Supporting Information

A Universal and High-Throughput Proteomics Sample Preparation Platform

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Supplemental Methods

Stock Solution Preparation. Ammonium bicarbonate (ABC) stock solution was prepared by dissolving ammonium bicarbonate powder in MS-grade water to a 1 M solution then diluting the 1 M solution to a 25 mM solution. IAA stock solution was prepared by dissolving IAA powder in 25 mM ABC to a final concentration of 109.5 mM in a 5 mL, round bottom, sterile test tube. Trypsin stock solution was prepared by dissolving 20 µg trypsin powder in 25 mM ABC to a final concentration of 1.145 ng/µL. This was done by first dissolving trypsin in 2 mL of 25 mM ABC (2 mL of Rapid Digest Buffer for the Promega Rapid Digest protocol) in the commercially purchased sequencing grade trypsin vial then dissolving 1 mL of this trypsin solution in 7.73 mL in a Falcon 15 mL Conical Centrifuge Tube by manual pipetting. For the Promega Rapid Digest protocol, Rapid Trypsin Gold was used in place of sequencing grade trypsin. FA stock solution was prepared by diluting formic acid in a 5 mL, round bottom, sterile test tube with water to a final concentration of 10% v/v. The 1% RapiGest stock solution was prepared by dissolving 10 mg RapiGest powder in 1 mL of 25 mM ABC. The lysis-denaturation-reduction (LyDeR) rapid buffer stock solution was formulated by adding TCEP (5 mM), protease inhibitor cocktail (PIC; 1X), and RapiGest (0.1% m/v) to NP40 Cell Lysis Buffer with final component concentration shown is parentheses. A fresh LyDeR stock was made before each sample preparation batch with the required total volume depending on the number of samples, as well as additional amount to account for the dead volume present in liquid transfers from tubes, reagent reservoirs, and plates.

Consumables. The 384ST tips used for the Agilent Bravo Automated Liquid Handling System were obtained from Agilent Technologies (Santa Clara, CA, U.S.A.). Expell plus 0.2-50 µL low retention, sterile, filter pipette tips were obtained from Capp (Nordhausen, Thuringia, DE). Corning CellBIND 384-well flat clear bottom black, polystyrene, sterile, microplates with lids were obtained from Corning (Corning, NY, U.S.A.). The 0.5 mL and 1.5 mL Protein LoBind tubes were obtained from Eppendorf (Hauppauge, NY, U.S.A.). Falcon 15 mL conical centrifuge tubes were obtained from Fisher Scientific (Waltham, MA,

U.S.A.). The 5 mL, round-bottom, sterile test tubes were obtained from Greiner Bio-One (Monroe, NC, U.S.A.). Low retention, sterilized, RT-LTS tips (10, 250, and 1000 µL) were obtained from Mettler-Toledo (Columbus, OH, U.S.A.). Nunc 384-well polypropylene storage microplates and 25 mL Matrix reagent reservoirs with and without dividers were obtained from Thermo Fisher Scientific (Waltham, MA, U.S.A.). DMSO-resistant foil plate seals were obtained from Thomas Scientific (Swedesboro, NJ, U.S.A.). A full consumables list containing vendors and catalog numbers can be found in **Table S2**.

Equipment. Samples in microtiter plates were incubated, chilled, and vortexed on a Thermal Mixer with a 96-well microplate block (Thermo Fisher Scientific, Waltham, MA, U.S.A.) modified with a custom-made conductor block (NCATS Prototype Instrument Lab, Rockville, MD, U.S.A.). Some reagent dispensing was performed using a Multidrop Combi Reagent Dispenser (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Solvent removal was performed on a Centrivap Complete Vacuum Concentrator (Labconco, Kansas City, MO, U.S.A.). Solutions were mixed on the Vortexer (Heathrow Scientific, Vernon Hills, IL, U.S.A.). A full instrument list containing vendors and catalog numbers can be found in **Table S3**.

Cell Culture. HeLa cells (ATCC CCL-2) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, U.S.A.). Six different densities of cells (500, 1K, 3K, 5K, 7K, 10K per well) were dispensed into a 384-well black clear-bottom assay plate (Corning, Corning, NY, U.S.A.) with 30 µL of culture medium containing 5% fetal bovine serum (FBS). After a 24 h incubation at 37 °C and 5% CO_2, the plate was washed 3 times with cold 1XPBS by BioTek EL406 Plate Washer (Winooski, VT, U.S.A.), and was stored in -80 °C freezer prior to use.

Human primary neonatal keratinocytes and keratinocyte growth media were purchased from Sciencell (Carlsbad, CA, U.S.A.). HEK 293 cells were purchased from ATCC (Manassas, VA, U.S.A.) and cultured in ATCC-formulated EMEM supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA, U.S.A.), 100 U/ml penicillin, and 100 µg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA,

