Store at 4°C and -20°C

PTMScan® HS Pan-Methyl Lysine Kit

#28411

1 Kit
(10 assays)

Cell Signaling TECHNOLOGY®

Support: +1-978-867-2388 (U.S.)
cellsignal.com/support

Orders: 877-616-2355 (U.S.)
orders@cellsignal.com

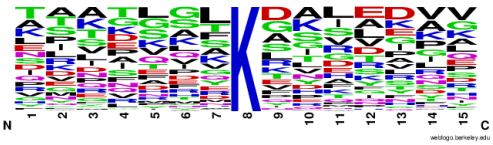
For Research Use Only. Not for Use in Diagnostic Procedures.

Product Includes	Product #	Kit Quantity	Storage Temp
PTMScan® HS Pan-Methyl Lysine Magnetic Immunoaffinity Beads	17815	1 x 200 µL	4°C
PTMScan® IAP Buffer (10X)	9993	10 x 600 µL	-20°C

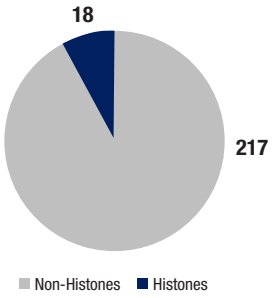
Description: PTMScan® HS is an enhanced PTMScan® methodology with improved identification of post-translationally modified peptides. PTMScan® technology employs a proprietary methodology from Cell Signaling Technology (CST) for peptide enrichment by immunoprecipitation using a specific bead-conjugated antibody in conjunction with liquid chromatography tandem mass spectrometry (LC-MS/MS) for quantitative profiling of post-translational modification (PTM) sites in cellular proteins. PTMs that can be analyzed by PTMScan® technology include phosphorylation, ubiquitination, acetylation, and methylation, among others. The technology enables researchers to isolate, identify, and quantitate large numbers of post-translationally modified cellular peptides with a high degree of specificity and sensitivity (HS), providing a global overview of PTMs in cell and tissue samples without bias about where the modified sites occur. For more information on PTMScan® products and services, please visit cellsignal.com/applications/proteomics.

Background: Methylation of lysine residues is a common regulatory post-translational modification (PTM) that results in the mono-, di-, or tri-methylation of lysine at ε-amine groups by protein lysine methyltransferases (PKMTs). Two PKMT groups are recognized based on structure and catalytic mechanism: class I methyltransferases or seven β strand enzymes, and SET domain-containing class V methyltransferases. Both use the methyl donor S-adenosyl-L-methionine to methylate histone and non-histone proteins. Class I methyltransferases methylate amino acids, DNA, and RNA (1,2). Six methyl-lysine-interacting protein families are distinguished based on binding domains: MBT, PHD finger, Tudor, PWWP, WD40 repeat, and chromodomains. Many of these display differential binding preferences based on lysine methylation state (3). KDM1 subfamily lysine demethylases catalyze demethylation of mono- and di-methyl lysines, while 2-oxoglutarate-dependent JmjC (KDM2-7) subfamily enzymes also modify tri-methyl lysine residues (4).

Most PKMT substrates are histone proteins and transcription factors, emphasizing the importance of lysine methylation in regulating chromatin structure and gene expression. Lys9 of histone H3 is mono- or di-methylated by G9A/GLP and tri-methylated by SETDB1 to activate transcription. JHDM3A-mediated demethylation of the same residue creates mono-methyl Lys9 and inhibits gene transcription (5). Tumor suppressor p53 is regulated by methylation of at least four sites. p53-mediated transcription is repressed following mono-methylation of p53 at Lys370 by SMYD2; di-methylation at the same residue further inhibits p53 by preventing association with 53BP1. Concomitant di-methylation at Lys382 inhibits p53 ubiquitination following DNA damage. Mono-methylation at Lys382 by SET8 suppresses p53 transcriptional activity, while SET7/9 mono-methylation at Lys372 inhibits SMYD2 methylation at Lys370 and stabilizes the p53 protein. Di-methylation at Lys373 by G9A/GLP inhibits p53-mediated apoptosis and correlates with tri-methylation of histone H3 Lys9 at the p21 promoter (1,6). Overexpression of PKMTs is associated with multiple forms of human cancer, which has generated tremendous interest in targeting protein lysine methyltransferases in drug discovery research.



