



Design and synthesis of fluorescent activity probes for protein phosphatases

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Abstract

Protein phosphatases act in concert with protein kinases to regulate and maintain the phosphoproteome. However, the catalog of chemical tools to directly monitor the enzymatic activity of phosphatases has lagged behind their kinase counterparts. In this chapter, we provide protocols for repurposing the phosphorylation-sensitive

sulfonamido-oxine fluorophore known as Sox to afford direct activity probes for phosphatases. With validated activity probes in-hand, inhibitor screens can be conducted with recombinant enzyme and the role of phosphatases in cell signaling can be investigated in unfractionated cell lysates.

1. Introduction

Protein phosphorylation plays a critical role in cellular communication (Vlastaridis et al., 2017). Consequently, there is a need to probe the enzymatic activities that maintain the integrity of the phosphoproteome in order to understand fundamental aspects of cellular communication as well as human disease. The key enzymes that regulate protein phosphorylation are the protein kinases (PKs) (Manning, Whyte, Martinez, Hunter, & Sudarsanam, 2002), that act as writers and the protein phosphatases (PPs) (Chen, Dixon, & Manning, 2017), that act as erasers. While significant efforts have been focused on developing chemical tools to interrogate PK function (Bishop et al., 2000; Gonzalez-Vera, 2012; Karginov et al., 2011; Oldach & Zhang, 2014; Ranjitkar, Brock, & Maly, 2010; Sharma, Agnes, & Lawrence, 2007), relatively little attention has been given to PPs (Brautigan, 2013; Casey & Stains, 2018; De Munter, Kohn, & Bollen, 2013; Fahs, Lujan, & Kohn, 2016; Tonks, 2013). Our laboratory has recently described an approach to afford peptide-based activity probes for PPs (Beck, Lawrence, Tung, Harris, & Stains, 2016; Beck, Truong, & Stains, 2016) by repurposing the phosphorylation-sensitive Sox fluorophore (Fig. 1) (Pearce, Jotterand, Carrico, & Imperiali, 2001; Shults, Pearce, & Imperiali, 2003). This strategy can be applied to both protein tyrosine

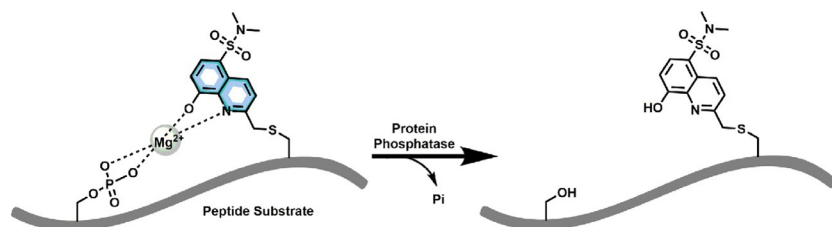


Fig. 1 Sox-based probes for protein phosphatase activity. The Sox fluorophore is placed at the +2 or –2 position relative to the phosphoamino acid (Ser, Thr, or Tyr). Chelation of magnesium enhances Sox fluorescence (ex. = 360 nm, em. = 485 nm). Enzymatic removal of the phosphoryl group decreases affinity for magnesium and results in a concurrent decrease in Sox fluorescence. The rate of decrease in Sox fluorescence is proportional to the enzymatic activity of the target protein phosphatase.

phosphatases (PTPs) (Beck, Lawrence, et al., 2016) and protein serine/threonine phosphatases (PSPs) (Beck, Truong, et al., 2016). Furthermore, if sufficiently selective substrate sequences can be identified, PP activity can be monitored in unfractionated cell lysates providing insight into the temporal dynamics of PP signaling under relevant biological stimuli (Beck, Truong, et al., 2016). In this chapter, we present our laboratory's routine procedures for design, synthesis, and validation of Sox-based fluorescent activity probes for PPs.

2. Synthesis of Sox-Br and Fmoc-CSox

Peptides containing the Sox fluorophore are commercially available through AssayQuant Technologies (<http://www.assayquant.com/>). Alternatively, Sox-based probes can be accessed via on-resin alkylation of peptides using Sox-Br (**1**, Fig. 2) or direct incorporation of a Sox-containing cysteine analog termed Fmoc-CSox (**2**, Fig. 2). Procedures for the synthesis of Sox-Br and Fmoc-CSox have been published previously (Lukovic, Gonzalez-Vera, & Imperiali, 2008; Pearce et al., 2001; Shults et al., 2003). Below, we outline the synthesis of these reagents (Figs. 3 and 4). This procedure assumes knowledge of standard organic chemistry techniques and the availability of basic organic synthesis laboratory equipment.

2.1 Synthesis of Sox-bromide (Sox-Br)

On-resin alkylation of peptides with Sox-Br requires fewer synthetic steps relative to procedures utilizing Fmoc-CSox. Thus, we recommend first attempting the synthesis of Sox-based probes using on-resin alkylation with Sox-Br. However, in certain cases where modifications in SPPS procedures

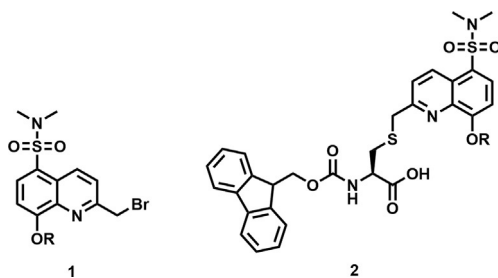


Fig. 2 Sox reagents used for alkylation (Sox-Br, **1**) of or direct incorporation (Fmoc-CSox, **2**) into peptide sequences. R = *tert*-butyldiphenylsilyl.

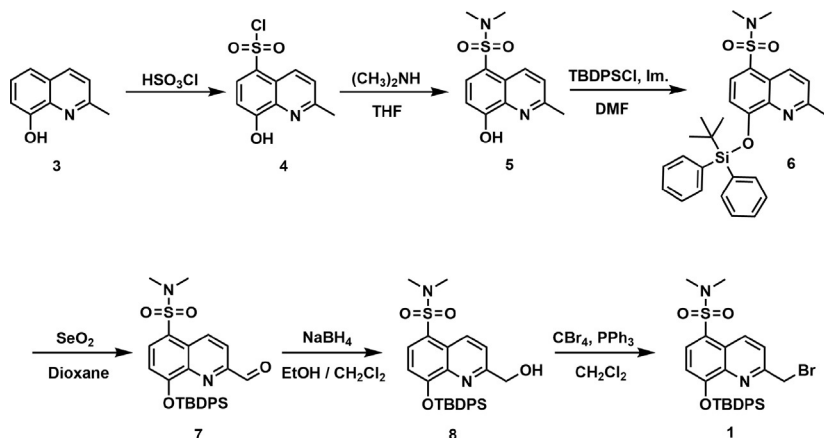


Fig. 3 Synthetic scheme for Sox-Br (1).

are required (such the use of acid labile resins) (Lukovic, Vogel Taylor, & Imperiali, 2009) or on-resin alkylation proves unsuccessful (Beck, Zhou, Casey, & Stains, 2015), Sox can be incorporated into the peptide sequence using Fmoc-CSox.

