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Enrichment of Tyrosine Phosphorylated Peptides for Quantitative Mass Spectrometry Analysis of RTK Signaling Dynamics

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Abstract

Cells sense and respond to mitogens by activating a cascade of signaling events, primarily mediated by tyrosine phosphorylation (pY). Because of its key roles in cellular homeostasis, deregulation of this signaling is often linked to oncogenesis. To understand the mechanisms underlying these signaling pathway aberrations, it is necessary to quantify tyrosine phosphorylation on a global scale in cancer cell models. However, the majority of the protein phosphorylation events occur on serine (86%) and threonine (12%) residues, whereas only 2% of phosphorylation events occur on tyrosine residues (Olsen *et al.*, 2006). The low stoichiometry of tyrosine phosphorylation renders it difficult to quantify cellular pY events comprehensively with high mass accuracy and reproducibility. Here, we describe a detailed protocol for isolating and quantifying tyrosine phosphorylated peptides from drug-perturbed, growth factor-stimulated cancer cells, using immunoaffinity purification and tandem mass tags (TMT) coupled with mass spectrometry.

Keywords: Tyrosine phosphorylation, Receptor tyrosine kinases, Signal transduction, Phosphoproteomics, Phosphotyrosine enrichment, Tandem mass tag labeling, Growth factor stimulation

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Background

Tyrosine phosphorylation (pY)-mediated signaling networks regulate important cellular processes like cell growth, migration, differentiation, and aging. Given the importance of this post-translational modification, it is not surprising that nearly half of all protein tyrosine kinases (PTKs) are deregulated in cancer (Del Rosario and White, 2010). Although the function of PTKs as oncogenes has been well established, protein tyrosine phosphatases (PTPs) can have either positive or negative effects on cell proliferation. These deregulated enzymes can modulate the tyrosine phosphorylation landscape of cells, resulting in aberrant cellular signaling and cancer. Therefore, research over the past decade has focused on the development of target-based therapeutics that inhibit these enzymes.

Receptor tyrosine kinases (RTKs) are a sub-class of PTKs that mediate cellular responses to growth factors. RTKs can be aberrantly activated by gain-of-function mutations, genomic amplification, chromosomal rearrangements, or autocrine signaling (Du and Lovly, 2018). These activated RTKs can initiate a wide range of downstream signaling pathways, such as RAS/MAPK or PI3K/AKT signaling, which elicit oncogenic responses in cells (Du and Lovly, 2018). Understanding these complex dynamic signaling networks and identifying ways to attenuate this aberrant signaling has important implications for cancer treatment.

Phosphoproteomics is a powerful method that can be used to measure the global tyrosine phosphorylation status of cancer cells in a site-specific manner. It can be used to characterize novel small molecule inhibitors and improve our understanding of signaling networks in disease contexts that aid in the translation of these findings into clinical benefit. Recently, the effects of a SHP2 allosteric inhibitor (SHP099) on the pY network in response to cell stimulation with epidermal growth factor (EGF) identified two classes of phosphosites – sites that are dephosphorylated by SHP2 and sites that are protected by the SH2 domains of SHP2 from dephosphorylation by other PTPs (Vemulapalli *et al*., 2021). These findings explain how SHP2 has both positive and negative effects on signaling. Here, we present a detailed workflow on sample preparation, enrichment, and quantitative profiling of phospho-tyrosine peptides, via immobilized metal affinity chromatography (IMAC) and tandem mass tag (TMT) labeling, followed by a final immunoaffinity purification of the phospho-tyrosine proteome using a pY monoclonal antibody, adapted from Vemulapalli *et al*. (2021).

Materials and Reagents

1. PCR tubes (Denville Scientific, catalog number: C18064)
2. Chloroform-resistant 15 mL centrifuge tubes (Falcon, catalog number: 352196)
3. Low protein binding tubes (Thermo Fisher Scientific, catalog number: 90410)
4. 50 mL reagent reservoir (Corning, catalog number: 4870)
5. 96-well U-bottom assay plate (Falcon, catalog number: 353910)
6. C18 47 mm Extraction Disc (Empore, catalog number: 2215-C18)
7. Sep-Pak C18 50 mg sorbent cartridges (Waters, catalog number: WAT054960)
8. SOLA HRP 10 mg Sep-Pak cartridges (Thermo-Fisher, catalog number: 03-150-391)
9. Autoradiography films (Ece Scientific, catalog number: E3018)
10. Plunger for preparing StageTip (Hamilton, catalog number: 1122-01)
11. mColorpHast pH (2.0-9.0) test strips (MilliporeSigma, catalog number: 109584)
12. Amersham Hybond 0.45 µm PVDF (GE, catalog number: 10600023)
13. 35 cm Sepax GP-C18 resin (1.8 µm, 150 A, Thermo Fisher Scientific)
14. MDA-MB-468 cells (ATCC, catalog number: HTB-132)
15. Liquid Nitrogen
16. 10× Tris Buffered Saline (Boston BioProducts, catalog number: BM-300)
17. EPPS (Sigma, catalog number: E9502)
18. Sodium dodecyl sulfate (Sigma, catalog number: L3771)
19. NaCl (Fisher chemical, catalog number: S671)
20. 1 M Tris-HCl buffer, pH 7.5 (ThermoFisher, catalog number: 15567027)
21. Tris base (Fisher Chemical, catalog number: BP152-500)

