Published in final edited form as:

Methods Mol Biol. 2015 ; 1295: 65–74. doi:10.1007/978-1-4939-2550-6_6.

Urinary Pellet Sample Preparation for Shotgun Proteomic Analysis of Microbial Infection and Host Pathogen Interactions

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Abstract

Urine is one of the most important biofluids in clinical proteomics, and many potential disease biomarkers have been identified using mass spectrometry-based proteomics in the past decades. Current studies mainly perform analyses of the urine supernatant devoid of cells and cell debris, and the pellet (sediment) fraction is discarded. However, the pellet fraction is biologically of interest. It may contain whole human cells shed into the urine from anatomically proximal tissues and organs (e.g., kidney, prostate, bladder, urothelium and genitals), disintegrated cells and cell aggregates derived from such tissues, viruses and microbial organisms which colonize or infect the urogenital tract. Knowledge of the function, abundance and tissue of origin of such proteins can explain a pathological process, identify a microbe as the cause of urinary tract infection, and measure the human immune response to the infection-associated pathogen(s). Successful detection of microbial species in the urinary pellet via proteomics can serve as a clinical diagnostic alternative to traditional cell culture-based laboratory tests. Filter aided sample preparation (FASP) has been widely used in shotgun proteomics. The methodology presented here implements an effective lysis of cells present in urinary pellets, solubilizes the majority of the proteins derived from microbial and human cells, and generates enzymatic digestion-compatible protein mixtures using FASP followed by optimized desalting procedures to provide a peptide fraction for sensitive and comprehensive LC-MS/MS analysis. A highly parallel sample preparation method in 96-well plates to allow scaling up such experiments is discussed as well. Separating peptides by nano-LC in one dimension followed by online MS/MS analysis on a Q-Exactive mass spectrometer, we have shown that more than 1,000 distinct microbial proteins and 1,000 distinct human proteins can be identified from a single experiment.

Keywords

Urine proteomics; Urinary pellet; microbial infection; host pathogen interaction; filter aided sample preparation (FASP); 96FASP

1. Introduction

Urine is a sample source of high importance for biomarker discovery because it is easily available and collected non-invasively in large quantities (1,2). The identity and quantity of proteins excreted into urine may reflect pathological conditions that can be traced to

different organs in the body, particularly the kidneys, prostate and urogenital tract (3). Currently, most urine proteomic studies focus on the analysis of the urinary supernatant, that is the soluble fraction of the collected urine sample following centrifugation at 1,500 to 5,000 x g for 5~15 min (4,5). The urinary pellet is frequently discarded. However, the urinary pellets, especially those from patients with urinary tract infection (UTI), which is one of the most common conditions that lead to hospital visits (6), contain not only pathogenic microbes, in most cases bacteria that colonize the urinary tract of the patient, but also host proteins associated with the inflammatory process following colonization with the microbial pathogen. Inflammation may include activities such as recognition of pathogen molecular patterns, cytokine release, leukocyte recruitment, lymphocyte recruitment, complement activation, immunoglobulin secretion, fibrin deposition, release of iron-sequestering proteins and direct microbial killing via enzymatic activities and permeabilization or disintegration of bacterial membranes (6–8). The presence and relative quantity of such proteins serve as a diagnostic indicators of infection and inflammation (7). Non-pathogenic bacteria not inducing inflammatory responses may also be identified from urinary pellets (9).

Our laboratory reported the first metaproteomic analysis of urinary pellets derived from patients diagnosed with either asymptomatic bacteriuria or UTI, and identified the microbial causes of bacteriuria (9). Most recently, our laboratory developed a high throughput urinary sample preparation approach, 96FASP (96-well filter aided sample preparation), for quantitative shotgun proteomic analysis (10). This method promises to be the prototype of an economical method for the diagnosis of urinary tract infections and inflammation in the future. This article describes an extensively used, robust step-by-step procedure pertaining to the preparation of urinary pellet samples using the FASP approach. The optional 96FASP method that is adapted to process multiple samples simultaneously is described as well.

2. Materials

2.1 Cell Lysis and FASP

1. Sartorius Vivacon 500 filter device (30,000 MWCO).
2. MultiScreen Ultracel-10 Filter Plate 10 kD (Merck-Millipore, USA).
3. Bench top centrifuge (for example, Eppendorf 5415R or equivalent).
4. (optional) Plate centrifuge (for example, Eppendorf 5810R or equivalent).
5. Misonix Sonicator 3000 Ultrasonic Cell Disruptor.
6. Pre-casted SDS PAGE gel (for example, NUPAGE 4–12%).
7. SpeedVac.
8. TMN buffer: 40 mM Tris-HCl, pH 8.1, 5 mM MgCl₂ and 100 mM NaCl.
9. Lysostaphin solution: 10 mg/ml in water (AMBI Products; from *Staphylococcus simulans*).

