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Simple Tip-Based Sample Processing Method for Urinary Proteomic Analysis

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Abstract

Mass spectrometry-based urinary proteomics is one of the most attractive strategies to discover proteins for diagnosis, prognosis, monitoring, or prediction of therapeutic responses of urological diseases involving the kidney, prostate, and bladder; however, interfering compounds found in urine necessitate sample preparation strategies that are currently not suitable for urinary proteomics in the clinical setting. Herein, we describe the C4-tip method, comprising a simple, automated strategy utilizing a reverse-phase resin tip-based format and “on-tip” digestion to examine the urine proteome. We first determined the optimal conditions for protein isolation and protease digestion on the C4-tip using the standard protein bovine fetuin. Next, we applied the C4-tip method to urinary proteomics, identifying a total of 813 protein groups using LC–MS/MS, with identified proteins from the C4-tip method displaying a similar distribution of gene ontology (GO) cellular component assignments compared to identified proteins from an ultrafiltration preparation method. Finally, we assessed the reproducibility of the C4-tip method, revealing a high Spearman correlation *R*-value for shared proteins identified across all tips. Together, we have shown the C4-

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ASSOCIATED CONTENT

Supporting Information

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Extended methods section, chemicals and materials, information related to in-solution digestion, nano-ESI-LC-MS/MS analysis, data analysis for protein identification and quantification, bar graphs of C4-tip binding time and capacity, overlap of protein identifications between disparate digestion methods of urinary proteomics, PANTHER GO assignments of protein identifications between disparate digestion methods of urinary proteomics, reproducibility of C4-tip digestion method, direct analysis of urine proteome using C4-tip TMT labeling schematic, and bar graph of measured urine peptide recovery (PDF)

Measured peptide recovery and peptide missed cleavages rates in various C4-tip digestion buffers, top 20 abundant proteins from disparate digestion methods, normal urine sample information utilized for direct analysis of the C4-tip methodology, reported Pearson correlation values for direct analysis of normal urine samples, C4 Tips MaxQuant search results, C4 Tip BE MaxQuant search results, in-solution BE digestion MaxQuant search results, 30% ACN in-solution BE digestion MaxQuant search results, PANTHER gene ontology assignment results for disparate digestion methods, direct analysis of normal urine Proteome Discovered 2.2 search results (including identified proteins, reported TMT ratios), TMT plex #1, direct analysis of normal urine Proteome Discovered 2.2 search results (including identified proteins, reported TMT ratios), and TMT plex #2 (XLSX)

The authors declare no competing financial interest.

tip method to be a simple, robust method for high-throughput analysis of the urinary proteome by mass spectrometry in the clinical setting.

Graphical Abstract

Urine is considered an attractive sample source for diagnosis, prognosis, monitoring, or prediction of responses to treatments of urological diseases due to its proximity, availability, and noninvasive, simple collection procedure. In addition, the biological function of the kidneys to filter circulating plasma allows for the abundance of proteins secreted into urine to reflect the systemic physiological status of the patient, such as in diabetes and heart diseases.¹ Dissimilar to plasma, which displays a dynamic range of protein concentration up to 10-orders of magnitude and is dominated by several highly abundant proteins (i.e., albumin, immunoglobulins, and fibrinogen), the urine proteome is viewed with a less dynamic range and is comprised of a smaller percentage of plasma-derived proteins, making it an attractive human biological fluid for protein sourcing.^{2,3}

In addition to proteins, the composition of urine includes urea, inorganic salts, and other biomolecules and compounds that can confound proteomic analyses. To address this, a variety of urine sample preparation methods have been explored, such as protein precipitation, ultrafiltration, and analytical ultracentrifugation.⁴ However, due to inherent drawbacks of each of the methods, with few exceptions,⁵ these techniques have not been adapted for high-throughput urinary protein sample preparation, which is necessary for clinical analysis.

