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# A One-Step Sample Concentration, Purification and Albumin Depletion Method for Urinary Proteomics

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## **Abstract**

Workflows in urinary proteomics studies are often complex and require many steps to enrich, purify, deplete and separate the complex mixture. Many of these methods are laborious, time-consuming and have the potential for error. Although individual steps of these methods have been previously studied, their downstream compatibilities with fractionation technologies such as Off-Gel electrophoresis have not been investigated. We developed a *One-Step* sample preparation workflow that simultaneously i) concentrates proteins, ii) purifies by removing salts and other low molecular weight compounds and iii) depletes (albumin) from urine samples. This simple and robust workflow can be multiplexed and is compatible with a diverse range of downstream multidimensional separation technologies. Additionally, due to its high reproducibility and flexibility in processing samples with different volumes and concentrations, it has the potential to be used for standardization of urinary proteomics studies, as well as for studying other body fluids of similar complexity.

#### **Keywords**

Shotgun Proteomics; Off-Gel electrophoresis; Albumin Depletion; Urinary Proteomics

# Introduction

The field of urinary proteomics presents an opportunity to discover clinically relevant diagnostic and prognostic biomarkers, and potential therapeutic targets of disease. Due to urine's importance as a biomarker source, numerous separation methods have been employed to reduce its complexity and to find potential low-abundance biomarkers<sup>1</sup>. Some of these include: Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE)<sup>2</sup>, Two-Dimensional Gel Electrophoresis (2DE)<sup>3, 4</sup>, GeLC-MS<sup>5, 6</sup>, Size Exclusion Chromatography<sup>7</sup>, Gas Phase Fractionation<sup>8</sup>, Hydrophilic Interaction Liquid Chromatography-Reversed Phase Liquid Chromatography (HILIC-RPLC)<sup>9</sup>, protein

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Supporting Information Supporting Information is Available: This material is available free of charge via the Internet at http://pubs.acs.org.

Supplementary Data The complete list of all proteins and peptides identified in samples 1, 2, 3, 5 and 7 using the *One-Step* method and <u>Off-Gel electrophoresis</u> are listed in individual sheets. The technical repeat data is listed in a separate sheet. The complete list of all proteins and peptides identified in sample 1 using the *One-Step* method and <u>High pH separation</u> is listed in the final sheet.

Equalizer bead technology<sup>10</sup>, Strong Cation Exchange-Reversed Phase Liquid Chromatography (SCX-RPLC)<sup>11</sup>.

Methods are continuously being refined and improved in an effort to achieve maximum protein coverage. Off-Gel electrophoresis is a recent advance in separation technology that fractionates proteins or peptides according to their isoelectric point (*pI*) and facilitates the recovery of the focused species in the liquid phase<sup>12</sup>. The high resolution orthogonal separation, excellent reproducibility, and increased analytical throughput allowing multiple samples to be run in parallel, are among some of the advantages of the Off-Gel technology. The superiority of isoelectric focusing as the first dimension of separation has been shown in comparison with more widely used methods such as GeLC-MS<sup>13</sup> and SCX<sup>14</sup>, <sup>15</sup>.

The majority of methodology studies on urine have involved improving steps of gel separation<sup>1, 2, 16</sup>. We sought to apply the Off-Gel technology as the first dimension of separation of urinary peptides prior to the conventional LC-MS/MS step of the shotgun proteomics. Despite our initial success with low urine volumes (1 and 5 ml), the separation of higher volumes (> 5 ml) were precluded because of a sharp increase in conductivity during the focusing because of incompatible salt concentrations. Due to the concomitant effects of diluted protein concentration of urine samples (particularly for non-renal disease studies), high-salt content and interfering compounds (urea, metabolites, etc.), purification is an absolute prerequisite for efficient application of the Off-Gel to urinary proteomics. Moreover, depletion of human serum albumin may help reduce the complexity of urine to improve detection of low-abundance proteins<sup>16</sup>. Typically, albumin depletion is performed in series with purification steps, which further complicates workflows and increases the potential for sample loss.

With the rapidly growing interest in the human urine proteome, we developed a fast, easy and reproducible *One-Step* method to simultaneously concentrate urinary proteins, remove salts and other contaminates, and deplete albumin. We present the application of the *One-Step* method in combination with the Off-Gel technology on both adult and pediatric urine samples.