22. Glycine (Millipore-Sigma, catalog number: 56-40-6)
23. MOPS (Sigma, catalog number: M5162)
24. Sodium hydroxide (VWR, catalog number: BDH7225)
25. Sodium phosphate, dibasic (Sigma, catalog number: S9763
26. Ni-NTA Magnetic Agarose beads (Qiagen, catalog number: 36113)
27. Leibovitz's L-15 Medium (Gibco, catalog number: 11415064)
28. TC treated 150 mm dishes (Thermo Fisher Scientific, catalog number: 168381)
29. 1× HBSS (Gibco, catalog number: 14025092)
30. 1× PBS (Corning, catalog number: 21-031-CV)
31. 1× Trypsin-EDTA, 0.25% phenol red (Corning, catalog number: 25-050-Cl)
32. Fetal bovine serum (GeminiBio, catalog number: 100-106)
33. Penicillin-streptomycin 100× solution (Gibco, catalog number: 15140-122)
34. Gibco Trypan blue solution, 0.4% (Thermo Fisher Scientific, catalog number: 15250061)
35. SHP099 (DC chemicals, catalog number: DC9737)
36. DMSO (Corning, catalog number: 25-950-CQC)
37. EGF (Gibco, catalog number: PHG0311)
38. Phospho-Tyr-1000 antibody (Cell Signaling Technology, catalog number: 8954)
39. Protease inhibitor cocktail (Roche, catalog number: 04693159001)
40. PhosSTOP (Roche, catalog number: 04906837001)
41. Sodium orthovanadate (NEB, catalog number: P0758L)
42. Dithiothreitol (DTT) (Thermo Fisher Scientific, catalog number: R0861)
43. Iodoacetamide (IAA) (Thermo Fisher Scientific, catalog number: A39271)
44. Bicinchoninic acid assay kit (Pierce, catalog number: 23225)
45. Water, HPLC-grade (MilliporeSigma, catalog number: 270733)
46. Methanol, HPLC-grade (MilliporeSigma, catalog number: 34860)
47. Chloroform, HPLC-grade (MilliporeSigma, catalog number: 528730)
48. Urea (MilliporeSigma, catalog number: U4883)
49. HEPES (MilliporeSigma, catalog number: H3375)
50. Lysyl Endopeptidase, Mass Spectrometry Grade (FUJIFILM Wako, catalog number: 125-05061)
51. Trypsin, sequencing-grade (Promega, catalog number: V511C)
52. Acetonitrile, LiChrosolv® (MilliporeSigma, catalog number: 103725)
53. Formic acid, LiChroPur™ (MilliporeSigma, catalog number: 543804)
54. Trifluoroacetic acid (TFA), HPLC-grade (MilliporeSigma, catalog number: 302031)
55. Quantitative Colorimetric Peptide Assay (Pierce, catalog number: 23275)
56. Ammonium hydroxide, LiChroPur™ (MilliporeSigma, catalog number: 543830)
57. EDTA, LiChroPur™ (MilliporeSigma, catalog number: 79884)
58. $FeCl_3$ (MilliporeSigma, catalog number: 451649)
59. Ethanol, HPLC-grade (MilliporeSigma, catalog number: 09-0851)
60. TMT11plex isobaric label reagents (Thermo Fisher Scientific, catalog number: A37725)
61. 50% Hydroxylamine (MilliporeSigma, catalog number: 159417)
62. Protein A agarose (MilliporeSigma, Roche, catalog number: 11134515001)
63. Phospho-Thr202/Tyr204-Erk1/2 antibody (Cell Signaling Technology, catalog number: 9101)
64. Erk1/2 antibody (Cell Signaling Technology, catalog number: 9102)
65. β-actin antibody (Sigma-Aldrich, catalog number: A1978)
66. Rabbit IgG, HRP-linked whole Ab (from donkey) (GE Healthcare, catalog number: NA934V)
67. Mouse IgG, HRP-linked whole Ab (from sheep) (GE Healthcare, catalog number: NXA931V)
68. Novex WedgeWell 12% Tris Glycine gels (Thermo Fisher Scientific, catalog number: XP00122BOX)
69. 6× Laemmli sample buffer (Boston BioProducts, catalog number: BP-111R)
70. Precision Plus protein dual color standards (Bio-Rad, catalog number: 1610374)
71. Bovine Serum Albumin Fraction V (MilliporeSigma, Roche, catalog number: 10735094001)
72. SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, catalog number: 34580)
73. PTMScan IAP buffer 10× (Cell Signaling Technology, catalog number: 9993)