U.S.A.). HepG2 cells, purchased from ATCC (Manassas, VA, U.S.A.), were cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC, Manassas, VA, U.S.A.) supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA, U.S.A.), 100 U/mL penicillin, and 100 µg/mL streptomycin. The human cardiomyocyte cell line (AC16) was purchased from MilliporeSigma (Burlington, MA, U.S.A.). AC16 cells were cultured in DMEM/F12 supplemented with 2 mM EmbryoMax L-glutamine, 10% EmbryoMax FBS and 1% of EmbryoMax Penicillin-Streptomycin solution, which were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All the cells were maintained at 37 °C under a humidified atmosphere and 5% CO_2. All the cultures were routinely monitored for mycoplasma contamination using MycoAlertTM PLUS mycoplasma detection kit (Lonza, Walkersville, MD, U.S.A.). ADPKD patient's primary renal tubular epithelial cells were received from Baltimore PKD center human ADPKD kidney biobank (UMD SOM, Baltimore, MD, U.S.A.). On arrival, frozen cells were rapidly thawed and contents diluted by slow addition of warm medium to the vial. Cell suspension was spun in a conical centrifuge tube at ~200 g for 5 min followed by removal of the supernatant fluid. The cells were resuspended with 5 mL of culture medium in a T25 culture flask using a 37 °C incubator. Primary renal tubular epithelial cells culture media consisted of DMEM/F12 culture media (Invitrogen, Carlsbad, CA, U.S.A.), 5% FBS, 0.5% Insulin-transferrin-selenium 100X (Invitrogen, Carlsbad, CA, U.S.A.), 0.01 µg/mL recombinant human epidermal growth factor (Invitrogen, Carlsbad, CA, U.S.A.), 5 nM Dexamethasone, 1 nM 3,3′,5-Triiodo-L-thyronine (MilliporeSigma, Burlington, MA, U.S.A.), 0.5% Pen/Strep (50 U/mL penicillin, 50 µg/mL streptomycin) (Thermo Fisher Scientific, Waltham, MA, U.S.A.), and 0.25 µg/mL Amphotericin B (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Six different densities of cells (500, 1K, 3K, 5K, 7K, 10K per well) were dispensed into a 384-well of black clear-bottom assay plate (Corning, Corning, NY, U.S.A.) for all the cell types except AC16, which included nine densities (100, 200, 400, 500, 1K, 3K, 5K, 7K, 10K per well). The assay plates were incubated at 37 °C and 5% CO_2 for 24 h. Subsequently, the cells were washed in 1X cold PBS for 3 times and residual medium was removed by gentle spinning in Centrifugal Blue Washer (BlueCatBio, Concord, MA, U.S.A.). The plates were stored in -80 °C freezer prior to use.

High-Throughput Sample Handling.

Cell Lysis, Protein Denaturation, and Reduction

The plated cells were placed in a 4 °C refrigerator to thaw (~45 min) while preparation of the LyDeR Rapid Buffer stock solution and experimental setup took place. LyDeR Rapid Buffer stock solution was prepared as described and 20 µL was added to each well of the 384-well LyDeR stock plate. The Agilent Bravo system was then used to transfer 10 µL of the LyDeR Rapid Buffer (deck position 8) to each well of the 384-well cell culture plate (deck position 6). The automation system parameters were as follows: aspiration height: 0.2 mm; aspiration speed: 10 µL/s; dispense height: 0.2 mm; dispense speed: 10 µL/s. The cell plate was then manually transferred to the Thermal Mixer modified with the custom copper conducting block for lysis incubation (4 °C, 850 rpm, 1 hour). Afterwards, the cell plate was centrifuged (4 °C, 3,700 g, 10 min) to settle cellular debris and then placed back onto deck position 6 of the Agilent Bravo. The supernatant from the cell lysis (6.3 µL) was transferred from the cell plate to the analyte plate (deck position 4) using the following parameters: aspiration height: 0.2 mm; aspiration speed: 2 µL/s; pre-aspirate: 2 µL; post-aspirate: 2 µL; dispense height: 0.5 mm; dispense speed: 10 µL/s. The analyte plate was sealed with DMSO-resistant foil and manually transferred to the Thermal Mixer for reduction incubation (40 °C, 850 rpm, 20 min). At the 10-minute mark, IAA stock solution was prepared in a reduced light exposure environment as described. After reduction incubation, the analyte plate was centrifuged (4 °C, 2,250 g, 20 s) to spin down and consolidate the condensed droplets underneath the plate seal and then returned to deck position 4 on the Agilent Bravo with the plate seal removed.

Protein Alkylation

The IAA stock plate was set onto the Agilent Bravo at deck position 9 and 1 µL of the solution was transferred to each well of the analyte plate with 8 subsequent mix cycles using the following parameters: aspiration height: 0.2 mm; aspiration speed: 10 µL/s; pre-aspirate: 2 µL; post-aspirate: 2 µL; dispense height: 0.5 mm; dispense speed: 10 µL/s. The analyte plate was then sealed with DMSO-resistant foil, wrapped with aluminum foil and incubated in the dark (RT, 30 min). After, the analyte plate was returned

to deck position 4 on the Agilent Bravo. Tips were replaced to prevent cross-contamination as the upcoming MeCN transfer involved an open reservoir of MeCN not separated by individual wells.

Protein Cleanup

The MS-grade MeCN, which had been chilled overnight at -20 °C, was used to rinse the solvent reservoir three times, filled with MeCN, and then manually placed onto the Agilent Bravo at deck position 7. Immediately, an automated transfer of MeCN (63 µL) to the analyte plate was performed by the Agilent Bravo system. The automation system parameters were as follows: aspiration height: 0.5 mm; aspiration speed: 10 µL/s; pre-aspirate: 2 µL; post-aspirate: 2 µL; dispense height: 4 mm; dispense speed: 10 µL/s. After dilution, the analyte plate was immediately sealed with DMSO-resistant foil and manually transferred to the Thermal Mixer for incubation (4 °C, 15 min). Upon completion, the analyte plate was centrifuged to precipitate the unfolded protein into a protein pellet (4 °C, 2,250 g, 10 min) and then returned to deck position 4 on the Agilent Bravo with the plate seal removed. A volume of 69 µL of MeCN was removed and discarded in waste at deck position 7 by Agilent Bravo automation using the following parameters: aspiration height: 0 mm; aspiration speed: 2 µL/s; pre-aspirate: 0 µL; post-aspirate: 0 µL; dispense height: 0.9 mm; dispense speed: 10 µL/s. The analyte plate was then manually transferred to the Centrivap Complete Vacuum Concentrator and evaporated for 10 min. During evaporation, the trypsin stock solution was prepared and plated as described followed by placement onto the Agilent Bravo at deck position 9. Once the evaporation was complete, the analyte plate was returned to deck position 4 of the Agilent Bravo.