2.1.1 8-Hydroxy-2-methyl-quinoline-5-sulfonyl chloride (4)

1. Add 8-hydroxy-2-methyl-quinoline (3, 10.0 g, 1 equiv.) to a 100–200 mL round bottom flask equipped with a rubber septum.
2. To the powder, add chlorosulfonic acid (50 mL, 12 equiv.) via direct pour from a graduated cylinder at room temperature with continuous magnetic stirring.
Tip: This addition should be performed slowly as this reaction is exothermic. Additionally, the rubber septum should be removed from the flask during this time to allow for the corrosive gas to escape into the fume hood.
3. Evacuate the flask with high vacuum and charge with N₂ (×3) following chlorosulfonic acid addition.
4. Stir at room temperature for 2–3 h.
5. Transfer the reaction mixture to a 2 L separatory funnel containing ice (3/4ths volume) and dichloromethane (CH₂Cl₂).
6. Shake vigorously and allow the CH₂Cl₂ to separate. The ice/dichloromethane slurry should display a light yellow/greenish hue.
7. Drain the bottom (CH₂Cl₂) layer into a bed of potassium carbonate in a large Erlenmeyer flask to remove residual water.

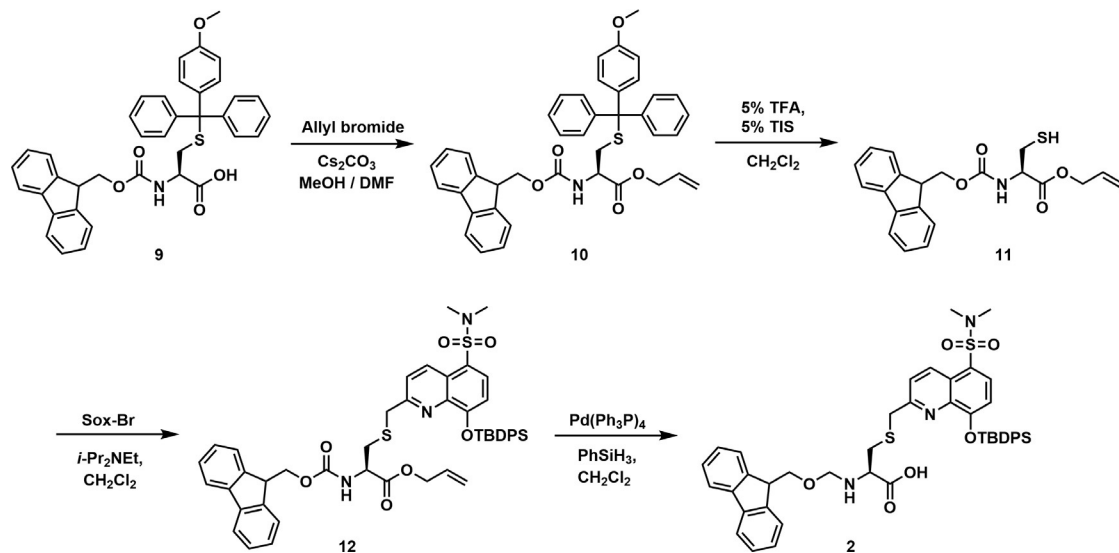


Fig. 4 Synthetic scheme for Fmoc-CSox (**2**).

8. Repeat $\times 3$ with the remaining ice/water layer, supplementing with fresh CH_2Cl_2 with each extraction.
9. The resulting combined organic fractions are then vacuum filtered, and solvent is removed in vacuo to yield a crude yellow powder.
10. The crude product can be used in the next step without further purification.

2.1.2 5-(*N,N*-dimethyl)sulfonamido-8-hydroxy-2-methylquinoline (5)

1. A solution of dimethylamine in tetrahydrofuran (2*M*, 5.5 equiv.), is added to tetrahydrofuran (150 mL) in a three-necked round bottom flask under N_2 and continual stirring.
2. 8-hydroxy-2-methylquinoline-5-sulfonyl chloride (**4**) (1 equiv.) is added in small portions over 3 h to the side-neck of the flask. After each addition, the atmosphere is evacuated with vacuum and recharged with N_2 .

Tip: The solution will turn a bright-yellow color and dissipate into a pale-yellow solution following evacuation of the flask. If residual powder is observed on the neck of the flask, use a syringe of fresh THF to wash off the neck of the round bottom flask to ensure the reactant washes into the reaction solution prior to atmospheric evacuation.

3. Following the final addition of **4**, allow the pale-yellow solution to stir for an additional 10–15 min.
4. Remove the round bottom flask from the stir plate apparatus and remove the solvent through rotary evaporation (low-vacuum).
5. Excess dimethylamine is removed by re-dissolving the sticky solid in CH_2Cl_2 followed by solvent removal using evaporation (3×50 mL).
6. Place the crude product under high-vacuum (minimum 2 h). The resulting crude product (**5**), a yellow powder, can be used in the next step without further purification.

2.1.3 8-*tert*-butyldiphenylsilyloxy-5-(*N,N*-dimethyl)sulfonamido-2-methylquinoline (6)

1. Add **5** (5.0 g, crude), imidazole (1.28 g, 1 equiv.), dry dimethylformamide (DMF, 28 mL), and *tert*-butyldiphenylsilyl chloride (5 mL, 1 equiv.) sequentially to a dry round bottom flask under N_2 .
2. Evacuate the atmosphere with vacuum and charge with N_2 ($\times 3$).
3. Stir the reaction mixture under N_2 atmosphere overnight at room temperature.
4. Remove DMF from the reaction mixture using high-vacuum rotary evaporation.

5. Dilute the resulting sticky solid with ethyl acetate (150 mL).
6. Wash with saturated ammonium chloride solution (50 mL), brine (2×50 mL), and dry over a bed of Na_2SO_4 .
7. Remove the solvent with rotary evaporation (low-vacuum followed by high-vacuum).
8. Purify the crude product (**6**) using flash chromatography (silica, 9:1 hexanes/ethyl acetate) to yield a white solid after removal of solvent.