74. Complete L-15 media (see Recipes)
75. Serum-free L-15 media (see Recipes)
76. Lysis buffer (see Recipes)
77. 1× SDS-PAGE Running buffer (see Recipes)
78. 1× Transfer buffer (see Recipes)
79. Blocking buffer (see Recipes)
80. IAP buffer (see Recipes)
81. Solvent A (see Recipes)
82. Solvent B (see Recipes)
83. Solvent C (see Recipes)
84. Solvent D (see Recipes)
85. Solvent E (see Recipes)
86. Solvent F (see Recipes)
87. Solvent G (see Recipes)
88. Solvent H (see Recipes)
89. Solvent I (see Recipes)
90. Solvent J (see Recipes)
91. Solvent K (see Recipes)
92. Solvent L (see Recipes)
93. Solvent M (see Recipes)
94. Solvent N (see Recipes)
95. Solvent O (see Recipes)
96. 1× TBST (see Recipes)
97. Digestion buffer (see Recipes)

Equipment

1. -80°C freezer
2. End-over-end tube rotator/shaker (Fisher Scientific, catalog number: 13-687-12Q)
3. Cell scrapers (VWR, catalog number: 734-2602)
4. Biosafety Cabinet
5. Nikon Eclipse TS100 microscope
6. 37°C water bath
7. 37°C, 0% CO_2 tissue culture incubator
8. 25°C and 37°C shakers
9. 1490 Reichert Bright-Line Hemocytometer (Hausser Scientific)
10. Refrigerated and room temperature microcentrifuges
11. XCell SureLock Mini-Cell Electrophoresis System (Invitrogen)
12. XCell II™ Blot Module (Invitrogen)
13. PowerPac Power supply (Bio-Rad)
14. X-ray film cassette
15. X-ray film processor
16. SpectraMax M5 plate reader (Molecular Devices)
17. Branson Digital Sonifier 250 with Tapered Titanium Microtip
18. Vacuum concentrator centrifuge (Thermo Fisher)
19. 20-position Extraction Manifold (Waters)
20. Mass spectrometer (Thermo Fisher)

Software

1. SoftMax Pro (Molecular Devices)
2. Microsoft Excel

Procedure

A. Ligand stimulation of MDA-MB-468 cells

SHP2 facilitates the full activation of RAS/MAPK signaling upon induction of EGFR or other receptor tyrosine kinases. It functions by interacting with tyrosine phosphorylated proteins and by dephosphorylating various substrates throughout the RTK signaling network. To capture the system-wide phosphorylation events regulated by SHP2, we allosterically inhibited SHP2 in a serum-starved breast cancer cell line, which was harboring EGFR amplification and stimulated with EGF ligand for various time periods.

1. Seed MDA-MB-468 cells in eleven 150 mm dishes (10 million cells in 20 mL media per dish) in pre-warmed complete L-15 media.
2. Grow the cells in a humidified tissue culture incubator at 37°C and 0% CO_2 until they reach 80% confluence (~48 h).

> *Note: The base medium for MDA-MB-468 cells is ATCC-formulated Leibovitz's L-15 Medium supplemented with 10% fetal bovine serum. L-15 medium is formulated for use in carbon dioxide (CO_2) – free systems, hence these cells were cultured in 0% CO_2.*

3. Aspirate the media and wash the cells twice with 15 mL of pre-warmed 1× HBSS buffer. Aspirate the HBSS buffer, add pre-warmed serum-free L-15 media (20 mL per dish) and incubate the cells at 37°C for 24 h.
4. The following day, treat the cells with DMSO carrier or 10 µM SHP099, and incubate at 37°C for 2 h.
5. Stimulate the cells with 10 nM EGF for 5, 10, or 30 min (as shown in Figure 1B, Vemulapalli *et al*., 2021).
6. To terminate ligand stimulation, gently wash the cells with 15 mL of ice-cold PBS. Aspirate PBS.
7. Using a cell scraper, scrape the cells off the dish in 1 mL of ice-cold PBS and transfer them to a 1.5 mL microcentrifuge tube.
8. Centrifuge the cells at 1,000 × *g* for 5 min at 4°C and aspirate the supernatant. Flash-freeze the cell pellets, by placing the tubes in a bath of liquid nitrogen for 10 s. Store the tubes at -80°C until further use.