Protein Digestion

An automated transfer of trypsin stock solution (22 µL) to the analyte plate was performed by the Agilent Bravo system with 8 subsequent mix cycles using the following parameters: aspiration height: 0.2 mm; aspiration speed: 10 µL/s; pre-aspirate: 2 µL; post-aspirate: 2 µL; dispense height: 0.5 mm; dispense speed: 10 µL/s. Upon completion, the analyte plate was sealed with DMSO-resistant foil and manually transferred to the Thermal Mixer for incubation (37 °C, 850 rpm, 15-18 hours). The Promega Rapid Digest

protocol samples were incubated at a much higher temperature for a lesser amount of time (70 °C, 850 rpm, 1-2 hours). After incubation, the analyte plate was centrifuged (4 °C, 2,250 g, 20 s) to spin down and consolidate the condensed droplets underneath the plate seal.

Formic Acid Quenching of Digestion

Formic acid stock solution was prepared and plated as described followed by placement onto the Agilent Bravo at deck position 7. An automated transfer of FA (2 µL) to the analyte plate was performed by Agilent Bravo system with 8 subsequent mix cycles using the following parameters: aspiration height: 0.2 mm; aspiration speed: 10 µL/s; pre-aspirate: 2 µL; post-aspirate: 2 µL; dispense height: 0.5 mm; dispense speed: 10 µL/s. Upon completion, the analyte plate was centrifuged (4 °C, 2,250 g, 20 s) and the pH measured to confirm the reaction was quenched, then the analyte plate was transferred to the autosampler of the UltiMate 3000 RSLC nano System coupled to an Orbitrap Fusion Lumos Tribrid Mass Spectrometer with Nanospray Flex ion source for LC-MS/MS analysis. All samples were analyzed with 15 µL injections.

NanoHPLC-MS/MS Analysis. Peptides were loaded onto the trap column by autosampler using a loading solvent (2% MeCN in 98% MS-grade water with 0.1% FA) at a flow rate of 4 µL/min. Elution of peptides from the analytical column was performed using a 120 min method (~90 min gradient) starting at 98% A (0.1% FA in MS-grade water) at a flow rate of 250 nL/min. The mobile phase was maintained at 2% B (0.1% FA in MS-grade MeCN) for 8 min, 2 – 10% B for 2 min, 10 – 32% B for 84 min, 32 – 90% B for 1 min, maintained at 90% B for 10 min, followed by re-equilibration of the column with 2% B for 15 min. Column oven temperature was set at 40 °C. Nanospray Flex ion source was operated in positive-ionization mode with the spray voltage set at 2,400 V, and ion transfer tube temperature set at 300 °C. The MS scan was operated in data-dependent acquisition mode with full MS scans over a mass range of m/z 350 – 2,000 with detection in the Orbitrap (60 K resolution), auto gain control (AGC) set to 4.0×10^5, and maximum injection time set to 50 ms. The fragment ion spectra were acquired in the linear ion trap

(IT) with a normalized collision energy of 28% at HCD activation mode. In each cycle of data-dependent acquisition analysis, the most intense ions above a threshold of 5,000 ion count were selected for the MS/MS analysis, and the cycle time for MS and MS/MS analysis was set as 1 second. The AGC for MS/MS was $1.0x10^4$ and a maximum injection time was 25 ms. Precursor ions with charges of +2 to +7 were isolated for MS/MS. The MS/MS isolation window was 1.2 Da, and the dynamic exclusion time was set at 60 s (after one MS/MS acquisition) with a mass tolerance of ±10 ppm.

HPLC-Multiple Reaction Monitoring (MRM) analysis. Peptides were loaded onto the C18 analytical column by autosampler using 92% A (0.1% FA in MS-grade water) and 8% solvent B (0.1% FA in MS-grade MeCN) at a flow rate of 400 µL/min. Elution of peptides from the analytical column was performed using a 10 min gradient starting at 98% A. The mobile phase was maintained at 8% B for 0.20 min, 8 – 25% B for 5.8 min, 25 – 80% B for 0.2 min, maintained at 90% for 1.8 min, followed by re-equilibration of the column with 8% B for 2 min. The column compartment temperatures were set at 50.0 °C. Source parameters were set as follows; gas temp: 325 °C; gas flow: 10 L/min; nebulizer: 20 psi; sheath gas temp: 375 °C; sheath gas flow: 11 L/min; capillary positive voltage: 3500V; nozzle positive voltage: 1000V. MRM transitions for two selected peptides and heavy labelled internal standards (IS) are shown in **Table S4**. Each lysine of the IS peptides was isotope-labeled at 13C and 15N. (New England Peptide, Gardner, MA, U.S.A.). The integrated peak Area Ratio, which was calculated using HeLa cell samples divided by heavy labeled internal standard through Agilent MassHunter Quantitative Analysis B.07.00 was used for the HeLa sample quantitative analysis (Linearity curve and regression calculation) across all the samples with different cell numbers.

