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Labeling and Identification of Direct Kinase Substrates

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Abstract

Identifying kinase substrates is an important step in mapping signal transduction pathways, but remains a difficult and time-consuming process. Analog-sensitive kinases (AS-kinases) have been used to selectively tag and identify direct kinase substrates in lysates from whole cells. In this approach a gamma-thiol ATP-analog and AS-kinase are used to selectively thiophosphorylate target proteins. Thiophosphate is used as a chemical handle to purify peptides from a tryptic digest, and target proteins are identified by liquid chromatography and tandem mass spectrometry (LC-MS/MS). Here, we describe an updated strategy for labeling AS-kinase substrates, solid-phase capture of thiophosphorylated peptides, incorporation of stable-isotopic labeling in cell culture (SILAC) for filtering nonspecific background peptides, enrichment of phosphorylated target peptides to identify low-abundance targets, and analysis by LC-MS/MS.

Introduction

Identification of protein substrates that are phosphorylated by a particular kinase has been time-consuming and prone to experimental artifacts. Analog-sensitive kinases (AS-kinases) have provided a system to label, visualize, and identify kinase substrates (Fig. 1). AS-kinases are engineered with “gatekeeper” mutations in their ATP-binding pocket such that they can accept bulky ATP analogs, generally with a large alkyl group at the N6 position (Fig. 1).¹ A wide range of AS-kinases have been generated and extensive validation has shown that the gatekeeper mutation does not affect their substrate specificity.

The Shokat and Clurman groups have each used γ -thiol-substituted ATP analogs to thiophosphorylate substrates of cyclin/cyclin-dependent kinase (CDK) complexes.^{2,3} In each case, the labeled substrates have been isolated by solid-phase capture through the thiophosphate group, followed by elution and subsequent identification of captured peptides by liquid chromatography and tandem mass spectrometry (LC-MS/MS). These approaches allow discovery of unknown kinase substrates, but have been challenging to implement. We have developed an updated method to those used by Blethrow *et al.*² With this approach, we have identified substrates of the extracellular signal-regulated protein kinase 2 (ERK2) in NIH 3T3-L1 fibroblasts.⁴ Although a large number of ERK2 substrates were identified, only a small fraction of these substrates had been previously reported, raising the possibility that additional high-throughput screens performed on this and other kinases will reveal signaling networks that are much more interconnected than has been appreciated so far.

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This protocol updates earlier procedures in several ways to improve yield, account for nonspecific labeling by endogenous kinases, and reduce nonphosphorylated background so that very low abundance substrates can be identified (Fig. 2). To minimize losses on tube and tip surfaces, the entire process is conducted using capillary columns. The capillary format is also used to perform small-scale phosphopeptide enrichment on immobilized metal affinity chromatography (IMAC) columns.⁶ This additional phosphopeptide enrichment step substantially reduces nonphosphorylated peptides and is critical for identifying less abundant proteins. Application of Stable Isotope Labeling in Cell Culture (SILAC) provides a quantitative comparison between AS-kinase labeling and the negative control reaction.⁵ SILAC also enables a quantitative comparison of substrate abundance between different cells types and conditions. We believe data collected with this methodology will help illuminate how signaling pathways are rewired in different biological contexts.

Materials

Cell Culture and Stable Isotope Labeling

Note: SILAC reagents are from Thermo Fisher Scientific/Pierce Protein Research Products (Thermo/Pierce).

Phosphate buffered saline (PBS) (#10010-023, Life Technologies)

DMEM for SILAC (#89985, Thermo/Pierce)

Note: Other SILAC base medium may be substituted for other cell lines.

Dialyzed FBS (#89986, Thermo/Pierce)

L-Arginine HCl (#89989, Thermo/Pierce)

¹³C₆ L-Arginine HCl (#88210, Thermo/Pierce)

¹³C₆¹⁵N₄ L-Arginine HCl (#88434, Thermo/Pierce)

Note: This reagent is optional.

L-Lysine 2HCl (#89987, Thermo/Pierce)

¹³C₆ L-Lysine 2HCl (#89988, Thermo/Pierce)

¹³C₆¹⁵N₂ L-Lysine 2HCl (#88432, Thermo/Pierce)

Note: This reagent is optional.

L-Proline (#88211, Thermo/Pierce)

Cell line expressing AS-ERK2 or another validated AS-kinase

Note: High expression and activity of the AS-kinase is critical for effective substrate labeling.

Substrate Labeling

Phorbol-12-myristate-13-acetate (PMA) (#4174, Cell Signaling Technologies, Danvers, MA)

Note: Other stimulating ligands or agonists may be used to activate other AS-kinases.

Halt Protease/Phosphatase Inhibitor Cocktail (#78441, Thermo/Pierce)

Phenylmethanesulfonyl fluoride (PMSF, #93482, Sigma-Aldrich)

Ethylene glycol tetraacetic acid (EGTA)

Tris(2-carboxyethyl)phosphine (TCEP) in 500 mM stock solution (#77720, Thermo/Pierce)

Note: Sulfur-based reducing agents may not be substituted for TCEP because they are not compatible with thiophosphate capture.

Sodium chloride (NaCl)

Magnesium chloride (MgCl₂)

Triton X-100

Glycerol

BCA Protein Assay Reagent (#23225, Thermo/Pierce)

Guanosine 5'-triphosphate (GTP) (#G8877, Sigma-Aldrich)

Gamma-thiol ATP analog (Bio-Log, Bremen, Germany)

Note: N6-phenyl-2-ethyl gamma-thiol ATP works well for AS-ERK2, another substituent at the N6 position may be better for other kinases.

Gamma-thiol ATP (#A1388, Sigma-Aldrich)

p-nitrobenzyl mesylate (PNBM) (#3700-1, Epitomics, Burlingame, CA)

Thiophosphate Ester Antibody (#2686-1, Epitomics)

Sample Processing

Methanol

Chloroform

Trypsin, sequencing grade (#V5113, Promega, Madison, WI)

Ammonium acetate

Urea (#U5378, Sigma-Aldrich)

Sep-Pak cartridges (WAT020515, Waters, Milford, MA)

Glacial acetic acid

Acetonitrile (ACN)

Thiophosphate Capture

Sulfolink beads (#20401, Thermo/Pierce)

Formic acid

Dithiothreitol (DTT)

Note: DTT is used for its nucleophilic thiol groups; other reducing agents may not be substituted.

Bovine serum albumin 2 mg/mL solution (#23209, Thermo/Pierce)

Potassium peroxymonosulfate (Oxone) (#228036, Sigma-Aldrich)

pH paper

HPLC Columns

POROS MC 20 beads (for immobilized metal affinity chromatography) (#1-5428-10, Invitrogen)

Note: Other phosphopeptide enrichment strategies, such as titanium dioxide, may be substituted.

POROS R2 10 μm beads (#1-1118-02, Invitrogen)

Reverse-phase (ODS-A) beads with 12 nm pores and 10 μm diameter (C18 beads, YMC, Allentown, PA)

Note: An alternative to C18 beads is an HPLC autosampler with in-line reverse-phase cartridge.