Motif analysis using nonredundant mono-, di-, and tri-methylated lysine sites enriched by PTMScan® HS Pan-Methyl Lysine Magnetic Immunoaffinity Beads starting from one milligram of mouse liver derived tryptic peptides. Orbitrap Fusion Lumos mass spectrometer analysis identified a total of 235 unique singly modified sites. Within this set of 235 sites, the number of mono-methylated sites is 129, di-methylated sites is 66, and tri-methylated sites is 40. The motif logo suggests that the PTMScan® HS Pan-Methyl Lysine Magnetic Immunoaffinity Beads recognize the methylated lysine motif independent of protein context, without other amino acid preferences.



The chart shows the relative category distribution of proteins with mono-, di-, and tri-methylated lysine sites derived from mouse liver peptides identified from PTMScan® HS Pan-Methyl Lysine Magnetic Immunoaffinity Beads.

Storage: All components in this kit are stable for at least 12 months when stored at the recommended temperature. Upon receipt, 17815S should be stored at 4°C. 9993S should be stored at -20°C. *Do not aliquot the antibody.*

Please visit cellsignal.com for validation data and a complete listing of recommended companion products.

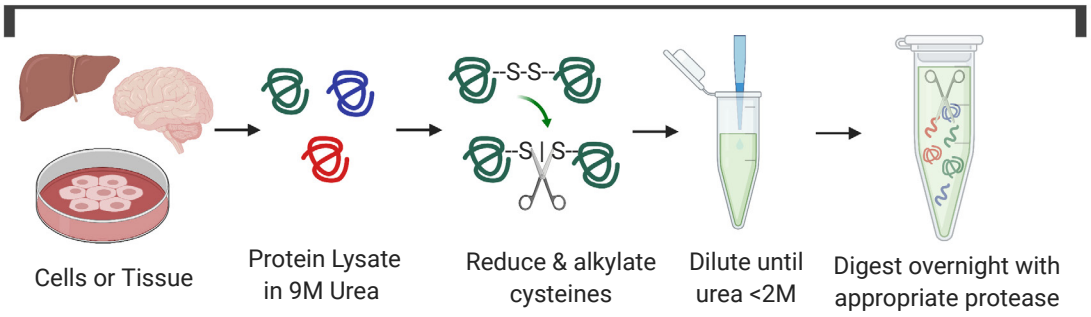
Background References:

(1) Lanouette, S. et al. (2014) *Mol Syst Biol* 10, 724.
(2) Clarke, S.G. (2013) *Trends Biochem Sci* 38, 243-52.
(3) Herold, J.M. et al. (2011) *Curr Chem Genomics* 5, 51-61.
(4) Thinnes, C.C. et al. (2014) *Biochim Biophys Acta* 1839, 1416-32.
(5) Klose, R.J. et al. (2006) *Nature* 442, 312-6.
(6) Yost, J.M. et al. (2011) *Curr Chem Genomics* 5, 72-84.

Applications: **W**—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **ChIP**—Chromatin Immunoprecipitation **IF**—Immunofluorescence **F**—Flow cytometry **FC-FP**— Flow cytometry-Fixed/Permeabilized **FC-L**— Flow cytometry-Live **E-P**—ELISA-Peptide
Species Cross-Reactivity: **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **Dm**—D. melanogaster **X**—Xenopus **Z**—zebrafish **B**—bovine **Dg**—dog **Pg**—pig **Sc**—S. cerevisiae **Ce**—C. elegans **Hr**—Horse
All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

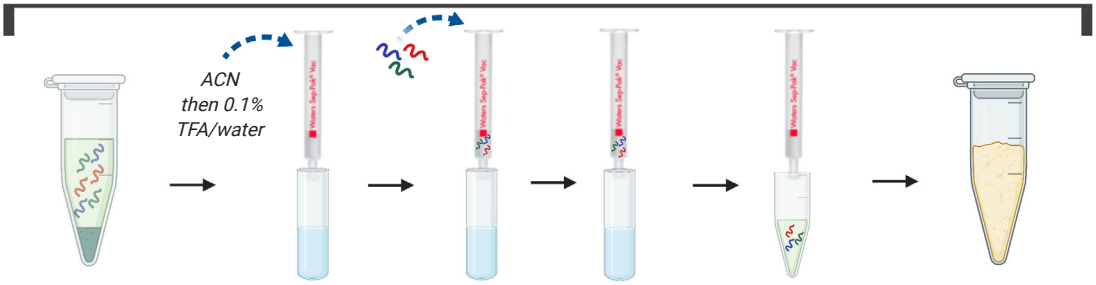
PTMScan® HS Urea Digestion & IAP Buffer Enrichment Protocol

