MStern Blotting–High Throughput Polyvinylidene Fluoride (PVDF) Membrane-Based Proteomic Sample Preparation for 96-Well Plates*§

Sebastian T. Berger‡¶, Saima Ahmed‡¶, Jan Muntel‡¶, Nerea Cuevas Polo‡¶**, Richard Bachur§, Alex Kentsis‡‡, Judith Steen, and Hanno Steen‡¶§§

We describe a 96-well plate compatible membrane-based proteomic sample processing method, which enables the complete processing of 96 samples (or multiples thereof) within a single workday. This method uses a large-pore hydrophobic PVDF membrane that efficiently adsorbs proteins, resulting in fast liquid transfer through the membrane and significantly reduced sample processing times. Low liquid transfer speeds have prevented the useful 96-well plate implementation of FASP as a widely used membrane-based proteomic sample processing method. We validated our approach on whole-cell lysate and urine and cerebrospinal fluid as clinically relevant body fluids. Without compromising peptide and protein identification, our method uses a vacuum manifold and circumvents the need for digest desalting, making our processing method compatible with standard liquid handling robots. In summary, our new method maintains the strengths of FASP and simultaneously overcomes one of the major limitations of FASP without compromising protein identification and quantification. Molecular & Cellular Proteomics 14: 10.1074/mcp.O115.049650, 2814–2823, 2015.

Mass spectrometry (MS)-based proteomics is moving increasingly into the translational and clinical research arena, where robust and efficient sample processing is of particular importance. The conventional sample processing methods in proteomics, namely SDS-PAGE, or in-solution-based sample processing, are slow and laborious and thus do not easily provide the reproducibility and throughput to meet current demands. A paradigm shift was the introduction of a filter-aided sample processing method (FASP), which is initially described by Manza et al. (1) and then fully realized in practice by Wisniewski et al. (2). These filter-aided methods make use of ultrafiltration membranes with molecular weight cut offs (MWCO) in the 10 to 30 kDa range to efficiently remove small molecules and salts and to capture denatured proteins on a cellulose filter even if the molecular weight of the protein is much smaller than the nominal MWCO of the ultrafiltration membrane. Thus, the denaturation step is crucial to ensure that proteins much smaller than the nominal MWCO are efficiently retained by, e.g. a 10 kDa MWCO filter.

In translational and clinical proteomics, which normally include large cohorts, the multititer-well plate is the preferred format for sample processing and storage. Although the application of FASP in the 96-well plate format has been described (3, 4), the major limitation of FASP in the 96-well plate is the much slower speed at which the 96-well plates have to be centrifuged: while a single ultrafiltration unit withstands up to 14,000 × g, the 96-well plate format can only be centrifuged at g-forces of up to 2,200 × g. This significantly lower g-force for 96-well plates results in a slow liquid transfer, which in turn considerably prolongs the required centrifugation times to hours instead of tens of minutes for the three to four necessary centrifugation steps (i) for the initial loading, reduction and alkylation, (ii) for the different washing steps, and (iii) for the elution (3).

Independent of the format FASP is performed in, the conventional FASP also requires relative large volumes of high salt concentration for efficient elution of the tryptic peptides. Hence, reversed-phase-based desalting of the samples is a prerequisite for subsequent LC/MS experiments. Apart from prolonging the entire FASP procedure, the numerous addi-
tional handling steps are potentially also associated with peptide losses (5).

In this study, we describe a novel sample processing workflow for MS-based proteomics that utilizes the strengths of filter-aided sample processing methods and at the same time overcomes their major limitations, without compromising the results, i.e. significantly reducing the number of identified peptides and/or proteins. The result is a significantly improved throughput as 96 samples (or multiples thereof) can be completely processed within a single workday.