Tip: The silica column should be pre-treated with acetone for 15–20 min prior to loading the crude product, in order to decrease decomposition in the column. Flush out acetone with hexanes vigorously before loading. Load the crude product onto the silica column with a minimal amount of CH_2Cl_2 using a Pasteur pipette.

2.1.4 8-tert-butylidiphenylsilyloxy-5-(N,N-dimethyl)sulfonamido-2-formylquinoline (**7**)

1. To a two-neck round bottom flask, add molecular sieves ($4 \text{ \AA}$, 5.0 g, 1 equiv.) activated at 180°C in high-vacuum for 5–6 h or through microwave activation (5×10 min, or until a burning plasma is observed in the beaker containing the molecular sieves).
2. Add **6** (4.82 g, 1 equiv.) to the dry flask with activated molecular sieves.
3. Add dry 1,4-dioxane (70 mL) to the reaction flask with continual stirring.
4. Heat the reaction flask to 95°C with a condenser (5°C water circulation) attached.
5. Add selenium dioxide (1.27 g, 1.2 equiv.) and evacuate atmosphere and charge with N_2 ($\times 3$).
6. Allow the reaction to proceed for 17–24 h and then remove from heat and allow to cool to room temperature.
7. Filter through a bed of celite to remove the black residue and molecular sieves.
8. The celite bed and molecular sieves are then washed with 1,4-dioxane until no more chromophore is detected by UV on TLC plates or orange color is observed in washes.
9. The 1,4-dioxane is removed by rotary evaporation.
10. The resulting yellow oil is re-dissolved in ethyl acetate (900 mL) and washed with brine (100 mL), nano-pure water (100 mL), saturated potassium carbonate (100 mL), and dried over a bed of Na_2SO_4 .
11. Remove the solvent by rotary evaporation to yield a sticky orange oil (**7**) which is used in the next step without further purification.

2.1.5 8-tert-butyl-diphenylsilyloxy-5-(N,N-dimethyl)sulfonamido-2-(hydroxymethyl)quinolone (**8**)

1. Sodium borohydride (456 mg, 1 equiv.) is dissolved in absolute ethanol (98 mL) and cooled to 0 °C in a round bottom flask. Evacuate the atmosphere and charge with N₂ (×3) under constant stirring at room temperature.
2. Crude **7** from the previous reaction (6.26 g) is dissolved in dry dichloromethane (203 mL) and ethanol (27 mL) in a separate round bottom flask under N₂, with constant stirring at room temperature. This mixture is then subjected to atmospheric evacuation and charged with N₂ (×3).
3. Add the solution of **7** drop-wise using an addition funnel, to the sodium borohydride solution.
Tip: The dark orange solution of **7** should be added slowly and take 1–3 h for completion.
4. Following the final addition of **7**, allow the reaction mixture to stir for 15 min.
5. Dilute the reaction mixture with diethyl ether (800 mL).
6. Wash the solution with saturated ammonium chloride (200 mL), nanopore H₂O (2 × 100 mL), brine (100 mL), and dry over a bed of Na₂SO₄ in a large Erlenmeyer flask.
7. Remove the solvent by rotary evaporation to yield a pale-yellow sticky solid.

2.1.6 2-bromomethyl-8-tert-butyl-diphenylsilyloxy-5-(N,N-dimethyl)sulfonamidoquinoline (*Sox-Br*, **1**)

1. The resulting crude alcohol (**8**) from the previous reaction (4.53 g, 1 equiv.) is dissolved in CH₂Cl₂ under N₂ and solid carbon tetrabromide, (CBr₄, 1 equiv.) is added.
Tip: Use 50 mL CH₂Cl₂ per 0.02 mol of **8**. In this example, 30 mL of CH₂Cl₂ was used to dissolve **8** and CBr₄, and 20 mL of CH₂Cl₂ was used to dissolve PPh₃ in step 3.
2. Cool the clear reaction mixture to 0 °C in an ice bath while stirring.
3. Triphenylphosphine (PPh₃, 1.5 equiv.) is then dissolved in CH₂Cl₂ and slowly added drop-wise. The resulting dark yellow-orange reaction mixture is stirred at 0 °C for 3 h.
4. Saturated NaHCO₃ (equal volume) is then added to the reaction mixture and the organic layer is separated, washed with brine (100 mL), dried over Na₂SO₄, and evaporated under reduced pressure.

5. The resulting oil is then purified by flash chromatography (Florisil, 100–200 mesh, 9:1 hexanes/ethyl acetate) to yield a clear oil (548 mg) after removal of solvent.
6. Product identity and purity should be assessed by MS and NMR, referring to previously published spectra (Shults et al., 2003).

2.2 Synthesis of Fmoc-Cys(Sox[TBDPS])-OH (Fmoc-CSox, 2)

Procedures for alkylation of peptides with Sox-Br can be found in Section 3.1.6. For certain sequences alkylation with Sox-Br has proven difficult (Beck, Zhou, et al., 2015), possibly due to inaccessibility of the nucleophilic cysteine. Moreover, modification of SPPS procedures may prohibit the use of acid labile protecting groups, which are needed for selective alkylation with Sox-Br (Lukovic et al., 2009). If these situations are encountered, Fmoc-Sox can be prepared using Sox-Br. The resulting Fmoc-protected amino acid can be used to incorporate Sox into peptides without the need for alkylation.

2.2.1 Fmoc-Cys-OAllyl (11)

1. To an oven-dried round-bottomed flask, equipped with a stir bar and under positive N₂ pressure, add Fmoc-Cys(Mmt)-OH (**9**, 1 equiv.) followed by 50 mL CH₂Cl₂ and 50 mL methanol (MeOH).
2. Allow the colorless solution to stir for 5 min at room temperature.
3. To this solution add cesium carbonate (Cs₂CO₃, 0.5 equiv.) and allow the mixture to stir at room temperature under N₂ (evacuate atmosphere with vacuum and charge ×3) for 45 min.
4. Remove the solvent by rotary evaporation and dissolve the resulting white, fluffy solid in DMF (100 mL, anhydrous).
5. Immediately add allyl bromide (3 equiv.) to the resulting solution.
6. The reaction mixture is then stirred at room temperature under positive N₂ pressure for 5 h. During this time, the reaction is monitored by TLC (R_f = 0.23, 20% EtOAc in hexanes).
7. Following completion, the reaction mixture is diluted with EtOAc (1 L), washed with 2% NaHCO₃ (200 mL), nanopore H₂O (250 mL ×3), brine (250 mL ×3), dried (Na₂SO₄), and concentrated in vacuo.
8. The crude product (**10**) is then dissolved in degassed, anhydrous CH₂Cl₂ (90 mL).
9. Triisopropyl silane (TIPS, 5 mL) and trifluoroacetic acid (TFA, 5 mL) are then added to the slightly yellow solution.