Day 1: Lyse Cells & Tissues, then Digest Proteins



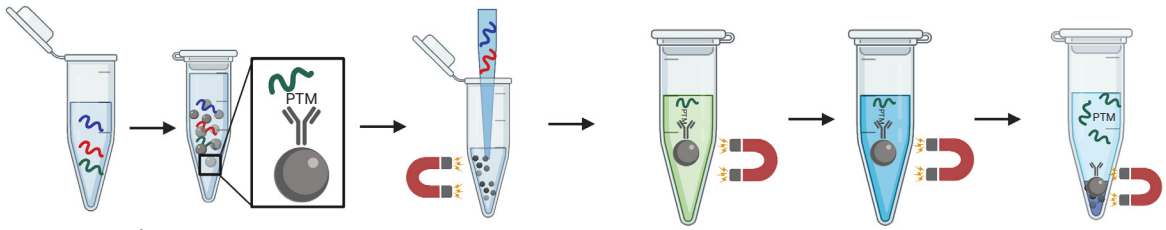
Cells or Tissue | Protein Lysate in 9M Urea | Reduce & alkylate cysteines | Dilute until urea <2M | Digest overnight with appropriate protease

Day 2: Peptide Purification on C18 SPE, then Dry

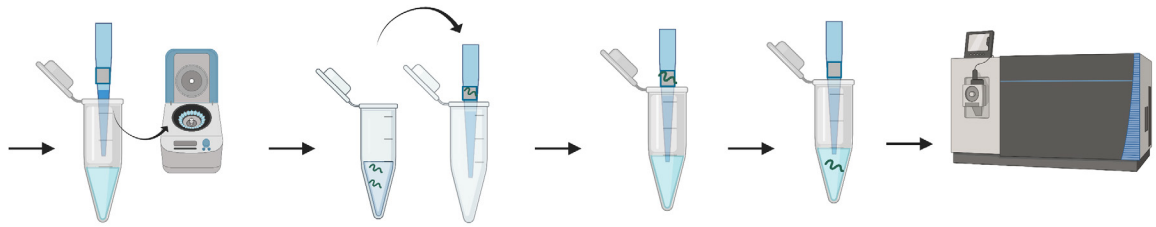


Add TFA; centrifuge 15 min 4000 xg | Wet & Equilibrate Sep-Pak C18 Column | Load Peptides | Wash column (3 steps) | Elute peptides in 50% ACN | Dry peptide solution in vacuum concentrator

PTM Peptide IP



Resuspend peptides in 1X IAP Buffer | Bind PTM peptides to magnetic antibody beads | Remove supernatant containing unmodified peptides | Wash 2x with 1X IAP Buffer | Wash 3x with LCMS-grade water | Elute PTM peptides in 0.15% TFA; Ab remains bound to beads



Wet & equilibrate Stagetip C18 bed, spin @ 2000 xg each step | Load enriched PTM Peptides | Wash 2x | Elute peptides in 40% ACN | Analyze by LCMS

PTMScan® HS Urea Digestion & IAP Buffer Enrichment Protocol

Materials & Reagents

Supplied Reagents:

1. PTMScan® IAP Buffer (10X) (Cell Signaling Technology, #9993)
2. PTMScan® HS Immunoaffinity Magnetic Beads, 20 µL per assay

Additional Reagents (Not Supplied):

1. 200 mM HEPES, pH 8.0 (Cell Signaling Technology, #44686)
2. Phosphatase Inhibitor Cocktail (100X) (Cell Signaling Technology, #5870)
3. Urea, Ultrapure, PTMScan® Qualified (Cell Signaling Technology, #60055)
4. Iodoacetamide, PTMScan® Qualified (Cell Signaling Technology, #88931)
5. DTT (Dithiothreitol) (Cell Signaling Technology, #7016)
6. PTMScan® Trypsin, TPCK-Treated (Cell Signaling Technology, #56296)
7. PTMScan® Wild Type Alpha-Lytic Protease (WaLP) (Cell Signaling Technology, #33036)
8. 1 mM Hydrochloric acid (HCl)
9. Sep-Pak Vac 1cc (50 mg) C18 Cartridges (Waters, #WAT054955)
10. Acetonitrile (Thermo Scientific, 51101)
11. Water, LC-MS Grade (Burdick and Jackson) (Cell Signaling Technology, #27732)
12. Trifluoroacetic acid (Thermo Fisher Scientific, 28904)
13. 20X Phosphate Buffered Saline (Cell Signaling Technology, #9808)
14. BCA Protein Assay Kit (Cell Signaling Technology, #7780)