#### **Methods**

#### **Urine Collection**

Sample 1 was a clean catch midstream urine sample obtained as the second void of the day from a healthy 38 year old male. Samples 2 to 7 were catheterized urine samples collected from 6 infant males under the age of 2 years with different renal and urologic diseases. Samples were collected in sterile specimen containers (Falcon 4.5 oz / 110 ml # 35-4013) under sterile conditions. Samples were centrifuged at 3500 g in 4°C for 10 minutes to clear the debris. 5 ml aliquots were stored at -80 °C.

# **Protein Concentration**

The concentration of the urine sample pre and post purification and depletion was estimated by Bradford assay (Bio-Rad) using a NanoDrop ND-1000 Spectrophotometer (Thermo). The Bradford assay was performed according to manufacturer's instructions. The measurements were performed in triplicate for each method and each biological replicate. The average value of the protein concentration is reported.

#### One dimensional gel electrophoresis (SDS-PAGE)

Samples from each experiment were re-suspended in 24  $\mu$ l of water and 8  $\mu$ l 4X LDS sample buffer (Invitrogen). Samples were heated at 70 °C for 10 minutes and then were

loaded into a 4–12% Bis-Tris precast gel (Invitrogen). Gels were run at 100 V and stained overnight in colloidal Coomassie blue as per manufacturer protocol (Invitrogen).

# One-Step (combined enrichment, purification and albumin depletion)

A 5 ml urine sample was reduced for 30 minutes with 30  $\mu$ l of 20 mM tris [2-carboxyethyl] phosphine (TCEP) and then alkylated for 30 minutes in the dark with 30  $\mu$ l of 150 mM iodoacetamide. Both reactions were carried out at room temperature. 200  $\mu$ l of the Anti-HSA resin (Sartorius Stedim) and a 5 ml urine sample were added to a Vivaspin 6 spin-filter. After a 10-minute incubation step, the column is placed in the centrifuge (20 minutes at 7000 g) and then washed 2 times each with 5 ml pure water for 10 minutes at 7000 g. The supernatant (30  $\mu$ l) was collected. In order to assure a good recovery of the sample from the resin, 100  $\mu$ l water was then added and vortexed with the resin. The supernatant was then aspirated and combined with the initial supernatant.

# **In-Solution Digestion**

After the *One-Step* method, the purified / depleted protein was dried by vacuum centrifugation and brought up to  $100~\mu l$  with 100~mM triethyl ammonium bicarbonate (TEAB), pH 8.5. The proteins were digested by addition of  $4~\mu l$  of 1~mg/ml trypsin (sequencing grade modified trypsin, Promega) to obtain a ratio of 1:50~m/m. Proteolysis was performed overnight at  $37~^{\circ}C$ .

# Off-Gel Electrophoresis

After digestion of 300 to 400  $\mu$ g of protein, peptides were separated using an Agilent 3100 Off-Gel Fractionator (Agilent). 24 cm, pH 3–10 IPG DryStrips (GE Healthcare) were used. Strips were rehydrated for 20 minutes with 20  $\mu$ l/well of the IEF buffer containing 0.2% IPG buffer pH 3–10. Samples were dissolved in 3600  $\mu$ l of the IEF buffer. 150  $\mu$ l of this peptide solution was loaded into each well, the cover seal was set into place and Immobiline DryStrip cover fluid (GE Healthcare) was added to both ends of the strip. Focusing was performed according to manufacturer's preset program up to 50 kV-h with maximum current of 50  $\mu$ A. Fractions were collected from each well. 100  $\mu$ l of 0.1% formic acid was added to each well and extracted after 10 minutes. The extracted peptides were combined and dried down in a speed vac.

# LC-MS/MS

All experiments were performed using an LTQ-Orbitrap mass spectrometer linked to a microautosampler and a nanoflow HPLC pump (both: Eksigent). The reversed phase columns were packed in-house by using Magic C18 particles (3  $\mu$ m, 200 Å; Michrom Bioresource) and PicoTip Emitters (New Objective, Woburn, MA). The peptides were eluted with a 60-minute linear gradient and data acquired in a data dependent fashion, fragmenting the 6 most abundant peptide species, which were then dynamically excluded for 60 seconds. Buffer A was 0.2% formic acid; buffer B was AcN/0.2% formic acid, and loading buffer was 5% formic acid/5% AcN.