360 μm outer diameter (o.d.) \times 100 μm internal diameter (i.d.) silica capillary (#TSP100375, Polymicro Technology, Phoenix, AZ)

360 μm o.d. \times 200 μm i.d. silica capillary (#TSP200350, Polymicro Technology)

700 μm o.d. \times 530 μm i.d. silica capillary (#TSP530700, Polymicro Technology)

Capillary cutting stone (LS-CUTTER, Labsmith)

Teflon tubing (PTFE 0.011 inch i.d. \times 0.0625 inch o.d., Zeus, Orangeburg, SC)

Formamide (#F9037, Sigma-Aldrich)

Potassium silicate (KASIL 1, PQ Corporation)

2 mL glass vials

Small magnetic stir bars

Angiotensin peptide (#A9650, Sigma-Aldrich)

β -casein (#C6905, Sigma-Aldrich)

HPLC-grade water (#95304, Sigma-Aldrich)

Immobilized Metal Affinity Chromatography and Mass Spectrometry

Iron (III) chloride (#451649, Sigma-Aldrich)

Ethylenediaminetetraacetic acid (EDTA)

Sodium phosphate dibasic

Equipment

Sample Preparation and Labeling

Standard molecular biology equipment (such as micropipettors, conical tubes, among others)

Standard equipment for mammalian cell culture

Western blotting apparatus

Cold room or refrigerated microcentrifuge (4°C)

30°C oven with rotator or rocking platform for microcentrifuge tubes for kinase reaction

Chemical fume hood

Ring stand

10 mL syringes
Vacuum centrifuge (Speed-Vac)
Lyophilizer
Dewar with liquid nitrogen

Thiophosphate Capture

Heat gun

Note: An oven set at 80°C may substitute.

50°C oven to prepare fritted capillary columns
Hand-drill with 72 gauge bit
Helium packing device (up to 1,000 PSI) with magnetic stir plate

Note: A low pressure alternative is a centrifuge column (#89868 or #89896, Pierce/Thermo).

Mass Spectrometry

HPLC-MS/MS system equipped with nanoflow electrospray ion source

Note: We use an Orbitrap XL from Thermo Scientific with 20 nL/min flow rate on a home-made tapered tip. Our HPLC gradient is 0% to 70% ACN in 0.2M acetic acid over two hours, and our instrument is set to perform a survey scan with 100,000 resolution followed by up to 10 MS2 experiments using CID fragmentation and analysis in the LTQ ion trap.

Analysis software for reverse database search (examples, MASCOT or MaxQuant)

Recipes

Recipe 1: Serum-Free SILAC Medium

500 mL DMEM SILAC base medium
50 mg L-arginine
50 mg L-lysine
115 mg L-proline

Dissolve each amino acid in 1 mL media and sterile filter back into the original bottle. Heavy amino acid SILAC is made by substituting $^{13}\text{C}_6$ L-arginine/L-lysine or $^{13}\text{C}_6^{15}\text{N}_4$ L-arginine/ $^{13}\text{C}_6^{15}\text{N}_2$ L-lysine.

Recipe 2: SILAC Growth Medium

450 mL Serum-Free SILAC Medium (Recipe 1)
50 mL dialyzed fetal bovine serum
Medium should be prepared under sterile conditions.

Recipe 3: PMA Working Solution

Dissolve 1 mg PMA in 1 mL DMSO to make a 1.6 mM stock solution. Add 62.5 μL to 937.5 μL DMSO to make a 100 μM working dilution. Separate the working solution into 100 μL aliquots and store at -20°C for six months.

Recipe 4: Lysis/Kinase Reaction Buffer

20 mM HEPES pH 7.5

137 mM NaCl

0.5 mM EGTA

25 mM MgCl₂

0.2% Triton X-100

10% Glycerol

Prepare 10 mL and store at 4°C for one month. Add PMSF (final concentration of 1 mM) and Halt Protease/Phosphatase inhibitor cocktail (final concentration 1×) immediately before use.

Recipe 5: GTP Stock Solution

Dissolve 10 mg of GTP in 191 µL deionized water to prepare a 100 mM stock solution and store 25 µL single-use aliquots at -20°C for up to one year.

Recipe 6: PNBM Stock Solution

Dissolve PNBM at 12 mg/mL in DMSO to prepare a 50 mM stock. Store at -20°C for one year as single use aliquots.

Recipe 7: PNBM Working Solution

Dilute 25 µL of Recipe 6 by adding 5 µL water five times mixing after each addition (stepwise dilution avoids precipitation). Prepare this solution immediately before use.

Recipe 8: Protein Resuspension Buffer

Prepare 200 µL per sample of 8M Urea solution by adding 0.48 g/mL of urea to water a volumetric flask or tube. Use this buffer the day it is prepared.

Recipe 9: Digest Buffer

100 mM ammonium acetate pH 8.9

1 mM CaCl₂

Prepare 500 mL and store at room temperature for six months.

Add TCEP to 2 mM immediately before use (1:250 dilution from 500 mM stock solution)

Recipe 10: 0.1% Acetic Acid

Add 1 mL glacial acetic acid to 1 L deionized water. Store at room temperature.

Recipe 11: Sep-Pak Equilibration Buffer

Add 0.2 mL glacial acetic acid to 20 mL deionized water and 180 mL ACN (90% ACN 0.1% acetic acid). Store in an air-tight glass bottle for up to one year.

Recipe 12: Sep-Pak Elution Buffer

Add 0.2 mL glacial acetic acid to 120 mL deionized water and 80 mL ACN (40% ACN in 0.1% acetic acid). Store in an air-tight glass bottle for up to one year.

Recipe 13: C18 Suspension Buffer

Add 2 mL isopropanol to 8 mL ACN and store at room temperature in a glass bottle for three months.

Recipe 14: Angiotensin Peptide Solution

Dissolve 1 mg angiotensin peptide in 771 μL 0.1% acetic acid (Recipe 10) to prepare a 1 nmol/ μL stock. Dilute the stock 1:1000 in 0.1% acetic acid to prepare a 1 pmol/ μL working solution. Store the stock at -20°C for one year; store the working solution at 4°C for one month.

Recipe 15: B-Casein Peptide Solution

Dissolve 2.4 mg (100 nmol) B-casein in 1 mL Digest Buffer (Recipe 9). Add 20 μg Trypsin, mix well and place at 37°C for six hours to overnight. Dilute the digest 1:100 into 0.1% acetic acid (Recipe 10) to make a 1 pmol/ μL solution. Divide into 50 μL single-use aliquots and store at -80°C .

Recipe 16: Thiophosphate Washing Buffer

25 mM HEPES, 50% ACN adjusted to pH 7.0

Prepare 100 mL and store in an air-tight glass bottle at room temperature for three months. Discard the solution if precipitate is visible.

Add 5 mM TCEP immediately before use (1:100 dilution from 500 mM stock solution).

Recipe 17: Thiophosphate Binding Buffer with BSA

Supplement 1 mL of Recipe 16 with 25 $\mu\text{g}/\text{mL}$ BSA (1:80 dilution from 2 mg/mL stock) and add 5 mM TCEP immediately before use (1:100 dilution from 500 mM stock solution). Prepare immediately before use and do not store this buffer.