EXPERIMENTAL PROCEDURES

Cell Culture—Human cervical cancer cells (HeLa) were propagated in Dulbecco's modified Eagle's medium (DMEM; 11965; Invitrogen, Waltham, MA). Upon achieving 85–90% confluence, the growth media were aspirated and the cells were washed three times with 5 ml ice-cold PBS. One ml of modified RIPA buffer (150 mM NaCl, 50 mM Tris/HCl (pH 7.4), 1% Nonidet P-40, 0.1% sodium deoxycholate, 1 mM EDTA) supplemented with 1X Roche Complete protease inhibitors was added to each plate of cells and incubated for 30 min on ice. Cells were scraped with a cell scraper, collected in Eppendorf tubes, and vortexed for 1 min. Cellular debris and other particulate matter was pelleted by centrifugation at 20,000 × g at 4 °C; the supernatant was recovered for further use.

Urine Collection for Ovarian Cyst Biomarker Study—Urine samples were collected from consenting patients visiting the emergency department at Boston Children's Hospital. The study was reviewed and approved by Boston Children's Hospital's Internal Review Board (Protocol Number X06-10-0493).

Protein Concentration Determination—Protein concentration was determined by using the Bradford Assay (6) (Bio-Rad DC™ Protein Assay) following the manufacturer's protocol. The standard curve was established using a stock solution of 20 mg/ml bovine serum albumin and final concentrations of 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, 1.5 mg/ml, and 2.0 mg/ml. After incubation at room temperature, the final measurement was performed in a microplate spectrophotometer (Bio-Rad Model 680) at a wavelength of 595 nm.

MSTern Blast—Undiluted neat urine (150 µl, i.e. ~15 µg of protein) was added to a mixture of 150 µg urea and 30 µl dithiothreitol (DTT) (100 µm in 1X Tris/HCl, pH 8.5). Diluted HeLa cell lysates (10 µg in 100 µl 50 mM ammonium bicarbonate (ABC)) or neat CSF (10 µl, i.e. ~10 µg of protein) was added to 100 µg urea and 20 µl DTT. The resulting solution was incubated for 20 min at 27 °C and 1,100 rpm in a thermos mixer. Reduced cysteine side chains were alkylated with 50 mM iodoacetamide (final concentration) and incubated for 20 min in the dark at 27 °C and 750 rpm.

The hydrophobic PVDF membrane in a 96-well plate format (MSIPS4510, Millipore, Milpore) was prewetted with 150 mM NaCl, 50 mM Tris/HCl (pH 7.4), 1% Nonidet P-40, 0.1% sodium deoxycholate, 1 mM EDTA) supplemented with 1X Roche Complete protease inhibitors and incubated for 2 h at 37 °C in a humidified incubator. After incubation for 2 h at 37 °C in a humidified incubator, the remaining digestion buffer was evacuated. Resulting peptides were eluted twice with 150 µl of 40% ACN (v/v)/0.1% (v/v) formic acid (FA) each. Upon pooling, the peptide solutions were dried in a vacuum concentrator. Lyophilized samples were stored at −20 °C until further analysis.

Of note: We tested different batches of filter plates and did not observe batch dependent performance differences.

Filter-Assisted Sample Preparation (FASP)—The filter-assisted sample preparation method was carried out as previously described (2). In short: Proteins were first denatured and reduced by adding 100 µl sample to 100 µl urea supplemented with 20 µl DTT. For the different sample types, namely urine, CSF, and HeLa lysate, we used a nominal protein content of 15 µg, 10 µg, and 10 µg, respectively, for analysis. After alklylation of reduced cysteine side chains with 50 mM iodoacetamide (final concentration), denatured peptides were captured on a 10 kDa MWCO spin filter (MRCPRT010, Millipore) and washed twice with 50 mM ABC. Protein digestion was performed with sequencing grade trypsin (V5111, Promega, Madison, WI) at a nominal enzyme to substrate ratio of 1:50. After incubation overnight with 100 µl digestion buffer (trypsin in 50 mM ABC), resulting peptides were eluted with 300 µl 0.5 M NaCl.