#### **Database Searching and Validation**

The 200 most intense fragment ions of each raw product ion spectrum were used for searches against the IPI human database (version 3.56) using Mascot version 3.2<sup>17</sup>. The following search parameters were applied: default charge states of 1+, 2+, 3+, and 4+ were used; a maximum of one missed cleavage was allowed with an average peptide mass tolerance of 5 ppm. A fragment ion search tolerance of 0.8 Da was permitted. Fixed modification on cysteine was carbamidomethylation and variable modifications were deamidation, oxidation of methionine, and N-terminal pyro-glutamic acid formation. All

data were searched against a merged target and decoy database. The peptide level score cutoff for each of the runs was automatically adjusted to ensure a 1% false discovery rate throughout the experiments. Peptides isoelectric points (pI) were calculated  $^{18}$ . In each fraction, peptides with calculated pI values beyond the  $\pm 0.5$  window around the average pI were discarded.

# **Results and Discussion**

# Challenges of shotgun urine proteomics

Although urine is abundant and easy to collect, it is a challenging sample for proteomics studies<sup>19, 20</sup>. The interfering salts and other contaminants can reduce the efficiency of digestion and effectiveness of multi-dimensional separation strategies that are critical for deep profiling using shotgun proteomics<sup>21</sup>. Obtaining pure protein samples from urine, without large sample loss, can be difficult and a major impediment to a successful gel-free approach.

Numerous protocols have been employed to concentrate and purify urinary proteins; e.g., lyophilization<sup>2, 8, 22</sup>, precipitation<sup>3, 23</sup>, ultracentrifugation<sup>3, 4</sup> and centrifugal filtration<sup>2, 7, 16, 24</sup>. The majority of these methods were developed for gel-based analysis, rather than for in-solution digestion and shotgun proteomics, which typically require more pure samples. Some of these methods, such as ethanol precipitation, are biased towards hydrophilic proteins and do not remove salts from the precipitated sample and thus alone prohibit proper digestion and separation in the downstream steps. Other methods, such as reverse-phase trapping columns, process the samples in sequence and therefore are prone to carry-over contamination<sup>2</sup>.

We initially anticipated that a reverse-phase trapping column (C4 macrotrap, Michrom Bioresources) purification would permit separation using IEF. The C4 macrotrap is a large pore polymeric reversed-phase trapping column designed to desalt and concentrate protein mixtures. At low urine volumes (e.g. 1 and 5 ml), the C4 macrotrap purified proteins (peptides after in-solution digestion) were separated by IEF, but at high-currents and overheating of the IPG strip (Data not shown). These findings are typically secondary to interfering compounds. At larger starting volumes (e.g. 20 ml) the C4 macrotrap purified urine proteins/peptides could not be separated by IEF. This implies that C4 purification did not clear enough of the interfering compounds from urine, and further highlights the challenges of urine.

As in serum and other discovery experiments, low-abundance urinary proteins are challenging to identify and quantify without appropriate depletion of abundant proteins. The ability to specifically and reproducibly remove high-abundant proteins may improve the identification of low abundant proteins<sup>25</sup>. Human Serum Albumin (HSA) is particularly troubling for urinary proteome analysis, especially in patients with proteinuria<sup>26</sup>. A variety of methods have been developed to remove albumin and other high abundance proteins from samples prior to analysis<sup>27, 28</sup>. However, most of these methods have been originally designed for blood-derived body fluids and add more steps to the workflow. Similarly, urine proteomics workflows typically require multiple steps to concentrate, purify, deplete and separate proteins or peptides. Our *One-Step* method permits simultaneous enrichment of urinary proteins, removal of salts and other contaminants, and depletion of albumin. Additionally, it is fast, reproducible, efficient, and allows parallel analysis of multiple samples with no risk of carryover.

#### One-Step (simultaneous concentration, purification and albumin depletion)

The overall *One-Step* integrated workflow is presented in Figure 1. Initially, we employed the Vivapure Anti-HSA columns due to their high recovery, effective albumin depletion and ease in processing multiple samples in parallel. However, Vivapure columns are intended for serum and have a small volume capacity of 500 µl. Because the protein concentration in urine is dramatically lower than serum and it varies significantly based on renal function and disease, spin columns that can accommodate a range of volumes are needed to recover the desired amount of protein.

To accommodate larger volumes we used a Vivaspin column because of its variable loading capacity (500  $\mu$ l to 20 ml). These filters have a low-binding poly-ether-sulfone vertical membrane, which affords high recovery and minimal membrane blockage. Another advantage is that they have an integrated dead-stop-volume of 30  $\mu$ l, eliminating the risk of concentrating to dryness. Importantly, multiple samples can be processed at the same time with no risk of cross contamination or sample carryover. To integrate albumin depletion, we utilized the same Anti-HSA resin used in Vivapure columns. The Anti-HSA resin has a high specificity and recovery and can be added directly to the sample.