Recipe 18: Sulfolink Quenching Buffer

25 mM HEPES, 50% ACN adjusted to pH 8.5

Prepare 50 mL and store in an air-tight container at room temperature for three months.

Add DTT to a final concentration of 5 mM to 0.5 mL of the solution immediately before use.

Recipe 19: Formic Acid Wash

Add 2.5 mL formic acid to 47.5 mL deionized water (5% formic acid). Store in an air-tight glass bottle for three months.

Recipe 20: Oxidizing Elution Buffer

Dissolve 10 mg Oxone to 1 mL deionized water to prepare a 10 mg/mL stock solution, and then add 200 μL of the stock solution to 800 μL deionized water to yield a 2 mg/mL working solution. Prepare both solutions immediately before use.

Recipe 21: IMAC EDTA Buffer

Dissolve 292 mg of EDTA in 10 mL deionized water (100 mM) and adjust the pH to 8.9. Store in an air-tight glass bottle for three months.

Recipe 22: IMAC Iron Chloride Solution

Dissolve 1 g iron (III) chloride in 61 mL deionized water [100 mM iron (III) chloride].

Store in an air-tight glass bottle for up to one month. Prepare a fresh solution if any precipitate is visible.

Recipe 23: IMAC Organic Rinse

Add 0.5 mL glacial acetic acid and 12.5 mL ACN to 47 mL deionized water (1% acetic acid 25% ACN). Store in an air-tight glass bottle for three months.

Recipe 24: IMAC Elution buffer

Prepare 50 mL of 250 mM sodium phosphate with pH adjusted to 9.0. Store in an air-tight glass bottle for three months.

Recipe 25: HPLC Aqueous Solvent

Add 12 mL glacial acetic acid to 988 mL HPLC-grade water (0.2 M acetic acid) and store in a glass bottle for six months.

Recipe 26: HPLC Organic Solvent

Add 700 mL ACN and 12 mL glacial acetic acid to 288 mL HPLC-grade water (0.2 M acetic acid, 70% ACN) and store in a glass bottle for six months.

Instructions**Cell Culture and SILAC Labeling**

We describe the conditions for SILAC labeling 3T3-L1 cells expressing wild-type and AS-ERK2. Growth medium and conditions for serum-starvation and stimulation can be adjusted to analyze other kinases. The kinase reaction should be performed with 4-6 mg of protein in approximately 600 μ L of Lysis/Kinase Reaction Buffer (Recipe 4). This concentration allows in vitro labeling to proceed efficiently, and matches the capacity of the Sep-Pak cartridge. Western blotting can be used to determine the extent of thiophosphate labeling.

1. Culture cells expressing wild-type kinase (WT-kinase) and AS-kinase in light and $^{13}\text{C}_6$ arginine/lysine SILAC Growth Medium (Recipe 2), respectively, for at least seven doublings.

Note: Appropriate SILAC base medium can be selected for the cell-type of interest. We generally grow one 15-cm plate to 80% confluence for the labeling reaction.

2. Serum-starve the cells for between 3 and 24 hours in Serum-Free SILAC Medium (Recipe 1).

Note: AS-ERK2-expressing 3T3-L1 cells only tolerate 3 hour serum-starvation. Serum starvation attenuates the activity of the kinase being studied prior to the labeling reaction.

3. Put PBS and Lysis/Kinase Reaction Buffer (Recipe 4) on ice for at least 15 minutes
4. Add PMA Working Solution (Recipe 3) directly to the cells in culture to a final concentration of 100 nM (1:1000 dilution) and incubate for 2.5 minutes in a cell culture incubator.

Note: This step rapidly activates the ERK2 kinase while minimizing the time available for substrate phosphorylation. Use an appropriate stimulation reagent for the cell type and kinase of interest.

- 5 Quickly aspirate the media, wash with 2 mL cold PBS, and aspirate very thoroughly.
- 6 Place the cells on ice, add 400 μ L of Lysis/Kinase Reaction Buffer (Recipe 4) to each plate, scrape cells thoroughly, and transfer lysate to a 1.5 mL tube. Leave the lysates on ice until step 11.

Note: The lysis buffer is very mild and leaves a considerable amount of cell debris. Debris may contain kinase substrates and should not be removed from the reaction.

- 7 After using the BCA assay kit to determine the protein concentration of the WT- and AS-expressing cell lysates, add Lysis/Kinase Reaction Buffer (Recipe 4) to the lysates so that they have the same volume and protein concentration.
- 8 Take a 13.5 μ L aliquot of each sample for Western blotting.

Note: This step is optional and is used for troubleshooting purposes.

- 9 Add in order to the sample on ice: TCEP to 2 mM (1:250 dilution from 500 mM stock solution), GTP to 1 mM [1:100 from GTP Stock Solution (Recipe 5)], and ATP analog to 50 μ M.
- 10 Vortex briefly to mix the reactions.
- 11 Place the reactions at 30°C on a rotor or rocking table for 1 hour.

Note: If desired for troubleshooting purposes, 13.5 μ L aliquots can be taken every 10-15 minutes for analysis by Western blotting. Stop the reaction by adding EDTA to 50 mM and placing the samples ice.

- 12 Take another 13.5 μ L aliquot of each sample.
- 13 Add 1.5 μ L of PNB Working Solution (Recipe 7) to the Western blot samples from steps 8 and 12, vortex briefly, and incubate at room temperature for 2 hours.

Note: This step is optional. Western blotting is used to estimate the extent of phosphorylation before and after thiophosphate labeling reactions. Additional samples taken during step 11 can be used to optimize the time for the labeling reaction to minimize background labeling.

- 14 Perform Western blotting with the Thiophosphate Ester Antibody diluted according to the manufacturer's instructions.

Note: This step is optional, but it may be helpful for troubleshooting the thiophosphate labeling.

Protein Precipitation and Digestion

The proteins in the lysates are precipitated to remove detergents, then denatured with urea, followed by digestion with trypsin. We describe precipitation with chloroform and methanol, but other protein precipitation methods, such as precipitation by ammonium sulfate, can also be used. Precipitated proteins should be physically disrupted by pipetting, vortexing, or sonication, but they will not dissolve completely until after digestion with trypsin.

1. Split each sample of labeled lysate into 200 μ L aliquots and place in 1.5 mL tubes.
2. Add 640 μ L methanol and 160 μ L chloroform to each 200 μ L aliquot and vortex briefly.

Note: Chloroform is hazardous, use in a chemical fume hood.

- 3 Add 480 μ L deionized water to each tube and vortex.
- 4 Spin the tubes at 4° C in a microfuge at maximum speed for 5 minutes.
- 5 Carefully remove the top liquid layer and discard.
- 6 Add 300 μ L methanol to the pellet, vortex briefly, and spin in a microfuge for 15 minutes at 4° C.
- 7 Remove the supernatant (if necessary, use a gel-loading tip to remove all the liquid) and allow the pellet air-dry at room temperature for 10-15 minutes.
- 8 Add 50 μ L Protein Resuspension Buffer (Recipe 8) to each pellet and vortex for 30 seconds.

