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Quantification of Protein Kinase Enzymatic Activity in Unfractionated Cell Lysates Using CSox-Based Sensors

Jon R. Beck[1], **Laura B. Peterson**[2], **Barbara Imperiali**[2], and **Cliff I. Stains**[1]

[1]Department of Chemistry, University of Nebraska – Lincoln, Lincoln, Nebraska

[2]Department of Biology and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts

Abstract

Defining perturbations in protein kinase activity within biological samples can provide insight into disease mechanisms as well as potential targets for drug development. In this protocol, we present a method that utilizes a phosphorylation-sensitive amino acid, termed CSox, to afford kinase-selective biosensors capable of reporting on enzymatic activity directly in biological samples. These sensors produce an increase in fluorescence in response to phosphorylation of an amino acid residue adjacent to CSox. Probes can be designed for either serine/threonine or tyrosine kinases and analysis can be performed using standard fluorescence equipment. The procedures provided herein represent our optimized protocols for the design, validation and application of CSox-based protein kinase activity sensors.

Keywords

cell signaling; kinase activity assay; kinase activity profiling; phosphorylation; fluorescence-based biosensor

INTRODUCTION

Protein kinases are ubiquitous signaling enzymes that catalyze the transfer of the γ-phosphoryl group of ATP to a hydroxy-amino acid (Manning et al., 2002). Phosphorylation of a protein substrate can modulate its activity through a variety of mechanisms, including induction of a conformational change or altering the affinity towards protein binding partners. The >500 protein kinases encoded in the human genome regulate a variety of signaling pathways such as cell growth, cell survival, inflammation, metabolism, and cell migration. As a consequence, the dysregulation of kinase enzymatic activity can drive a variety of disease phenotypes. These observations have resulted in a concerted effort to develop kinase inhibitors for clinical applications (Cohen, 2002). Beyond the utility of small molecule protein kinase inhibitors in the clinic, chemical genetics has emerged as a powerful tool to elucidate the biological function of a kinase of interest (KOI) (Bishop et al., 2000). However, complementary methodologies to quantitatively assess the enzymatic activity of a KOI in biological samples containing numerous endogenous kinases have lagged behind. This has led to the standard practice of using activity proxies to infer kinase activity in biological samples.

Previously, we described an approach to directly monitor kinase activity in unfractionated cell lysates utilizing a phosphorylation-sensitive fluorophore termed Sox (Shults et al., 2005). In this protocol we present our most up to date usage of this fluorophore, which involves covalent linkage to an engineered cysteine within a synthetic kinase substrate (Lukovic et al., 2008). These CSox-based sensors are capable of detecting phosphorylation through a process known as chelation-enhanced fluorescence (CHEF, Fig. 1A). Importantly, this approach allows the user to tune the selectivity of the CSox-based substrate for the KOI and directly monitor the activity of that KOI in the presence of the endogenous kinome (Lukovic et al., 2009; Stains et al., 2011) (Fig. 1B).

The Basic Protocol provides our optimized procedures for utilizing CSox-based kinase activity sensors to quantify kinase signaling changes in cell lysates derived from samples treated with an external stimulus. This procedure relies on careful optimization of the kinase activity probe (Support Protocol 1) as well as validation of sensor selectivity in the presence of endogenous kinases (Support Protocol 2). Detailed procedures for the chemical synthesis of CSox-based activity sensors have been described elsewhere (Lukovic et al., 2008; Pearce et al., 2001; Shults and Imperiali, 2003). Herein we provide procedures for the synthesis of Sox-Br, a key intermediate required for the construction of CSox-based activity sensors, as well as alkylation of synthetic peptide substrates with Sox-Br (Support Protocol 3). Alternatively, CSox-based activity sensors are currently available under the Omnia® trademark from Life Technologies and can be custom synthesized to user specifications. Taken together, these resources allow for the successful design, synthesis, validation and application of CSox-based activity sensors by any lab.