B. Preparation of cell lysates

Whole cell extracts prepared from EGF-stimulated cells served as starting materials for enrichment of tyrosine phosphorylated peptides for mass spectrometry.

1. Thaw the cell pellets on ice and resuspend them in 1 mL of lysis buffer.
2. Sonicate the cell lysates on ice at 30% amplitude for a total of 30 s (three 10 s pulses), to shear DNA and reduce sample viscosity.
3. Centrifuge the cell extracts at 24,000 × *g* for 20 min at 4°C. Transfer the supernatant into new 1.5 mL tubes and discard the pellets (insoluble cell debris).
4. Perform a bicinchoninic acid (BCA) protein assay according to the manufacturer's instructions. Absorbance of the lysates and BSA standards were measured in a SpectraMax microplate reader, using SoftMax Pro software. A BSA standard curve was created using Microsoft Excel and used to normalize lysates to a concentration of 1 mg/mL.
5. Aliquot 50 µL from the normalized samples, boil them in 1× Laemmli sample buffer at 95°C for 5 min, and store them at -80°C until further use.

Notes:

a. *These samples will be used for testing the ERK phosphorylation response of MDA-MB-468 cells to SHP099 and EGF treatments by western blot.*
b. *ERK is a serine/threonine kinase whose dual phosphorylation at T202/Y204 is used as a readout for RTK activation.*

6. Add dithiothreitol (DTT) to the 950 µL of normalized cell lysate, to a final concentration of 5 mM. Incubate at 55°C for 30 min to reduce disulfide bonds. Allow the samples to cool to room temperature.
7. Add iodoacetamide across all samples, to a final concentration of 14 mM. Incubate at room temperature for 45 min in the dark to alkylate reduced cysteines.
8. Add DTT to a final concentration of 10 mM. Incubate in the dark at room temperature for 15 min to quench excess iodoacetamide.
9. Freeze samples at -80°C until further use.

C. Western blotting

To confirm the cellular response to drug/ligand treatment, western blotting was performed. As expected, EGF stimulation induced an ERK1/2 phosphorylation response in MDA-MB-468 cells and SHP099 treatment inhibited this (Figure 1A, Vemulapalli *et al*., 2021).

1. Load the samples (20-30 µg/lane) in 12% tris glycine gels, along with 5 µL of protein ladder.
2. Run the gels in 1× Tris-Glycine SDS buffer at 100 V. Stop the electrophoresis when the dye front reaches the bottom of the gel.
3. Transfer the proteins to a 0.45 µm PVDF membrane using XCell II Blot module following the manufacturer's instructions.
4. Incubate the membrane in 10 mL of blocking buffer for 1 h at room temperature with gentle agitation.
5. Decant the blocking buffer and incubate the membrane in 5-10 mL of primary antibody [Phospho-Thr202/Tyr204-Erk1/2 (1:2000 dilution), Erk1/2 (1:4000 dilution), and β-actin (1:10,000 dilution)] diluted in blocking buffer with gentle agitation overnight at 4°C.
6. Wash the membrane three times for 10 min each with 15 mL of 1× TBST.
7. Incubate the Phospho-Thr202/Tyr204-Erk1/2 and Erk1/2 membranes in 10 mL of rabbit IgG secondary antibody, and the β-actin membrane in 10 mL of mouse IgG secondary antibody, both diluted in blocking buffer at 1:5,000 dilution for 1 h at room temperature with gentle agitation.
8. Wash the membrane with 15 mL of 1× TBST for 10 min three times.
9. Incubate the membrane in 3 mL of a solution containing the SuperSignal West Pico PLUS Luminol/Enhancer and SuperSignal West Pico PLUS peroxide (1:1 dilution) for 5 min at room temperature with gentle agitation. Place the membrane in a plastic sheet protector in a film cassette and expose it to X-ray film (optimal exposure times vary for each primary antibody) and image using an X-ray film processor (Figure 1A, Vemulapalli *et al*., 2021).
10. After confirming appropriate cellular response to drug and/or ligand treatments, proceed to sample preparation for mass spectrometry.