Note: The pellet will not disperse completely until it is digested with trypsin.

- 9 Add 250 μ L of Digest Buffer (Recipe 9) to each pellet and combine all tubes (WT and AS-kinase samples) together into a single 15 mL Falcon tube.
- 10 Add trypsin to a substrate:enzyme ratio of 1:50 by mass (20 μ g trypsin per mg of protein determined from the protein assay) and place overnight on a rotor at room temperature.

Desalt and Lyophilize

Digested proteins must be desalted prior to thiophosphate capture. We use C18 Sep-Pak cartridges. Sep-Pak Plus cartridges can accommodate 4 mg of digested peptide; Sep-Pak Light cartridges should be used for samples with less than 1 mg of digested peptide. Divide the digest as necessary so that no more than 4 mg of peptide is applied to each Sep-Pak cartridge.

1. Acidify the digested sample by adding glacial acetic acid to a final concentration of 10%.
2. Centrifuge the digest at 4000g for 10 minute to pellet debris.

Note: Acidified samples may be stored indefinitely at -80°C.

- 3 Remove the plunger from a 10 mL syringe and attach the Sep-Pak(s) to the syringe.
- 4 Add 10 mL 0.1% Acetic Acid (Recipe 10) to the syringe and use the plunger to push it through the Sep-Pak at 2 mL/min, leaving approximately 0.1 mL in the syringe so that no air enters the Sep-Pak.

Note: We recommend using a ring stand to suspend the syringe over a waste container during this process.

- 5 Remove the Sep-Pak from the syringe, then remove the plunger from the syringe, and add 0.1 mL 0.1% Acetic Acid (Recipe 10) to the top of the Sep-Pak so that it does not dry out.
- 6 Reattach the Sep-Pak on the syringe, add 10 mL Sep-Pak Equilibration Buffer (Recipe 11), and use the plunger to push it through the Sep-Pak at 2 mL/min, leaving 0.1 mL in the syringe.
- 7 Remove the Sep-Pak from the syringe, then remove the plunger from the syringe, and add 0.1 mL 0.1% Acetic Acid (Recipe 10) to the top of the Sep-Pak.

- 8 Reattach the Sep-Pak to the syringe, add 10 mL 0.1% Acetic Acid (Recipe 10), and use the plunger to push it through the Sep-Pak at 2 mL/min, leaving 0.1 mL in the syringe.
- 9 Remove the Sep-Pak from the syringe, then remove the plunger from the syringe, and add 0.1 mL 0.1% Acetic Acid (Recipe 10) to the top of the Sep-Pak.
- 10 Reattach the Sep-Pak to the syringe, add the acidified digest, and use the plunger to push it through at a flow rate less than 1 mL/min.
- 11 Remove the Sep-Pak from the syringe, then remove the plunger from the syringe, and add 0.1 mL 0.1% Acetic Acid (Recipe 10) to the top of the Sep-Pak.
- 12 Reattach the Sep-Pak to the syringe, add 10 mL 0.1% Acetic Acid (Recipe 10), and use the plunger to push it through at 2 mL/min.
- 13 Remove the Sep-Pak from the syringe, then remove the plunger from the syringe, and add 0.1 mL 0.1% Acetic Acid (Recipe 10) to the top of the Sep-Pak.
- 14 Reattach the Sep-Pak to the syringe and add 5 mL Sep-Pak Elution Buffer (Recipe 12).
- 15 Place the syringe over a 15 mL Falcon tube and use the plunger to push Sep-Pak Elution Buffer through the Sep-Pak at 1 mL/min.
- 16 Reduce the eluate to less than 1 mL in a vacuum centrifuge.
- 17 Freeze the samples at -80°C.

Note: Samples may be stored indefinitely at -80°C.

- 18 Immerse samples in liquid nitrogen for 10 minutes and lyophilize to dryness.

Note: Lyophilized samples may be stored indefinitely at -80°C.

Packing of POROS R2, C18, and IMAC Capillary Columns for Thiophosphate Capture and Thiophosphate Enrichment

Substrate peptides eluting from the SulfoLink beads are captured with a capillary column packed with POROS R2 bead (Fig. 3). After washing to remove Oxone from the R2 column, peptides are transferred to a capillary column containing IMAC beads charged with iron (III) to further enrich phosphorylated peptides. Finally, peptides are transferred from the IMAC column to a capillary column containing C18 beads. The C18 column is then placed in-line for identification of substrate peptides by LC-MS/MS. We use the POROS R2 column for the initial reverse-phase capture and rinsing because it supports a flow rate of 4 μ L/min at pressure below 200 PSI, whereas the C18 column provides better chromatographic separation during LC-MS/MS. One of each capillary column should be prepared before proceeding with the protocol. Notes in the protocol indicate alternative approaches if capillary columns are not available.

1. Use a capillary cutter to cut a 20 cm piece of 100 μ m i.d. capillary for R2 and C18 columns, or 200 μ m i.d. capillary for the IMAC column.
2. Prepare frit solution by combining 20 μ L formamide with 80 μ L Kasil, vortex for 30 seconds, and centrifuge at maximum speed for 1 minute in a microfuge.
3. Place one end of the capillary in the frit solution and draw up about 0.5 cm by capillary force.

4. Heat the frit for 15 seconds with a heat gun or bake for 15 minutes at 80°C.
5. Wash the frit with ACN for about five minutes using a helium packing device at low pressure.

Note: ACN flow should be visible but it should not spray. Briefly apply additional heat to the frit if it does not flow freely with pressure below 100 PSI.

6. Use a small spatula to transfer approximately 40 μL of POROS R2, C18, or IMAC beads to 2 mL glass vial with magnetic stir bar; then for R2 and C18 beads add 0.5 mL of C18 Suspension Buffer (Recipe 13), for IMAC beads add 0.5 mL of deionized water. 7. Place the vial with beads into a helium packing device on a stir plate and use the stir plate at its lowest setting to keep the beads suspended.
7. Insert the fritted column into the helium packing device and slowly increase pressure to load beads in the capillary to a length of 10 cm.
8. Replace beads in the helium packing device with 1 mL of 0.1% Acetic Acid (Recipe 10) and wash the column for 10 minutes 200 PSI.

Note: After this step, the process for finalizing the columns diverges, with the R2 and C18 columns handled similarly and the IMAC column differently.

Finalizing and Blocking Nonspecific Binding Sites on the R2 and C18 Columns

We block nonspecific binding sites on the beads with angiotensin, which is an XX amino-acid peptide. This also reduces sample loss. Other standard peptides may be used as an alternative to angiotensin.

1. Replace the 0.1% acetic acid in the helium packing device with Angiotensin Peptide Solution (Recipe 14) and load 5 μL onto the column at 200 PSI.
2. Place the column on an HPLC and run a 20-minute gradient from 0 to 70% ACN in 0.2 M acetic acid (Recipes 25 and 26). Alternatively, put 1 mL HPLC Organic Solvent (Recipe 26) in a glass vial and place it in the helium packing device, and then insert the column and wash for 5 minutes at 200 PSI.