10. The resulting red solution is then allowed to react at room temperature under N_2 for 3 h and the progress is monitored by TLC ($R_f = 0.28$, 20% EtOAc in hexanes). The reaction mixture turns a clear yellow color upon completion.
11. The reaction mixture is then diluted in CH_2Cl_2 (300 mL), washed with 5% $NaHCO_3$ (250 mL $\times 2$), nanopore H_2O (250 mL), brine (300 mL), dried (Na_2SO_4), and concentrated in vacuo.
12. Perform flash column chromatography (SiO_2) by loading crude product in CH_2Cl_2 , elute with 20% EtOAc in hexanes to yield the product (**11**) as a fluffy white solid after removal of solvent.

2.2.2 *Fmoc-Cys(Sox[TBDPS])-OAllyl (12)*

1. To an oven-dried, round-bottomed flask equipped with a stir bar and under positive N_2 pressure, add Fmoc-Cys-OAllyl (1 equiv.) dissolved in anhydrous CH_2Cl_2 (70 mL).
2. To this colorless solution, add Sox-Br (**1**, 1 equiv.) followed by anhydrous *N,N*-Diisopropylethylamine, (DIPEA, 1.5 equiv.). The reaction (pale yellow in color) is then stirred at room temperature under positive N_2 pressure overnight.
3. The crude reaction mixture is then diluted with CH_2Cl_2 (400 mL), washed with saturated ammonium chloride (NH_4Cl , 2×100 mL), nanopore H_2O (100 mL), brine (100 mL), dried (Na_2SO_4), and concentrated in vacuo.
4. The resulting crude product is then purified by flash column chromatography (SiO_2 equilibrated with 10% EtOAc in hexanes). Load the crude product in CH_2Cl_2 and eluent with increasing amounts of EtOAc (10, 20, and 30%) in hexanes, yielding the product (**12**) as a white solid after solvent removal.

Tip: Load the crude product onto the column in a minimal amount of CH_2Cl_2 . Flush with pure hexanes to elute the dichloromethane before addition of ethyl acetate.

2.2.3 *Fmoc-Cys(Sox[TBDPS])-OH (2)*

1. To an oven-dried round-bottomed flask equipped with a stir bar under positive N_2 pressure, add a solution of Fmoc-Cys(Sox[TBDPS])-OAllyl (**12**, 1 equiv.) dissolved in anhydrous, degassed ($\times 3$) CH_2Cl_2 (100 mL).
2. To the resulting pale red solution, add phenylsilane (25 equiv.) followed by tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$, 0.04 equiv.). After 10–20 min, the reaction should turn a deep red color.

3. The resulting mixture is then stirred at room temperature under positive N_2 pressure for 3 h. Reaction progress is monitored by TLC ($R_f=0.36$, 10% MeOH in CH_2Cl_2).
4. Upon completion of the reaction, the solvent is removed in vacuo.
5. The crude mixture is then passed through a short flash chromatography column (SiO_2). Load the crude product in CH_2Cl_2 and eluent with 1, 2, 3, 4, 5, 10, 15% MeOH in CH_2Cl_2 to yield the product (2). The resulting Fmoc amino acid was used in SPPS without further purification.
6. Product purity and identity should be characterized by reverse-phase HPLC, MS, and NMR using previously published spectra ([Lukovic et al., 2008](#)).



3. Synthesis of Sox-based phosphatase substrates

While several small molecule probes for PPs have been described, increased selectivity can often be achieved utilizing peptide-based sensors ([Casey & Stains, 2018](#)). In this section we provide general procedures for the synthesis of peptide-based probes containing Sox using solid-phase peptide synthesis (SPPS). This procedure assumes a general familiarity with SPPS and access to standard SPPS equipment.

3.1 General SPPS conditions

Our laboratory typically employs Fmoc-PAL-PEG-PS resin (Applied Biosystems, 0.18 mmol/g) for SPPS. For alkylation of peptides with Sox-Br, Fmoc-Cys-(Mmt)-OH (Chem-Impex International, 03699) is used since the Mmt protecting group can be selectively removed prior to reaction with Sox-Br ([Section 3.1.6](#)). The Sox-fluorophore is generally placed at the +2 or -2 position relative to the site of dephosphorylation ([Fig. 1](#)) ([Beck, Lawrence, et al., 2016](#); [Beck, Truong, et al., 2016](#); [Lukovic et al., 2008](#)). In certain cases, addition of Sox to a peptide can alter the efficiency of that substrate for a target enzyme. Thus, our laboratory synthesizes both analogs (+2 and -2) and evaluates their efficiency using recombinant enzyme in order to choose an optimal substrate for cell lysates studies ([Section 4](#)). Lastly, our laboratory uses a low-cost setup for SPPS consisting of Bio-Rad Poly-Prep chromatography columns (9 cm height), Vac-Man Laboratory Vacuum Manifolds from Promega (A7231), and solvent resistant three-way stopcocks (Qosina, 99166, [Fig. 5](#)). Procedures

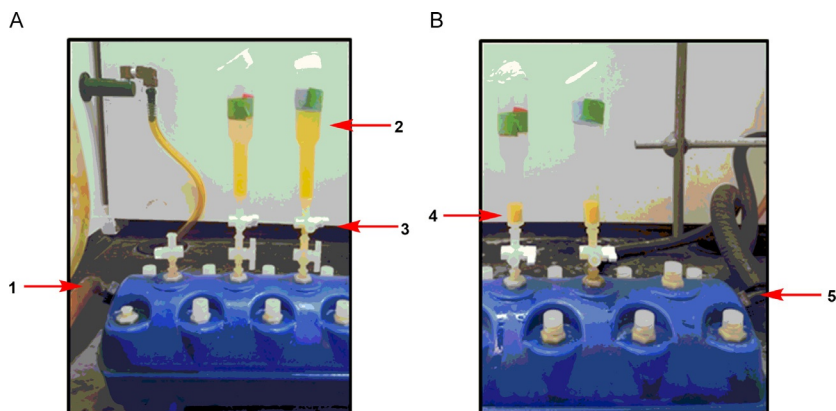


Fig. 5 Peptide synthesis apparatus. (A) A coupling reaction bubbling under nitrogen (1) in a column (2) with a (3) three-way stopcock. (B) A resin bed (4) after solvent removal by vacuum (5).

given below are tailored to this setup. In addition, it should be noted that peptides are synthesized in the C- to N-terminal direction via SPPS (Fig. 6), which is opposite to the standard convention for writing peptide sequences (N- to C-terminus).