D. Protein precipitation and digestion

After cell lysis, proteins were precipitated by the methanol-chloroform method to remove unwanted cellular material such as lipids, genomic DNA, and detergents present in the lysis buffer, which can interfere with downstream steps, including enzymatic digestion. The protein extract is enzymatically digested using proteases, such as trypsin and/or LysC, to generate peptides that are suitable for analysis via bottom-up proteomics and mass spectrometry.

1. Thaw and transfer the 950 µL of cell lysates to chloroform-resistant 15 mL Falcon tubes (see Materials and Reagents).
2. Add 4 volumes (3.80 mL) of methanol to the cell lysate, vortex for 5 s, and centrifuge at 2,000 × *g* for 30 s at room temperature. Keep the pellet and remove the supernatant.

3. Add 1 volume (950 µL) of chloroform, vortex for 5 s, and centrifuge at 2,000 × *g* for 30 s at room temperature. Keep the pellet and remove the supernatant.
4. Add 3 volumes (2.85 mL) of water and vortex for 5 s.
   Note: The solution will appear milky; this indicates protein precipitation.
5. Centrifuge the tubes at 4,000 × *g* for 20 min at room temperature.
   Note: A protein pellet will appear at the interphase between the bottom chloroform layer and the top aqueous layer.
6. Aspirate the top aqueous layer carefully with a 1 mL micropipette to waste, and add 4 volumes of ice-cold methanol to wash the protein pellet.
7. Vortex the pellet for 10 s and centrifuge at 4,000 × *g* for 20 min at room temperature.
   Note: The pellet settles to the bottom of the tube. Aspirate the supernatant using a micropipette to waste.
8. Add 4 volumes of ice-cold methanol, vortex for 10 s, and centrifuge at 4,000 × *g* for 20 min at room temperature. Repeat for a total of three methanol washes.
9. With the final methanol wash, aspirate the supernatant with a 1 mL micropipette to waste.
   Note: The pellet settles to the bottom of the tube. Aspirate the supernatant using a micropipette to waste.
10. Add 1 mL of digestion buffer: 8 M urea diluted in 25 mM HEPES, pH 8.5 to the pellet.
    Note: Always prepare fresh urea solution.
11. Resolubilize the pellet with physical disruption, such as sonication (40% amplitude, 5 s pulses, three times).
12. Dilute lysates to a final 4 M of urea concentration, with the addition of 1 mL of 25 mM HEPES pH 8.5.
13. Digest the protein sample in Lysyl endopeptidase (Lys-C), at an enzyme-to-protein ratio of 1:100 at 37°C for 2 h with shaking.
14. Dilute the 4 M urea to a final concentration of 2 M urea, by the addition of 2 mL of 25 mM HEPES pH 8.5.
15. Add Lys-C at an enzyme-to-substrate ratio of 1:50 and digest overnight while shaking at room temperature, to ensure that 100% of the protein is cleaved by Lys-C.
16. The following day, dilute the sample to 1 M urea, by the addition of 4 mL of 25 mM HEPES pH 8.5.
17. Continue protein digestion with trypsin added at an enzyme-to-substrate ratio of 1:100, and incubate at 37°C for 6-8 h with shaking.

E. Assessment of missed cleavages

Verify the completeness of protein digestion to ensure that the majority of identified peptides are suitable for analysis and peptide sequencing via mass spectrometry.

1. To assess the percentage of missed cleavages, de-salt a small fraction (~2-3 µg) of peptides from a few representative samples using C_{18} STAGE Tips, following Rappsilber *et al*. (2003).
2. Following the desalting steps, dry peptides via vacuum centrifugation.
3. Reconstitute the dried peptides in ~6 µL of Solvent A to analyze by LC-MS/MS.
   Note: A missed cleavage rate of less than 10% is acceptable. If the missed cleavage rate is >10%, repeat the tryptic digestion at 1 M urea.
4. After ensuring that the cleavage rate is >90%, aliquot 5 µL from each of the 11 samples to measure their corresponding peptide concentrations, using the Pierce quantitative colorimetric peptide assay, according to the manufacturer's instructions.
5. Store the peptide samples at -80°C until further use, or directly proceed to the next step.

F. De-salting and clean-up of peptide samples

De-salting by C18 Solid-Phase Extraction (SPE) will remove DNA, RNA, metabolites, and undigested protein from the peptide mixture.

1. Thaw and acidify samples to a final concentration of 1% TFA (v/v; pH ~2) and incubate on ice for 15 min.
   Note: Acidification will improve peptide interaction with the stationary phase of the SPE cartridges.
2. Centrifuge the samples at 1,780 × *g* for 15 min at room temperature, and transfer the supernatant to new 15 mL tubes.