Equipment Not Included:

1. 1.5 mL microcentrifuge tubes
2. Magnetic rack for 1.5 mL microcentrifuge tubes (Cell Signaling Technology, #7017 or #14654)
3. End-over-end rotator
4. Centrifuge capable of handling 1.5 mL tubes
5. Vacuum concentrator (Speed-Vac)
6. pH indicator strips for pH 0-14
7. Pierce C18 Spin Tips (Thermo Fisher Scientific, 84850)

I. Cell Lysis and Protein Digestion

PTMScan® enrichment kits are compatible with many protein extraction, digestion, and purification protocols. Compatible workflows include in-solution digestion, those that use centrifugal reactors (FASP[1], S-Trap cartridges[2], or iST[3]) or magnetic bead precipitation (SP3[4]). Regardless of the particular method selected, ensure that peptides are completely dry and free of lysis buffer components, lipids, and excess salts prior to using the immunoaffinity purification kit. Below is a general protocol that uses in-solution digestion followed by solid phase extraction on Sep-Paks.

References:

(1) Wiśniewski, J. (2018) *Methods Mol Biol* 1841, 3-10. PMID: 30259475

(2) Zougman A. et.al. (2014) *Proteomics* 14(9), 1006-0. PMID: 24678027

(3) Kulak N.A. et.al. (2014) *Nat Methods* 11(3), 319-24. PMID: 24487582

(4) Hughes C.S. et. al. (2019) *Nat. Protocols* 14(1), 68-85. PMID: 30464214

NOTE: Prepare solutions for cell lysis (Section I), C18 column purification (Section II), and peptide binding and washing during IAP enrichment (Section III) with reverse osmosis deionized (RODI) or equivalent grade water. Prepare solutions using HPLC grade water (Burdick and Jackson water) for the peptide elution (Section III) and the peptide concentration steps (Section IV).

A. Solutions and Reagents

NOTE: Prepare solutions with RODI or equivalent grade water.

1. Urea Lysis Buffer: 9 M urea, 20 mM HEPES pH 8.0, 2X Phosphatase Inhibitor Cocktail (Cell Signaling Technology, #5870). For 10 mL, weigh out 5.41 g of urea powder and dissolve it in 1 mL of 200 mM HEPES pH 8.0 stock and 4 mL RODI water. Mix well until the urea goes into solution completely. Add 200 µL of "100X" Phosphatase Inhibitor Cocktail (to be used at 50X for PTMScan®), and adjust the final volume to 10 mL total.

   NOTE: The Urea Lysis Buffer should be prepared fresh prior to each experiment. Do not include protease inhibitors.

   NOTE: Dissolving urea is an endothermic reaction. Urea Lysis Buffer preparation can be facilitated by placing a stir bar in the beaker and by using a warm (not hot) water bath on a stir plate. 9 M urea is used so that upon lysis, the final concentration is approximately 8 M. The urea lysis buffer should be used at room temperature. Placing the urea lysis buffer on ice will cause the urea to precipitate out of solution.

2. DTT solution (1.25 M): Dissolve the 192.8 mg supplied in the product tube in 1.0 mL of RODI water. Divide into 200 µL aliquots. Store at -20°C for up to one year. Thaw one aliquot for each experiment.

PTMScan® HS Urea Digestion & IAP Buffer Enrichment Protocol

3. Iodoacetamide solution: Dissolve 19 mg of iodoacetamide in water to a final volume of 1 mL. After weighing the powder, store in the dark and add water only immediately before use. The iodoacetamide solution should be prepared fresh prior to each experiment.
4. Trypsin-TPCK: Store dry powder for up to 2 years at -80°C. Prepare 1 mg/mL stock in 1 mM HCl. Divide into 1 mL aliquots. Store aliquots at -80°C for up to one year.

B. Preparation of Cell Or Tissue Lysate

Suspension Cells

1. Grow approximately 1 x 10^7 cells for each experimental condition or enough cells to produce approximately 1 mg of soluble protein. (If desired, scale up to 5 x 10^7 cells.)
2. Harvest cells by centrifugation at 130 x g, for 5 min at room temperature. Carefully remove supernatant, wash cells with 20 mL of cold 1X PBS, centrifuge, remove PBS wash, and add 1 mL Urea Lysis Buffer (room temperature) to the cell pellet. Pipet the slurry up and down a few times (do not cool lysate on ice as this may cause precipitation of the urea).