Table S1. List of reagents

Reagent	Cat. No.	Vendor
NP40 Cell Lysis Buffer	FNN0021	Thermo Fisher (Waltham, MA)
TCEP	51805-45-9	Millipore Sigma (Burlington, MA)
Formic acid (FA)	85178	Thermo Fisher (Waltham, MA)
RapiGest	186001861	Waters (Milford, MA)
Protease Inhibitor Cocktail (PIC)	P8340	Millipore Sigma (Burlington, MA)
HeLa commercial digest	88328	Thermo Fisher (Waltham, MA)
MeCN MS grade	900667	Millipore Sigma (Burlington, MA)
Iodoacetamide (IAA)	144-48-9	Millipore Sigma (Burlington, MA)
Trypsin	V5111	Promega (Madison, WI)
Promega Rapid Digestion Kit-Trypsin	VA1060	Promega (Madison, WI)
Water MS grade	900682	Millipore Sigma (Burlington, MA)
Ammonium bicarbonate	A6141	Millipore Sigma (Burlington, MA)

Table S2. List of labware

Labware	Cat. No.	Vendor
Falcon 15 mL Conical Centrifuge Tubes	14-959-49D	Fisher Scientific (Waltham, MA)
Protein LoBind Tubes (0.5 &1.5 mL)	022431064 (0.5 mL), 022431081 (1.5 mL)	Eppendorf (Hauppauge, NY)
384-Well Polypropylene Storage Microplates	264573	Thermo Fisher (Waltham, MA)
Reagent Reservoirs	8095, 8093-11	Thermo Fisher (Waltham, MA)
Expell Plus 0.2-50 ul pipette tips	5030006	Capp (Nordhausen, Germany)
Tips-RT-LTS low retention, sterilized	30389229, 30389246, 303892216	Mettler-Toledo (Columbus, OH)
Agilent Bravo 384ST tips	19133-112	Agilent Technologies (Santa Clara, CA)
DMSO Resistant Foil	4ti-0512	Thomas Scientific (Swedesboro, NJ)
384-well Flat Clear Bottom Microplates	3770BC	Corning (Corning, NY)
Tube, 5 mL, Round Bottom, Sterile	115262	Greiner Bio-One (Monroe, NC)
0.3 mL clear glass limited volume vial w/ snap seals	0010105/0015775	Worldwide Glass Resources (Vineland, NJ)

Table S3. List of instruments and accessories

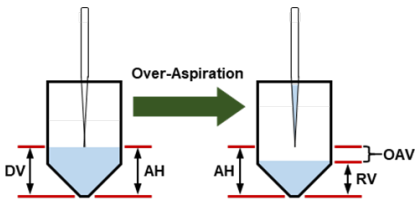
Labware	Cat. No.	Vendor
Falcon 15 mL Conical Centrifuge Tubes	14-959-49D	Fisher Scientific (Waltham, MA)
Protein LoBind Tubes (0.5 &1.5 mL)	022431064 (0.5 mL), 022431081 (1.5 mL)	Eppendorf (Hauppauge, NY)
384-Well Polypropylene Storage Microplates	264573	Thermo Fisher (Waltham, MA)
Reagent Reservoirs	8095, 8093-11	Thermo Fisher (Waltham, MA)
Expell Plus 0.2-50 ul pipette tips	5030006	Capp (Nordhausen, Germany)
Tips-RT-LTS low retention, sterilized	30389229, 30389246, 303892216	Mettler-Toledo (Columbus, OH)
Agilent Bravo 384ST tips	19133-112	Agilent Technologies (Santa Clara, CA)
DMSO Resistant Foil	4ti-0512	Thomas Scientific (Swedesboro, NJ)
384-well Flat Clear Bottom Microplates	3770BC	Corning (Corning, NY)
Tube, 5 mL, Round Bottom, Sterile	115262	Greiner Bio-One (Monroe, NC)
0.3 mL clear glass limited volume vial w/ snap seals	0010105/0015775	Worldwide Glass Resources (Vineland, NJ)

Table S4. Peptide sequence, precursor, and product ions for each multiple reaction monitoring transition

Peptide Name	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	RT (min)
LSDGVAVLK	451.5	Wide	788.5	Wide	175	17	5	4.22
LSDGVAVLK	451.5	Wide	701.4	Wide	175	17	5	4.22
LSDGVAVLK	451.5	Wide	586.5	Wide	175	18	5	4.22
LSDGVAVLK_IS	455.5	Wide	796.5	Wide	175	17	5	4.22
LSDGVAVLK_IS	455.5	Wide	709.4	Wide	175	17	5	4.22
LSDGVAVLK_IS	455.5	Wide	594.5	Wide	175	18	5	4.22
VGEVIVTK	422.9	Wide	745.6	Wide	175	18	5	3.01
VGEVIVTK	422.9	Wide	688.4	Wide	175	19	5	3.01
VGEVIVTK	422.9	Wide	286.1	Wide	175	21	5	3.01
VGEVIVTK_IS	426.9	Wide	753.6	Wide	175	18	5	3.01
VGEVIVTK_IS	426.9	Wide	696.4	Wide	175	19	5	3.01
VGEVIVTK_IS	426.9	Wide	286.1	Wide	175	21	5	3.01

Abbreviations: IS – internal standard; Prec Ion – precursor ion; MS1 Res – MS1 resolution; Prod Ion – product ion; MS2 Res – MS2 resolution; Frag (V) – Fragmentor (V); CE (V) – collision energy (V); Cell Acc (V) – cell acceleration (V); RT (min) – retention time (min).