3. Label and mount 50 mg C_{18} Sep-Pak cartridges to a vacuum manifold connected to a waste collection vessel. Turn on the vacuum.
   Note: Use 50 mg sorbent per 1 mg of protein per sample.
4. Activate the C_{18} Sep-Pak with 1 mL of Solvent B.
5. Equilibrate the Sep-Pak with 1 mL of Solvent C.
6. Wash the Sep-Pak three times with 1 mL of Solvent D.
7. Load the acidified peptides diluted in Solvent D onto the cartridge. Apply vacuum starting at the lowest setting and gradually increase, to allow a dropwise flow rate of the sample.
   Note: A slower flow rate allows adequate interaction between the sample and the sorbent.
8. Perform four 1 mL washes with 1 mL of Solvent D.
9. Turn off the vacuum, discard the waste liquid, insert clean labeled 2 mL collection tubes, and replace the manifold cover.
10. Elute the peptides with 450 µL of Solvent E.
11. Perform a second elution in the same collection tubes, using 300 µL of Solvent F.
12. Dry the desalted peptides by vacuum centrifugation overnight. Store peptides at -80°C until further use, or directly proceed to next step.

G. Immobilized Metal Affinity Chromatography (IMAC)

IMAC is the most commonly method for phosphopeptide enrichment. It works on the principle that negatively charged phosphate groups on peptides have affinity towards positively charged metal ions. Here, we used agarose beads that have strong metal-chelating nitrilotriacetic acid (NTA) groups to chelate with Fe^{3+} ions and enriched phosphorylated peptides from the peptide mixture.

1. Fill 2 mL tubes with Solvent H to reduce peptide-to-surface binding and set tubes aside until the elution step.
2. Prepare Ni-NTA magnetic agarose beads (Qiagen):
   a. Transfer 500 µL (500 µL/mg protein) of bead slurry into a 1.5 mL tube.
   b. Wash the beads with 1 mL of water, centrifuge briefly, and aspirate supernatant into waste. Wash beads twice more with 1 mL of water to remove all traces of ethanol.
   c. Add 1 mL of 40 mM EDTA to the beads and incubate at room temperature for 30 min with shaking.
   d. Wash the beads with 1 mL of water thrice and aspirate supernatant into waste.
   e. Chelate the beads with 1 mL of 100 mM $FeCl_3$ and incubate at room temperature for 30 min with shaking.
   f. Wash the beads with 1 mL of Solvent G and aspirate supernatant into waste. Repeat this step for a total of four washes.
3. Reconstitute the peptides in Solvent G to achieve a final concentration of 1 mg/mL. Adjust the Solvent G volumes based on the concentrations determined from the previous colorimetric peptide assay. Transfer the peptides to the tubes containing the beads. Incubate the peptide bead mixture via end-over-end rotation for 30 min at room temp.
   Note: Prior to sample adsorption, ensure that the pH of the peptide samples is acidic.
4. After 30 min, briefly centrifuge the peptide-bead slurry and collect the supernatant containing non-phosphorylated peptides into new 2 mL tubes. Do not disturb or touch the magnetic bound phosphopeptides. Dry the non-phosphorylated peptides by vacuum centrifugation and store at -80°C until needed for proteomic analyses.
5. Wash the phosphopeptide bound-bead slurry with 1 mL of Solvent G three times and aspirate the supernatant to waste.
6. Remove Solvent H from the 2 mL elution tubes from the previous step 1 into waste. Add 36 µL of Solvent I to a final concentration of 2% formic acid of your total elution volume (below step) in these same elution tube(s).
7. Add 300 µL of Solvent J to the phosphopeptide bound-bead slurry and shake for 3 min at room temperature. Centrifuge briefly and transfer the phosphopeptide eluate (supernatant) to the acidified 2 mL elution tubes from step 6.

8. Repeat the above elution step twice to ensure that all phosphopeptides have eluted off the IMAC beads. All three eluates (900 µL) should be collected in a single acidified 2 mL tube(s) at 2% formic acid.
   Note: The phosphopeptide yield can vary from on average ~2-4% of the initial protein input concentration. For example, using these protocols, 1 mg of digested peptides can yield ~20 µg of enriched phosphopeptides.
9. Dry the phosphopeptides via vacuum centrifugation and solubilize the phosphopeptides in 500 µL of Solvent D.
10. Desalt the phosphopeptides using SOLA HRP 10 mg cartridges (Thermo Fisher):
    a. Activate with 500 µL of Solvent H.
    b. Equilibrate with 500 µL of Solvent K.
    c. Wash with 500 µL of Solvent D three times.
    d. Load 500 µL of acidified phosphopeptides (ensure pH <4).
    e. Wash with 500 µL of Solvent D three times.
    f. Elute phosphopeptides twice with 500 µL of Solvent F, collect the eluate in a single 2 mL tube, and dry using vacuum centrifugation.
    g. Store desalted phosphopeptides at -80°C until further use, or directly proceed to TMT labeling.

