Management of Tamm–Horsfall Protein for Reliable Urinary Analytics

Xiaomeng Xu, Karina Barreiro, Luca Musante, Oliver Kretz, Hanfei Lin, Hequn Zou,* Tobias B. Huber, and Harry Holthofer*

Purpose: Urinary extracellular vesicles (uEVs) are a novel source of biomarkers. However, urinary Tamm–Horsfall Protein (THP; uromodulin) interferes with all vesicle isolation attempts, precipitates with normal urinary proteins, thus, representing an unwanted “contaminant” in urinary assays. Thus, the aim is to develop a simple method to manage THP efficiently.

Experimental design: The uEVs are isolated by hydrostatic filtration dialysis (HFD) and treated with a defined solution of urea to optimize release of uEVs from sample. Presence of uEVs is confirmed by transmission electron microscopy, Western blotting, and proteomic profiling in MS.

Results: Using HFD with urea treatment for uEV isolation reduces sample complexity to a great extent. The novel simplified uEV isolation protocol allows comprehensive vesicle proteomics analysis and should be part of any urine analytics to release all sample constituents from THP trap.

Conclusions and clinical relevance: The method brings a quick and easy protocol for THP management during uEV isolation, providing major benefits for comprehensive sample analytics.

1. Introduction

Urinary extracellular vesicles (uEVs) are small size (30–400 nm in diameter) phospholipid bilayered structures actively secreted by all cell types facing the urinary space. Originating either from the endosomal compartment or directly from evagination of cellular plasma membrane, diversity of extracellular vesicles (EV) found in urine can be considered as a snapshot of physiopathological activity upstream in the kidney and entire body as reflected by EVs filtered from circulation through the kidney.[2] Thus, EV profiling represents a lucrative platform for novel biomarkers to provide dynamic molecular information for early disease mechanisms, prediction of outcome and response to therapy.[3] uEVs carry a wide variety of molecules including specialized proteins, lipids, and (deoxy) ribonucleic acids uniquely protected from deleterious actions of ubiquitous proteases, ribonucleases, and lipases present abundantly in crude urine.[4] The precise functions of uEVs are not fully understood. Interestingly, they may be shuttled from upstream cell types to more distant target cells allowing a novel way of cell-to-cell communication.[5] Despite the exploding interest to EV biology, there are still many elementary challenges related, e.g., to overlapping nomenclature of EV categories and, especially, to the methods applied to isolate uEVs efficiently for downstream applications. Notably, the
traditional uEV isolation methods have been challenging, labor-intensive, costly, and time consuming. Furthermore, they have not met the inherent challenges of urine: wide ionic concentration and pH range, intra- and interindividual variability, artifacts caused by naturally occurring urinary pigments and proteins as well as protease effects all complicating their isolation and downstream analytical processes. Particularly, Tamm–Horsfall protein (THP; uromodulin), the most abundant and glycan-rich glycoprotein in urine[6] can naturally entrap a large portion of uEVs to form filamentous networks.[7] Hence, THP represents a significant challenge as it distorts the spectrum of uEV classes recovered. For MS analysis, overabundant contaminant proteins take up a significant total mass fraction and may bind low abundance peptides to cause serious interference.[8]

Several methods have been proposed to eliminate THP contamination. These include, e.g., use of detergents such as dithiothreitol (DTT) and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS).[10] Salt precipitation followed by differential centrifugation,[31] and a dilution protocol using alkaline pH and low ionic concentration called KeepEX.[12] Although these methods can be successful in reducing THP level in uEV preparations, they have obvious shortcomings. DTT reduces disulfide bridges, which affects original protein structure. This directly influences proteomic analysis of EVs. CHAPS reasonably preserve protein conformation and enzymatic activities but its use is more time consuming than the DTT method. KeepEX substantially increases the volume of sample thus increasing the processing time.

In this study, we used the Hydrostatic Filtration Dialysis in combination with various chaotropic reagents to yield an optimized protocol for THP denaturation, depolymerization, and subsequent EV release and characterized the uEVs freed from the bulk of THP.

2. Experimental Section

Majority of reagents were purchased from Sigma–Aldrich (St. Louis, MI) unless specified otherwise in the text.