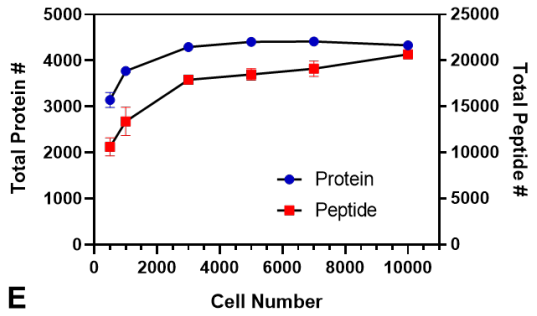
A



B

Liquid Transfer Steps	Aspirate Parameters				Dispense Height (mm)	Mix Cycles
	Height (mm)	Speed (µL/sec)	Pre-Aspirate (µL)	Post-Aspirate (µL)		
LyDeR Rapid Buffer added to cells	0.2	10.0	2.0	2.0	0.2	–
Supernatant to new plate	0.2	2.0	2.0	2.0	0.5	–
IAA added to supernatant	0.2	10.0	2.0	2.0	0.5	–
IAA mixing	0.1	10.0	2.0	2.0	0.5	8
MeCN added to supernatant	0.5	10.0	2.0	2.0	4.0	–
MeCN/supernatant removal	0.0	2.0	0.0	0.0	2.0	–
Trypsin added to proteins	0.2	10.0	2.0	2.0	2.0	–
Trypsin mixing	0.2	10.0	2.0	–	0.9	8
FA added to digested proteins	0.2	10.0	2.0	2.0	0.5	–
FA mixing	0.2	10.0	2.0	–	0.5	8

C



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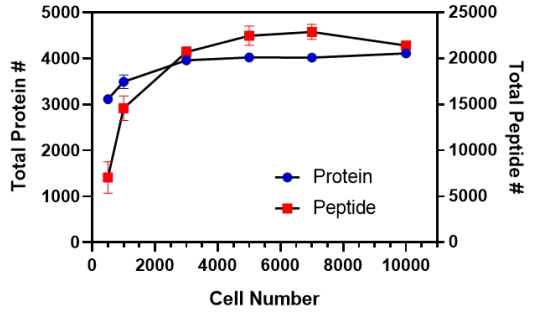

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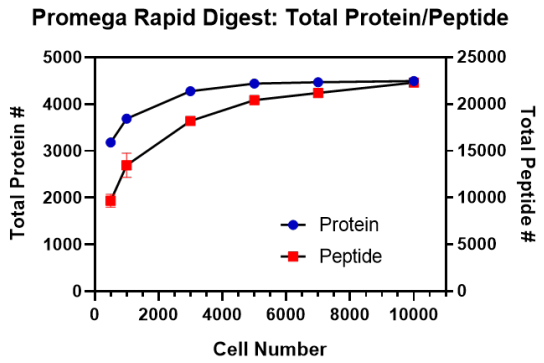


Figure S1. Method development and evaluation using Hela cells. (**A**) Over-aspiration effect; dead volume (DV), aspiration height (AH), residual volume (RV), over-aspiration volume (OAV). (**B**) Liquid transfer steps and parameters. (**C-E**) Scatter plots summarizing the total protein and peptide identifications for User 1-Plate 2 digest (**C**)**,** User 2-Plate 3 digest (**D**), and Promega Rapid Digest (Plate 4) (**E**).