STRATEGIC PLANNING

Designing and Evaluating Initial Sensor Constructs

Sequences for initial sensor designs can be drawn from the primary literature. However, since the selectivity of CSox-based sensors is largely dependent on the substrate sequence, careful attention should be given to any available information concerning the kinetic parameters as well as selectivity for the KOI. Consensus phosphorylation sites often do not provide adequate selectivity amongst endogenous kinases; well-characterized substrates should be employed when possible. As a general guideline, we have found that peptide substrates with k_{cat} values of >20 min^{-1} provide a sufficient turnover in order to visualize endogenous kinase activity in cell lysates within one hour (Stains et al., 2012). In addition, K_M values in the low μM range (<5 μM) typically provide selectivity for the KOI while minimizing off-target activity if appropriate sensor concentrations are used (i.e. 1 – 2x K_M). Once a substrate sequence is chosen, the position of CSox within the substrate as well as *in vitro* assay conditions should be optimized (Support Protocol 1). Substrates should be evaluated against recombinant kinases with known overlapping sequence specificity. If off-target activity is observed at this stage the culprit kinase can be easily identified, since the reactions are run in parallel with each recombinant enzyme, and appropriate small molecule inhibitors can be added to the assay. These initial assays allow for the optimization of reaction conditions before transitioning to more complex systems such as cell lysates.

Validation of Sensors in Cell Lysates

> Following *in vitro* assay optimization, the selectivity of CSox-based activity sensors should be evaluated in cell lysates containing endogenous kinases. First, the amount of lysate used in the assay is optimized to provide the highest signal-to-noise between samples stimulated for KOI activity and controls. Following this step, the KOI is immunodepleted from the lysate allowing for the contribution of the remaining endogenous kinases to be determined. Antibodies that are compatible with immunoprecipitation should be utilized for this experiment. Lastly, known small molecule inhibitors of the KOI can be employed to further delineate sensor selectivity. If possible, multiple inhibitors should be employed to establish that signal from the sensor is not the result of an off-target kinase that is also inhibited by the chosen small molecule.

BASIC PROTOCOL 1: GENERAL CELL LYSATE ASSAY FORMAT

> The hallmark of a validated CSox-based kinase activity sensor is its ability to selectively report on perturbations in kinase activity in complex biological samples, such as unfractionated cell lysates. Kinase activity can be stimulated according to standard protocols for the KOI. Once lysate samples are obtained, alterations in the enzymatic activity of a KOI can be determined using a fluorescence plate reader by analysis of the increase in fluorescence of the CSox-based kinase activity sensor with respect to time.

Materials

> Appropriate mammalian cell line (ATCC, etc.)
>
> Corning 150 mm culture dishes (Sigma, CLS430599)
>
> Tissue culture media (appropriate for the cell line in use)
>
> Ice-cold PBS
>
> Cell scrapers (Fisher Scientific, 08-100-242)
>
> Non-denaturing lysis buffer (see Reagents)
>
> Temperature-controlled micro-centrifuge
>
> Liquid nitrogen dewar flask
>
> Fluorescence plate reader (SynergyH2 or similar)
>
> 10x Mg^{2+} assay buffer (see Reagents, the concentration of Mg^{2+} is optimized in Support Protocol 1)
>
> 10x CSox peptide sensor solution (see Reagents, the optimal concentration of CSox-based sensor is determined in Support Protocol 1)
>
> 10x ATP solution (10 mM, see Reagents)
>
> Corning 96-well half-area flat-bottom white microplate (Sigma, CLS3693)

Activation of a KOI and Lysate Preparation

> **1** Grow appropriate mammalian cells to 80% confluency in two 150 mm dishes.

2   Stimulate cells from one dish with the method of choice for the KOI.

> For example, PKA can be activated by addition of forskolin to the culture media.

3   Maintain the second dish under normal growth conditions to serve as a non-stimulated control.

4   After stimulation, place both dishes on ice, remove media and rinse 3x with 10 mL ice-cold PBS, making sure to remove all liquid following each rinse.