Note: Blocked and finalized columns may be stored at XX for XX.

3. Run another 20-minute gradient from 0 to 70% ACN in 0.2 M acetic acid (Recipes 25 and 26) over the column immediately before use.

Finalizing and Blocking Nonspecific Sites on the IMAC Column

We condition IMAC capillary columns prior to use by loading and eluting 5 picomoles of digested B-casein. This blocks nonspecific binding sites and improves recovery and specificity of the phosphopeptide enrichment. Immediately prior to use a conditioned IMAC capillary column must be loaded with iron (III) chloride. We recommend beginning this process during step 16 of the next section.

1. Remove the 0.1% Acetic Acid from the helium packing device, and push air through the IMAC capillary at 100 PSI for 1 minute.
2. Prepare frit solution by combining 20 μL formamide with 80 μL Kasil, vortex for 30 seconds, and centrifuge at maximum speed for 1 minute in a microfuge.
3. Place the unpacked end of the capillary in the frit solution and draw up about 0.5 cm by capillary force.
4. Heat the frit for 15 seconds with a heat gun or bake for 15 minutes at 80°C.

5. Wash the frit with ACN for about five minutes using a helium packing device at low pressure.

Note: ACN flow should be visible but it should not spray. Briefly apply additional heat to the frit if it does not flow freely with pressure below 100 PSI.

- 6 Place a glass vial containing 1 mL deionized water into the helium packing device, insert the finished IMAC column, and wash for 5 minutes at 100 PSI.
- 7 Place a vial containing 1 mL IMAC EDTA Buffer (Recipe 21) in a helium packing device and insert the IMAC column into the device. Adjust the pressure for a flow rate of approximately 10 $\mu\text{L}/\text{min}$ for 10 minutes.
- 8 Replace the IMAC EDTA Buffer with a vial containing deionized water and flow over the IMAC column at 10 $\mu\text{L}/\text{min}$ for 10 minutes.
- 9 Replace the vial containing deionized water with a vial containing IMAC Iron Chloride Solution (Recipe 22) and flow over the IMAC column at 10 $\mu\text{L}/\text{min}$ for 15 minutes.
- 10 Remove the IMAC column from the helium packing device and reinsert upside down.
- 11 Flow IMAC Iron Chloride Solution (Recipe 22) at 10 $\mu\text{L}/\text{min}$ for 5 minutes.
- 12 Return the column to its original direction, replace the IMAC Iron Chloride Solution with a vial containing 0.1% Acetic Acid (Recipe 10) and flow at 10 $\mu\text{L}/\text{min}$ for 10 minutes.
- 13 Replace the 0.1% Acetic Acid (Recipe 10) with a vial containing B-casein Peptide Solution (Recipe 15) and flow at approximately 1 $\mu\text{L}/\text{min}$ for 10 minutes.
- 14 Replace the B-casein with IMAC Organic Rinse (Recipe 23) and flow at 10 $\mu\text{L}/\text{min}$ for 5 min, and then reverse the direction of the column and flow again at 10 $\mu\text{L}/\text{min}$ for 5 min.
- 15 Replace the IMAC Organic Rinse with 0.1% Acetic Acid (Recipe 10) at and flow at 10 $\mu\text{L}/\text{min}$ for 10 minutes.
- 16 Replace the 0.1% Acetic Acid with IMAC Elution Buffer (Recipe 24) for and flow at approximately 4 $\mu\text{L}/\text{min}$ for 20 minutes.

Note: The conditioned IMAC column may be stored for three months at room temperature.

Thiophosphate Capture

We use SulfoLink beads to capture thiol-containing peptides, including both thiophosphate-labeled substrate peptides and cysteine-containing peptides. These beads are light sensitive until after the quenching step and must be protected from light until that step.

1. Resuspend the lyophilized peptides to a final concentration of 25 mg peptide/mL in Thiophosphate Binding Buffer with BSA (Recipe 17).

Note: The buffer pH will drop when sample peptides are brought into solution. The buffer pH should not be readjusted at this step.

- 2 Invert the sealed container of SulfoLink beads until they are suspended uniformly, then transfer SulfoLink bead slurry to a 1.5 mL tube (25 μL for each mg of protein in the starting lysate, up to 150 μL), pellet the beads in a microfuge at 1000 rpm for 10 seconds, and discard the supernatant.

Note: SulfoLink beads degrade once they have been exposed to air and should remain sealed until immediately before use (discard the remainder, do not reseal).

- 3 Wash the SulfoLink beads twice in 500 μ L Thiophosphate Washing Buffer (Recipe 16) for 5 minutes with end-over-end rotation in the dark, pelleting the beads in a microfuge at 1000 rpm for 10 seconds and discarding the supernatant after each wash.
- 4 Add the dissolved peptide sample to the beads and rotate overnight at room temperature in the dark.
- 5 Pellet the beads the beads for 10 seconds at 1000 rpm in a microfuge and remove the supernatant.
- 6 Wash the beads twice in 500 μ L of Thiophosphate Washing Buffer (Recipe 16) with end-over-end rotation for 5 minutes, pelleting the beads in a microfuge at 1000 rpm for 10 seconds and discarding the supernatant after each wash.
- 7 Wash the beads in 500 μ L of SulfoLink Quenching Buffer (Recipe 18) for 10 minutes at room temperature with rotation or rocking, pellet the beads in a microfuge at 1000 rpm for 10 seconds, and discard the supernatant.
- 8 Wash the beads in 500 μ L of Thiophosphate Wash Buffer (Recipe 16) for 5 minutes with rotation, pellet the beads in a microfuge at 1000 rpm for 10 seconds, and discard the supernatant.
- 9 Wash the beads in 500 μ L of Formic Acid Wash (Recipe 19) for five minutes without rotation, pelleting the beads for 1 min at 2000 rpm in a microfuge and discarding the supernatant.

Note: The stronger centrifugation is necessary because the Formic Acid Wash is more viscous than the other binding and washing buffers.

- 10 Wash the beads briefly in 500 μ L of Thiophosphate Wash Buffer (Recipe 16), pellet the beads in a microfuge at 1000 rpm for 10 seconds, and discard the supernatant.

Thiophosphate Cleaning and Elution

We use a helium packing device to transfer SulfoLink beads loaded with labeled substrate peptides to a capillary column for additional washing and elution to a POROS R2 column (Fig. 3). Thiophosphorylated peptides are converted to normal phosphate during the elution step, making them suitable for phosphopeptide enrichment. If capillary columns are not available the beads may instead be transferred to a 2 mL centrifuge column and washed by gravity-flow. Details about how this changes the procedures are indicated in notes associated with specific steps where necessary.

1. Cut a 45-cm piece of 530 μ m i.d. capillary.
2. Prepare a frit solution by combining 20 μ L formamide with 80 μ L KASIL, vortexing 30 seconds, and centrifuging at maximum speed in a microfuge for 1 minute.
3. Use capillary action to draw about 0.5 cm of frit solution into the capillary, and place the capillary in an oven at 50° C for 10 minutes.
4. Heat the frit for 15 seconds using a heat gun to completely polymerize the material.