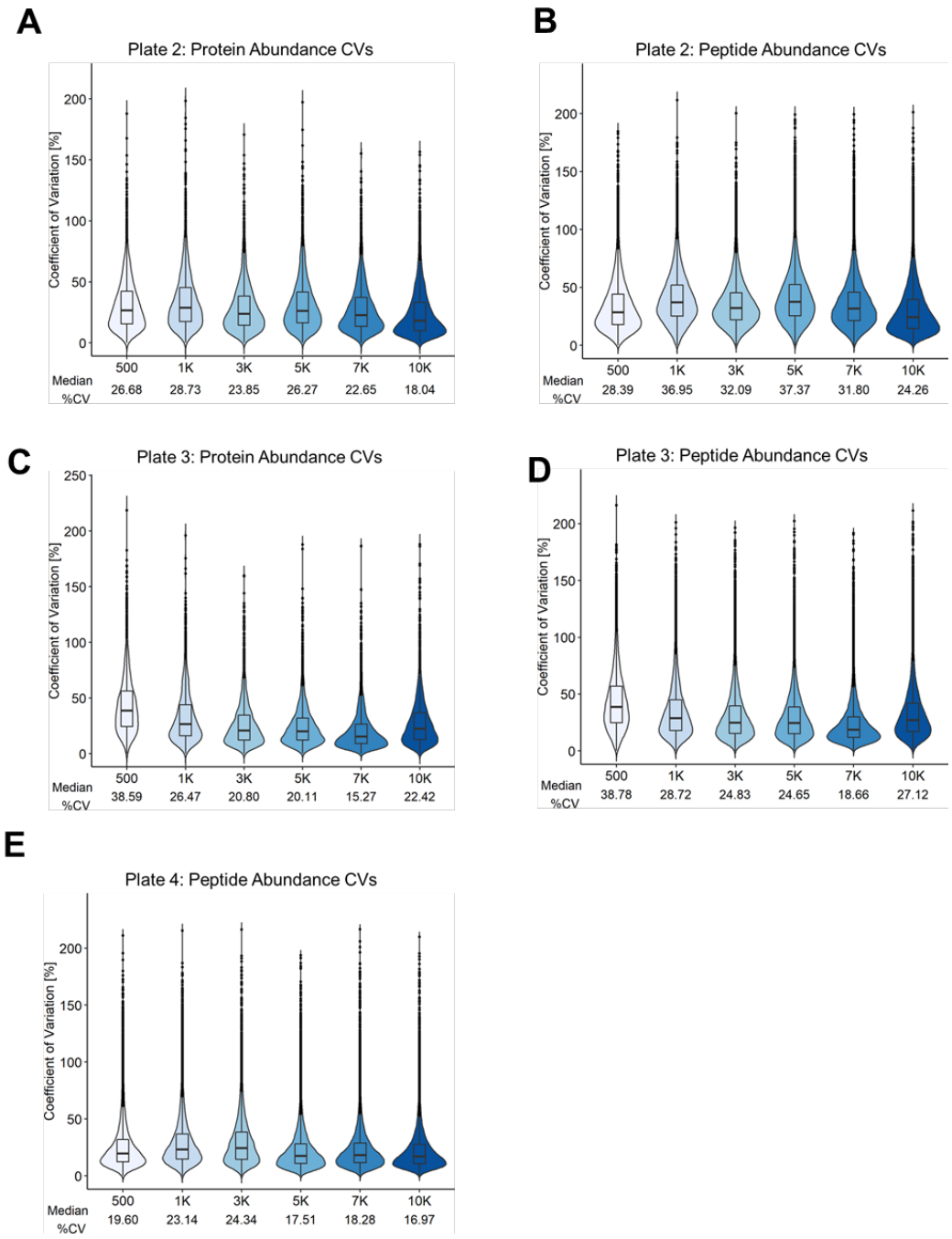


Figure S2. Protein and Peptide intraplate reproducibility by CVs. Violin plots of the Coefficient of Variation (CV) for the 384-well plate platform for abundance of Plate 2 proteins (**A**), Plate 2 peptides (**B**) Plate 3 proteins (**C**), Plate 3 peptides (**D**), and Plate 4 peptides (**E**) from four separate digestions of HeLa cells: Plate 1 and 2, individual plates performed by User 1-Plate 3, an individual plate performed by User 2-Plate 4, an adapted Rapid Digest protocol.

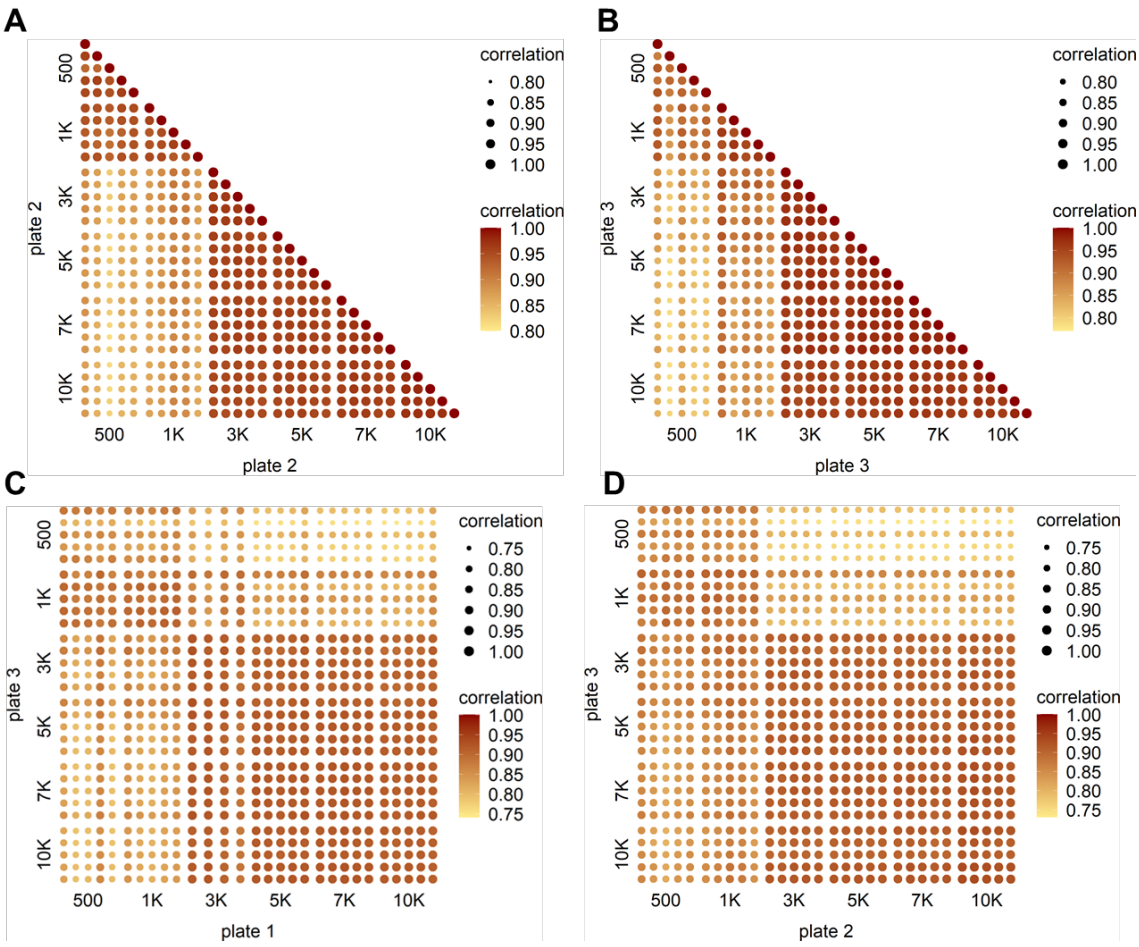


Figure S3. Protein reproducibility of intra-plate and inter-user replicates. Heat maps in which each cell corresponds to a scatter plot of a linear regression model and each data point within that scatter plot represents the abundance of an individual protein between Plate 2 and Plate 2 (intraplate replicates) (**A**), Plate 3 and Plate 3 (intraplate replicates) (**B**), and Plate 1 and Plate 3, Plate 2 and Plate 3 (interuser replicates) (**C, D**).