Adherent Cells

1. Grow approximately 1 x 10^7 cells for each experimental condition or enough cells to produce approximately 1 mg of soluble protein. (If desired, scale up to 5 x 10^7 cells.) The cell number corresponds to approximately one 150 mM culture dish (depending on the cell type), grown to 70-80% confluence.
2. To harvest, remove media from the first dish by decanting, and let stand in a tilted position for 30 sec so the remaining medium flows to the bottom edge. Remove the remainder of the medium at the bottom edge with a P-1000 micropipettor. Rinse each dish with 5 mL of cold PBS. Remove PBS as described above.
3. Use 1 mL of lysis buffer per dish; e.g., start with 5 mL if five dishes will be harvested. Add 1 mL of Urea Lysis Buffer (at room temperature) to the first dish, scrape the cells into the buffer, and let the dish stand in tilted position after scraping the buffer to the bottom edge of the tilted dish.
4. If multiple dishes will be combined to make a larger sample, remove the medium from the second dish as above.Transfer the lysis buffer from the first dish to the second dish using a pipette, then tilt the first dish with the lid on for 30 sec and remove the remaining buffer from the dish and collect. Scrape cells from the second dish and repeat the process until the cells from all the dishes have been scraped into the lysis buffer. Collect all lysate in a 50 mL conical tube.

   NOTE: DO NOT place Urea Lysis Buffer or culture dishes on ice during harvesting. Harvest cells using Urea Lysis Buffer at room temperature. During lysis, the buffer becomes viscous due to DNA released from the cells.

Tissue Specimens

1. Collect tissue specimens by flash freezing in liquid nitrogen. A specimen of approximately 20-50 mg (wet weight) should provide sufficient material for PTMScan® (1 mg of soluble protein or more).
2. Place the still-frozen tissue in a 50 mL conical tube. Add a minimum of 2 mL of lysis buffer (use ~5 mL per 100 mg of wet tissue for larger specimens), or enough to submerge it completely.
3. Lyse the tissue using a homogenizer. Wash the tool with RODI water in between each sample.

   NOTE: If desired, the PTMScan® protocol may be interrupted at this stage. The lysed cells or tissues can be frozen and stored at -80°C for several weeks.

C. Sonication and Centrifugation

1. Using a microtip, sonicate lysate at 15 W output with 3 bursts of 15 sec each. Cool on ice for 1 min between each burst.
2. Clear the lysate by centrifugation at 20,000 x g for 15 min at room temperature and transfer the protein extract (supernatant) into a new tube.

   NOTE: Centrifugation is performed at room temperature to prevent urea from precipitating out of solution.

   NOTE: Lysate sonication fragments DNA and reduces sample viscosity. Ensure that the sonicator tip is submerged in the lysate. If the sonicator tip is not submerged properly, it may induce foaming and degradation of your sample.

D. Protein Quantitation

1. Measure soluble protein concentration using a colorimetric assay kit that is compatible with urea lysis buffer. The BCA assay is recommended.
2. Normalize all samples so that equal amounts of protein are prepared for each condition and replicate. Alternatively, design the experiment so that equal numbers of cells are prepared for each sample. Add urea lysis buffer to bring up the volume of all samples to the same amount.

E. Reduction and Alkylation of Proteins

1. Add 1/278 volume of 1.25 M DTT to the cleared cell supernatant (e.g., 3.6 µL of 1.25 M DTT for 1 mL of protein extract), mix well and incubate at room temperature for 60 min.
2. Add 1/10 volume of iodoacetamide solution to the cleared cell supernatant (100 µL IAA for 1 mL lysate), mix well, and incubate for 15 min at room temperature in the dark.
3. Dilute 4-fold with 20 mM HEPES pH 8.0 to a final concentration of approximately 2 M urea, 20 mM HEPES, pH 8.0. For example, add 3 mL 20 mM HEPES pH 8.0 for 1 mL of lysate.

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F. Protease Digestion

NOTE: WaLP is used for SUMOylated peptide analysis in combination with the Ubiquitin/SUMO Remnant Kit (Cell Signaling Technology, #59322). All other PTMScan® HS samples should be prepared with trypsin as the protease.