2.1. Urine Samples

First morning void urine samples were collected from healthy laboratory staff, aged 20–44 (N = 4), during three consecutive days. First morning void urine was processed within 3 h without adding protease inhibitors. Urine was anonymously labeled and pooled together. The urine collection and isolation of vesicles were done in Dublin City University. The study design and sample collection were approved by the Research Ethics Committee of Dublin City University (DCU REC/2014/222). All the experiments were performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all participants.

2.2. Vesicle Purification

uEVs were enriched by HFD according to our previous protocol[13] with minor modifications.[14] Briefly, urine samples were centrifuged at 2000 × g for 30 min at room temperature (RT). Before storing at ~80 °C, 50 mL of a stock solution of 1 mM sodium citrate and 5 mM sodium EDTA disodium salt was added to 950 mL supernatant 2000 g (SN 2000). A total of 0.1 mg silver colloid and 4.5 mg sodium dichloroisocyanurate were mixed with the sample before HFD was performed. Then 5 mL concentrated SN 2000 was left in the membrane, 200 mL treated Milli-Q water (0.1 mg silver colloid and 4.5 mg sodium dichloroisocyanurate L−1) was filled to wash the membrane. Concentrated SN 2000 named as HDFa was collected.

2.3. Tamm–Horsfall Denaturation

A summary of the uEV isolation and THP elimination protocol is shown in Figure 1A and Figure S1, Supporting Information. One milligram of HDFa was treated with 5 mL (final volume) of a solution made of:

a) 200 mM dithiothreitol (DTT) in 10 mM triethanolamine,[7]
b) 1% w/v 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS).[10]
c) 6 mM Urea in 50 mM citrate buffer, pH 3.5, and 10 mM arginine;
d) 200 mM DTT in 10 mM triethanolamine plus 6 mM urea,
e) 1% CHAPS and 200 mM DTT in 10 mM triethanolamine.

All samples were mixed in end-over-end agitation (40 revolutions min−1) for 1 h at RT and placed again in the HFD system using a 1000 kDa molecular weight cut-off (MWCO) dialysis membrane. Fifty milliliters of the same buffer followed by 200 mL of deionized (DI) water was used to remove all the proteins below the MWCO (HFDb).

2.4. Transmission Electron Microscopy, Protein assay, Gel Electrophoresis, and Western Blotting

A full description of the methods is provided in Supporting Information (see Supporting Information Methods)
Figure 1. Workflow of the uEV isolation and THP elimination (A) and sample preparation for MS analysis (B).
2.5. Tunable Resistive Pulse Sensing

Tunable Resistive Pulse Sensing (TRPS) measurements were performed with qNano instrument (Izon Ltd, Christchurch, New Zealand) according to manufacturer’s instructions. Polyurethane nanopore membrane NP150 (analysis range 85–300 nm) and NP300 (analysis range 150–900 nm; Izon Ltd) were utilized, membranes were stretched at 46 mm (NP150) and 44.5 mm (NP300); the voltage was set at 0.46 V (NP150) and 0.34 V (NP300), Multipressure was set at 2, 5, and 8 mBar (NP150) and at 3, 6, and 9 mBar (NP300), respectively, to determine particle concentration. Electrolyte solution was made of PBS and 0.05% (v/v) Triton X100 filtered with a Millipore Millex GS 0.22 µm syringe filter (Merck Millipore). Current pulse signals were collected using Izon Control Suite 3.1.2.268 software (Izon Ltd). Blockade counts setting in this study was fixed at minimum of 1000 events. Calibration was made using standard polystyrene particles of 100, 200, and 330 nm (CPC100B; CPC200B, and CPC400B; Izon Ltd.). uEV fractions were sonicated for 5 min and before the analysis with the NP150 samples were filtered with a Millipore Millex GS 0.45 µm syringe filter (Merck Millipore). A full description of the method is provided in Supporting Information (see Supporting Information Methods).