5   Add 400 μL of ice-cold non-denaturing lysis buffer to the dish and, using a cell scraper, vigorously spread the lysis buffer across the entire surface.

6   Angle the dish slightly downward and begin scraping the lysate toward the bottom of the dish, making sure to scrape the entire surface of the dish.

7   Collect the accumulated lysate from the bottom of the dish with a micro-pipette and place in a 1.6 mL micro-centrifuge tube on ice.

8   Mark each sample as "control" or "stimulated" as appropriate.

9   Incubate both sets of lysate on ice for 15 minutes.

10   Centrifuge samples for 5 min at 10,000 × g at 4 °C to pellet the insoluble material. Carefully collect the soluble fraction, transfer to a labeled micro-centrifuge tube and place on ice.

> Certain cell lines produce a noticeable lipid layer that will appear as a cloudy material on top of the soluble fraction. If a significant lipid layer is present, pierce the material with a pipette tip to recover the soluble fraction.

11   Aliquot each sample into 20 μL fractions in micro-centrifuge tubes on ice.

12   Flash freeze the aliquoted samples in liquid nitrogen and store at −80 °C.

> For kinases studied thus far, we have not detected any appreciable loss of activity over a 6 month period in this lysis buffer formulation. However, if long-term storage is desired, the stability of kinase enzymatic activity in lysis buffer should be determined.

Data Acquisition and Work-up

13   Turn on the fluorescence plate reader and allow the instrument to auto-calibrate.

14   Create a kinetic protocol using the following parameters

> Temperature: 30 °C
>
> Delay: 10 minutes (allows for thermal equilibration of the assay solution)
>
> Kinetic Read: 10 minutes (30 second intervals, $\lambda_{excitation}$ = 360 nm, $\lambda_{emission}$ = 485 nm)
>
> Eject Plate: For manual addition of lysate

Kinetic Read: 2 hours (30 second intervals, $\lambda_{excitation}$ = 360 nm, $\lambda_{emission}$ = 485 nm)

> The initial 10 minute read without the addition of lysate allows the user to define the starting fluorescence of the CSox probe. This data can be used to identify reactions that have reached completion after addition of lysate but prior to reading on the plate reader (generally observed as a flat line with a total fluorescence >2-fold higher than the initial read).

> Read time can be increased or reduced based on individual kinase kinetics.

15 Make a master mix consisting of water, Mg^{2+} assay buffer, CSox peptide sensor and ATP. The final volume in each well should be 120 μL. Therefore, the total volume of the master mix should be equivalent to the number of reactions to be run multiplied by 120 μL, minus the total volume of lysate to be added to each well. The final concentration of Mg^{2+} assay buffer and CSox peptide sensor should be 1 x (the optimal concentrations of Mg^{2+} and CSox peptide are determined in Support Protocol 1 and will vary depending on the sensor sequence). The final concentration of ATP during the assay should be 1x (1 mM).

> When making the master mix take into account the possible loss of solution due to pipetting. For example, if a total of 6 wells are to be analyzed during an experiment make enough master mix for 6.5 wells in order to ensure that there will be sufficient solution for each well to be assayed. Each sample type should be assayed in triplicate.

16 Aliquot an appropriate amount of master mix into individual wells of the 96-well half-area plate, taking into account the cell lysate solution to be added later in order to reach the final volume of 120 μL.

> For example, in an assay of 120 μL total volume of which 6 μL would be cell lysate, one would add 114 μL of master mix to each well.

17 Place the 96-well plate into the fluorescence plate reader and begin the kinetic protocol.

18 Remove the appropriate number of lysate samples from the −80 °C freezer and place on ice.

> Avoid subjecting samples to freeze/thaw cycles as this can decrease kinase activity and lead to inconsistent data. Freeze/thawed samples may be used for western blotting or other experiments that are not reliant on preserved kinase activity.

19 Dilute the lysate samples with non-denaturing lysis buffer to the appropriate concentration as previously determined (see Support Protocol 2). Keep samples on ice.