NOTE: Alternative proteases such as GluC, chymotrypsin, and others can be used in addition to the protease digests outlined in the reference table to expand the coverage of modified peptides from each Motif Antibody. When considering the use of additional protease digests it should be compatible with the respective Motif Antibody by not cleaving residues within the designated sequence motif. Alternate protease digests that generate larger proteolytic peptides may not be ideal if the resulting peptides do not ionize well in the mass spectrometer.

Trypsin Digestion

1. Add Trypsin-TPCK at 1 mg : 37.5 mg (enzyme:substrate). This requires 27 µL of 1 mg/mL Trypsin (Cell Signaling Technology, #56296) stock for 1 mg lysate. Digest overnight at room temperature with mixing.
2. Analyze the lysate before and after digest by SDS-PAGE to check for complete digestion.

WaLP Digestion

NOTE: Use WaLP only for generating KGG remnants in SUMOylated protein experiments.

1. Use 25 µL of WaLP stock (#33036) for each 1 mg of protein lysate. This equates to 1:100 enzyme:substrate mg:mg ratio.
2. Analyze the lysate before and after digest by SDS-PAGE to check for complete digestion.

II. C18 Purification of Lysate Peptides

NOTE: Purification of peptides is performed at room temperature on C18 reversed-phase Sep-Pak columns from Waters (#WAT054955).

NOTE: C18 purification uses reversed-phase (hydrophobic) solid-phase extraction. Peptides and lipids bind to the chromatographic material. Large molecules such as DNA, RNA, and most protein, as well as hydrophilic molecules such as many small metabolites are separated from peptides using this technique. Peptides are eluted from the column with 50% acetonitrile (ACN) and separated from lipids and proteins, which elute at approximately 60% ACN and above.

NOTE: About 2.5 mg of protease-digested peptides can be purified from one C18 column. Purify peptides immediately after proteolytic digestion.

A. Solutions and Reagents

NOTE: Prepare solutions with HPLC grade water and the highest grade organic solvents, such as Pierce Trifluoroacetic Acid (TFA), Sequencing grade (Thermo Fisher Scientific, 28904) and Pierce Acetonitrile (ACN), LC-MS Grade (Thermo Scientific, 51101). All percentage specifications for solutions are vol/vol.

1. 20% trifluoroacetic acid (TFA): add 1 mL TFA to water to a total volume of 5 mL.
2. Solvent A (0.1% TFA): add 1 mL of 20% TFA to 199 mL water.
3. Solvent B (0.1% TFA, 50% acetonitrile): add 5 mL of acetonitrile (ACN) and 50 µL of 20% TFA to 4.95 mL of water.
4. Wash buffer (0.1% TFA, 5% acetonitrile): For 10 mL of wash buffer, add 50 µL of 20% TFA to 9.45 mL of water, then add 500 µL of acetonitrile.

   NOTE: Organic solvents are volatile. Tubes containing small volumes of these solutions should be prepared immediately before use and should be kept capped as much as possible because the organic components evaporate quickly.

B. Acidification of Digested Cell Lysate

NOTE: Before loading the peptides from the digested sample on the column, they must be acidified with TFA for efficient peptide binding. The acidification step helps remove fatty acids from the digested peptide mixture.

1. Add 1/20 volume of 20% TFA to the digest for a final concentration of 1% TFA. Check the pH by spotting a small amount of peptide sample on a pH strip (the pH should be under 3). After acidification, allow precipitate to form by allowing the sample to stand for 15 min on ice.
2. Centrifuge the acidified peptide solution for 15 min at 4000 x g at room temperature to remove the precipitate. Transfer peptide-containing supernatant into a new 50 mL conical tube without dislodging the precipitated material.

C. Peptide Purification

NOTE: Application of all solutions can be performed with a vacuum manifold or by gravity flow. If using vacuum, keep flow rates below approximately 0.33 mL/min for most steps. Sample loading should be done by gravity flow to maximize recovery.

1. Pre-wet the column with 0.5 mL 100% ACN.
2. Equilibrate column with 1 mL of Solvent A (0.1% TFA). Repeat this step once.
3. Load acidified and cleared digest (from Section II.B) one mL at a time until all of the sample has been loaded. Load sample by gravity flow, without applying vacuum.
4. Wash with 1 mL of Solvent A (0.1% TFA). Repeat this step once.
5. Wash with 0.5 mL of Wash buffer (5% ACN/0.1%TFA).
6. Place columns above new 1.5 mL polypropylene tubes to collect eluate. Elute peptides with a sequential wash of 3 x 0.2 mL of Solvent B (0.1% TFA, 50% acetonitrile).