H. Tandem Mass Tag (TMT) labeling

To enable relative quantification of phosphopeptides from the eleven EGF/SHP099 treatments simultaneously, samples were labeled with 11 TMT reagents, to allow for multiplexed quantitative measurements via mass spectrometry.

1. Reconstitute desalted phosphopeptides in 200 mM EPPS pH 8.5 at room temperature.
2. Perform isobaric labelling of the enriched phosphopeptides using TMT reagents (0.8 mg) that are dissolved in 40 µL of Solvent H, for a final concentration of 20 µg/µL. Label each phosphopeptide sample with one TMT reagent at a ratio of 4:1 (TMT:phosphopeptide) by mass, to assure efficient labeling of all phosphopeptides (see below for labeling efficiency criteria). For example, 30 µg of phosphopeptides will use 6 µL of TMT reagent.
3. Allow the TMT reaction to proceed at room temperature for ~1.5 h prior to performing a quality control check, often referred to as a TMT labeling ‘ratio check’, to assess labeling efficiency and phosphopeptide enrichment efficiency. Aliquot approximately 2 µL of each phosphopeptide-TMT reaction into a combined mixture and desalt using a C18 StageTip, as described previously for the digestion and missed cleavage check (section E). Freeze the stock phosphopeptide labeling reactions at -80°C, until the ratio check has been acquired and analyzed.
4. If the labeling efficiency is >98%, the labeling reactions can be quenched individually with Solvent L to a final concentration of 0.5% hydroxylamine for 15 min, acidified, and all TMT labeled peptides combined into a single mixture of TMT labeled phosphopeptides to be dried by vacuum centrifugation.
5. Desalt the combined TMT labeled phosphopeptide mix with a 50 mg Sep-Pak, using the method previously discussed for unenriched peptides (section F).
6. Lyophilize the final desalted TMT labeled phosphopeptide mix for up to ~48 h, to ensure that all traces of trifluoroacetic acid have been removed from the phosphopeptides. Store the phosphopeptides at -80°C until further use, or directly proceed to next step.

I. Immunoaffinity purification (IAP) of tyrosine phosphorylated peptides

Phosphopeptide mixtures enriched by IMAC majorly contain phospho-serine and phospho-threonine peptides and very low levels of phosphotyrosine peptides. Since SHP2 is an SH2 domain-containing protein tyrosine phosphatase that binds and dephosphorylates phospho-tyrosine (pY) residues, we enriched pY peptides by immunoprecipitating the IMAC-purified peptides using a phospho-tyrosine antibody.

1. Aliquot 60 µL of protein A-agarose bead slurry (which is equal to 30 µL of packed beads) using a precut 1 mL micropipette tip into a clean 1.5 mL tube.
   Note: Invert the bottle well before pipetting beads.