For mass spectrometry-based proteomic analysis, sample preparation includes protein extraction from the sample source and removal of contaminants, followed by protease digestion to generate peptide fragments. For high-throughput sample analysis, these steps must be optimized and then integrated to generate a method that is robust and reproducible. Previously, reverse-phase chromatography has been utilized to isolate small sets of urinary proteins,^{6–8} but the reverse-phase matrix has not been fully explored in the context of urinary proteomics, including protein isolation and protease digestion, followed by mass spectrometry analysis. Although reversephase C-18 resin has routinely been used in the proteomics field for peptide binding, resin material with shorter alkyl chains (e.g., C4) shows better recovery of proteins relative to resin materials with longer alkyl chains (e.g., C8 and C18).⁹ In this study, we evaluated the utility of an automated tip-based format (C4-tip)

for urinary protein isolation using C4 reversephase resin paired with “on-tip” protease digestion and mass spectrometry to examine the urine proteome.

EXPERIMENTAL SECTION

C4-Tip Fabrication.

A polyethylene sheet (2 mm diameter) was inserted into a Thermo Scientific matrix D.A.R.T.s tip with a volume capacity up to 300 μL . For C4-tips, 30 mg of C4 reverse-phase resin in 300 μL of methanol was loaded into the tip, with a second polyethylene sheet (5 mm diameter) inserted to seal the resin material. The packed C4-tips were then ready for use in the Versette Liquid Handler (Thermo Scientific).

Isolation and Protease Digestion of Urinary Proteins Using C4-Tip.

Each liquid aspiration/dispense cycle was performed using 200 μL of buffer in approximately 2 min at room temperature, unless otherwise noted. C4-Tips were conditioned with 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile (ACN), followed by 0.1% TFA (10 cycles each). Solubilized proteins were acidified with formic acid ($\text{pH} < 3$) and coupled to C4-tips (15, 30, and 60 cycles for Figure S1A and 60 cycles for rest of other experiments) in a total volume of 300 μL . C4-Tips were rinsed with 0.1% TFA and then 100 mM triethylammonium bicarbonate (TEAB) to remove unbound and contaminant material (10 cycles each). Bound proteins were reduced with 10 mM Tris 2-carboxyethyl phosphine (TCEP) and alkylated with 15 mM iodoacetamide (20 cycles each). Protease digestion was performed (1:40 enzyme/protein) in 50 mM TEAB and 30% ACN with 120 cycles of aspirating/dispensing to recover digested peptides directly from C4-tips to solution. C4-Tips were subsequently rinsed with 50% ACN and 0.1% TFA to recover the remaining peptides. Peptides directly released from C4-tips by protease digestion and peptides eluted by 50% ACN and 0.1% TFA were pooled, dried, reconstituted in 0.1% formic acid (FA), and subjected to C-18 cleanup via the StageTip method.¹⁰ Desalted peptides were labeled with 10-plex Tandem-Mas-Tag (TMT) reagents following the manufacturer's instructions. Peptides were resuspended in 50 mM HEPES, pH 8.5, and labeled with TMT reagent resuspended in anhydrous ACN for 1 h at RT. The reaction was quenched using 5% hydroxylamine at RT for 15 min, and differentially labeled samples were combined. The pooled peptides were then subjected to cleanup using C-18 SepPak columns and dried down.

RESULTS AND DISCUSSION

Workflow of C4-Tips.

To develop a sample preparation methodology that would be high-throughput for urinary protein extraction and digestion for MS-based proteomics, we devised a reverse-phase tip-based format using C4 resin. Previous reports have indicated the utility of “in-tip/on-tip” digestion methodologies,^{11–13} but few have been adapted for urinary proteomic analysis due to the challenges inherent to urine's unique composition. As illustrated in Figure 1, urine samples were acidified, and proteins were bound to the C4-tips using repeated aspiration/dispensing steps. After being bound, proteins are washed with 0.1% TFA to remove contaminants (desalting), followed by 100 mM TEAB to adjust the pH in each of the tips.

Proteins are then subjected to washes with TCEP and iodoacetamide (IAA), followed by an additional 50 mM TEAB rinse step (reduction and alkylation). Proteins were then tryptically digested, releasing peptides directly from the C4-tip, followed by elution of the remaining peptides with 50% ACN and 0.1% TFA. Two released peptide fractions were then pooled and then subjected to C-18 cleanup prior to nano-ESI-LC-MS/MS.

Assessment of Binding Time for Protein on C4-Tips.