2.6. MS Analysis

One hundred microgram as dry pellet of HFDa U (Figure 1B) was resolubilized and reduced in 100 µL of 10 mM Tris (carboxyethyl) phosphine (TCEP), 8 mM urea, 0.1 mM EDTA, and 0.2 mM Tris buffer pH 8.8 in dark for 1 h at RT, followed by alkylation with 20 mM iodoacetamide (IAA) in dark for 2 h. Finally, 40 mM N-acetyl cysteine (NAC) was used to quench the excess of IAA. The sample was then precipitated by TCA-acetone to eliminate the excess of salts. Samples were digested by sequencing grade trypsin in presence of 1% v/v sodium deoxycholate (DOC) in 50 mM Tris-HCl pH 7.8 in a ratio of 1 µg of trypsin for 50 µg of total protein, at +37 °C overnight. After the digestion samples were acidified with 1% v/v formic acid (FA) to precipitate DOC, the supernatant, along with the external proteome, was cleaned up by Sep-Pak C18 cartridge (Waters Associates, Milford, MA) according to manufacturer’s instructions.

Cleaned up samples were dissolved in 0.1% v/v TFA and 2% v/v acetonitrile (ACN) solution, directly loaded onto an RP analytical column (300µm i.d x 5 mm, packed with Acclaim PepMap RSLC C18, 5 µm, 100 A, nanoViper). The gradient was composed of an increase from 5% to 50% solvent B (0.1% FA in 80% ACN) over 65 min, and climbing to 90% in 4 min, then holding at 90% for 6 min. All was done at a constant flow rate of 300 nL min⁻¹. The MS analysis was performed on Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific).

The peptides were subjected to NSI source followed by MS/MS in Q ExactiveTM (Thermo Fisher) coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 70 000. Peptides were selected for MS/MS using NCE setting as 27; ion fragments were detected in the Orbitrap at a resolution of 17 500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 1E4 in the MS survey scan with 30.0 s dynamic exclusion. The electrospray voltage applied was 2.0 kV. Automatic gain control (AGC) was used to prevent overfilling of the ion trap; 1E5 ions were accumulated for generation of MS/MS spectra. For MS scans, the m/z scan range was 350 to 1800 m/z. Fixed first mass was set as 100 m/z. Protein identification were performed with Protein Pilot 5.0 (AB Sciex) software by searching Uniprot-Human (http://www.uniprot.org/proteomes/UP000005640).

3. Results

3.1. THP Denaturation

DTT, urea, CHAPS, and a combination of urea-DTT and CHAPS-DTT were used to compare THP polymer denaturation and release THP complexing with uEVs and then harvested with a second HFD step.

Figure 2 shows the partitioning of THP, the large band at 100 kDa (Figure 2A,B), TSG101 as a general vesicle marker (Figure 2C,D), and human serum albumin (HSA) as a soluble control protein (Figure 2E,F).

Among the chaotropic reagents and their combinations tested, only urea in acidic buffer, urea, and DTT at pH 7.6 were effective to remove the bulk of THP from the vesicles fraction (Figure 2A, lanes 3a and 4a). This fraction was flushed in the flow-through (Figure 2B, lanes 3b and 4b) without affecting the integrity of vesicles as indirectly evidenced by presence of TSG101 in the retenant (Figure 2C, lanes 3a and 4a) but not in the flow-through (Figure 2D, lanes 3b and 4b). Notably, while both treatments successfully removed THP, they were practically ineffective to strip vesicles from HSA as only a small fraction of HSA was recovered in the flow-through with the DTT–urea treatment. A minimum of 6 µm urea (Figure S2, Supporting Information) in acidic pH (Figure S3, Supporting Information) was necessary to depolymerize THP filaments at a molecular weight smaller than the MWCO of the dialysis membrane (1000 kDa).

3.2. Transmission Electron Microscopy

Electron microscopy analysis confirmed the presence of a heterogeneous population of vesicles in respect to size and morphology (Figure S4, Supporting Information). This shows there is no apparent change in vesicle class proportions. Notably, THP significantly entrapped uEV into its filamentous meshwork in HFDa samples (Figure S4A, Supporting Information), but in HFDa after urea treatment (HFDa U) samples this THP protein meshwork was not seen (Figure S4B, Supporting Information).