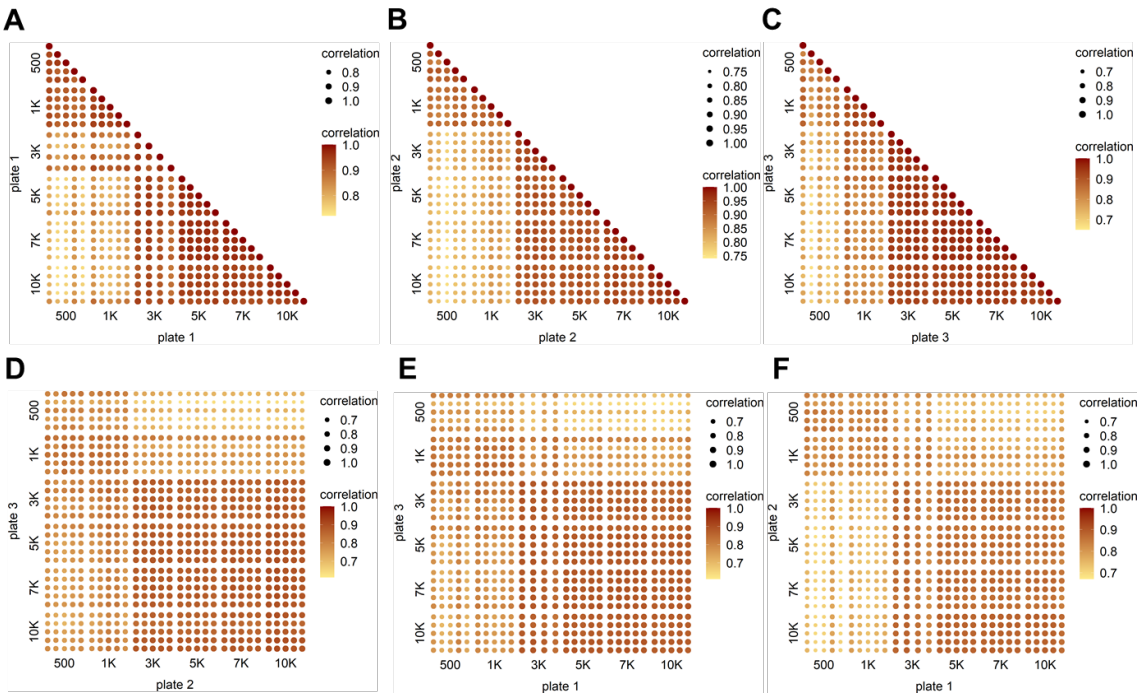


Figure S4. Peptide reproducibility of intraplate, interplate, and interuser replicates. Heat maps in which each cell corresponds to a scatter plot of a linear regression model and each data point within that scatter plot represents the abundance of an individual peptide between Plate 1 and Plate 1 (intraplate replicates) (**A**), Plate 2 and Plate 2 (intraplate replicates) (**B**), Plate 3 and Plate 3 (intraplate replicates) (**C**), Plate 2 and Plate 3 (interuser replicates) (**D**), Plate 1 and Plate 3 (interuser replicates) (**E**), Plate 1 and Plate 2 (interplate replicates) (**F**).

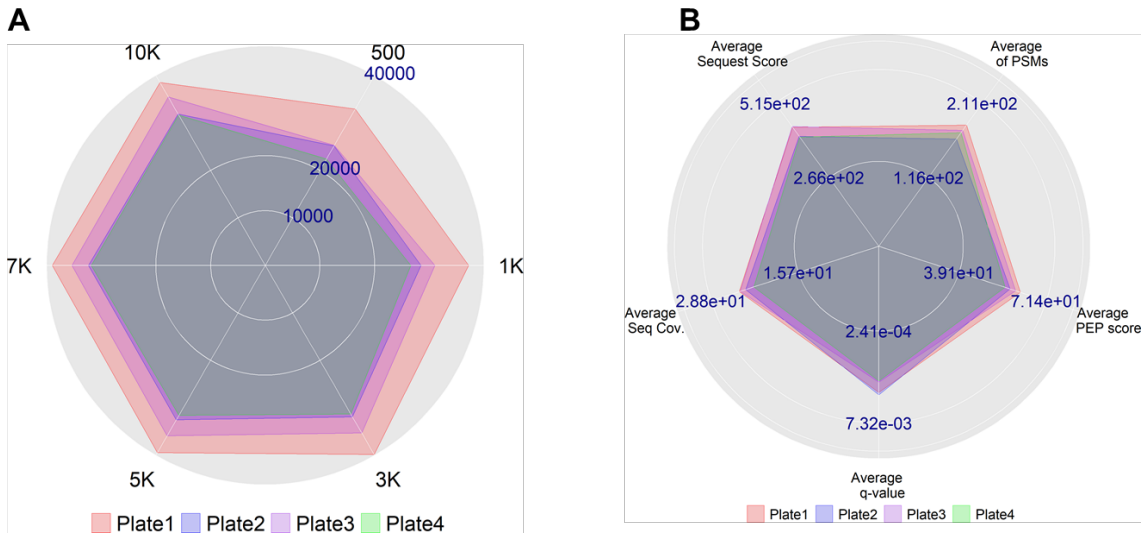


Figure S5. Reproducibility of peptide abundance between different plates, users, and protocols. Radar plots of total peptide identifications (**A**) and proteomic metrics (**B**) from four separate digestions of HeLa cells: Plate 1 and 2, individual plates performed by User 1-Plate 3, an individual plate performed by User 2-Plate 4, an adapted Rapid Digest protocol.