To determine the amount of time necessary for sufficient binding of proteins from solution to C4-tips, we utilized the standard protein fetuin and measured unbound protein material via BCA assay. As seen in Figure S1A, 300 μg of fetuin in 250 μL of 0.1% TFA was aspirated/dispensed through the C4-tips and the remaining protein in the solution was measured at 30 min (15 cycles), 1 h (30 cycles), and at 2 h (60 cycles). We observed approximately 80% of the protein binding after 30 min, with 90% bound after 2 h. These results mirror a previous study, showing rapid adsorption of proteins onto C4 material after 30 min, and adsorption plateauing afterward when evaluated over 24 h,¹⁴ suggesting that 2 h was sufficient for protein binding. We next evaluated the maximum capacity of the C4-tip by binding various amounts of fetuin (100–600 μg) onto a C4-tip for 2 h, and measuring the amount of unbound material (Figure S1B). Our results indicated that 30 mg of the C4 resin could bind almost 400 μg of protein material. The protein concentration of normal urine is estimated to be between 0 and 14 mg/dL (0–140 $\mu\text{g/mL}$),¹⁵ and the binding capacity of the C4-tip would be sufficient to allow for direct analysis of urine; even in cases of increased protein urine concentrations (proteinuria).¹⁶ Next, we examined the impact of serial binding of protein samples on the C4-tips. We bound 100, 150, or 300 μg of fetuin in three, two, and one periods of sample binding (each period consisted of a total of 2 h (60 cycles)), respectively, observing almost 90% of material bound (Figure S1C). Finally, we assessed the amount of material eluted from the C4-tips. After washing the C4-tips with 0.1% TFA, proteins were eluted with 50% ACN and 0.1% TFA. The BCA assay was used to measure the recovery of the fetuin, indicating 50% of protein was released from the C4-tips (data not shown). This latter observation suggested that protein material remained bound on the C4 resin after elution and prompted us to consider an alternative method that would yield equivalent or better sample recovery, and also further simplify our sample preparation method.

“On-Tip” Protein Digestion.

We next wanted to explore utilizing an “on-tip” automated digestion format via the C4-tips for processing protein samples. Protein sample preparation for tryptic digestion generally involves a reduction step, to break the disulfide bonds of proteins, followed by alkylation, to prevent the reforming of the disulfide bonds, with the inclusion of these steps improving protease cleavage and sequence coverage.¹⁷ Following fetuin protein binding to the C4-tips (2 h), reduction and alkylation were carried out (40 min each) prior to protease digestion. To determine the optimal buffer for “on-tip” protein digestion and sample recovery, we examined a total of five buffer compositions. Following protein digestion (6 h total), recovered peptides were then analyzed via mass spectrometry. The entire automated C4-tip procedure (Figure 1) could be performed in a total of 9 h. We observed that the buffer composition influenced the recovery of peptides, as well as the efficiency of protease

cleavage of fetuin (Table S1). Inclusion of 30% ACN in our digestion buffer not only resulted in a higher recovery of peptides more consistently but also greatly improved the efficiency of trypsin enzyme activity, as indicated by the reduced missed cleavage rate (Table S1). It has been previously shown that the presence of ACN can serve to denature a protein,¹⁸ and protein digest of individual proteins has been carried out in high organic buffers with improved peptide recovery.¹⁹ We found higher organic composition (50% ACN) to be more varied in peptide recovery and digestion efficiency, possibly due to the buffer composition changing as the organic solvent evaporated during the digestion step. With these results, we decided to apply our C4-tip methodology with 50 mM TEAB buffer with 30% ACN to examine the proteome of normal urine.

Comparison of C4-Tip Digestion.