3.3. TRPS for Vesicle Size Distribution

With the particle size distribution (PSD) method used for PSD analysis, it was evident that their number and size were closely similar for both HFDa and HFDaU (Table S1, Supporting Information).
Figure 2. Colloidal Coomassie staining and Western blotting of the fraction after the chaotropic treatments. A) Retentate from the dialysis tube (a: above the membrane molecular cut-off). B) Flow through (b: below the membrane molecular cut-off). Ten micrograms of total protein per lane was loaded. C and D) Detection of TSG101; E and F) Detection of human serum albumin and TSG101. Lane 1, HFDa; Lane 2a and 2b, DTT; Lane 3a and 3b, urea pH 3.5; Lane 4a and 4b, urea + DTT pH 7.6; Lane 5a and 5b, CHAPS; Lane 6a and 6b, CHAPS + DTT.

3.4. Western Blot Analysis of uEV Markers Before (HFDa) and After (HFDaU) Urea Treatment

Information) uEVs. This result directly shows the good recovery of uEVs after urea treatment without effect to any vesicle categories.

3.5. Mass Spectometry

Protein profile of uEVs recovered by the HFDaU was done by in-solution trypsin digestion proteomic analysis.[15] Accordingly, we found 1171 (HFDaU1), 1176 (HFDaU2), and 1021 (HFDaU3) proteins identified by at least two peptides with 95% of
Figure 3. Western blot analysis of uEV markers (B–J) and non EV-associated proteins (K–O) before (HFDa) and after (HFDaU) urea treatment. A) Coomassie Staining; B) CD9; C) TSG101; D) ALIX; E) β-Actin; F) dipeptidyl peptidase 4 DPP4; G) Nephriylsin NEP; H) Podocin; I) Calbindin (CALB); J) Rab5α; K) Human serum albumin (HSA); L) IgG γ chain; M) IgA α chain; N) IgM µ chain; O) α1 antitrypsin inhibitor.
Figure 4. Venn diagram of the protein identification: A) Common proteins in HFDa U1, HFDa U2, and HFDa U3; B) Common and unique proteins in Vesiclepedia (788), urinovesiclepedia (10130) and urinovesiclepedia (4835).

confidence, respectively, for the three replicas of same preparations. Out of 1477 non-redundant protein identification (HFDa U1+U2+U3), 788 proteins were common in all three datasets (Figure 4A). Interestingly, 771 of our protein yield were found in the Vesiclepedia repository (Figure 4B; www.microvesicles.org), which collects all published information EV findings, while nine proteins have not been reported in this repository before.

Gene ontology classification (http://www.pantherdb.org/; large-scale gene function analysis with the PANTHER classification system; Table 1A) of those 788 proteins showed that 20 of the proteins identified in HFDaU belong to the endosomal sorting complex required for vesicle transport (ESCRT) pathway itself.[16] This shows that our urea treatment does not appear to damage vesicles. Altogether around 69% of all ESCRT machinery components were detected from HFDaU in our study, showing that exosomes remain abundant in the uEV fraction after urea treatment.

Tetraspanin-enriched microdomains are yet another class of known proteins involved in the intracellular sorting machinery to yield microvesicles and exosomes.[17] Notably, 11, eight, and four out of the 33 human tetraspanin proteins were identified in HFDaU1, -U2, and -U3, respectively (see Table 1B). This directly shows that urea treatment is delicate enough not to destroy the fragile protein components of microvesicles and exosome biogenesis.

Using the GO biological process analysis approach, we could show that additional ten of our found HFDaU proteins are involved in cytoskeletal trafficking of vesicles (Tables S2 and S3, Supporting Information).

In summary our results show that urea treatment is delicate enough and can release uEV proteins when THP contaminant is carefully managed.

4. Discussion

Tamm–Horsfall glycoprotein is the most abundant “normal” physiological protein found in urine, with tendency to aggregate and form filamentous meshworks to actively bind urinary particles, apoptotic cells[18] and interact directly with uropathogenic bacteria.[19] Accordingly, THP appears to protect against urinary tract infections but also prevents, e.g., calcium crystal formation and kidney stones.[20] Moreover, it regulates innate immunity by binding to immunoglobulins and cytokines[21] evidencing the multitude of its actions in the urine. Beyond these data, the full physiological role of THP still remains incompletely understood.

Notably, THP level in urine appears to be a valuable indicator of functional kidney mass[22] and the respective UMOD gene is associated with chronic kidney disease and, especially, diabetic kidney damage[23] by mechanisms to be fully elucidated.