# Protein recovery and albumin depletion

As shown in Figure 2A, efficient depletion and good recovery was obtained with the *One-Step* method. Sample 1 was used for this initial experiment. Both the neat urine sample and the neat sample with 20  $\mu$ g spiked-in HSA were efficiently depleted. The concentration was reduced from 50.1  $\mu$ g/ml of the neat urine to 38.6  $\mu$ g/ml after the *One-Step* process. The sample with spiked-in HSA (72.5  $\mu$ g/ml) also had excellent depletion (42.3  $\mu$ g/ml). Measuring the concentration of three technical repeats of the same samples showed a variability of only 5% indicating good reproducibility.

Since albumin is a common carrier of low-molecular weight proteins, we investigated whether depletion of albumin resulted in a co-depletion phenomena  $^{29}$ . We examined the effect of reducing and alkylating our samples to disrupt the bonds between albumin and other proteins prior to albumin depletion and purification to determine if recovery would improve. Using our combined method, we measured the protein concentration of sample 1 with reduction & alkylation to shift from 50.1  $\mu g/ml$  (before the depletion), to 38.6  $\mu g/ml$  (after the depletion); and to 35.0  $\mu g/ml$  without reduction & alkylation (Figure 2B). Although this does not definitively demonstrate the co-depletion phenomena, upfront reduction and alkylation does not diminish the effectiveness of the method. Additionally, the most beneficial advantage of performing reduction and alkylation prior to purification is that it allows removal of the excess of reduction/alkylation reagents along with other impurities. This modification reduces the overall number of sample handling steps and risk of sample loss.

#### Effect of urine pH

Since the pH of urine is highly variable as a function of normal physiology and diet<sup>30</sup>, we investigated the effect of variable pH on depletion and recovery. As shown in Figure 2C, in the acidic pH range (3–5), high depletion efficiency is achieved but the recovery is poor. In the basic pH range (9–11) zone, high recovery is achieved, with suboptimal depletion. Neutral pH (7–8) condition was the ideal condition to achieve both efficient depletion and high recovery. Consequently, we recommend that all urine samples be balanced to a pH of 7 prior to the initiation of the workflow.

# Reproducibility

In order to evaluate the applicability of the *One-Step* method to urine samples of varying composition, six patients (samples 2–7) with varying renal and urologic diseases were chosen from our urine sample repository. As shown in Figure 3, despite differences in starting protein concentration, proteome composition, and concentration of albumin, all samples were effectively depleted and a good recovery was obtained. Albumin depletion appeared to significantly improve the intensity of other proteins within the sample.

# Applying the One-Step urine sample preparation to Off-Gel electrophoresis

As a method of separation, Off-Gel has been demonstrated to be a high resolution and reproducible alternative to the traditional multi-dimensional LC separations employed in shotgun workflows<sup>12, 31, 32</sup>. It has the advantage of true orthogonality and providing complimentary *pI* information. However, because urine has a high amount of interfering salts and metabolites, it has been difficult to implement IEF technology towards the investigation of the urinary proteome. We investigated whether the *One-Step* workflow would provide a desalted sample that is compatible with Off-Gel separation regardless of the starting volume.

Since lower volumes of urine (1–5 ml) worked with Off-Gel, we first used 30 ml of sample 1 to evaluate the application of the *One-Step* method to Off-Gel electrophoresis. Urine protein concentration is often low and large volumes may be required for shotgun proteomic discovery studies. 30 ml provides an appropriate amount of protein for optimal IEF separation (400ug) and should adequately test the efficacy of the *One-Step* method. The run was completed in less than 12 hours with no current problems during the focusing. Peptides were separated into 24 fractions. In total, we identified 703 proteins corresponding to 2698 peptides using a 1% false discovery rate. 16% of these proteins were identified with a single peptide. The number of proteins and peptides identified is presented in Table 1 and their complete list is provided in Supplementary Data 1.

As shown in Figure 4A, the average standard deviation of the peptides pI values from the average pI per fraction indicates good focusing quality for the majority of the fractions. Only a few basic fractions demonstrated higher deviations. This can be partly explained by the well-known low focusing quality in the basic region of the IPG strips<sup>33</sup> and partly attributed to the pI prediction algorithm used, which is specifically optimized for peptides in the acidic region<sup>18</sup>.