Peptide elutes were desalted with reversed-phase-based TARGA C-18 spin tips (SEMSS18R, Nest Group, Southborough, MA) prior to LC-MS/MS analysis. Lyophilized samples were stored at −20 °C until further analysis.

LC-MS/MS Analysis—Peptides were reconstituted in loading buffer (5% ACN (v/v), 5% FA (v/v)). LC-MS/MS analysis was performed on a microfluidic chip system (EK425) coupled to a TripleToF 5600+ mass spectrometer (both Sciex, Framingham, MA). Tryptic digests (~1 µg) were loaded onto a trap column (ReproSil-Pur C18-AQ, 200 µm × 0.5 mm, 3 µm) and subsequently separated on a ReproSil-Pur C18-AQ analytical column chip (75 µm × 15 cm, 3 µm) at a flow rate of 300 nL/min. A linear gradient from 95% to 65% buffer A (0.2% FA in HPLC water; buffer B: 0.2% FA in ACN) within 60 min was applied. Samples were ionized applying 2.3 kV to the spray emitter. Analysis was carried out in a data-dependent mode. Survey MS1 scans were acquired for 200 ms. The quadrupole resolution was set to “UNIT” for MS2 experiments, which were acquired for 50 ms in “high sensitivity” mode. The following switch criteria were used: charge: 2+ to 4+; minimum intensity: 100 counts per second. Up to 35 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 17 s.

Samples for the ovarian cyst biomarker study were analyzed using the same LC-chip system (Sciex, trapping column: 200 µm × 0.5 mm ReproSil-Pur C18-AQ 3 µm; analytical column: 75 µm × 15 cm ReproSil-Pur C18-AQ 3 µm) coupled online to a Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Peptides (4 µl of digest) were separated by a linear gradient from 93% buffer A (0.2% FA in HPLC water)/7% buffer B (0.2% FA in ACN) to 75% buffer A/25% buffer B within 75 min. The MS was operated in a data-dependent mode. Survey MS1 scans were acquired for 200 ms. The quadrupole resolution was set to “UNIT” for MS2 experiments, which were acquired for 50 ms in “high sensitivity” mode. The following switch criteria were used: charge: 2+ to 4+; minimum intensity: 100 counts per second. Up to 35 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 17 s.

Acquired MS raw files (WIFF) were analyzed using ProteinPilot (version 4.5.1, revision 2768; Paragon™ Algorithm 4.5.1.0, 2765; Sciex) using the human UniProtKB database (Homo sapiens).
FASP versus MStern blot—Filter-based sample processing in general and FASP in particular have established themselves as widely used proteomics sample processing/digestion methods due to their improved sensitivity and throughput when compared with gel-based digestion methods and due to their improved compatibility with salts, detergents, and other denaturants in comparison to conventional solution-based processing methods. Despite a multitude of advantages, FASP or FASP-like methods have the drawback of not being readily compatible with 96-well plate formats because of the small pore size of the cellulose-based ultrafiltration membranes. This gives rise to very long centrifugation times when used in the 96-well plate format due to the limited g-force 96-well plates can withstand: 2,200 × g for 96-well plates versus 14,000 × g for individual ultrafiltration units. Cellulose ultrafiltration membranes that are used for the FASP approach feature a pore size of 1–3 nm (10 to 30 kDa MWCO), giving rise to this significant flow restriction.

We reasoned that switching to large pore hydrophobic PVDF membranes, which have a proven high affinity to proteins that is utilized in Western blotting on a daily basis, would overcome the problem of slow liquid transfer through the membrane. These hydrophobic PVDF membranes are used for sterilization and/or filtration feature pores in the size range of 220 to 450 nm (Fig. 1A), which is 100 times larger than the pores found in the 10 or 30 kDa MWCO ultrafiltration cellulose membranes used for, e.g. FASP. The capacity of PVDF-based protein processing is theoretically in the 25 μg/well range (100
µg/cm² (12)), which is lower than in cellulose-membrane-based filtration units. Given the sensitivities of current LC/MS systems, this amount is still plentiful where rarely more than 1 µg is injected per analysis run. The main difference between cellulose membrane and PVDF for membrane-proteomics sample processing is the mode of protein retention: while ultrafiltration cellulose membranes retain the proteins by size-based filtration, PVDF retains the proteins by efficiently adsorbing them onto its large hydrophobic surface, which is widely exploited in e.g. Western blotting, giving rise to our suggested name “MSTern blotting.”