Here, we show how THP directly affects recovery of uEV harvest and distorts its downstream protein analysis. Many known artifacts in urinary analytics are caused by THP aggregation, which harvests free proteins, reduces the number of urinary cells, as well as distorts extracellular vesicle yield.[14] As shown in this study, better management of THP in urine adds new proteins to those reported in earlier uEV studies (see www.microvesicles.org). Notably, artifacts caused by THP similarly distort analytics directly from crude urines.

Several approaches have been used earlier to manage the known THP effects on uEVs. These have attempted to eliminate the bulk of urinary THP. In some approaches, reagents including dithiothreitol (DTT)[7] or deuterated water to create sucrose cushion or gradient[24] have been used. Other methods to rid THP include size exclusion chromatography[25] or serial combination of DTT, centrifugation, centrifugal ultrafiltration, and size exclusion chromatography.[26] Yet another alternative to eliminate THP artifacts is to induce THP polymerization with diatomaceous earth filter.[28] Each of these approaches may have some benefits but in general they are inefficient, tedious, time consuming, and costly. According to our hypothesis the interference with THP occurs due to aggregation. Solubilization of these aggregates could also reduce contamination of uEV. For this, we tested three chaotropic agents, DTT, CHAPS, and urea. DTT disrupts the polymeric network presumably by reduction of disulfide bonds linking monomers.[7] CHAPS is a non-denaturating zwitterionic detergent suited for the disruption of nonspecific protein interactions, while also protecting conformation of protein(s).[10]
Table 1. List of proteins identified in HFDa Urea belonging to ESCRT machinery (A) and tetraspanin family (B).

A

| Complex   | Accession number | Gene name | Protein name                                      | HFDa U1 | HFDa U2 | HFDa U3 |
|-----------|-----------------|-----------|--------------------------------------------------|---------|---------|---------|
| ESCRT-0   | O14964          | HGS       | Hepatocyte growth factor-regulated tyrosine kinase substrate | 1       |         |         |
| ESCRT-I   | Q99816          | TSG101    | Tumor susceptibility gene 101 protein             | 7       | 9       |         |
|           | Q9UK41          | VPS28     | Vacular protein sorting-associated protein 28 homolog |         |         |         |
|           | Q9H9H4          | VPS37B    | Vacular protein sorting-associated protein 37B    |         |         |         |
|           | A5DBV6          | VPS37C    | Vacular protein sorting-associated protein 37C    |         |         |         |
|           | Q86XT2          | VPS37D    | Vacular protein sorting-associated protein 37D    |         |         |         |
|           | Q96EY5          | MVB12A    | Multivesicular body subunit 12A                   |         |         |         |
|           | Q9H7P6          | MVB12B    | Multivesicular body subunit 12B                   |         |         |         |
| ESCRT-II  | Q98RG1          | VPS25     | Vacular protein-sorting-associated protein 25     | 2       | 3       |         |
|           | Q86VN1          | VPS36     | Vacular protein-sorting-associated protein 36     |         |         |         |
| ESCRT-III | O43633          | CHMP2A    | Charged multivesicular body protein 2a            | 6       | 11      |         |
|           | Q9UQ3N          | CHMP2B    | Charged multivesicular body protein 2b            |         |         |         |
|           | Q9EFZ7          | CHMP6     | Charged multivesicular body protein 6             |         |         |         |
|           | Q9HH44          | CHMP4B    | Charged multivesicular body protein 4b            |         |         |         |
|           | Q9N2Z3          | CHMP5     | Charged multivesicular body protein 5             |         |         |         |
|           | Q7LBR1          | CHMP1B    | Charged multivesicular body protein 1b            |         |         |         |
| Vps4-Vta1 | P53990          | IST1      | IST1 homolog (hIST1) (Putative MAPK-activating protein PM28) | 4       | 4       |         |
|           | Q9UN37          | VPS4A     | Vacular protein sorting-associated protein 4A     |         |         |         |
|           | O753S1          | VPS4B     | Vacular protein sorting-associated protein 4B     |         |         |         |
|           | Q9NP79          | VTA1      | Vacular protein sorting-associated protein VTA1 homolog |         |         |         |