Note: Alternatively, bake the frit for 15 minutes at 80° C.

- 5 Place a glass vial containing 1 mL of ACN into the helium packing device and insert the capillary with fritted side up. Carefully raise the pressure until ACN begins to flow and allow it to flow for 10 seconds.

Note: This step requires extremely low pressure to avoid spraying ACN from the column.

- 6 Cut a 2 cm piece of Teflon tubing and, using a hand-drill with 72-gauge bit, drill one cm through one side of the Teflon tubing (this matches the larger capillary).

Note: One end of the tubing will be drilled to match the gauge of the SulfoLink capillary, and the other will be drilled to match the smaller POROS R2 column.

- 7 Cut a 5 cm piece of 100 μm i.d. capillary and thread this through the drilled Teflon tube to widen the smaller end of the connector and remove debris from the drilling. Discard the 5 cm capillary.
- 8 Attach the wide end of the connector to the fritted end of the 530 μm i.d. capillary.
- 9 Suspend the SulfoLink beads bound to the peptides in 1 mL Thiophosphate Wash Buffer (Recipe 16), transfer to a 2 mL glass vial with a magnetic stir bar, and place the vial in a helium packing device over a stir plate to keep the beads suspended.
- 10 Insert the fritted 530 i.d. capillary into the helium packing device so that it sits in the bead suspension without contacting the stir bar (Fig. 3A), and then slowly increase the pressure until beads begin to flow (they will be visible through the capillary). Periodically add binding buffer to the vial to avoid running dry. Continue until >90% of the beads have been loaded.
- 11 Reduce any unused volume of the capillary using a capillary cutter to trim the capillary 3 cm below the end of packed beads.
- 12 Replace the vial that contained beads with a vial containing 1 mL 0.1% Acetic Acid (Recipe 10) and wash the beads by flowing 0.1% acetic acid for 5 minutes at 200 $\mu\text{L}/\text{min}$ (~ 100 PSI). Add additional 0.1% acetic acid to the vial as necessary to avoid running dry.
- 13 Attach the prepared POROS R2 column to the SulfoLink column using the Teflon connector (Fig. 3B).

Note: From this point all buffers flowing over the SulfoLink beads will also pass through the POROS R2 column. This allows peptides to be captured as they are eluted while minimizing losses from sample handling or adsorption of peptides onto surfaces. If capillaries are not being used, peptides may be eluted by incubating SulfoLink beads in one bead volume of Oxidizing Elution Buffer (Recipe 20) for five minutes at room temperature with rotation. Eluted peptides should be further enriched by desalting on a reverse-phase surface followed by phosphopeptide enrichment by IMAC (desalting is necessary because Oxone is incompatible with phosphopeptide enrichment), or else eluted peptides may be desalted and analyzed directly by LC-MS/MS.

- 14 Adjust the pressure for a flow rate of 4 $\mu\text{L}/\text{min}$ using 0.1% Acetic Acid (Recipe 10) (~ 200 PSI).
- 15 Replace 0.1% Acetic Acid in the helium packing device with a vial containing 1 mL Oxidizing Elution Buffer (Recipe 20) and flow for 10 minutes at 4 $\mu\text{L}/\text{min}$.
- 16 Replace Oxidizing Elution Buffer in the helium packing device with a vial containing 0.1% Acetic Acid (Recipe 10) and flow at 4 $\mu\text{L}/\text{min}$ for 25 minutes.

Note: It is important to flush the entire volume of the column so that all peptides are collected on the R2 column. The duration of this step can be adjusted as necessary.

- 17 Remove the R2 column from the SulfoLink capillary, place it into a helium packing device with 0.1% Acetic Acid (Recipe 10), and wash 5 minutes at 200 PSI to remove Oxone prior to phosphopeptide enrichment. The capillary containing SulfoLink beads may be discarded.

Immobilized Metal Affinity Chromatography

1. Attach a Teflon connector to the fritted end of the R2 column carrying substrate peptides. Use the connector and attach the R2 column to either end of the prepared IMAC column.
2. Place a vial containing 0.1% Acetic Acid (Recipe 10) into a helium packing device, insert the R2 column into the device (Fig. 3C), and adjust the pressure until liquid flows at between 0.5 and 1 $\mu\text{L}/\text{min}$ (the solution will be flowing through both the R2 and IMAC columns).
3. Replace the 0.1% acetic acid with a vial containing 1 mL HPLC Organic Solvent (Recipe 26) and flow at the same pressure as in the previous step until 10 μL has passed over the two columns. Discard the R2 column.

Note: This step transfers peptides from the R2 to the IMAC column and a slow flow rate is critical so that phosphopeptides have time to bind to the IMAC column.

- 4 Place a vial containing 1 mL IMAC Organic Rinse (Recipe 23) in a helium packing device, insert the IMAC column, flow for 5 minutes at 10 $\mu\text{L}/\text{min}$, then reverse the direction of the column and repeat the wash. Return the column to its original direction.
- 5 Replace the IMAC Organic Rinse with a vial containing 0.1% Acetic Acid (Recipe 10) and flow at 10 $\mu\text{L}/\text{min}$ for 10 minutes.
- 6 Attach a prepared C18 column to the front of the IMAC column with a Teflon connector (Fig. 3D).
- 7 Flow IMAC Elution Buffer (Recipe 24) through the columns for 10 minutes at 4 $\mu\text{L}/\text{min}$. Peptides eluting from the IMAC column will be captured on the C18 column.

Note: Alternatively, peptides may be eluted from the IMAC column without a C18 column attached and loaded manually or with an autosampler onto an HPLC-MS/MS system. There is very little peptide at this stage so take care to avoid adsorption on surfaces or loss during transfers.

- 8 Remove the C18 column from the Teflon connector.

Note: The IMAC column may be stored and reused for up to three months.

- 9 Place a vial containing 0.1% Acetic Acid (Recipe 10) into a helium packing device, insert the C18 column, set the pressure to 200 PSI, and wash for 10 minutes.

Note: The C18 column is ready to be placed in-line for peptide identification by LC-MS/MS.

- 10 Process the eluted peptides by LC-MS/MS.

Note: The LC-MS/MS protocol will depend on the MS equipment and configuration. We recommend consulting an experienced mass spectrometry facility about how to analyze the

samples. Peptides phosphorylated on serine or threonine often have a dominant neutral loss of 98 Dalton. Fragmenting the neutral loss ion with MS3 or multistage activation may improve peptide identification.

- 11 Analyze the MS spectra using software such as MASCOT⁷ or MaxQuant⁸ to identify phosphorylated peptides from the MS/MS spectra.

Note: Because of the oxidizing elution conditions, methionine residues are always oxidized to sulfoxide or sulfone. Follow-up experiments on a previously unknown substrate are time-consuming and difficult; therefore, we recommend checking automatic assignments by manually assigning every peak to a predicted fragment ion.

- 12 Extract the SILAC quantitation for each peptide manually or using software appropriate for the MS instrument and then use the SILAC ratios for nonphosphorylated peptides to establish a threshold for identifying kinase substrates.