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7. Dry the peptide solution in a vacuum concentrator (Speed-Vac) set to ambient temperature overnight or until completely dry. The pellet should be visible at the end.

   NOTE: Peptide solutions may be frozen at -80°C for 1 hr or longer before placing in the Speed-Vac; this will prevent full tubes from spilling when placed at an angle to dry.

   NOTE: A standard lyophilization apparatus is also acceptable in place of a vacuum concentrator.

   NOTE: Dry, digested peptides are stable at -80°C for several months (seal the closed tube with parafilm for storage). The PTMScan® procedure can be interrupted before or after drying. Once the dry peptide is dissolved in 1X IAP Buffer (see next step), continue to the end of the procedure.

III. Immunoaffinity Purification (IAP)

A. Prepare solutions

1. PTMScan® IAP Buffer (10X) #9993: Dilute with RODI or equivalent water to 1X concentration before use. Prepared 1X IAP Buffer can be stored up to one month at 4°C.
2. IAP Elution Buffer: 0.15% TFA + 99.85% water. Add 11.3 µL of 20% TFA stock to 1.5 mL of LCMS grade water. Prepare this solution in a container that has never been exposed to soap, as detergents will interfere with LCMS analysis.

B. Procedure

1. Centrifuge the dry peptides (approximately 1 mg is recommended for a PTMScan® HS experiment) for 5 min at 2,000 x g at room temperature to collect all material at the bottom of the tube. Add 1.5 mL of 1X IAP Buffer to dried peptides. Resuspend pellets mechanically by pipetting repeatedly, taking care not to introduce excessive bubbles into the solution.

   NOTE: Tubes can be shaken gently at room temperature using a vortexer or thermomixer for 5 min or placed in a sonicator bath for 2 min to ensure complete solubilization, if necessary.

   NOTE: After dissolving the peptide, check the pH of the peptide solution by spotting a small volume on pH indicator paper. The pH should be close to neutral (no lower than 7.0). If necessary, add 2 µL of 1M Tris base at a time until the pH is at 7.0.

2. Clear solution by centrifugation for 5 min at 10,000 x g at 4°C. Cool on ice.

   NOTE: There may be a small, insoluble pellet. Transfer supernatant to a clean tube and discard the pellet.

3. Briefly spin the vial of antibody-bead slurry at no more than 2,000 x g for 2-5 sec to bring down any buffer and beads clinging to the sides and cap of the vial. Pipet the antibody bead slurry gently to obtain a uniform suspension of beads, then take out 20 µL of bead slurry and place into a 1.5 mL microcentrifuge tube for each sample. Remix the bead stock before each pipetting step. Verify that each sample gets an equal aliquot of beads to ensure reproducible results.
4. Transfer 1 mL of ice-cold 1X PBS into the 1.5 mL microcentrifuge tube, mix buffer with beads by inverting the tube five times. Place the tube on a magnetic separation rack. Wait 10 sec or until beads are attracted to the magnet. Carefully remove the PBS buffer. Repeat bead washing with 1 mL of 1X PBS three times for a total of FOUR PBS washes.
5. Transfer the soluble peptide solution into the tube containing antibody beads and discard the insoluble pellet. Avoid creating bubbles upon pipetting.
6. Tighten the cap and seal the top of the tube with plastic or a caplock clip to avoid leakage. Incubate on an end-over-end rotator for 2 hr at 4°C.

   NOTE: Ensure the beads remain in suspension while rotating and that bubbles do not collect at the bottom of the tube as this will prevent proper bead and sample mixing.

7. Briefly spin the tube at no more than 2,000 x g for 2-5 sec to bring down the beads and solution clinging to the sides and cap. Place the tube in the magnetic separation rack for 10 sec. Transfer the unbound peptide solution to a microcentrifuge tube. Optional: Save this supernatant at -80°C for future use in subsequent IPs for other PTMs.

   NOTE: Keep the 1X IAP Buffer and LCMS Water on ice for the subsequent steps.