After establishing an initial protocol for MSTern blotting, we evaluated whether the theoretical processing speed advantage can be realized in practice. Indeed, the use of low-grade (e.g. house) vacuum was sufficient for the liquid transfer through the PVDF membrane in 10 s to 2 min (depending on the numbers of wells used). In contrast, the centrifugation-based liquid transfer through ultrafiltration cellulose membranes in the 96-well plate format, which requires swinging-bucket rotors, requires 1 to 2 h (3, 4) for each liquid transfer step. In summary, the fast liquid transfers through the PVDF membrane can be realized in practice and is the major source of time savings for the MSTern blotting sample processing in comparison to FASP (Fig. 18).

Besides a faster liquid transfer through the membrane, further time savings are realized by the post digestion peptide elution, which uses a simple mixture of acetonitrile and formic acid instead of concentrated salt solutions as in the case of FASP. The residual amount of ammonium bicarbonate salts are further reduced by the subsequent quick vacuum centrifugation, such that the samples are ready for LC/MS analysis once they have been evaporated to dryness. In contrast, FASP requires a prolonged vacuum centrifugation and subsequent lengthy and expensive reversed-phase-based desalting of the digests. Together with the faster liquid transfers, all time savings add up to more than 9.5 h when processing samples with MSTern blotting instead of FASP. In addition, the use of vacuum manifolds also allows for easier automation when compared with FASP, which requires centrifugation.

Performance of MSTern Blot—After establishing that using hydrophobic PVDF for membrane-based proteomic sample processing instead of hydrophilic-regenerated cellulose as in the case of FASP results in significant time-savings, we investigated the compatibility of adsorption of complex protein mixtures with tryptic digestion. The digestion of individual proteins adsorbed onto PVDF membranes had been described before (13–16). However, these previous publications used electroblotting to transfer proteins out of an SDS-PAGE onto the PVDF membrane prior to proteolysis and mass spectrometric analysis. Thus, it was not evident that proteins from complex protein mixtures could be quickly adsorbed onto PVDF membrane by forcing dilute protein solutions through the membrane within seconds or minutes using vacuum instead of electroblotting, which operates in the hour timescale (16). To test whether fast adsorption of proteins to hydrophobic PVDF is compatible with proteomic studies on complex protein mixtures, we used three different types of samples that are either considered as a good source for biomarker (neat urine and neat cerebrospinal fluid (CSF)) as well as a highly complex fractionated whole (HeLa) cell lysate.

The initial digestion optimization resulted in conditions that match or exceed the performance of FASP; thus, we expect that a more thorough optimization will provide even better results (for details, see Material and Methods). We processed four aliquots for each sample type and analyzed tryptic digests of the different samples types by LC-MS/MS using a 1 h gradient. These analyses identify 497 ± 58, 2,733 ± 160, and 676 ± 143 proteins from neat CSF, HeLa cell lysate, and neat urine, respectively (Fig. 2A and Supplemental Tables 1 to 6). The FASP-based processing of four aliquots of the same samples resulted in 561 ± 40, 2,473 ± 89, and 622 ± 133 proteins for neat CSF, HeLa cell lysate, and neat urine, respectively. Also, the dynamic ranges of the identified proteins as determined using the iBAQ method (8) was similar for both sample processing methods: ~5 orders of magnitude of the two neat body fluids and ~6 orders of magnitude for the HeLa cell lysate (Fig. 2B). These numbers clearly showed that our MSTern blotting approach gives protein identification rates at least as good as FASP, irrespective of the nature and complexity of the sample.