20   Following the initial 10 minutes of thermal equilibration and 10 minute kinetic read without lysate, remove the 96-well plate from the fluorescence plate reader and add the appropriate type and amount of lysate dilutions to the desired wells.

21   Mix the assay solution in each well multiple times with a multi-channel pipette.

> The presence of lysis buffer in the assay solution can lead to the formation of bubbles in the assay wells. Bubbles must be removed prior to data collection as they can interfere with optical assays. Large bubbles may be readily dissipated with a hypodermic needle, however small bubbles are extremely difficult to remove. Avoid introducing small bubbles to the assay wells by not injecting air into the assay solution during mixing with the multi-channel pipette.

22   Place the 96-well plate back into the fluorescence plate reader and initiate the 2 hour data collection phase.

23   Following data collection, remove the 96-well plate from the plate reader and cover with parafilm to protect from dust or other contaminants.

> 96-well plates may be used in multiple experiments but individual assay wells should not be reused.

24   Plot the collected data for each assay well as a function of Arbitrary Fluorescence Units (AFU) and time (minutes) in Microsoft Excel or other preferred spreadsheet program.

25   Select the time points for the initial, linear portion of data and fit to a trend line.

> *The entire data set should graph as a hyperbolic plot. However, only the initial linear portion of the plot should be analyzed. There are two situations that can arise in which obtained data may not produce a hyperbolic plot. First, the data may exhibit an initial lag phase prior to the linear region of the data that should be used for work-up* (Lukovic et al., 2009; Shults et al., 2005; Stains et al., 2011; Stains et al., 2012). *In this case, the initial lag phase should not be included in the analysis as this will bias the fit. Second, the data may exhibit a linear, positive slope but may not become asymptotic. This occurs when the assay has not been monitored for a sufficient time to allow for the observation of the asymptotic portion of the data. This does not pose a problem as the initial linear portion of the plot represents the data of interest and the reaction rate can be obtained without the asymptotic portion. In all data work-up, it is important to determine the reaction slope over the same time period for each assayed sample.*

26   Find the average reaction slope (slope of trend lines from step 25) and calculate the standard deviation of each triplicate sample set. The average reaction slope is directly proportional to the enzyme activity.

27   Plot the average reaction slopes of each sample type as a bar graph with error bars consisting of the standard deviation of each sample set.

SUPPORT PROTOCOL 1: DESIGN AND EVALUATION OF INITIAL SENSOR CONSTRUCTS

The initial design, characterization and *in vitro* evaluation of potential substrates are essential steps in the development of optimized CSox-based kinase activity sensors. Following the design and synthesis of potential substrates, certain physical properties of the sensor (Mg^{2+} K_D, fold fluorescence signal increase) and kinetic parameters of the enzyme/substrate complex (K_M, V_{max}) should be determined in order to guide further assay optimization. Furthermore, identification of off-target interactions between the sensor and highly homologous kinases in a controlled, *in vitro* environment provides for troubleshooting opportunities prior to analyzing the relatively more complex reaction conditions encountered in cell lysate assays.

Materials

Fluorescence plate reader (SynergyH2 or similar)

Corning 96-well half-area flat-bottom white microplates (Sigma, CLS3693)

10x CSox peptide sensor solution (see Reagents, the optimal concentration of CSox-based sensor will be determined in the protocol below)

10x ATP solution (10 mM, see Reagents)

500 mM Tris-HCl (10x) (pH = 7.5 at 22 °C)

1.5 M NaCl (10x)

10x Mg^{2+} assay buffer (see Reagents, the concentration of Mg^{2+} will be optimized in in the protocol below)

Recombinant KOI (Life Technologies, EMD Millipore, etc.)

Enzyme dilution buffer (see Reagents)

Recombinant panel of kinases (Life Technologies, EMD Millipore, etc.)

0.1 M NaOH with 1 mM Na_2EDTA

$MgCl_2$ (Alfa Aesar, 10797)

Position of CSox and Recognition Elements

1 Select a peptide substrate sequence based on literature reports or native substrates for the KOI.

> The chosen substrate should, ideally, have some element of specificity for the KOI beyond simple consensus sequence recognition motifs common to the KOI family.