3.1.1 Swelling

1. Weigh out 50–200 mg of resin into the column.
2. Swell the resin by allowing it to incubate in CH_2Cl_2 for 5–15 min.
3. Drain CH_2Cl_2 using vacuum.
4. Wash ($\times 5$) with the appropriate reaction solvent (Table 1).

3.1.2 Deprotection

1. Add 2 mL of deprotection solution (20% 4-methylpiperidine (v/v) in the appropriate solvent, Table 1) and bubble nitrogen through the column for 5 min.
2. Remove the solvent using vacuum.
3. Repeat steps 1–2 ($\times 3$).
4. After the final deprotection, wash the resin ($\times 5$) with 2–5 mL of the appropriate solvent (Table 1) followed by 2–5 mL of CH_2Cl_2 .
5. Using a spatula, remove a small sample of resin and place in an Eppendorf tube.
6. Perform the TNBS (Habeeb, 1966), chloranil (Vojkovsky, 1995), or Kaiser (Kaiser, Colescott, Bossinger, & Cook, 1970) test for free amines.

Tip: For proline residues, use the chloranil test for secondary amines.

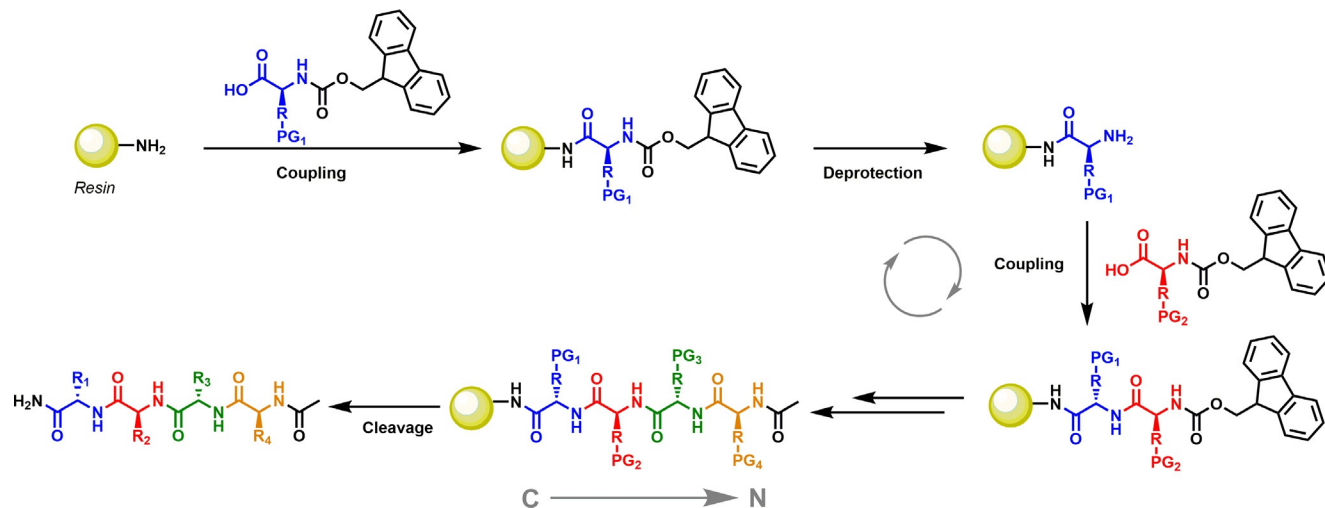


Fig. 6 General scheme for solid-phase peptide synthesis. Peptides are synthesized in the C- to N-terminal direction through a repetitive series of deprotection and coupling steps. Cleavage provides the final peptide sequence. R represents amino acid side chains and PG represents protecting groups.

Table 1 SPPS coupling cocktails utilized by our laboratory.

Situation	Coupling reagent	Coupling additive	Solvent	Base
Basic coupling	PyBOP or HBTU	HOBt	DMF	DIPEA
Repetitive Asp and Glu residues	COMU	Oxyma	NMP	TMP
Fmoc-CSox/difficult couplings	PyAOP	HOAt	DMF	Collidine

7. If the free amine test is negative (minimal or no color), repeat steps 1–6.
8. If the test for free amines is positive (orange–TNBS, orange—chloranil primary amine, green—chloranil secondary amine, blue—Kaiser), proceed directly to the next section.

3.1.3 Activation and coupling

1. Weigh out 6 equiv. of Fmoc-amino acid, coupling reagent, and coupling additive (Table 1) into a 1.5 mL Eppendorf tube.
Tip: Equivalents of Fmoc-amino acid, coupling reagent, coupling additive, and base should be calculated based on the loading capacity of the selected resin.
2. Dissolve the reagents in a minimal volume of the appropriate solvent (Table 1).
3. Add 12 equiv. of the appropriate base (Table 1) and incubate for 1–2 min.
4. Add the contents of the Eppendorf tube directly to the resin. Add additional solvent to bring total reaction volume to ~5 mL.
5. Bubble the reaction mixture with nitrogen for 45 min–1 h.
Tip: Most amino acid couplings are complete within 1 h. However, certain residues, such as arginine, may require longer coupling times.
6. Following completion of the reaction remove the coupling cocktail by vacuum.
7. Wash the resin with the solvent used for the reaction ($\times 5$) followed by CH_2Cl_2 ($\times 5$).
8. Perform a free-amine test. Successful coupling should result in no color change (negative result).
9. In the case of partial or incomplete coupling, repeat steps 1–8 with fresh reagents.
Tip: For particularly difficult sequences, free amines can be capped as in Section 3.1.5.
10. In the case of a complete reaction (negative free amine test), continue with successive couplings by repeating steps 1–8 above.

3.1.4 Pausing SPPS

1. Following a successful coupling reaction, wash the resin with the solvent used for the reaction ($\times 5$), CH_2Cl_2 ($\times 5$), and MeOH ($\times 5$).
2. Resin-bound peptides can be stored in the reaction column at 4°C for weeks.
3. Restart the synthesis by swelling the resin as in [Section 3.1.1](#), step 2.
4. Proceed with deprotection, activation, and coupling steps until peptide sequence is complete.