Next, we tested the loading capacity of the PVDF membrane. To this end, we loaded 5, 10, 15, and 30 µg of HeLa cell lysate into individual wells of the 96-well plate with the hydrophobic PVDF membrane at the bottom of each well. The flow throughs of the loading and washing solutions were collected and subsequently processed using FASP. In parallel, identical amounts of protein were directly processed with FASP (Fig. 2C).

In summary, FASP and MSTern blotting resulted in similar number of identified proteins (while MSTern consistently identified more than FASP as already shown in Fig. 2A), irrespective of the amount of protein processed. In contrast, the number of proteins identified in the flow through of the MSTern-blotting-based processing steadily increases such that, at a nominal loading of 30 µg, as many proteins are identified in the flow through as in the adsorbed fraction. Based on these numbers, we concluded that the optimal loading capacity is in the 10 to 15 µg per well range, plenty of material for today’s LC/MS instrumentation. These amounts of proteins correspond to e.g. 150 µl of urine or 15 µl of CSF, which are easily available from primary body fluids. Thus, we did not perform additional experiments to test the lower loading limits of MSTern blotting.

Detecting Biases in Proteins Identified in Method-Specific Samples—Since MSTern blotting and FASP have very different modes of retention, we reasoned that both methods will exhibit different preferences for protein identification. To look into this issue, we compared the identification overlap from
the combined search results of the four MStern blots and four FASP preparations of neat urine, HeLa cell lysate, and neat CSF, which were used to generate Fig. 2A. The Venn diagrams clearly show that 2/3 to 3/4 of the identified proteins are shared between the MStern blotting and the FASP method, while 1/4 to 1/3 of the proteins are unique to either MStern blotting or FASP (Fig. 3A-3C). We also compared the commonalities and differences at the peptide level. Here, specific peptides were in the 50 to 60% range such that only down to 40% of the observed peptides were in common.

For the subsequent GO annotation of the method-specific proteins, we used the funrich.org tool, which uses more broadly defined ontologies to make comparisons more generalizable. Figure 3 shows the results of these comparative protein localizations, whereby only the 12 most populated GO terms are listed. For neat urine and HeLa cell lysates, only minor differences are observable for the major GO terms. Slightly bigger differences are observable for the neat CSF, such as MStern blotting’s biases against plasma membrane and extracellular proteins and preferences for nucleolar, mitochondrial, and/or cytosolic proteins. However, a clear trend is not obvious.

Physicochemical Properties—To better understand the process-specific differences in the identified proteins and peptides, we took a closer look at the physicochemical properties of the unique and shared proteins and peptides (Fig. 4—the graphs for the HeLa cell lysates are shown; the graphs for CSF and neat urine can be found as Supplemental Figs. 1 and 2). In particular, we compared the molecular weight, the pI and the hydrophobicity/GRAVY score. For reference purposes, we also show the theoretical distribution of all human proteins found in SwissProt. Comparing the plots for the proteins (Fig. 4A), it is apparent that FASP is biasing in favor of small (low molecular weight), charged (higher and lower pI) and more hydrophilic (lower GRAVY score) proteins. In contrast, MStern blotting has a slight preference for larger and less-charged proteins. These observed dissimilarities match
the differences in the binding modes used for the two sample processing strategies.

Comparing the physicochemical properties of the peptides (Fig. 4B) identified a major shift of the molecular weight of the MStern-blotting-specific peptides. The MStern-blotting-specific peptides also showed a shift away from lower pI values in favor of higher pI values above a pI of 6.8 and a minor shift toward less-hydrophilic peptides. The latter was unexpected, as larger peptides are generally assumed to be more hydrophobic. For reference purposes, we also show the distributions for the theoretical tryptic peptides from all human proteins found in SwissProt assuming no missed cleavage or two missed cleavages.