10. Mutanolysin solution: 2 mg/ml in water (Sigma-Aldrich; from *Streptococcus globisporus*).
11. NaOH solution: 100 mM in water.
12. UA buffer: 8 M urea in 50 mM Tris-HCl, pH 8.1. UA buffer should be prepared freshly each day.
13. USED lysis buffer: 8M Urea, 1% SDS, 5 mM Na-EDTA, 50 mM DTT. USED buffer should be prepared freshly each day.
14. IAA solution: 0.05 M iodoacetamide in 50 mM Tris-HCl, pH 8.1. IAA solution should be prepared freshly each day.
15. ABC buffer: 50 mM ammonium bicarbonate in water.
16. Trypsin solution: trypsin (Promega sequencing grade); stock concentration 0.1 µg/µl and stored at -80°C.

2.2. StageTip desalting

1. Empore C₁₈ Extraction disks (3M, catalog number: 2215).
2. Activation buffer: 100% methanol.
3. Wash and equilibration buffer: 0.5% acetic acid in H₂O.
4. Elution solution-I: 0.5% acetic acid, 60% acetonitrile and 40% H₂O.
5. Elution solution-II: 0.5% acetic acid, 80% acetonitrile and 20% H₂O.

2.3. LC-MS/MS

1. XCalibur software (version 2.2, Thermo Scientific).
2. Proteome Discoverer (version 1.4, Thermo Scientific).
3. HPLC solvent A: 0.1% formic acid in water.
4. HPLC solvent B: 0.1% formic acid in acetonitrile.
5. Trap column: C18 PepMap100, 300 µm × 5 mm, 5µm, 100 Å (Thermo Scientific, USA).
6. Analytical column: PicoFrit, 75 µm × 10 cm, 5 µm BetaBasic C18, 150 Å (New Objective, USA)
7. Ultimate 3000 nano-LC system coupled to Q-Exactive mass spectrometer (Thermo Scientific, USA).

3. Methods

An overview of the protein sample preparation for urinary pellets is provided in Figure 1, as explained in detail in the following procedures. The schematic also shows the downstream applications (e.g., LC-MS/MS and database search) for urinary proteome analysis.

3.1. Lysis of urinary pellets and collection of lysates

1. Take urine samples (see Note 1), centrifuge at 3,000 x g for 15 min at 4°C, discard the urine supernatant and recover the pellet, retaining ~1.0 ml of residual urine supernatant to avoid disturbing the pellet.
2. Add ~10 ml ice-cold phosphate -buffered saline PBS, gently shake the tube and centrifugation for another 15 min at 3,000 x g. Discard the supernatant and store the wet pellet in –80°C or process immediately.
3. Add USED buffer (see Note 2) consistent with an approximate volume ratio of 4:1 (buffer volume/urinary pellet volume) to lysis cells and solubilize the contents in the urinary pellet. If the urinary pellet volume is very small, a minimum of 100 µl USED buffer volume can be added.
4. Vortex vigorously for 10 sec a few times to re-suspend the pellet; pipette up and down to re-suspend the pellet if the vortexing step was not sufficient to homogenize the pellet. Incubate for 30 min at room temperature and vortex or flick tube occasionally.
5. Using Misonex Sonicator (with the water bath is attached, not the probe), put the urinary lysate into an ice/water filled water bath. Set the program on amplitude 9 for nine 45-sec cycles with 45-sec intermittent cooling of the urinary lysate sample(s).
6. Let the urinary lysate sample(s) sit for approximately five more minutes. Spin urinary lysate sample(s) in a bench-top centrifuge at maximum speed (14,000 x g) for 10 min, and transfer lysate supernatant to a new 1.7 ml microtube (see Note 3). Freeze the urinary lysate at –80°C if necessary.

3.2. FASP processing urinary pellet samples for shotgun proteomics

1. Before processing pellet lysate using FASP, flush the filters with 200 µL NaOH (100 mM) and 200 µL UA buffer once separately.
2. Aliquot a volume equivalent to 10~100 µg total protein quantity into Vivacon 500 filter device, and mix with 200 µL UA buffer.
3. Spin at 14,000 x g for 10 to 30 min.
4. Add 200 µL UA buffer and repeat the spin at 14,000 x g to concentrate until the volume in the filter unit is reduced to ~10 µl.
5. Add 100 µL of the IAA solution (final concentration 50 mM) and mix by vortexing filter unit for 1 min. Incubate without mixing for 20 min in the dark at ambient temperature.
6. Spin at 14,000 x g for 10 to 30 min. Discard flow-through from collection tube.
7. Add 200 µL of UA buffer and spin at 14,000 x g for 10~30 min. The final sample volume in the filter unit should be 20 µL or less.