3.1.5 Capping

1. Following completion of the amino acid sequence, deprotect the terminal Fmoc amino acid using 20% 4-methylpiperidine in the appropriate solvent.
2. Wash the resin with solvent ($\times 5$).
3. Test for free-amines, if the test is negative repeat the deprotection.
4. If the test is positive, add 20 equiv. of pyridine and acetic anhydride (Ac_2O) to the resin and fill to 5 mL with solvent.
5. Bubble under nitrogen for 30–45 min.
6. Remove the capping cocktail via vacuum and wash with solvent ($\times 5$) followed by CH_2Cl_2 ($\times 5$).
7. Test for free-amines, the test should be negative, if not repeat the capping procedure.

3.1.6 Selective side-chain deprotection and Sox-Br alkylation

1. Conduct a test cleavage ([Section 3.1.7](#)) and characterization by MS ([Section 3.1.8](#)) to ensure that the desired product is present before alkylation with Sox-Br.
2. Prepare 100 mL of deprotection solution containing TFA (1% v/v) and triisopropylsilane (TIPS, 5% v/v) in CH_2Cl_2 .
3. Swell the resin with DMF (5 min). Remove the solution by vacuum and incubate with CH_2Cl_2 (45 min).
4. Remove the solution by vacuum and wash with CH_2Cl_2 ($\times 5$).
5. Add 3–4 mL of the deprotection solution to the peptide column and bubble with nitrogen for 20 min.
6. Remove the deprotection solution using vacuum and repeat step 5 until the bright yellow color, corresponding to the liberated Mmt protecting group, completely disappears.
7. To ensure complete Mmt removal, perform an additional round of deprotection following the loss of yellow color.

8. Wash the resin with CH_2Cl_2 ($\times 5$) and DMF ($\times 5$).
9. Add 200 μL of anhydrous DMF (per 50 mg of resin) and 6 equiv. of freshly distilled tetramethylguanidine (TMG). Allow this solution to incubate for 3–5 min.
10. Add 3 equiv. of Sox-Br (dissolved in anhydrous DMF) and agitate the suspension on a mechanical rocker overnight.
11. Following the reaction, remove the solution and wash with DMF ($\times 5$) followed by CH_2Cl_2 ($\times 5$).
12. Perform a test cleavage on a small sample of resin (Section 3.1.7) and characterize using HPLC and MS analysis (Section 3.1.8).
13. If minimal alkylation product is observed, repeat steps 1–11.

3.1.7 Complete deprotection and cleavage

The following procedure outlines how to perform a complete side-chain deprotection and cleavage from the resin. To perform a test cleavage, scale the reaction back to 1 mL of cleavage cocktail Reagent K* (King, Fields, & Fields, 1990) and use a minimal amount of resin.

1. Prepare a 10 mL solution of cleavage cocktail Reagent K* (TFA/phenol/water/thioanisole/1,2-ethanedithiol/TIPS; 80:5:5:5:2.5:2.5 v/v).
2. Transfer the resin to a 15 mL conical tube using a spatula.
3. Add the Reagent K* cocktail to the resin.
4. Rotate at room temperature for 3 h.
5. Evaporate off most of the solution under a gentle stream of N_2 .
6. Precipitate the peptide out of the remaining solution by addition of ice-cold diethyl ether (Et_2O). For large-scale cleavages, a white precipitate should be observed.
7. Pellet the resin and precipitated peptide by centrifugation at 13.3 rcf for 5 min.
8. Remove the supernatant using a Pasteur pipette.
9. Add 5 mL of fresh, ice-cold Et_2O and vortex.
10. Pellet and repeat steps 7–9 ($\times 2$).
11. Following the final removal of the supernatant, dry the pellet by setting the open tube in a fume hood for 5–20 min.
12. Dissolve the white precipitate in nano-pure water (2–3 mL).
13. Filter the dissolved peptide using a 10 mL syringe, equipped with a filter (0.2 μm , Waters), into a fresh 15 mL conical tube.
14. Wash the conical tube containing the original cleavage reaction with an additional 2–3 mL of nano-pure water and filter this solution into the tube containing the peptide.
15. Purify the peptide by semi-preparative HPLC (Section 3.1.8).

3.1.8 Purification and characterization of peptides

1. Our laboratory utilizes semi-preparative reverse-phase HPLC (solvent A: acetonitrile with 0.1% TFA; solvent B: water with 0.1% TFA) and monitors UV absorption at 228 nm (amide bond) and 316 nm (Sox) (Lukovic et al., 2008, 2009). Typical gradients consist of 5–95% solvent A over 30 min on a C₁₈ YMC-PACK Pro (5 μ m, 250 $\times$ 20 mm, flow rate = 15 mL/min) column. Gradients should be optimized based on the purity of each sample.

Tip: Before semi-preparative HPLC, an analytical HPLC run can be performed with 40 μ L of sample to determine purity. For test cleavages conducted prior to incorporation of Sox, monitor absorbance at 228 nm (amide bond) and 280 nm (Fmoc, Trp, Tyr, and Phe).

2. Collect fractions in 15 mL conical tubes and freeze at -80°C . Multiple runs may be required for large-scale synthesis.
3. Lyophilize each fraction to remove solvent. Fractions containing large amounts of acetonitrile should be placed on a rotary evaporator prior to freezing and lyophilization.
4. After lyophilization, a white powder will generally be visible in the tube. Dissolve this in 1–2 mL of nano-pure water.
5. Verify the identity of the peptide using MS and assess purity using analytical HPLC. This procedure should result in samples with >95% purity. If significant contamination is observed, gradient conditions for semi-preparative HPLC can be modified in order to obtain pure samples.
6. Determine the concentration of the purified peptide by UV–Vis spectrometry using the extinction coefficient of the Sox fluorophore, $\epsilon = 8247 \text{ M}^{-1} \text{ cm}^{-1}$ at 355 nm in 0.1 M NaOH with 1 mM Na₂EDTA (Shults et al., 2003).
7. Stock solutions can generally be stored at 4°C for at least 6 months or -20°C for longer periods.



4. In vitro characterization of peptide substrates

Following probe synthesis, it is essential to determine optimal assay conditions for each new sensor prior to conducting lysate assays. These experiments involve determining the affinity for Mg^{2+} , the optimal concentration of Mg^{2+} , and the efficiency and selectivity of a substrate for a target enzyme. With these optimized assay conditions in-hand, the utility of the probe for monitoring PP activity in cell lysates can be assessed (Section 5).

