766	3488	16623	75.0%	590
Average	**188**	**1843**	**3639**	**16870**	**76.9%**	**626**
%CV	**1.7%**	**2.4%**	**2.5%**	**3.1%**	**1.2%**	**2.4%**

D.

	1	2	3	4	5	6	7	8	9	10	11	12	%CV
A	93.6%	86.5%	97.2%	93.3%	100.0%	100.0%	100.0%	100.0%	92.3%	92.2%	100.0%	93.9%	4.6%
B	92.1%	79.9%	91.5%	94.3%	99.0%	99.6%	99.3%	100.0%	91.0%	96.9%	100.0%	100.0%	6.3%
C	82.4%	78.3%	90.7%	82.4%	86.9%	89.0%	91.4%	89.6%	87.6%	80.9%	100.0%	97.5%	7.4%
D	84.8%	70.6%	81.4%	83.1%	85.7%	98.0%	87.5%	100.0%	90.4%	91.5%	100.0%	91.9%	9.6%
E	81.3%	82.3%	80.3%	89.8%	95.7%	85.3%	93.5%	95.3%	88.6%	97.2%	95.9%	98.0%	7.3%
F	75.3%	84.2%	89.1%	82.1%	92.8%	88.0%	92.6%	97.6%	92.8%	98.2%	99.7%	100.0%	8.4%
G	84.3%	85.2%	83.1%	85.0%	91.8%	85.8%	90.6%	97.1%	96.6%	99.7%	100.0%	100.0%	7.4%
H	82.1%	82.6%	88.6%	79.8%	88.6%	84.3%	89.8%	96.8%	95.1%	94.1%	95.7%	91.9%	6.5%
%CV	7.0%	6.2%	6.6%	6.4%	5.8%	7.4%	4.8%	3.6%	3.4%	6.4%	2.0%	3.7%	**7.6%**

Figure 5. Well-to-well reproducibility study. A) Peptide recovery efficiency for 48 replicates of pooled normal serum sample processed with the uHTPPP workflow. %CVs of peptide recovery efficiency for each row and column are displayed in green highlighted cells down the far-right column and last row, respectively. Average %CV of peptide recovery efficiency for all 48 samples was 7.2%. B) Box plots summarize protein and peptide ID results and %CV from 12 randomly selected serum samples for LC-MS/MS analysis. Locations of these samples are highlighted in yellow. C) Extended LC-MS/MS results from samples selected in B. D) Peptide recovery efficiency for 96 replicates of pooled normal serum sample processed with the uHTPPP workflow after further optimization.

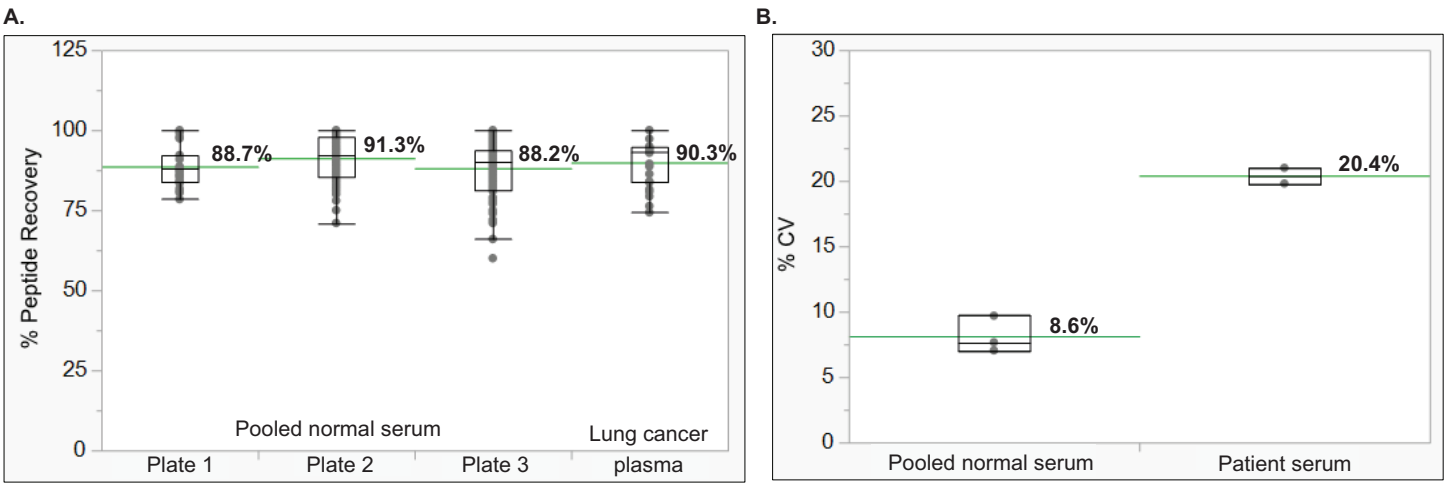


Figure 6. Plate-to-plate reproducibility study. A) Box plots summarize peptide recovery efficiency of replicates within each plate from either pooled normal serum or pooled lung cancer plasma using the uHTPPP workflow. B) A comparison of variability (%CV) of replicates from three plates of pooled normal serum and 2 plates of individual patient serum.

For plate-to-plate reproducibility, we tested three different EasyPep 96 filter plates using either pooled normal serum or pooled lung cancer plasma on three different days. A summary of plate-to-plate variability is shown in Figure 6A based on the averaged peptide recovery efficiency from each filter plate. Over 88% peptide recovery efficiency was observed for serum and for plasma (Figure 6A). We also compared averaged %CV of peptide recovery efficiency among three plates from pooled normal serum samples and %CV of peptide recovery efficiency from two plates of patient serum samples (individual patients) using the uHTPPP workflow. We observed an average %CV of 8.6% for pooled normal serum and an average %CV of 20.4% for individual patient serum (Figure 6B). It is not surprising that patient samples showed larger variations than pooled normal serum samples because protein concentrations could vary among individuals in a fixed volume of blood. Therefore, it is important to measure peptide recovery efficiency from the sample preparation prior to LC-MS/MS analysis to record patient variability.

Conclusion

We have developed a fully automated workflow (uHTPPP) using commercially available components for high-throughput sample preparation of plasma and serum for mass spectrometry analysis. This workflow uses an automated script to process plasma or serum using an EasyPep 96 MS kit on the Hamilton liquid handling robotic platform as well as proper QA/QC operations. The results demonstrate the following:

- Automated 96-well filter plate sample preparation with the [MPE]^2 achieves better peptide recovery for both serum and plasma compared to the manual spin column method.
- uHTPPP workflow shows good well-to-well and plate-to-plate reproducibility with less than 10% CV.
- It is important to measure individual patient variability from the sample preparation workflow before LC-MS/MS analysis to avoid reporting compounded errors from sample preparation and analytical variability.

The workflow can be coupled to other low flow HPLC systems (e.g., Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano systems) and to other Thermo Scientific™ Orbitrap MS or quadrupole-Orbitrap series. Furthermore, the automated workflow can be further customized by incorporating Thermo Scientific™ Tandem Mass Tag™ labeling and peptide fractionation into the workflow.

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