8. Add 200 μ L of ABC buffer and spin at 14,000 x g for 10~15 min. Add 200 μ L of ABC buffer and repeat spin step once more.
9. Add 100 μ L of ABC buffer to filter unit and trypsin so that the final protein:trypsin ratio is 100:1. Mix by vortexing for 1 min. Transfer the filter to a new filter collection tube.
10. Incubate at 37°C overnight in a temperature-controlled incubator (12~16 hours).
11. On the next day, add 200 μ L of ABC buffer, spin the filter unit at 14,000 x g for 10 to 15 min and collect the filtrate (with the peptide mixture) into a maximum recovery tube.
12. Add 100 μ L of ABC buffer to filter unit and trypsin so that the final protein:trypsin ratio is 100:1, and re-incubate the tube at 37°C for another 4 to 6 hours.
13. Add 200 μ L of ABC buffer, spin the filter unit after this second digestion step at 14,000 x g for 10 to 15 min and collect the second filtrate (with the peptide mixture) into the same maximum recovery tube;
14. Add another 200 μ L of ABC buffer, spin the filter unit after the wash step at 14,000 x g for 10–15 min and collect the wash (with residual peptides) into the same maximum recovery tube. The final volume in the collection tube is ~600 μ L.
15. Dry the peptide solution using a Speed-Vac (this may take 2~3 hours). The tryptic peptide mixture is ready now for desalting with the StageTip method.

3.3. (optional) 96-well filter plate processing urinary pellet samples for shotgun proteomics

1. Instead of using individual Vivacon filters to process urinary pellet samples separately, one can use 96-well filter plate to process multiple samples simultaneously. This could be a good option when tens or hundreds of samples have to be analyzed (10).
2. All the following procedures are the same as described above for single filter processing; there are a couple of changes:
 1. Each spin takes place on plate centrifuge at 2,600 x g for 45~90 min;
 2. The collection plate should be polypropylene based (e.g., to reduce possible non-specific bindings);
 3. The lid of the 96-well filter plate cannot seal the plate very well; to avoid sample drying out after overnight incubation, parafilm could be used to warp and tightly seal the lid. In the meantime, at least 100 μ L ABC buffer should be added into each well for overnight digestion.

3.4 Peptide desalting using StageTip protocol

1. This method is adapted from a published protocol (11); several changes have been made to optimally fit the preparation of urinary pellet samples (see Note 4).

2. Prepare StageTip by punching out small discs (1~3 layers depending on the sample amount) of C18 Empore filter using a 22 G flat-tipped syringe and ejecting the discs into P200 pipette tips. Ensure that the disc is securely wedged in the bottom of the tip.
3. Activate a tip by forcing 200 μ L methanol through the tip. Use this step to check if the StageTip is leaky or overtight.
4. Force 200 μ L Elution Solution-II through the tip.
5. Equilibrate tip by forcing 200 μ L Wash and equilibration buffer through the tip. The tip is now ready for sample loading.
6. Resuspend the dried peptides into 100 μ L of Wash and equilibration buffer, and vortex for 10 min.
7. Load the 100 μ L peptide solution - made in step (5) above - by forcing them through the C18-StageTip. Do not discard the flow-through, but collect it into the original tube.
8. Reload the peptide solution onto the tip. This step may be repeated 2 or 3 times.
9. Wash the tip with 200 μ L Wash buffer, and repeat this step 1 or 2 times. The flow-through during this step can be discarded.
10. Elute the peptides with 200 μ L Elution Solution-I (once) and 200 μ L Elution Solution-II (twice). Collect all of the eluates (~600 μ L) into one maximum recovery microtube.
11. Dry the peptide eluates in the Speed-Vac (this may take 1~2 hours).
12. Store then at -80°C , or re-suspend it with LC solvent A for immediate LC-MS/MS analysis.

3.5 Nano-LC-MS/MS and computational analysis

1. Resuspend the dried peptide samples into 20~50 μ L LC solvent A, centrifuge at maximal speed for 3~5 minutes, transfer to sample vials and load to HPLC autosampler.
2. The LC-MS/MS analysis is operated by an Ultimate 3000 nano LC system and Q Exactive mass spectrometer. Around 2~5 μ L of the samples were first loaded onto a trap column at high flow rate 5 μ L/min, and then separated on a PicoFrit analytical column at a nano flow rate of 300 nL/min. For a two-hour LC-MS run, a linear gradient was applied from 100% solvent A to 35% solvent B over 100 min, followed by a steeper gradient to 80% solvent B over 15 min. The column was re-equilibrated with solvent A for 5 min. Eluting peptides were acquired in data-dependent mode using XCalibur software and top10 method as described before (10).
3. The acquired raw files are then processed using the Proteome Discoverer software. The protein database involved in this study contain UniProt human protein sequences and common urinary tract pathogens (12). They can be

downloaded from UniProt website (<http://www.uniprot.org/>). MS search parameters are similar to published previously (10).