B

| Protein accession | Gene name | Protein name | HFDa U1 | HFDa U2 | HFDa U3 |
|-------------------|-----------|--------------|---------|---------|---------|
| O60637            | TSPAN3    | Tetraspanin-3| √       |         |         |
| O43657            | TSPAN6    | Tetraspanin-6| √       |         |         |
| P19075            | TSPAN8    | Tetraspanin-8| √       |         |         |
| O00322            | UPX1A     | Tetraspanin-21| √       |         |         |
| O60635            | TSPAN1    | TSPAN1      | √       |         |         |
| O14817            | TSPAN4    | TSPAN4      | √       |         |         |
| P60033            | CD81      | TSPAN28     | √       |         |         |
| P60862            | CD63      | TSPAN30     | √       |         |         |
| Q86UF1            | TSPAN33   | TSPAN33     | √       |         |         |
| P48309            | CD151     | TSPAN24     | √       |         |         |
| P27701            | CD82      | TSPAN27     | √       |         |         |

Urea disrupts noncovalent bonds in proteins, thus working as protein denaturant, and it can be used to increase solubility of proteins.\(^{[29]}\) In addition, arginine was experimented as a cosolvent with urea, as it is known to increase solubility and contribute to protein folding.\(^{[29]}\)

The principles behind urea effects on proteins have been actively discussed for decades but still remain unknown. Urea may exert its effect directly, by binding to the protein and disrupting van der Waals interactions resulting in denaturation. Alternatively, urea acts as a chaotrope, altering the hydrogen-bond structure of water thus promoting the solvation of hydrophobic groups. Urea can also bind to the amino acid backbone to compete with native interactions in the unfolding process. Why pH 3.5 is optimum for urea action can be due to ionizable groups like tyrosine (pK\(_a\) = 10.07), cysteine (pK\(_a\) = 8.33), histidine (pK\(_a\) = 5.97), aspartic (pK\(_a\) = 3.65), and glutamic acid (pK\(_a\) = 4.25), which could lose the negative charge, respectively. In general low pH includes very low electrostatic interactions among charged groups while the protein backbone remains positive. The pH effect on protein-glycan moieties may also play a role.

We have shown earlier that failure to manage THP properly may lead to up to 40% loss of uEVs, thus loosing
unselectively important uEVs of different classes.\[30\] Resulting lower uEV yields may very potentially lose valuable biomarkers. Furthermore, most of the earlier approaches have been limited by urine volumes: if up to 40% of uEVs are lost in THP precipitation, bigger starting volumes are needed to compensate losses in harvesting. Elimination of THP typically also adds one extra step to EVs isolation protocols. As we demonstrate here, management of THP can be efficiently integrated into EVs isolation process with Hydrostatic Filtration Dialysis with major benefits of vastly simplified and less time consuming procedure.

Existence of valuable biological material entrapped within vesicles of variable sizes has been reported since late 1980s.\[31\] The seminal reports of EVs found also in urine and, especially, of their valuable contents of structural and functional proteins, lipids, DNA, and a variety of RNA species have created a fundamentally new opportunity for simplified biomarker identification. Notably, EVs appear to consist qualitatively 4–10x more of potential biomarkers compared to use of crude urine and should thus be preferred. Furthermore, the protein and RNA diversity of uEVs appears well protected against, e.g., free urinary ribonuclease and protease activities.

With the recent development of a new approach called HFD with its optimization by urea treatment many known caveats of conventional EV isolation techniques can be avoided as shown here. HFD is simple, inexpensive, and easy to adopt in any laboratory without costly machinery or ultracentrifugation device. It also easily handles big volumes of urine rapidly, by itself equals laboratory without costly machinery or ultracentrifugation device. It can also easily handle big volumes of urine rapidly, by itself equals without costly machinery or ultracentrifugation device. It can also easily handle big volumes of urine rapidly, by itself equals without costly machinery or ultracentrifugation device. It can also easily handle big volumes of urine rapidly, by itself equals without costly machinery or ultracentrifugation device. It can also easily handle big volumes of urine rapidly, by itself equals without costly machinery or ultracentrifugation device. It can also easily handle big volumes of urine rapidly, by itself equals without costly machinery or ultracentrifugation device. It can also easily handle big volumes of urine rapidly, by itself equals without costly machinery or ultracentrifugation device. It can also easily handle big volumes of urine rapidly, by itself equals without costly machinery or ultracentrifugation device. It can also easily handle big volumes of urine rapidly.