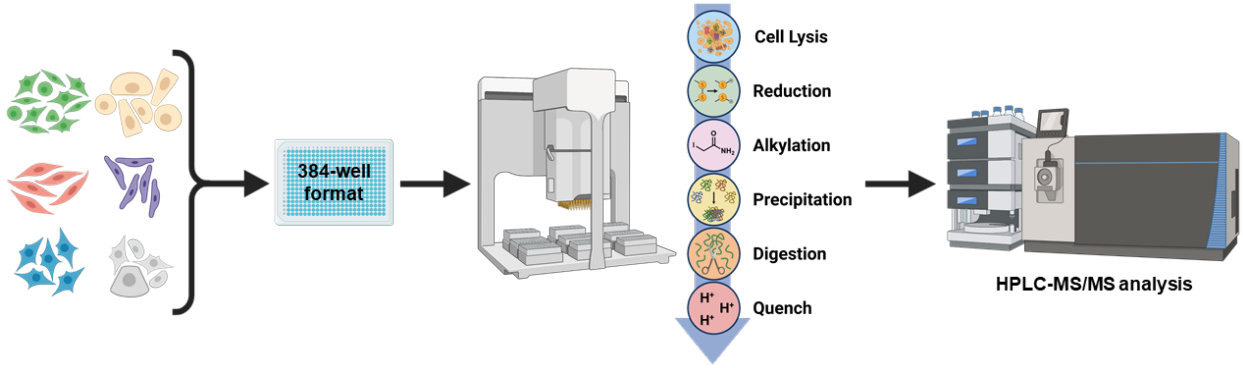
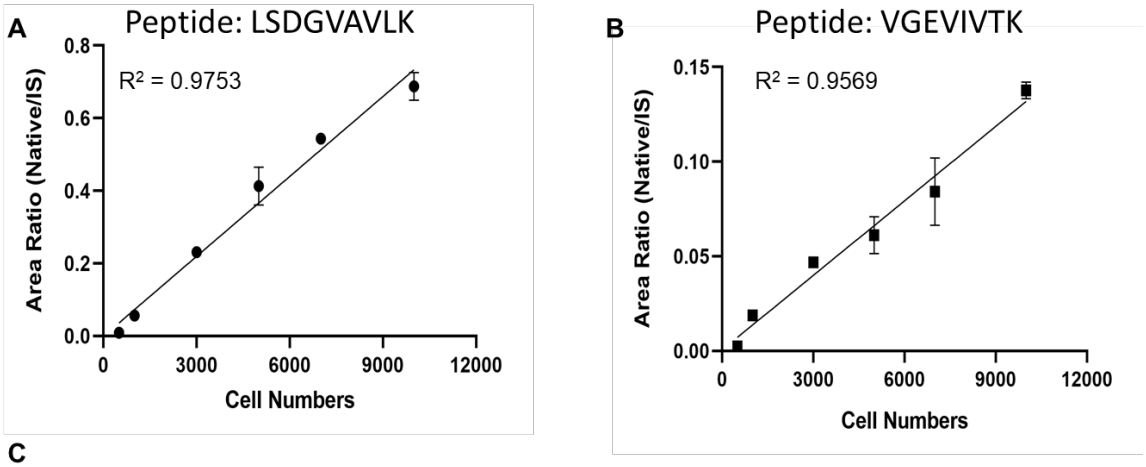


Figure S6. Universal applicability of sample preparation platform. Experimental workflow for 384-well digestion of six different cell types.



C

Cell Count	Bio Rep	Pep A	Pep A IS	Ratio A/IS	Median	CV %	Pep B	Pep B IS	Ratio B/IS	Median	CV%
	B01	25.17	2613.92	0.010			3.00	688.73	0.004		
500	B02	25.25	2393.12	0.011	0.0096	15.7	2.24	655.91	0.003	0.0034	5.3
	B03	17.59	2518.75	0.007			2.21	706.53	0.003		
	B01	148.85	2413.74	0.062			11.24	715.35	0.016		
1K	B02	135.16	2900.08	0.047	0.0591	11.1	15.41	747.77	0.021	0.0183	10.9
	B03	151.21	2558.02	0.059			12.72	693.83	0.018		
	B01	592.67	2655.00	0.223			31.45	705.84	0.045		
3K	B02	599.39	2538.72	0.236	0.2331	2.4	33.21	695.82	0.048	0.0477	3.2
	B03	570.51	2447.27	0.233			34.29	717.50	0.048		
	B01	1062.43	2302.67	0.461			32.35	625.19	0.052		
5K	B02	817.47	2283.63	0.358	0.4219	10.1	44.70	625.80	0.071	0.0648	12.6
	B03	990.24	2347.04	0.422			43.15	665.29	0.065		
	B01	1168.28	2154.42	0.542			41.70	600.89	0.069		
7K	B02	1238.45	2296.32	0.539	0.5423	0.8	58.48	562.19	0.104	0.0792	18.3
	B03	1172.73	2134.79	0.549			49.09	619.35	0.079		
	B01	1626.68	2266.25	0.718			85.33	639.09	0.134		
10K	B02	1698.17	2425.72	0.700	0.7001	4.4	78.19	549.36	0.142	0.1372	2.6
	B03	1391.50	2156.97	0.645			71.81	523.09	0.137		

Figure S7. HPLC-MRM data for selected two peptides (60 kDa mitochondrial heat shock protein) with C13 and N15 labeled Internal Standards (IS) from 500 to 10K cells. (**A**) Selected peptide LSDGVAVLK (Pep A). (**B**) Selected peptide VGEVIVTK (Pep B). (**C**) MRM calculations for two peptides including three biological replicates for each cell count.