We then compared the C4-tip method to other methods of sample preparation of urine for proteomic analysis. Two 25 mL aliquots of normal urine were processed using a concentrator unit with a 10 kDa mass cutoff membrane. The volumes for both were reduced 40-fold. From one aliquot, approximately 300 μg (1 $\mu\text{g}/\mu\text{L}$) of protein from the concentrated urine sample was acidified and bound to the C4-tip. The second aliquot was buffer exchanged (BE) into 50 mM Tris-HCl, pH 7.5, which allowed for a protein concentration measurement that could then be extrapolated to the nonbuffer exchanged sample (concentrated sample). SDS-PAGE analysis indicated a similar protein profile in each urine preparation (Figure S2A). As previously noted, there are a variety of preparation methods for evaluating urine protein composition. Ultrafiltration is a useful technique, as it facilitates sample volume concentration, while simultaneously removing salts and other molecules,⁴ in addition to allowing the exchange of the urine sample into a digestion compatible buffer. To determine whether the C4-tip method was a comparable method of urine sample preparation, we examined the performance between ultrafiltration and our described technique, utilizing the BE urine aliquot and concentrated urine aliquot, respectively. In parallel, 300 μg (1 $\mu\text{g}/\mu\text{L}$) of BE urine was bound to a C4-tip, in addition to a 200 μg aliquot of BE urine subjected to in-solution digest in two conditions, 8 M urea and 30% ACN. In assessing these four digestions conditions, we could evaluate the performance of the C4-tip compared to the well-established method of ultrafiltration, followed by in-solution digestion, as well as determining any bias that may be introduced by the C4-tip format, including the C4 matrix as well as the digestion buffer composition. Upon analyzing ~ 1 μg of peptide material via nano-ESI-LC-MS/MS using the C4-tip format, we identified 813 proteins groups from the concentrated urine sample and 725 from the BE urine sample, respectively; 787 protein groups from the in-solution digest of the BE urine sample and 807 protein groups from the 30% ACN in-solution digest of the BE urine sample. When comparing the gene annotations of the proteins identified between the concentrated urine sample digested via the C4-tip and BE urine sample digested in-solution, we observed 547 proteins commonly identified between the two methods (Figure S2B). Overall, we observed an overlap range of 54–62%, depending on the total number of proteins identified in the respective four conditions, with 108, 27, 108, and 59 uniquely identified proteins in the C4-tip, C4-tip BE, in-solution BE, and 30% ACN BE digests, respectively. Utilizing PANTHER cellular component gene ontology (GO) assignments,²⁰ the identified proteins from the individual sample digestions displayed a similar distribution of classified proteins regardless of the sample preparation

methodology (Figure S3). Previously, it has been shown, that the method of urinary protein extraction can impact both yield and proteins recovered;²¹ however, the overlap of identified proteins and the result of our GO analysis indicate an equivalent representation of the urine proteome regardless of the digestion method employed.

In order to assess the distribution of individual protein abundance in each of the four digestion conditions, we utilized the intensity-based absolute quantitation (iBAQ) algorithm that is part of MaxQuant software suite.^{22–24} We found a similar dynamic range of protein abundance between all three sample preparations, spanning ~5-orders of magnitude (Figure 2), which is similar to another report utilizing a filter plate-based method.⁵ Highly abundant proteins observed across all four preparations, many of which are glycoproteins, included AMBP, ALB, CD59, UMOD, PTGDS, and immunoglobulins IGKC and IGHA1 and are in agreement with other studies relating overall urine protein abundance (Table S2).²⁵ In our iBAQ results, we did not observe albumin as the most abundant protein in the urine proteome in any of the four preparations, as reported elsewhere.^{25,26} Although we would have to consider the systemic impact of variant digestion methods employed (e.g., C4 resin hydrophobicity, membrane absorption, and protein solubility), the reported iBAQ values for the top urine proteins and albumin were proportional between the C4-tip method and buffer-exchanged in-solution digestion method, and without a calibration curve of protein/peptide standards to ascertain accurate protein abundance, the use of iBAQ to infer proportional abundance could inaccurately report specific protein abundance within a sample.²⁷ Other studies have utilized a lectin-affinity approach to circumvent the challenge of serum protein abundance (e.g., albumin) and identify glycoproteins of interest in the urine proteome;²⁸ however, our method enables a global protein profiling of urinary samples and would be applicable for monitoring albuminuria resulting from chronic kidney diseases.²⁹ Inherent to our described method is the potential loss of endogenous urine peptides due to the use of C4 for protein-level binding, which is not optimal for peptide-level binding. Although this was not explored in our current investigation due to the unique challenges of peptidome data acquisition and bioinformatic analysis,³⁰ subsequent studies can evaluate the utility of the C4-tip method for protein-depletion prior to direct peptidome profiling of urine.