Investigating the major shift in the molecular weight distributions of the observed process-specific peptides revealed an increase in peptides with missed cleavages from 12.5% to 37.4% for the MStern blotting versus FASP. Attempts to modulate the degree of missed cleavages by varying the content of organic solvent (17) and/or the digestion time had only minor effects, which might indicate that the adsorption of the proteins seems to interfere with the trypsinization.

**Protein Quantification**—Since this degree of missed cleavages can affect the quantification of individual peptides that are affected by being missed cleaved, we investigated the effect on the quantification of proteins, which normally uses the combined information from numerous peptides. To this end, we took a closer look at two technical repeats of the HeLa cell lysates, neat urine, and neat CSF digested using the MStern blotting and the FASP process (Fig. 5).

Next, we extracted the peptide ion signal intensity for each protein, prior to correlating the intensities for MStern blotting versus MStern blotting (blue), for FASP versus FASP (yellow), and for FASP versus MStern blotting (green). The correlations for MStern versus MStern and FASP versus FASP were very tight, with R²-values ranging from 0.85 to 1.0. The lower correlation for the HeLa lysate had to be expected given the complex nature of the samples, which results in undersam-
pling and highlights the negative effect of the stochastic nature of unbiased data-dependent acquisition routines on protein quantification, which is particularly limiting in the case of low abundant proteins. However, this limitation is independent of the sample processing but can probably be improved when using e.g. nonstochastic data-independent acquisition routines.

The correlation of MStern versus FASP showed a slightly broadened scatter, with $R^2$-values ranging from 0.92 to 0.99 for urine and CSF and 0.67 for the HeLa cell lysate. Such slight reduction in correlation is expected when comparing two independent sample processing method; nevertheless, the good to excellent correlations of the MStern versus FASP-based quantification clearly show that the increase in missed cleavage sides as observed for MStern-blotting-based processing still provides solid quantitative information comparable to and compatible with FASP-based processing.

**Ovarian Cyst Biomarker Study**—Using this newly developed MStern blotting strategy, we processed 89 individual urine samples (150 µl of each) collected from pediatric patients of the Emergency Department at Boston Children’s Hospital seen for abdominal pain with the aim to identify urinary mark-
ers for the different causes of abdominal pain. In this particular study, we were looking for urinary markers for ovarian cysts. While ovarian cysts in children and adolescent are common, normally asymptomatic, and easily detected by ultrasound, it is crucial to differentiate in a timely fashion ovarian cysts from other conditions that require surgical intervention such as appendicitis or ovarian torsion. Thus, other means than slow imaging to quickly differentiate nonsurgical conditions such as ovarian cysts from surgical conditions are highly desirable. To this end, neat urine samples, i.e. samples without any preprocessing and/or protein concentrations determination, were processed using our MStern blotting strategy. The entire processing from the neat urine sample to the LC/MS ready sample took less than a workday (Fig. 1).

After analyzing these 89 samples (additional information in Supplemental Table 7) with a 75-min gradient using a Q-Exactive mass spectrometer, the data were searched with MaxQuant (9) and quantified using the iBAQ approach (8). Before further analysis, we excluded five samples (three ovarian cyst samples) in which we identified less than 200 proteins, probably due to low urinary protein concentrations. Of the remaining 84 urine samples, 10 were associated with ovarian cysts and 74 with alternative causes of abdominal pain. In total, 2,070 urinary proteins were identified in this sample set. A following statistical analysis (t test) identified 11 proteins, which indicated statistical significance even after extremely conservative Bonferroni correction for multiple testing.