8. Add 1 mL of chilled 1X IAP Buffer to the beads, mix by inverting the tube 5 times. Briefly centrifuge the tube, place the tube in the magnetic stand for 10 sec and remove all IAP buffer. Repeat one time for a total of TWO washes with IAP Buffer.
9. Add 1 mL chilled LCMS water to the beads, mix by inverting the tube 5 times. Briefly centrifuge the tube, place the tube in the magnetic stand for 10 sec and remove all water. Repeat two more times for a total of THREE water washes.
10. Add 50 µL of IAP Elution Buffer (0.15% TFA) to the beads, use a mixer such as an Eppendorf ThermoMixer set at <500 rpm to keep beads in suspension at room temperature for 10 min. Do not mix so vigorously that beads are splashed to the sides and cap of the tube. If there is no mixer available, tap the bottom of the tube several times (do not vortex), and let stand at room temperature for 10 min, mixing gently every 2-3 min.
11. Briefly centrifuge the tube and place it in the magnetic rack. Transfer eluted sample to a new microcentrifuge tube.
12. Add another 50 µL of IAP Elution Buffer (0.15% TFA) to the beads to repeat the elution step. Combine both eluents in the same microcentrifuge tube. Verify that no beads are transferred to the collection tube by setting the elution on the magnet for a few seconds.

    NOTE: In this step, the post-translationally modified peptides of interest will be in the eluent.

PTMScan® HS Urea Digestion & IAP Buffer Enrichment Protocol

IV. Concentration and Purification of Peptides for LCMS Analysis on C18 Tip

NOTE: We recognize there are many routine methods for concentrating peptides using commercial products such as C18 tips (see below) that have been optimized for peptide desalting/concentration. Regardless of the particular method, we recommend that the method of choice be optimized for recovery and be amenable for peptide loading capacities of at least 10 µg.

C18 tips: Pierce C18 Spin Tips (Thermo Fisher Scientific, 84850)

A. Solutions and Reagents

NOTE: Prepare solutions with Burdick and Jackson water or other LCMS grade water. Organic solvents (trifluoroacetic acid, acetonitrile) should be of the highest grade.

Recommended: Pierce Trifluoroacetic Acid (TFA), Sequencing grade (Thermo Fisher Scientific, 28904) and Pierce Acetonitrile (ACN), LCMS Grade (Thermo Scientific, 51101).

Prepare all solutions in containers that have never been exposed to soap, as detergents will interfere with LCMS analysis.

1. Stagetip Wetting Solution (0.1% trifluoroacetic acid, 50% acetonitrile): add 50 µL of 20% trifluoroacetic acid to 4.95 mL LCMS water, then add 5 mL acetonitrile for 10 mL final volume.
2. Stagetip Equilibration & Washing Solution (0.1% trifluoroacetic acid): add 50 µL of 20% trifluoroacetic acid to 10 mL LCMS water.
3. Stagetip Elution Solution (0.1% trifluoroacetic acid, 40% acetonitrile): add 50 µL of 20% trifluoroacetic acid to 5.95 mL LCMS water, then add 4 mL acetonitrile, for a final volume of 10 mL.

   NOTE: Organic solvents are volatile. Tubes containing small volumes of these solutions should be prepared immediately before use and should be kept capped as much as possible, to prevent evaporation of organic components.

B. Procedure

NOTE: All centrifugation steps in this section should be carried out at room temperature. Spin at 2,000 x g or a speed that passes all the solution through the tip in approximately 3 min.

1. Cut the lid off of a 1.5 mL tube and place an adapter on top. Place a C18 tip in the adapter. There should be enough room in the tube to collect ~100 µL of liquid without touching the C18 material at the bottom of the tip.
2. Equilibrate the C18 tip by pipetting 50 µL of Wetting Solution onto the top and centrifuging at 2,000 x g for approximately 3 min or until all the solution has passed through. Transfer the C18 tip to a clean collection tube.
3. Add 50 µL of Equilibration & Washing Solution (0.1% TFA) and centrifuge 3 min. Repeat this step once for two total Equilibration & Washing steps.
4. Load sample by passing IAP eluent through the C18 tip. Load IAP eluent in two steps using 50 µL in each step.
5. Wash the C18 tip by passing 50 µL of Equilibration & Washing Solution through two times.
6. Elute peptides off the C18 tip by passing 10 µL of Stagetip Elution Solution (40% ACN, 0.1% TFA) through two times. Pool the two resulting eluents.
7. Dry down the C18 tip eluents from the C18 tip purification in a vacuum concentrator (Speed-Vac) and store the peptides dry at -20°C until LCMS analysis will begin. At that time, re-dissolve the peptides in an appropriate solvent for LCMS analysis such as 5% acetonitrile, 0.1% TFA, 94.9% water (MS grade).