Next, we employed label-free quantitation (LFQ) using MaxQuant, to quantify the relative abundance of proteins across all four sample preparation conditions. MaxQuant LFQ intensity values were reported for 340 proteins across all methods, with reported Spearman correlation *R*-values being the highest between the two C4-tip digestion conditions (*R* = 0.932) (Figure S2C). The correlation values between the C4-tip and in-solution and 30% ACN digestions was 0.778 and 0.802, respectively, which is comparable to a previous report exploring protein-level correlation of digestion replicates utilizing either an in-solution or FASP-based sample preparation strategy.^{31,32} Overall, these results indicate that the performance of the C4-tip is comparable to the previously established method of ultrafiltration followed by in-solution digestion for examining the urinary proteome.

Reproducibility of C4-Tips.

To assess the reproducibility of the C4-tip format, three C4-tip digests were performed using normal, concentrated urine. In total, 948 protein groups were identified across all three C4-

tip digestions, with 814, 790, and 801 protein groups identified from tip 1, tip 2, and tip 3 digestions, respectively. (Figure S4A). In total, almost 70% of the proteins were commonly identified in all three tips, with differences in identified proteins most likely related to the biases of data-dependent MS2 analyses.^{33,34} In this data set, we observed an average of 70% of peptides having no missed cleavages, which is comparable to other studies reporting missed cleavage rates ranging from 30 to 40% using in-solution digestion.^{31,32} Using the LFQ intensity values reported for the 614 protein groups identified across all three tips, we assessed the reproducibility of the C4-tip method. The Spearman correlation *R*-values ranged from 0.975 to 0.978 among the C4-tip replicates, revealing excellent reproducibility across all three C4-tips (Figure S4B,C). In addition, we observed a similar distribution of the LFQ log intensity between all three tips, when plotted against total protein number (Figure S4D). Taken together, these results indicate the C4-tip method displays a high level of tip-to-tip repeatability for the analysis of the urinary proteome.

Application of C4-Tip to Urinary Proteomics.

Next we applied the C4-tip method to directly analyze urine specimens from healthy donors (Table S3). Three 300 μ L volumes of urine were removed from each sample and subjected to the C4-tip methodology. Following protein digestion, we determined the starting protein concentration of each urine sample and the recovered amount of peptide material for each of the C4-tips ($n = 3$ for each sample) using a Bradford assay. We observed variable protein concentrations for each of the urine samples and also measured variable digestion efficiencies of certain samples (Figure S5A). Several factors that may have contributed to this observed variability of sample recovery could include the starting protein concentration, which would have impacted the total amount of protein digested, or the amount of insoluble material present in the individual urine sample. In addition, the manual construction of the C4-tips could be variable in itself and influence the recovery of peptide material; however, this could be addressed by incorporating appropriate QC metrics. Regardless, for all of the samples, we found that we had sufficient peptide material ($>1 \mu$ g) for TMT-labeling and mass spectrometry analysis and devised a TMT-labeling strategy that would enable us to determine sample preparation reproducibility, in addition to determining sample labeling accuracy (Figure S5B). Two TMT sets were generated and subjected to mass spectrometry analysis, wherein we identified 1150 and 1032 protein groups, respectively, which is comparable to the average number of urinary proteins identified without fractionation.³⁵ Utilizing the reported TMT intensities for each of the proteins, we were able to calculate the correlation of protein abundance in each of the sample preparations, observing values greater than 0.91 for all technical replicates (Figure 3; Table S4), indicating a high level of reproducibility irrespective of the variability of measured urine protein concentration and amount of recovered peptide material. This is a promising result in the context of urinary proteomics, as the protein concentration and composition of urine is extremely variable, and our results suggest that the C4-tip methodology sufficiently binds enough urinary protein material even from samples with extremely low protein concentrations (e.g., 0.017 μ g/mL) for downstream proteomic analysis. Alternatively, for urine samples with extremely low protein concentration, serial loading (Figure S1C) could be employed to maximize sample binding and recovery. Overall, the results mirror our previous analysis using LFQ to assess

tip-to-tip repeatability and show that the automated C4-tip methodology can facilitate direct analysis of urine proteome without significant preprocessing steps.