Note: For example, use the AS-kinase sample versus the wild-type sample at three standard deviations above the mean for nonphosphorylated peptides.

Troubleshooting No Thiophosphate Labeling Detected by Western Blotting

First, verify that the activated AS-kinase is present in the sample by either Western blotting for phosphorylation on the activation loop (if applicable to the kinase) or adding a recombinant substrate protein and checking for thiophosphorylation by *in vitro* kinase reaction. If the kinase is present and active, determine whether the AS-kinase accepts thiophosphate by performing kinase reactions with purified AS-kinase and substrate protein in the presence of gamma-thiol ATP. Check for activity by Western blotting for thiophosphate. Some kinases do not accept gamma-thiol ATP analogs. If the AS-kinase accepts thiophosphate, then determine whether the AS-kinase accepts the particular ATP analog used in the experiment. A number of N6-substituted gamma-thiol analogs are available and it may be necessary to optimize the reaction by empirically testing various analogs with the AS-kinase of interest.

It is possible that a weak signal is due to extensive phosphorylation prior to cell lysis, which would leave few sites available for the AS kinase reaction and thiophosphate labeling. Western blotting or MS analysis of the samples before and after the kinase reaction should provide an indication of the extent of phosphorylation or lysate. If the AS-kinase is active prior to lysis, then many of its substrates will be phosphorylated and not available for thiophosphate labeling.

Strong Thiophosphate Labeling in the Negative Control

Excess, nonspecific labeling is the most common cause of background signal in the negative control reaction. One way to minimize this problem is to lower the concentration of ATP analog to reduce nonspecific utilization by other kinases. Conducting a time-course experiment by taking aliquots from the labeling reactions every 10-15 minutes can aid in determining the optimal duration of the labeling reaction.

Strong Labeling by the AS-Kinase by Western Blotting but No Substrate Peptides Detected by LC-MS/MS

Sometimes the aliquots taken after labeling for Western blotting may show a strong signal, yet the MS data does not have labeled peptides. Loss of signal in the MS samples can result from inefficient thiophosphate capture. The pH of the peptide sample in Thiophosphate

Binding Buffer with BSA (Recipe 17) should be between 5 and 5.5 after addition of peptide in order for the reaction with the SulfoLink beads to proceed efficiently. If the pH is too high then cysteine competes for binding to the beads. The pH of the peptide solution may be adjusted with dilute HCl if necessary (we recommend adjusting the pH of an aliquot of binding buffer to 5.0 and then adding the necessary volume). Inefficient capture due to degradation of the SulfoLink beads can also cause a loss of the signal in the MS data. The SulfoLink beads should not be unsealed until immediately before use.

Loss of signal in the MS samples can result from using TCEP that is too old or that was added to the buffers too early. TCEP stock solution should be replaced every three months. Loss of the MS signal can also result from inefficient phosphopeptide enrichment. To verify this process, digest a known phosphoprotein, such as B-casein, and perform phosphopeptide enrichment followed by LC-MS/MS to ensure that phosphopeptides are efficiently recovered.

A thiophosphate positive control can be generated using gamma-thiol ATP (no alkylation at N6) with a recombinant kinase and its substrate, such as Jun N-terminal kinase 1 (JNK1) and c-Jun. Spike 10 picomole of thiophosphorylated c-Jun into the AS-kinase substrate labeling reaction before the precipitation step. Use this to verify that the known substrate peptides are recovered and to determine an approximate limit of detection the protocol.

Related Techniques

This protocol is derived from the Shokat lab protocol published in Blethrow *et al.*² An alternative capture chemistry was described by the Clurman lab in Chi *et al.* in which thiophosphorylated peptides are bound to a bead surface by formation of a disulfide linkage.³ Treatment with base selectively hydrolyzes the phosphorous-sulfur bond of the thiophosphate, releasing the bound peptides with normal phosphate in place of the labeled group. Cysteine-containing peptides are bound through a thioether bond that is not affected by base. In our hands, the Clurman protocol suffered from substantial binding of nonphosphorylated peptides, but we believe that the protocol could be optimized to work effectively. As an alternative to capturing labeled peptides, thiophosphorylated proteins may be alkylated with PNBM and immunoprecipitated using the thiophosphate ester antibody, followed by SDS-PAGE and identification by LC-MS/MS.^{1, 9}

Notes and Remarks

Even with efficient substrate labeling, capture, and identification; these approaches still have substantial limitations. The LC-MS/MS approach used here is only suitable for phosphorylation sites that fall within tryptic peptides suitable for the analysis. Many kinases include arginine or lysine in their recognition motif and may produce peptides too short for identification. Alternative proteases may be used as appropriate. Peptides containing cysteine are also likely to be captured on SulfoLink beads through the cysteine thiol instead of through thiophosphate. We have not observed any cysteine-containing peptides that we believe to be bona fide substrate sites.

The protocol remains limited by the requirement to use *in vitro* kinase reactions in cell lysate instead of performing labeling in intact cells. We have experimented extensively with permeabilization strategies to get ATP analogs into the cell. Although Banki *et al.* have reported in-cell labeling using permeabilized cells⁹, we have achieved very limited labeling in cells, sufficient for identification of the most abundant sites, but not at an amount suitable for low-level substrate identification. An alternative approach would be to conduct labeling reactions in isolated organelles (note that the low-detergent lysis buffer in this protocol may leave protein complexes and some organelles intact).

There is also a biological limitation in that the kinase substrates must not be phosphorylated when the cells are lysed. This is especially challenging in cases with high endogenous kinase activity (for example, kinase substrates downstream of constitutively activating mutations). These systems require extensive optimization to reversibly inhibit kinase activity long enough for substrates to be dephosphorylated, followed by rapid reactivation of the kinase prior to lysis.

Despite all limitations and caveats, unbiased identification of kinase substrates can reveal a huge number of novel targets. Application of this approach to a variety of kinases and biological systems has the potential to reveal previously unknown components of signaling networks.