Materials

Fluorescence plate reader
 384-well flat-bottom plates (Corning, 3824, 40 μ L assay volume)
 Nano-pure water
 Sodium chloride (NaCl)
 MgCl_2
 10 $\times$ Assay Buffer: 500 mM Tris-HCl (pH 7.5 at 22 $^\circ\text{C}$), 10 mM DTT, 20 mM EGTA (not used in PSP assays), 0.1% Brij-35P (v/v)
 Enzyme dilution buffer: Tris-HCl 50 mM (pH 7.5 at 22 $^\circ\text{C}$), 2 mM EGTA (not used in PSP assays), 1 mM DTT, 0.01% Brij-35P, 0.1% glycerol (v/v)
 Tris-HCl Buffer: 50 mM, pH 7.5 at 22 $^\circ\text{C}$

4.1 Mg^{2+} K_D determination

1. Determine the fluorescence of Sox peptides (1 μM) in the presence of Tris-HCl (50 mM, pH 7.5 at 22 $^\circ\text{C}$), NaCl (150 mM), and varying concentrations of MgCl_2 (0–250 mM) in a total volume of 40 μL . Excite samples at 360 nm and measure emission from 400 to 650 nm.
2. The fluorescence of each peptide should be measured in triplicate for each concentration of MgCl_2 and averaged.
Tip: Most peptides will display an emission maximum at 485 nm, although we have observed that certain sequences can shift the emission maximum of Sox. This can be readily observed using the emission scan above and the maximum emission wavelength can then be empirically determined.
3. Subtract the fluorescence of the sample with no MgCl_2 (background) from the fluorescence of samples containing varying concentrations of MgCl_2 .
4. Plot the background corrected fluorescence data versus the concentration of Mg^{2+} and fit the curve to a one-site binding isotherm:

$$\text{AFU} = \frac{B_{\text{max}} [\text{Mg}^{2+}]}{K_D + [\text{Mg}^{2+}]}$$

where AFU is arbitrary fluorescence units, B_{max} is the maximal fluorescence at saturating concentrations of Mg^{2+} , K_D is the equilibrium dissociation constant, and $[\text{Mg}^{2+}]$ is the concentration of Mg^{2+} .

Tip: In order to discriminate between the phosphorylation state of the peptide, the K_D of the phosphopeptide for Mg^{2+} should be tighter than

the K_D of the corresponding non-phosphopeptide for Mg^{2+} . If a minimal difference in K_D is observed, alternative peptide sequences should be pursued.

4.2 Fold fluorescence increase (FFI) determination

1. Measure the fluorescence of the phosphorylated and non-phosphorylated peptides ($1\ \mu M$) in solutions containing 50 mM Tris-HCl, pH 7.5 at 22 °C, 150 mM NaCl, and varying Mg^{2+} concentrations based on the K_D of the phosphorylated peptide for Mg^{2+} (0.5, 1.0, 1.5, and $2.0 \times$ the observed K_D).
2. The fold fluorescence increase is defined as the maximum emission of the phosphorylated peptide divided by the fluorescence of the corresponding non-phosphorylated peptide.
3. The concentration of Mg^{2+} that yields the highest FFI should be used for all subsequent assays.

4.3 Determination of kinetic parameters with recombinant phosphatase

1. Wells containing varying concentrations of phosphorylated-peptide and non-phosphorylated peptide ($1\text{--}250\ \mu M$) in assay buffer ($1 \times$ in well) with the optimal $MgCl_2$ concentration are prepared in triplicate.
2. Record the emission of the assay mixture (prior to recombinant phosphatase addition) at 485 nm for 10 min.
3. Determine the average fluorescence emission of wells containing varying concentrations of phosphorylated and non-phosphorylated peptides without enzyme.
4. By plotting the average fluorescence as a function of peptide concentration and fitting to a linear trend line, the slope values (AFU/ μM) for samples containing phosphorylated (f_p) and non-phosphorylated (f_{np}) peptide can be determined. The difference between the slopes of these two samples ($f_p - f_{np}$) yields a correction factor that can be used to calculate the concentration of the phosphorylated peptide remaining in solution at a given time after addition of enzyme (step 6).
5. Following the initial 10 min read, recombinant phosphatase (25 nM stock solution in enzyme dilution buffer) is added to a final concentration of 2.5 nM in wells containing phosphorylated peptide. Fluorescence emission is monitored for 1 h.

6. Plot the fluorescence change versus time for wells containing phosphorylated substrate peptide at each concentration. Select time points for the initial linear portion of the data corresponding to <10% substrate turnover (by comparison to fluorescence prior to enzyme addition in step 2) and fit to a linear trend line. The resulting reaction slope (AFU/min) is divided by the correction factor ($f_p - f_{np}$) obtained in step 4 above, yielding the rate of substrate depletion ($\mu\text{M}/\text{min}$).

Tip: If reactions have consumed >10% substrate, repeat the experiment using less enzyme.

7. Rates of substrate depletion are converted to rates of product formation by multiplying by -1 , plotted versus the concentration of phosphorylated peptide, and fit to the following equation to determine kinetic parameters (Nath & Atkins, 2006):

$$\text{Velocity} = \frac{d[P]}{dt} = -\frac{d[S]}{dt} = \frac{V_{\max} [S]}{[S] + K_M}$$

where $d[P]/dt$ is the change in product concentration versus time, $d[S]/dt$ is the change in substrate concentration versus time, $V_{\max}$ is the maximal velocity of the reaction, K_M is the Michaelis-Menten constant, and $[S]$ is the concentration of substrate. k_{cat} can be determined by dividing $V_{\max}$ ($\mu\text{M}/\text{min}$) by the concentration of phosphatase (μM). The specificity constant (k_{cat}/K_M) can be used to compare the efficiency of different substrates for a given phosphatase. Generally, substrates displaying larger specificity constants should be used for further assays.

4.4 Determination of limit of detection for recombinant phosphatase

1. Add optimal concentrations of phosphorylated peptide substrate (at least $1 \times$ to $2 \times K_M$) and MgCl_2 to assay buffer ($1 \times$ final concentration in $40 \mu\text{L}$ total) in a series of wells. Allow extra volume for subsequent addition of enzyme, such that the final assay volume is $40 \mu\text{L}$.
2. Monitor the fluorescence emission of each well for 10 min prior to addition of enzyme.

Tip: Comparing the fluorescence of wells before and after addition of enzyme can be used to determine if the initial portion of the reaction has been missed. Reaction rates can be decreased by using less enzyme.