The resolution obtained by analytical IEF is amongst the highest from present biochemical separation techniques<sup>33</sup>. Figure 4B shows the number of fractions each peptide was identified as a measure for resolution. 79% of the peptides were uniquely identified in one fraction followed by 9% in 2, 5% in 3 and 3% in 4 fractions. These results demonstrated good resolution of the Off-Gel separation, and imply that the One-Step methodology is providing a purified sample. The heterogeneous distribution of the identified peptides per fraction is demonstrated in Figure 4C. In some fractions more than 900 peptides have been identified, in others less than 50 peptides were detected. Unfortunately, the current fixed well-sizes of the Off-Gel system does not permit improvement in the distribution of collected fractions by adapting fraction size<sup>34</sup>. Nevertheless, the use of Off-Gel is advantageous due to the extra information provided by pI. The expected pI of a peptide can be used to support or refute its identification as determined by mass spectrometry, as well as detect possible post-translational modifications<sup>35</sup>.

# Reproducibility

We applied the One-Step / Off-Gel workflow to 4 different biological samples with varying renal and urologic disease (Samples 2, 3, 5 and 7). Four IPG strips (24 cm, 3–10) were run in parallel and 24 samples were collected from each strip (96 total). Figure 5 demonstrates that the degree of separation and depth of interrogation obtained with our initial test sample (sample 1) were reproduced in these 4 diseased samples. All 4 samples showed a similar pattern of their average pI per fraction along the IPG strips. High resolution separation is shown in Figure 5B with 76%, 80%, 82% and 78% of peptides in samples 2, 3, 5 and 7 uniquely identified in a single fraction. Finally, the distribution of the peptides per fraction showed minor differences among the samples, as expected due to the high variation of the urinary proteome among different individuals. To verify the reproducibility of the workflow, a technical replicate of sample 2 was run from the first stage of the One-Step method. Four random fractions were chosen and the identified proteins were compared to the first experiment. As expected, new proteins were identified in each run<sup>36</sup>. 76%, 83%, 85% and 73% reproducibility was obtained for the identified proteins in fractions 4, 9, 12 and 22, respectively, confirming good reproducibility. Overall, using the One-Step method we were able to process a range of urine volumes from 1 ml to 30 ml and compositions without observing any problems with digestion efficiency, OFF-gel focusing, or LC-MS/MS.

# Applying the One-Step urine sample preparation to High pH reversed phase separation

The primary goal for the development of the *One-Step* method was to produce and develop a fast, reproducible and reliable method for urine sample preparation to be compatible with Off-Gel electrophoresis. To determine the compatibility of the *One-Step* method with other 1<sup>st</sup> dimension separation methods, we tested prepared samples using High pH reversed phase separation<sup>37</sup>. Without performing optimization of pH gradients and the High pH workflow, we identified 499 unique proteins corresponding to 1729 unique peptides. 74 of these proteins were detected with a single peptide. The complete list of the identified proteins and peptides is provided in Supplementary Data 2. This preliminary data demonstrates that the *One-Step* method can produce a sample that is applicable to various 1<sup>st</sup> dimension separation techniques.

# **Conclusion and Perspective**

To identify clinically useful urinary biomarkers, it is essential to have a fast and reliable urinary proteomics workflow. Many methods of protein enrichment, purification and depletion are effective but not necessarily compatible with the downstream steps in a multi-dimensional shotgun proteomics workflow. We introduce a *One-Step* method that simultaneously concentrates urinary proteins, removes salts and other contaminates, and depletes albumin. The application of our workflow to the analysis of complex pathological urine samples showed the efficiency of this method in handling different urine samples independent of the volume and the amount of proteins and salts. Recently, in our laboratory, we have successfully applied chemical labeling methods (iTRAQ and TMT) to the samples prepared by the *One-Step* method (manuscript in preparation). Chemical labeling has allowed for multiplexing using different 1<sup>st</sup> dimension separation methods. In the near future we plan to expand this workflow to allow the quantitative analysis of post-translational modifications in the urinary proteome, and other biologic fluids of similar complexity.