CONCLUSION

With its noninvasive and simple collection procedures, urine is considered an ideal biological fluid for disease monitoring and diagnosis. However, the composition of urine requires extensive sample preparation strategies prior to proteomic analyses, many of which are difficult to adapt for high-throughput analysis in the clinical setting. Reverse-phase chromatography has been utilized for analyzing small sets of urinary proteins, but we sought to adapt this technique into an automated, tip-based format. First, we evaluated our technique using a standard protein to determine optimized protein binding and digestion conditions. Next, we compared the C4-tip format to the established technique of ultrafiltration followed by in-solution digestion for examining the urinary proteome, revealing that the C4-tip is comparable to the previously used technique. We then examined the reproducibility of the C4-tip format for analyzing the urine proteome, revealing a high level of repeatability between individual C4-tip experiments. Finally, we applied the C4-tip method to enrich and digest urinary proteins from unprocessed urine, indicating that the C4-tip method is a robust methodology for urinary proteomics. Overall, we have shown that the C4-tip format is a simple, reproducible technique for proteomic sample processing, and it can have applications in the clinical setting for investigating the urinary proteome, as well as being expanded to include other biological samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- (1). Beasley-Green A Int. Neurourol. J. 2016, 20, S70–S75. [PubMed: 27915473]
- (2). Hu S; Loo JA; Wong DT Proteomics 2006, 6 (23), 6326–6353. [PubMed: 17083142]
- (3). Thomas S; Hao L; Ricke WA; Li L Proteomics: Clin. Appl. 2016, 10 (4), 358–370. [PubMed: 26703953]
- (4). Olszowy P; Buszewski BJ Sep. Sci 2014, 37 (20), 2920–2928.
- (5). Yu Y; Suh MJ; Sikorski P; Kwon K; Nelson KE; Pieper R Anal. Chem. 2014, 86 (11), 5470–5477. [PubMed: 24797144]
- (6). Lehman-McKeeman LD; Caudill DJ Pharmacol. Methods 1991, 26, 239–247.
- (7). Arai H; Tomizawa S; Maruyama K; Seki Y; Kuroume T Nephron 2004, 66 (3), 278–284.
- (8). Turpeinen U; Koivunen E; Stenman UH Clin. Chem. 1987, 33 (10), 1756–1760. [PubMed: 3311463]
- (9). Astefanei A; Dapic I; Camenzuli M Chromatographia 2017, 80 (5), 665–687. [PubMed: 28529348]
- (10). Rappsilber J; Mann M; Ishihama Y Nat. Protoc. 2007, 2 (8), 1896–1906. [PubMed: 17703201]