3. Dilute recombinant phosphatase in enzyme dilution buffer and add to wells at varying concentrations (e.g., 2.5–0.1 nM). A separate reaction without enzyme serves as the blank.
4. Monitor the fluorescence emission immediately after addition of enzyme for 1 h.
5. Readings can be background corrected using wells without enzyme (blank).
6. The linear region of the resulting fluorescence data is plotted versus time for each enzyme concentration and fit to a linear trend line. For ease of interpretation, the absolute values of these reaction slopes are used from here on. Reaction slopes for each enzyme concentration should be averaged.
7. Plotting the reaction slope versus enzyme concentration allows for the determination of the limit of detection using the following equation:

$$\text{Limit of detection} = \frac{3 \times \text{Standard deviation of the blank}}{\text{Slope of the linear trend line}}$$

4.5 Panel assays with recombinant phosphatases

Defining the selectivity of phosphatase probes is a critical step in determining the utility of a sensor for cell lysate assays. Testing a panel of homologous phosphatases, or those known to have overlapping substrate specificity, can highlight potential issues with off-target activity. If off-target activity is observed, inhibitors can be used to suppress off-target signal (Beck, Truong, et al., 2016). Alternatively, if off-target activity overwhelms target signal, new peptide substrates can be pursued or the target enzyme can be enriched from lysates (Beck, Lawrence, et al., 2016).

1. Add optimal concentrations of phosphorylated peptide substrate (at least $1 \times$ to $2 \times K_M$) and MgCl_2 to assay buffer ($1 \times$ final concentration in 40 μL total) in a series of wells. Allow extra volume for subsequent addition of enzyme, such that the final assay volume is 40 μL .
2. Monitor the fluorescence emission of each well for 10 min prior to addition of enzyme.
3. Prepare recombinant phosphatase stocks by dilution in enzyme dilution buffer and add to the well. A reasonable starting concentration is 5 nM of each enzyme (this concentration can be adjusted based on the observed reaction slopes).
4. Monitor the fluorescence emission immediately after addition of enzyme for 1 h.

5. Plot the linear region of the fluorescence emission versus time and determine the average, absolute reaction slope for each enzyme sample.
6. Data can be background corrected using wells without enzyme.
7. Plot the average reaction slope, and corresponding standard deviation, for each enzyme in bar graph format.



5. Validation in cell lysates

Once a selective probe is identified, validation studies in cell lysates can be performed. Target selectivity can be probed using siRNA knock-down of phosphatase expression (Beck, Truong, et al., 2016). A general protocol for preparation and analysis of cell lysates is given below. Reducing reagents are excluded from PTP assays in order to preserve oxidative modifications to the catalytic cysteine. Catalase and superoxide dismutase are included in lysis buffers for PTPs in order to prevent non-specific oxidation (Beck, Lawrence, et al., 2016). PSP assays do not contain chelating reagents to preserve PSP catalytic activity.

Materials

Fluorescence plate reader

384-well flat-bottom plates (Corning, 3824, 40 μ L assay volume)

Corning 150 mm culture dishes (Sigma, CLS430599)

Cell scrapers (Fisher Scientific, 08-100-242)

Liquid nitrogen in Dewar flask

Phosphate-buffered saline (PBS; Life Technologies, 10010-023), ice cold

Refrigerated microcentrifuge

MgCl₂

10 $\times$ PTP Assay Buffer: 500 mM Tris-HCl (pH 7.5 at 22 $^{\circ}$ C), 20 mM EGTA, 0.1% Brij-35P (v/v)

PTP Lysis Buffer: 50 mM Tris-HCl (pH 7.5 at 22 $^{\circ}$ C), 150 mM NaCl, 1% Triton X-100 (v/v), 1% (v/v) Protease Inhibitor Cocktail (Life Tech, 78430), 2 mM EGTA, 100 μ g/mL catalase (Millipore, 219261), and 100 μ g/mL superoxide dismutase (Millipore, 574594)

10 $\times$ PSP Assay Buffer: 500 mM Tris-HCl (pH 7.5 at 22 $^{\circ}$ C), 10 mM DTT, 0.1% Brij-35P (v/v)

PSP Lysis Buffer: 50 mM Tris-HCl (pH 7.5 at 22 $^{\circ}$ C), 1 mM DTT, 150 mM NaCl, 1% Triton X-100 (v/v), 1% (v/v) Protease Inhibitor Cocktail (Life Tech, 78430)

5.1 Preparation of cell lysates

1. Culture cells as specified by the supplier.
2. Seed cells onto 150 mm culture dishes. A control sample (no treatment) as well as a sample for subsequent siRNA knockdown should be prepared.
3. Allow cells to grow to the desired confluency (e.g., 80%).
4. Add siRNA knockdown reagents to the treatment sample and culture according to manufacturer recommendations.
5. After sufficient time has passed for knockdown, place culture dishes on ice and remove the media. Wash $3 \times$ with ice cold PBS.
6. Lyse cells by adding 200 μ L of the appropriate lysis buffer and scrap using a cell scraper.
7. Incubate for 15 min and collect lysate in an Eppendorf tube.
8. Centrifuge at $10,000 \times g$ for 5 min at 4°C .
9. Collect the supernatant and flash freeze aliquots in liquid nitrogen.
10. Use one aliquot to determine total protein concentration. Our laboratory routinely uses the BioRad protein assay (BioRad, 500-0006).
11. Verify knockdown of the target PP via Western blotting according to supplier protocols.

5.2 Cell lysate assays

1. Mix optimized concentrations of phosphorylated peptide substrate and MgCl_2 with assay buffer ($1 \times$ final concentration in 40 μ L total) in a series of wells. Allow extra volume for subsequent addition of lysate, such that the final assay volume is 40 μ L.
2. Monitor the fluorescence emission of each well for 10 min prior to addition of lysate.
3. Lysate stocks can be diluted using lysis buffer and are generally assayed at 5–20 μ g total protein per well where the lysate is $<5\%$ of the total volume of the assay. Optimal amounts of total protein may vary and should be optimized empirically.
4. Monitor the fluorescence emission for 1 h.
5. Plot the linear region of the fluorescence emission versus time and determine absolute reaction slopes for each sample. Average the reaction slopes for each sample and determine standard deviations.
6. Reaction slopes and corresponding standard deviations can be plotted in bar graph form. A selective probe should yield a reduced reaction slope upon siRNA knockdown that is proportional to the extent of target PP knockdown.



6. Conclusions

Recent work has demonstrated the importance of PPs in cell signaling as well as the potential of these enzymes as drug targets. However, chemical tools to study the activity dynamics of these enzymes have lagged behind those available for protein kinases (Casey & Stains, 2018). The method described above provides an approach for the synthesis and validation of Sox-based, fluorescent activity probes for PPs. With a validated probe in-hand, the activity dynamics of PPs can be assessed under relevant biological conditions (Beck, Lawrence, et al., 2016; Beck, Truong, et al., 2016). Thus, these probes provide a new means to investigate the chemical biology of PPs, adding to our understanding of cellular signaling.

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