In summary, we provide here an easy method optimized to manage challenges with urinary THP caused artifacts in all urinary analytics.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements
X.X., K.B., and L.M. contributed equally to this study. The skilful assistance of Ms Dorota Tataruch is warmly acknowledged. This study was supported by grants from the EU FP7 Program (UroSense, agreement \#286386), the Paulo Foundation of Finland and the South Wisdom Valley Innovative Research Team Program (2014XTDD04). T.B.H. was supported by the DFG (CRC1410, CRC 992), BMBF (01G1513C), and European Research Council-ERC grant 616891. This project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking under grant agreement no. 115974 (BEAT-DKD; T.B.H., H.H.). This Joint Undertaking receives support from the European Union’s Horizon 2020 research and innovation programme and EFPIA and by the H2020-IMI2 consortium BEAT-DKD.

Conflict of Interest
The authors declare no conflict of interest.

Keywords
biomarker, mass spectrometry, Tamm–Horsfall protein, urea, urinary extracellular vesicles

Received: February 6, 2019
Revised: August 10, 2019
Published online: September 8, 2019

[1] P. A. Gonzales, T. Pitsikun, J. D. Hoffert, D. Tchapyjnikov, R. A. Star, R. Kleta, N. S. Wang, M. A. Knepper, J. Am. Soc. Nephrol. 2009, 20, 363.
[2] a) U. Erdbrugger, T. H. Le, J. Am. Soc. Nephrol. 2016, 27, 12; b) J. M. Street, E. H. Koritszinsky, D. M. Glispie, R. A. Star, P. S. Yuen, Adv. Clin. Chem. 2017, 78, 103.
[3] a) K. Barreiro, H. Holthofer, Cell Tissue Res. 2017, 369, 217; b) S. Bruno, S. Porta, B. Bussolati, Eur. J. Pharmacol. 2016, 790, 83; c) G. De Palma, F. Sallustio, F. P. Schena, Int. J. Mol. Sci. 2014, 16, 17; d) D. Karpmann, A. L. Stahl, I. Arvidsson, Nat. Rev. Nephrol. 2017, 13, 545.
[4] a) K. C. Miranda, D. T. Bond, J. Z. Levin, X. Adiconis, A. Sivachenko, C. Russ, D. Brown, C. Nusbaum, L. M. Russo, PLoS One 2014, 9, e96094; b) Z. Wang, S. Hill, J. M. Luther, D. L. Hachey, K. L. Schey, Proteomics 2012, 12, 329.
[5] a) J. J. Gildea, J. E. Seaton, K. G. Victor, C. M. Reyes, D. Bigler Wang, A. C. Pettigrew, C. E. Courtner, N. Shah, H. T. Tran, R. E. Van Schiver, J. M. Carlson, R. A. Felder, Clin. Biochem. 2014, 47, 89; b) J. M. Street, W. Birkhoff, R. I. Menzies, D. J. Webb, M. A. Bailey, J. D. Dear, J. Physiol. 2011, 589, 6119.
[6] M. Brunati, S. Perucca, L. Han, A. Cattaneo, F. Consolato, A. Andolfi, C. Schaeffer, E. Olinger, J. Peng, S. Santambrogio, R. Perrier, S. Li, M. Bokhove, A. Bachi, E. Hummler, O. Devuyst, Q. Wu, L. Jovine, L. Rampoldi, eLife 2015, 4, e08887.
[7] P. Fernandez-Llama, S. Khositseth, P. A. Gonzales, R. A. Star, T. Pitsikun, M. A. Knepper, Kidney Int. 2010, 77, 736.
[8] X. Liu, C. Chinello, L. Musante, M. Cazzaniga, D. Tataruch, G. Calzaferri, A. James Smith, G. De Sio, F. Magni, H. Zou, H. Holthofer, Proteomics Clin. Appl. 2015, 9, 568.
[9] K. Kobayashi, S. Fukushima, Annu. Rev. Biophys. 2001, 388, 113.
[10] L. Musante, M. Saraswat, E. Duriez, B. Byrne, A. Ravida, B. Domon, H. Holthofer, PLoS One 2012, 7, e37279.
[11] a) M. Kosanovic, M. Jankovic, BioTechniques 2014, 57, 143.
[12] P. Lin, J. Zhou, D. Pi, B. Chen, X. Wang, S. Liang, Anal. Biochem. 2008, 377, 259.
[13] a) J. H. Hurley, Crit. Rev. Biochem. Mol. Biol. 2010, 45, 463; b) J. H. Hurley, P. I. Hanson, Nat. Rev. Mol. Cell Biol. 2010, 11, 556.
[14] L. Mo, H. Y. Huang, X. H. Zhu, E. Shapiro, D. L. Hasty, X. R. Wu, Kidney Int. 2004, 66, 1159.
[15] M. D. Saemann, T. Weichhart, M. Zeyda, G. Stafferl, M. Schunn, K. M. Stuhlmeier, Y. Sobanov, T. M. Stulnig, S. Akira, A. von Gabain, U. von Ahsen, W. H. Horl, G. J. Zlabinger, J. Clin. Invest. 2005, 115, 468.
[22] M. Pruijm, B. Ponte, D. Ackermann, F. Paccaud, I. Guessous, G. Ehret, A. Pechere-Bertschi, B. Vogt, M. G. Mohaupt, P. Y. Martin, S. C. Youhanna, N. Nagele, P. Vollenweider, G. Waeber, M. Burnier, O. Devuyst, M. Bochud, Clin. J. Am. Soc. Nephrol. 2016, 11, 70.