- (11). Kulak N.a; Pichler G; Paron I; Nagaraj N; Mann M Nat. Methods 2014, 11 (3), 319–324. [PubMed: 24487582]
- (12). Hahn HW; Rainer M; Ringer T; Huck CW; Bonn GK J. Proteome Res. 2009, 8 (9), 4225–4230. [PubMed: 19639939]
- (13). Nguyen MTN; Verhelst SHL; Solari FA; Venne AS; Sickmann A; Shema G; Zahedi RP; Loroch S; Kollipara L Mol. Cell. Proteomics 2018, 17 (4), 826–834. [PubMed: 29358340]
- (14). Aguilar MI; Clayton DJ; Holt P; Kronina V; Boysen RI; Purcell AW; Hearn MT W. Anal. Chem. 1998, 70 (23), 5010–5018.
- (15). Tietz N Fundamentals of Clinical Chemistry, 3rd ed; WB Saunders Co: Philadelphia, PA, 1987.
- (16). Julian BA; Suzuki H; Suzuki Y; Tomino Y; Spasovski G; Novak J Proteomics: Clin. Appl. 2009, 3 (9), 1029–1043. [PubMed: 20161589]
- (17). Gundry RL; White MY; Murray CI; Kane LA; Fu Q; Stanley BA; Van Eyk JE Current Protocols in Molecular Biology 2009, 1–23.
- (18). Griebenow K; Klibanov AM J. Am. Chem. Soc. 1996, 118 (47), 11695–11700.
- (19). Strader MB; Tabb DL; Hervey WJ; Pan C; Hurst GB Anal. Chem. 2006, 78 (1), 125–134. [PubMed: 16383319]
- (20). Mi H; Casagrande JT; Thomas PD; Muruganujan A Nat. Protoc. 2013, 8, 1551–1566. [PubMed: 23868073]
- (21). Thongboonkerd V; Chutipongtanate S; Kanlaya RJ Proteome Res. 2006, 5, 183–191.
- (22). Cox J; Mann M Nat. Biotechnol 2008, 26 (12), 1367–1372. [PubMed: 19029910]
- (23). Tyanova S; Temu T; Cox J Nat. Protoc. 2016, 11 (12), 2301–2319. [PubMed: 27809316]
- (24). Schwanhaussier B; Busse D; Li N; Dittmar G; Schuchhardt J; Wolf J; Chen W; Selbach M Nature 2011, 473 (7347), 337–342. [PubMed: 21593866]
- (25). Nagaraj N; Mann MJ Proteome Res. 2011, 10 (2), 637–645.
- (26). Zhao M; Li M; Yang Y; Guo Z; Sun Y; Shao C; Li M; Sun W; Gao Y Sci. Rep. 2017, 7 (1), 3024. [PubMed: 28596590]
- (27). Ahrné E; Molzahn L; Glatter T; Schmidt A Proteomics 2013, 13 (17), 2567–2578. [PubMed: 23794183]
- (28). Yang N; Feng S; Shedden K; Xie X; Liu Y; Rosser CJ; Lubman DM; Goodison S Clin. Cancer Res. 2011, 17 (10), 3349–3359. [PubMed: 21459797]
- (29). Molinaro R; Pocino K; Daloiso PD; Giannace A; Spirito G; Zuppi C; Antenucci MJ Clin. Lab. Anal. 2016, 30 (6), 888–891.
- (30). Klein J; Vlahou A; Zürbig P; Carrick E; Pejchinovski M; Krochmal M; Pape L; Schanstra JP; Magalhães P; Pontillo C; Jankowski J; Mischak H; Mullen W; Siwy J; Makridakis M Proteomics: Clin. Appl. 2018, 12 (5), 1700163.
- (31). Ludwig KR; Schroll MM; Hummon AB J. Proteome Res. 2018, 17 (7), 2480–2490. [PubMed: 29754492]
- (32). Chiva C; Ortega M; Sabidó EJ Proteome Res. 2014, 13 (9), 3979–3986.
- (33). Tabb DL; Vega-Montoto L; Rudnick PA; Variyath AM; Ham AJL; Bunk DM; Kilpatrick LE; Billheimer DD; Blackman RK; Cardasis HL J. Proteome Res. 2009, 9 (2), 761–776.
- (34). Chen J; Shah P; Zhang H Anal. Chem. 2013, 85, 10670–10674. [PubMed: 24079330]
- (35). Santucci L; Candiano G; Petretto A; Bruschi M; Lavarello C; Inglese E; Giorgio Righetti P; Marco Ghiggeri G. Data Br. 2014, 1, 25–28.

Figure 1.

Urinary proteomics using C4-tips. Protein sample is acidified, and proteins are bound onto the C4-tips. Next, tips are washed, and the bound proteins are subjected to reduction, alkylation, and “on-tip” protease digestion. Protease digested peptides are released directly during digestion, and the remaining peptides are recovered via 50% ACN washing and elution. Both fractions are pooled, and peptides are analyzed via LC–MS/MS.

Figure 2.

Dynamic range of protein abundance of the urine proteome using disparate digestion methods. Reported iBAQ values for proteins identified using the C4-tip digestion format (A), the C4-tip BE digestion (B), in-solution digestion (C), and in-solution digestion containing 30% ACN (D), respectively. iBAQ values were plotted against the protein's abundance rank.

Figure 3.

Direct analysis of urine protein using the C4-tip digestion method. The heat map of correlation coefficients using reported TMT protein intensities shows high reproducibility of the technical replicates for the five normal urine samples. Pearson correlation analyses revealed an R value of >0.90 .