Among these proteins that showed statistical significant abundance differences were two related serpins, namely serpin B3 and B4, also called squamous cell carcinoma antigen 1 and 2 (Fig. 6A). Although serpins, in particular serpin E1, have been associated with e.g. polycystic ovary syndrome (18, 19), serpin B3 and 4 have so far only been described in cystic teratomas of the ovary (20). Further studies beyond the scope of this work will be necessary to understand the biological and biomedical implications of this finding. However, further analysis of the receiver-operating characteristic curves

Fig. 5. Correlation of FASP- and MStern-blotting-based protein quantifications. Correlation of the ProteinPilot-derived signal intensities of the proteins identified in CSF, HeLa lysate and urine (see Fig. 2): MStern blot versus MStern blot (left), FASP versus FASP (middle), and MStern blot versus FASP (right).
and box plots (AUROC SPB3 = 0.920; SPB4 = 0.928; Fig. 6B) might indicate a promising application of these proteins as a rule-out test of ovarian cysts in pediatric patients.

**Conclusion and Perspective**—Exploiting the high protein-binding capacity of hydrophobic PVDF, which is also commercially available in the form of 96-well filtration plates, we devised a 96-well-plate-based sample processing method, which allows for the complete processing of multiples of 96 samples in a workday or less. The major time advantages compared with e.g. FASP-based protocols are the fast liquid transfers and the omission of the need for desalting digests prior to loading onto an LC/MS system. The former is the result of the 100 times larger pores when compared with ultrafiltration membranes with appropriate molecular weight cutoffs. The latter was enabled by the efficient elution with organic solvents instead of high salt concentrations. This accelerated sample processing allows generating LC/MS-ready peptide samples, starting from 89 urine samples were processed within a single workday. The subsequent analysis of these urine samples identified two serpins as potential biomarker candidates for ovarian cysts based on their highly significant differential abundance in urine from ovarian cyst patients versus abdominal pain controls.

In summary, MStern blotting is an ideal method to process dilute samples such as neat urine for downstream proteomic analysis, which lends itself to easy automation. Even though we notice a particularly advantageous application to dilute samples such as urine, MStern is applicable to a wide range of samples without sacrificing analytical depth or quantitative nature of the data.

**Acknowledgments**—We thank Waltraud Mair, Omar Barnaby, and Kevin Broadbelt for insightful discussions and critically reading the manuscript.

*The TripleTOF 5600 used for some of the experiments was funded by a US National Institute of Health Shared Instrumentation Grant S10OD010706 to HS. Part of this work was made possible by a grant from the Harvard Institute of Translational Immunology, and through support from the Leona M. and Harry B. Helmsley Charitable Trust. This article contains supplemental material Supplemental Tables 1–7 and Supplemental Figs. 1 and 2.*
REFERENCES

1. Manza, L. L., Stamer, S. L., Ham, A. J., Codreanu, S. G., and Liebler, D. C. (2005) Sample preparation and digestion for proteomic analyses using spin filters. Proteomics 5, 1742–1745

2. Wiśniewski, J. R., Zougman, A., Nagaraj, N., and Mann, M. (2009) Universal sample preparation method for proteome analysis. Nature Meth. 6, 359–362

3. Switzer, L., van Angeren, J., Pinkse, M., Kool, J., and Niessen, W. M. (2013) A high-throughput sample preparation method for cellular proteomics using 96-well filter plates. Proteomics 13, 2980–2983

4. Yu, Y., Suh, M. J., Sikorski, P., Kwon, K., Nelson, K. E., and Pieper, R. (2014) Urine sample preparation in 96-well filter plates for quantitative clinical proteomics. Anal. Chem. 86, 5470–5477

5. Naldrett, M. J., Zeidler, R., Wilson, K. E., and Kocourek, A. (2005) Concentration and desalting of peptide and protein samples with a newly developed C18 membrane in a microspin column format. J. Biomolecular Techniques 16, 423–428

6. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254

7. McKeon, T. A., and Lyman, M. L. (1991) Calcium ion improves electrophoretic transfer of calmodulin and other small proteins. Anal. Biochem. 193, 125–130

8. Schwanhäuser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen, W., and Selbach, M. (2011) Global quantification of mammalian gene expression control. Nature 473, 337–342