2 Design four peptides as pairs in which each peptide pair is delineated by a cysteine substitution at the (+)2 or (−)2 amino acid relative to the site of phosphorylation. Within each set of peptides, one sequence should contain the native amino acid that will serve as the site of phosphorylation (this will be the

substrate) and the other sequence should contain the corresponding phosphoamino acid at the site of phosphorylation (this sequence will serve as a positive control for complete phosphorylation). Fmoc-Cys(Mmt)-OH should be used at the (+)2 or (−)2 positions relative to the site of phosphorylation, this will allow for the covalent attachment of the Sox fluorophore to the peptide sequence. All other amino acids should use standard protecting groups for Fmoc solid-phase peptide synthesis (SPPS).

> For example, if the original substrate sequence is AHLQRQL**S**IAHH the designed sequences for synthesis would be: AHLQRCL**S**IAHH and AHLQRCLp**S**IAHH (pair 1) as well as AHLQRQL**S**ICHH and AHLQRQLp**S**ICHH (pair 2). In these sequences, **S** is the site of phosphorylation by the KOI and p**S** represents phosphoserine.

3   Synthesize the four peptides according to Support Protocol 3 (Lukovic et al., 2008; Pearce et al., 2001; Shults and Imperiali, 2003) or obtain the sequences commercially from Life Technologies.

4   Dissolve the purified peptide in water. Determine the peptide concentration by diluting a small aliquot of the peptide in 0.1 M NaOH containing 1 mM Na_2EDTA and measuring absorbance at 355 nm (ε_{355}= 8247 M^{-1} cm^{-1}) (Shults and Imperiali, 2003). Typically, averages of three independent dilutions measured in triplicate are used (i.e. 1:12.5, 1:25 and 1:50).

> Purified peptides can be stored for 6–12 months as solutions in water (4 °C) or indefinitely as lyophilized powders at −20 °C.

Determination of Mg^{2+} K_D

5   Set up a protocol on a fluorescence plate reader with the following parameters:

> Temperature: 30° C
>
> Single Point Read: $\lambda_{excitation}$ = 360 nm, $\lambda_{emission}$ = 485 nm

6   Set up ten assay wells for each peptide with 50 mM Tris-HCl (pH = 7.5 at 22 °C), 150 mM NaCl, and 1 μM peptide with $[Mg^{2+}]$ = 1 mM, 2.5 mM, 5 mM, 10 mM, 15 mM, 20 mM, 30 mM, 50 mM, 100 mM and 250 mM respectively for each well (total volume = 120 μL)

7   Collect fluorescence data for all four peptides.

8   Plot the data for each peptide as AFU versus the Mg^{2+} concentration using a nonlinear fitting program, such as KaleidaGraph. Fit the data to a one-site binding isotherm (Eqn 1):

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

> The K_D of the phosphopeptide for Mg^{2+} should be tighter than the K_D of the corresponding non-phosphopeptide for Mg^{2+}. This allows for the

discrimination of the phosphorylation state of the target amino acid using the appropriate concentration of Mg^{2+}, which is determined below.

Fold Fluorescence Increase of Phospho versus Non-Phospho Sensors

9   For each pair of phospho and non-phosphopeptides prepare solutions containing 50mM Tris-HCl (pH = 7.5 at 22 °C), 150 mM NaCl, 1 μM peptide, 1 mM ATP, and varying concentrations of Mg^{2+} equal to 0.5, 1, and 1.5 times the K_D of the corresponding phosphopeptide for Mg^{2+} within each pair (in a total of 120 μL per well). This assay should be performed in triplicate for each concentration of Mg^{2+}, yielding a total of 9 wells for each peptide.