4. Figure 2 shows a typical nanoLC-MS/MS analysis of the urinary pellet samples. The base peak of the TIC shows the eluted peptides from HPLC column are acquired by mass spectrometer in 120 min. Shown in the upper panel is a representative full MS scan collected at retention time 75.28 min. The mass of each peak (peptide) are accurately measured by Orbitrap-based mass analyzer at high resolution (e.g., 70,000), and then sequenced in HCD-based collision cell. The resulting fragments are measured again by Orbitrap analyzer with high resolution (e.g., 17,500). With high quality MS and MS/MS data (mass errors are shown for each peak the figure), database search engine (e.g., Sequest) will assign each peak a unique amino acid sequence and its corresponding protein, as indicated by yellow triangle (▼) in the MS scan. The peak $m/z=846.0721$ is magnified in the right window. The cysteine in protein TRFL has a carbamidomethyl modification. The gray diamond (◆) shows a keratin peptide (VDALMDEINFMK, $\delta = -2.74$ ppm; [K2C5_HUMAN]). In this single LCMS analysis, 2,198 proteins, including human (1,139) and bacterial proteins (1,059), were successfully identified.

4. Notes

1. Usually 5~50 ml of urine is collected from human subjects. The preferred approach is to process the urine sample immediately after collection by centrifugation (see step 2). It is possible to store the urine at 4°C for up to 6 hours before centrifugation. Finally, the entire urine sample may be stored at -80°C at the clinical site, shipped to the site of proteomic analysis and thawed prior to centrifugal separation of urinary supernatant and urinary pellet. The freeze-thaw step may alter the composition of the urinary pellet. For a given project with multiple samples, a distinct urine storage and processing method should be selected.
2. In the case that Gram positive bacteria with thick cell walls, such as *Streptococcus pneumoniae* and *Staphylococcus aureus*, are suspected to be present in urine samples, resuspend the urinary pellet samples in a volume of TMN buffer to have an approximate volume ratio of 1:10, usually less than 200 μ l. Pipette the suspension up and down a few times in a 1.5 ml tube. Add lysostaphin and mutanolysin to a final concentration of 20 μ g/ml. Mix gently to homogenize the enzymes and suspended cells. Incubate the sample in the 37°C shaker-incubator. Take out the tube out every 30 min and check if a pellet collects at the bottom. If so, briefly vortex every 30 min. Complete digestion after a 3h incubation.)

After pre-treatment with lysostaphin and mutanolysin, add up to 600 μ l USED buffer into the lysate.
3. To estimate the protein concentration, take 10 μ l aliquot of the supernatant to another new microtube, mix with SDS loading buffer and run it in a SDS-PAGE

gel. Load 2 µg and 5 µg BSA standards in the same gel. Coomassie Blue (CB)-G250 stain the gel followed by its destaining with standard procedures (13). From the overall CB-G250 staining intensity of urinary pellet lysate bands, estimate the total protein amount in the lane based on BSA staining intensities. The protein concentration could also be measured by tryptophan fluorescence as reported before (14).

4. Instead of using syringe to manually push solvents through the StageTips, one can use pipette tip adaptors (commercially available from The Nest Group, MA) which fit the 1.5-mL or 2.0-mL microtubes well. This way, all the processing steps with syringe can now be done using bench-top centrifuge (15).

Abbreviations

FASP	filter aided sample preparation
HCD	higher energy collision disassociation
LC	liquid chromatography
MS	mass spectrometry
TIC	total ion chromatogram
UTI	urinary tract infection
MWCO	molecular weight cut off

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Figure 1.

An overview of the urinary pellet sample preparation for shotgun proteomics. The procedures are explained in detail the chapter. Briefly, the urine samples are first spin down to collect pellet fraction. The pellets are then lysed and digested following FASP protocol. A protocol of 96-well format FASP is also illustrated. Afterwards, the protein digests are cleaned using StageTip and analyzed by nanoLC-MS/MS. The host and pathogen protein identifications can be obtained by searching a metaproteome database which includes human proteins as well as the proteins of common pathogens related to your study (in this case, the urinary tract infection).

Figure 2.

A typical LC-MS analysis of the urinary pellet sample. The full mass (MS) and fragmentation (MS/MS) are recorded by high resolution and high accuracy mass spectrometer. A representative MS scan at a given time (for example, $t = 75.3$ min, as indicated by the red bar and arrows in the lower panel) with mass and charge state information (for example, $m/z=668.3510$, $z=2$) is shown in the middle panel. Most of the ions in this spectrum will be isolated and fragmentized afterwards. Then, the bioinformatics tool (for example, Sequest algorithm) will assign the most confident amino acid sequence to each peak based on sophisticated scoring system. Detailed explanations of the figure are provided in the chapter.