# **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

#### **Abbreviations**

MS Mass Spectrometry

MS/MS Tandem Mass Spectrometry
LC Liquid Chromatography

**2DE** Two-Dimensional Gel Electrophoresis

CE Capillary Electrophoresis

FFE Free Flow Electrophoresis

**HILIC** Hydrophilic Interaction Liquid Chromatography

**RPLC** Reversed-Phase LC

SCX Strong Cation Exchange

IPG Immobilized pH Gradients

**IEF-AF4** Isoelectric Focusing and Asymmetrical Flow field-flow fractionation

**SDS-PAGE** Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis

**kV-h** kiloVolt-hour

**TEAB** triethyl ammonium bicarbonate

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# Figure 1. One-Step method workflow

The final workflow for preparation and analysis of urine samples. Urine is first spun down to remove debris and the pH is adjusted to neutral. Urine proteins are reduced and alkylated. This sample is then added to the Vivaspin 6 spin-filters. Anti-HSA resin is added. Samples are incubated and then spun down completing the protein concentration, purification and albumin depletion. Proteins are removed leaving the Anti-HSA resin behind. Proteins undergo in-solution digestion with trypsin. Samples could then be chemically labeled for quantitation and multiplexed\*, prior to undergoing 1st dimension separation (Off-Gel electrophoresis is currently utilized\*\*) and LC-MS/MS.

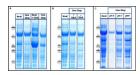


Figure 2. Efficiency, Recovery and pH sensitivity of the One-Step Method

**A.** The recovery and depletion efficiency of the *One-Step* enrichment, purification and depletion method applied to neat urine sample from a healthy individual with and without 20 μg spiked-in HSA. **B.** The effect of Reduction and Alkylation (R&A) on the depletion efficiency and recovery of the *One-Step* process. **C.** The effect of pH on the *One-Step* method recovery and depletion.

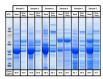


Figure 3. Reproducibility and efficiency of the One-Step method

The reproducibility and efficiency of the *One-Step* enrichment, purification and albumin depletion method is tested on urine samples 2 to 7. Protein concentrations before and after the *One-Step* method are presented.

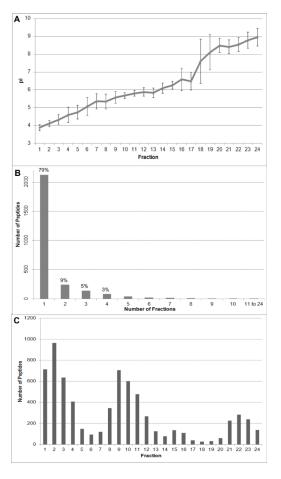
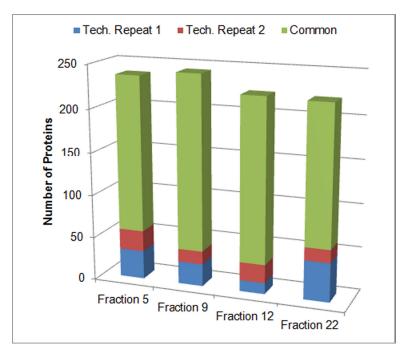


Figure 4. One-Step method and Off-Gel Electrophoresis

The application of the *One-Step* sample preparation method to the Off-Gel technology. **A.** Demonstrates the average *pI* of the peptides identified in each fraction with their average standard deviations. **B.** Depicts the number of fractions each peptide has been identified in as a surrogate for the resolution. **C.** Shows the distribution of the peptides along the IPG strip for each sample.



**Figure 5.** Application of the *One-Step* method and Off-Gel Electrophoresis to different samples The comparison of four urine sample (samples 2, 3, 5 and 7) prepared with the *One-Step* sample preparation method and analyzed using the Off-Gel technology. **A.** Demonstrates the average *pI* of the peptides identified in each fraction. **B.** Depicts the number of fractions each peptide has been identified in as a surrogate for the resolution. **C.** Shows the distribution of the peptides along the IPG strip for each sample.



**Figure 6. Reproducibility of the** *One-Step method and* **Off-Gel Electrophoresis** Technical reproducibility of the *One-Step* urine sample preparation method in combination with Off-Gel separation and data-dependent LC-MS/MS. Blue bars represent the number of proteins identified only in technical replicate 1 and red bars indicate the number of proteins identified exclusively in technical replicate 2. Green bars show the number of proteins identified in both technical replicates.

# Table 1

Number of unique proteins and peptides identified in each sample using the One-Step urine sample preparation method in combination with Off-Gel electrophoresis. The number of proteins identified with a single peptide is presented.

Sample	Number of proteins	Number of peptides	Number of single hits
1	703	2698	109
2	650	2093	155
3	666	2203	125
5	628	1988	145
7	695	2386	176