9. Cox, J., and Mann, M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nature Biotechnol. 26, 1367–1372

10. Vizcalno, J. A., Deutsch, E. W., Wang, R., Cserdos, A., Reisinger, F., Rios, D., Dianes, J. A., Sun, Z., Farrah, T., Bandeira, N., Binz, P. A., Xenarios, I., Eisenacher, M., Mayer, G., Gatto, L., Campos, A., Chalkley, R. J., Kraus, H. J., Albar, J. P., Martinez-Bartolomé, S., Apeviler, R., Omenn, G. S., Martens, L., Jones, A. R., and Hermjakob, H. (2014) ProteomeXchange provides globally coordinated proteomics data submission and dissemination. Nature Biotechnol. 32, 223–226

11. Baker, P. R., and Chalkley, R. J. (2014) MS-viewer: A web-based spectral viewer for proteomics results. Mol. Cell. Proteomics 13, 1392–1396

12. (2011) Sample Preparation in Biological Mass Spectrometry, 1st ed., Springer, New York

13. Sloane, A. J., Duff, J. L., Wilson, N. L., Gandhi, P. S., Hill, C. J., Hopwood, F. G., Smith, P. E., Thomas, M. L., Cole, R. A., Packer, N. H., Breen, E. J., Cooley, P. W., Wallace, D. B., Willams, K. L., and Gooley, A. A. (2002) High throughput peptide mass fingerprinting and protein macroarray analysis using chemical printing strategies. Mol. Cell. Proteomics 1, 490–499

14. Metho, R. M., Dufresne-Martin, G., Leclerc, P., Leduc, R., and Klarskov, K. (2005) Mass spectrometric peptide fingerprinting of proteins after Western blotting on polyvinylidene fluoride and enhanced chemiluminescence detection. J. Proteome Res. 4, 2216–2224

15. Fernandez, J., and Mische, S. M. (2001) Enzymatic digestion of proteins on PVDF membranes. Current Protocols in Protein Science, J. E. Coligan, B. M. Dunn, D. W. Speicher, P. T. Wingfield, and M. Chiu, eds., Wiley, Hoboken, NJ, Chapter 11, Unit 11.2

16. Eckerskorn, C., and Lottspeich, F. (1993) Structural characterization of blotting membranes and the influence of membrane parameters for electroblotting and subsequent amino acid sequence analysis of proteins. Electrophoresis 14, 831–838

17. Dickhut, C., Feldmann, I., Lambet, J., and Zahedi, R. P. (2014) Impact of digestion conditions on phosphoproteomics. J. Proteome Res. 13, 2761–2770

18. Lee, Y. H., and Song, G. G. (2014) Plasminogen activator inhibitor-1 4G/5G and the MTHFR 677C/T polymorphisms and susceptibility to polycystic ovary syndrome: A meta-analysis. Eur. J. Obstetrics Gynecol. Reprod. Biol. 175, 8–14

19. Koou, E., Tziomalos, K., Dinas, K., Katsikis, I., Kandaraki, E. A., Tsourdi, E., Mavridis, S., and Panidis, D. (2012) Plasma plasminogen activator inhibitor-1 levels in the different phenotypes of the polycystic ovary syndrome. Eur. J. Obstetrics Gynecol. Reproducive Biol. 159, 21–29

20. Miyazaki, K., Tokunaga, T., Katauchi, H., Ohba, T., Tashiro, H., and Okamura, H. (1991) Clinical usefulness of serum squamous cell carcinoma antigen for early detection of squamous cell carcinoma arising in mature cystic teratoma of the ovary. Obstetrics Gynecol. 78, 562–566

21. Kulak, N. A., Pichler, G., Paron, I., Nagaraj, N., and Mann, M. (2014) Minimal, encapsulated proteomic-sample processing applied to copy-number estimation in eukaryotic cells. Nature Meth. 11, 319–324