2. Wash the beads with 1 mL of ice-cold 1× PBS, vortex briefly, centrifuge at 2,000 × *g* for 30 s, and aspirate supernatant. Repeat the wash for a total of three times.
3. Conjugate 30 µL of phospho-tyrosine rabbit antibody (P-Tyr-1000) (equivalent to 100 µg antibody) with the pre-washed protein A-agarose beads and 1× PBS to a final volume of 1.5 mL.
   Note: Couple the phospho-tyrosine rabbit antibody (P-Tyr-1000; Cell Signaling Technology) to protein A agarose beads one day prior to the phosphotyrosine peptide enrichment.
4. Seal the tube with parafilm and incubate by end-over-end rotation overnight at 4°C.
5. Centrifuge the antibody-conjugated beads at 2,000 × *g* for 30 s, aspirate and discard supernatant.
6. Wash the antibody-conjugated beads thrice with 1 mL of ice-cold 1× PBS, aspirate and discard supernatant.
7. Resuspend antibody-conjugated beads in 40 µL of ice-cold 1× PBS and use immediately.
8. Reconstitute lyophilized TMT-labeled phosphopeptides in 500 µL of 1× IAP buffer.
   Note: Pipette gently using a 1 mL micropipette to avoid introducing air bubbles.
9. Ensure that the sample has a neutral pH (~7.4). If the sample is acidic (pH <6), add 5-10 µL of 1 M Tris buffer pH 8.0.
10. Centrifuge the sample at 10,000 × *g* for 5 min at 4°C.
11. Transfer the supernatant (TMT labelled phosphopeptides) directly atop the antibody-conjugated beads, to ensure immediate mixing while avoiding the introduction of air bubbles.
12. Seal the tube with parafilm and incubate for 2 h at 4°C.
13. Centrifuge at 2,000 × *g* for 30 s. Transfer the supernatant to a clean tube and store at -80°C.
    Note: The supernatant contains phospho-serine and phospho-threonine peptides which can be analyzed by mass spectrometry if desired.
14. Add 1 mL of 1× IAP buffer to the antibody-conjugated beads, gently invert the tube five times, centrifuge at 2,000 × *g* for 30 s at 4°C, and discard supernatant.
15. Add 400 µL of ice-cold water to the beads, transfer contents to a 0.2 µM filter spin column, and centrifuge at 1,000 × *g* for 30 s at 4°C. Discard the flow-through. Use 400 µL of ice-cold water to wash, collect any residual beads from the initial tube, and transfer them to the filter spin column with the previously transferred antibody-conjugated beads. Centrifuge at 2,000 × *g* for 30 s at 4°C and discard the flow-through.
16. Place the spin column in a new 1.5 mL collection tube, add 75 µL of 100 mM formic acid to elute the pY peptides from the antibody-conjugated beads, and incubate for 10 min at 25°C.
    Note: Gently tap/flick the tube every 5 min for mixing. Do not vortex. Centrifuge at 1,000 × g for 30 s.
17. Perform a second elution in the same collection tube. Add 75 µL of 100 mM formic acid to the beads, incubate for 10 min gently tapping the tube every 5 min, and centrifuge at 2,000 × *g* for 30 s.
18. Desalt the acidified eluate containing phospho-tyrosine peptides using a C_{18} StageTip as previously described. Elute the pY peptides with 25 µL of Solvent M, successively with 50 µL of Solvent N into a glass vial suitable for LC-MS/MS, and dry by vacuum centrifugation. Reconstitute the phospho-tyrosine peptides in 4.5 µL of Solvent O for analysis via liquid chromatography-mass spectrometry.

Recipes

1. Complete L-15 media
Leibowitz's L-15 media
10% FBS
1× Penicillin-streptomycin

2. Serum-free L-15 media
Leibowitz's L-15 media
1× Penicillin-streptomycin

3. Lysis buffer
2% SDS

150 mM NaCl
50 mM Tris (pH 8.5-8.8)
Protease inhibitor cocktail (1 tablet per 20 mL)
PhosSTOP (2 tablets per 20 mL)
2 mM Sodium orthovanadate

4. **1× SDS-PAGE Running buffer**
   25 mM Tris base
   192 mM Glycine
   0.1% SDS

5. **1× Transfer buffer**
   25 mM Tris base
   192 mM Glycine
   20% Methanol

6. **Blocking buffer**
   5% BSA
   1× TBST

7. **IAP buffer**
   50 mM MOPS/NaOH pH 7.2
   10 mM Na_2HPO_4
   50 mM NaCl

8. **Solvent A**
   5% acetonitrile
   5% formic acid
   90% water

9. **Solvent B**
   100% methanol

10. **Solvent C**
    50% acetonitrile
    0.1% trifluoroacetic acid
    49.9% water

11. **Solvent D**
    0.1% trifluoroacetic acid
    99.9% water

12. **Solvent E**
    40% acetonitrile
    0.1% trifluoroacetic acid
    59.9% water

13. **Solvent F**
    70% acetonitrile
    0.1% trifluoroacetic acid

29.9% water

14. Solvent G

80% acetonitrile
0.15% trifluoroacetic acid
19.85% water

15. Solvent H

100% acetonitrile

16. Solvent I

50% formic acid
50% water

17. Solvent J

50% acetonitrile
0.7% ammonium hydroxide
49.3% water

18. Solvent K

70% acetonitrile
29% water
1% trifluoroacetic acid

19. Solvent L

50% hydroxylamine
50% water

20. Solvent M

40% acetonitrile
0.1% formic acid
59.9% water

21. Solvent N

70% acetonitrile
0.1% formic acid
29.9% water

22. Solvent O

3% acetonitrile
0.5% formic acid
96.5% water

23. 1× TBST

For 1 L of 1× TBST, add:
100 mL of 10× Tris Buffered Saline
900 mL of water
1 mL of 100% Tween 20

24. Digestion buffer
8 M urea diluted in 25 mM HEPES pH 8.5

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Competing interests

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