[23] a) C. Pattaro, J. Nephrol. 2018, 31, 475; b) C. Pattaro, A. Teumer, M. Gorski, A. Y. Chu, M. Li, V. Mijatovic, M. Garnaas, A. Tin, R. Sorice, Y. Li, D. Taliun, M. Olden, M. Foster, Q. Yang, M. H. Chen, T. H. Pers, A. D. Johnson, Y. A. Ko, C. Fuchsberger, B. Tayo, M. Nalls, M. F. Feitosa, A. Issacs, A. Dehghan, P. d’Adamo, A. Adeyemo, A. K. Dieffenbach, A. B. Zonderman, I. M. Nolte, P. J. van der Most, et al., Nat. Commun. 2016, 7, 10023.

[24] a) C. Y. Chen, M. C. Hogan, C. J. Ward, Methods Enzymol. 2013, 524, 225; b) M. C. Hogan, K. L. Johnson, R. M. Zenka, M. C. Charlesworth, B. J. Madden, D. W. Mahoney, A. L. Oberg, B. Q. Huang, A. A. Laktionov, L. L. Nesbitt, J. L. Bakeberg, D. J. McCormick, H. R. Bergen, C. J. Ward, Kidney Int. 2014, 85, 1225.

[25] G. Corso, I. Mager, Y. Lee, A. Gorgens, J. Bulterma, B. Giebel, M. J. A. Wood, J. Z. Nordin, S. E. Andaloussi, Sci. Rep. 2017, 7, 11561.

[26] M. Wachalska, D. Koppers-Lalic, M. van Eijndhoven, M. Pegtel, A. A. Geldof, A. D. Lipinska, R. J. van Moorselaar, I. V. Bijnensdorp, J. Circ. Biomark. 2016, 5, 4.

[27] M. Y. Konoshenko, E. A. Lekchnov, A. V. Vlassov, P. P. Laktionov, Biomed. Res. Int. 2018, 2018, 1.

[28] F. Serafini-Cessi, G. Bellabarba, N. Malagolini, F. Dall’Olio, J. Immunol. Methods 1989, 120, 185.

[29] P. T. Wingfield, Curr. Protoc. Protein Sci. 2001, Appendix 3, Appendix 3A.

[30] L. Musante, M. Saraswat, A. Ravida, B. Byrne, H. Holthofer, Nephrol., Dial., Transplant. 2013, 28, 1425.

[31] J. C. Akers, D. Gonda, R. Kim, B. S. Carter, C. C. Chen, J. Neuro-Oncol. 2013, 113, 1.