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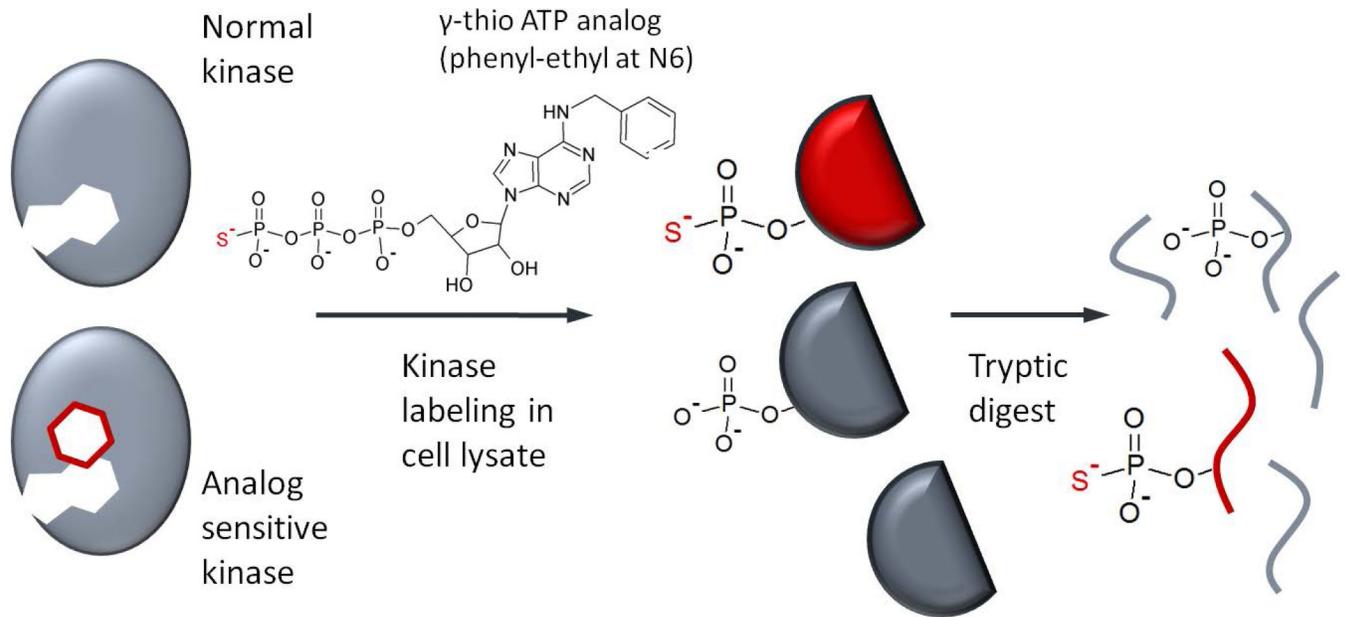


Fig. 1. Analog-sensitive kinases utilize N6-substituted ATP analogs to selectively thiophosphorylate their substrates. Processing and tryptic digest produces a mixture containing thiophosphorylated substrate peptides.

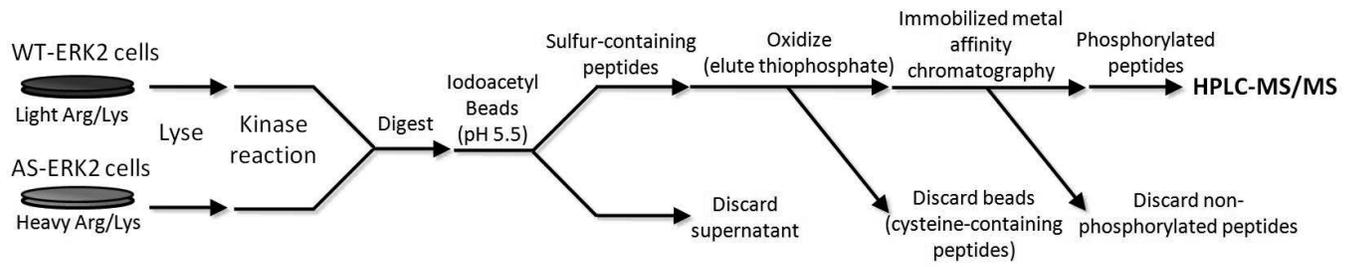


Fig. 2. Overview of AS-kinase substrate labeling and identification coupled with quantification by SILAC.

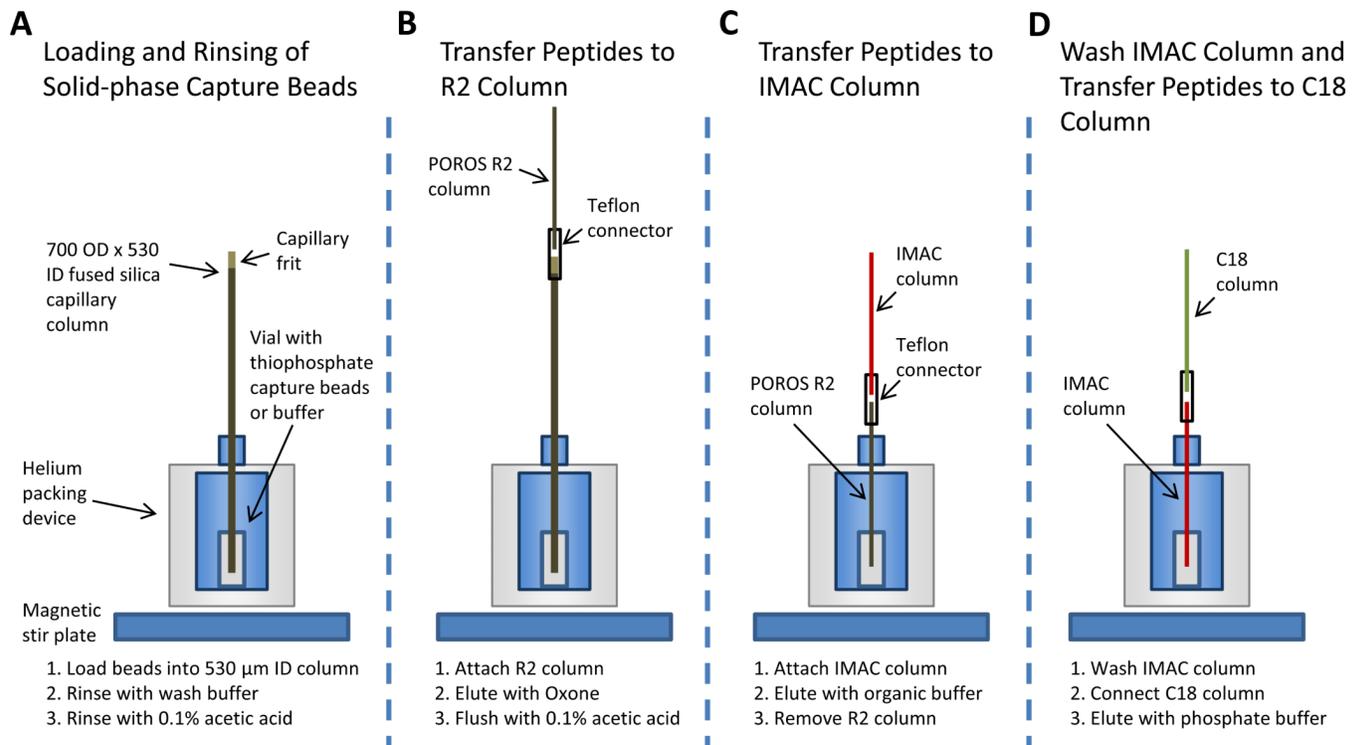


Fig. 3. Schematic of the helium packing device and capillary columns. **(A)** Sulfolink beads are packed into a 530 μm i.d. fused silica capillary column and rinsed. **(B)** Captured thiophosphorylated peptides are eluted from Sulfolink beads to the Poros R2 reverse-phase column with Oxone. **(C)** After rinsing to remove excess Oxone, peptides are eluted from the R2 column to the IMAC column, followed by rinsing to remove non-phosphorylated peptides. **(D)** Phosphorylated peptides are eluted from the IMAC column to the C18 column for identification by LC-MS/MS analysis.