10   Analyze the assay wells with the fluorescence plate reader using the same protocol from step 5.

11   Calculate the quotient of the average AFU of the phosphopeptide with the average AFU of the corresponding non-phosphopeptide at each $[Mg^{2+}]$. This value corresponds to the maximum fold change upon phosphorylation at each $[Mg^{2+}]$ and provides information regarding the sensitivity of each peptide sensor; typical values are 3 – 10-fold. The $[Mg^{2+}]$ that provides the highest fold increase in fluorescence between the non-phospho and phosphopeptide should be used for all subsequent experiments and incorporated into the 10x Mg^{2+} assay buffer.

Kinetic Parameters

12   Use the fluorescence plate reader protocol from step 14 of Basic Protocol 1.

13   Make master mix solutions of water, Mg^{2+} assay buffer (with the optimal $[Mg^{2+}]$ determined above) and ATP as in step 15 of Basic Protocol 1. Assemble the mixes such that final concentrations of 1 μM, 2.5 μM, 5 μM, 10 μM, 15 μM, 20 μM, 30 μM, 50 μM, 100 μM and 250 μM non-phosphopeptide (substrate) respectively for both peptide pairs 1 and 2 are achieved during the assay. Assume that you will add 12 μL of kinase to each well to reach a total volume of 120 μL. Assays should be performed in triplicate.

> The substrate concentrations given above represent a general starting point and should be modified based on the observed K_M for the substrate being studied. Final substrate concentrations should range from 0.2 – 5x the K_M.

14   Add 108 μL of each master mix to individual wells.

15   Make separate master mix solutions of water, Mg^{2+} assay buffer and ATP that will yield final concentrations of 1 μM, 2.5 μM, and 5 μM phosphopeptide (reaction product) respectively for both peptide pairs 1 and 2 as described in step 13. Assays with phosphopeptides should also be performed in triplicate.

16   Add 108 μL of each master mix type from step 15 to individual wells.

17 Place the 96-well plate into the fluorescence plate reader and begin the protocol.

18 Dilute the recombinant KOI with enzyme dilution buffer (see Reagents) to 10 nM.

19 After 20 minutes add 12 μL of recombinant KOI to each substrate well (1 nM final, in 120 μL total).

> In order to observe the initial portion of the reaction, the final concentration of the KOI may need to be adjusted depending on the turnover rate of the KOI for the substrate under investigation. For example, if a significant amount of substrate has been consumed prior to monitoring the reaction in the plate reader (>2-fold increase in fluorescence between the initial 10 minute read without the KOI and the first read after addition of the KOI), the concentration of the KOI should be lowered to allow for the visualization of the linear part of the reaction corresponding to <10% substrate turnover.

20 Mix each well using a multichannel pipette.

21 Place the plate back into the plate reader and continue the protocol.

22 For the initial 10 minute reading (before adding KOI), plot the average AFU versus [peptide] for each substrate and phosphopeptide pair in Microsoft Excel or preferred spreadsheet program.

23 Fit a linear trend line to each plot from step 22.

24 Calculate the difference between the average slopes of the phosphopeptide and substrate peptide for each pair, termed fp-fs.

25 Plot the collected data after addition of the KOI to each assay well as a function of AFU and time (minutes) in Microsoft Excel or other preferred spreadsheet program.

26 Select the time points for the initial linear portion of data corresponding to <10% product turnover and fit to a linear trend line.

27 Calculate the reaction velocity at each [substrate] by dividing the slope (AFU/min) by the appropriate fp-fs (AFU/μM) value corresponding to the substrate, product pair found in step 24.

> Division of the reaction slope at each concentration of substrate by fp-fs corrects for the loss of fluorescence due to conversion of the relatively less fluorescent substrate to the more fluorescent phosphopeptide product. Reaction velocities determined in this manner are in units of μM/min.

28 Plot the reaction velocities versus [substrate] for each pair in a nonlinear fitting program, such as KaleidaGraph. Fit each curve to the Michaelis-Menten equation (Eqn 2):

$$v = \frac{V_{max}[\text{S}]}{K_M + [\text{S}]}$$

This plot yields values for K_M (μM) and V_{max} (μM/min). k_{cat} (min^{-1}) can be calculated by dividing V_{max} by the concentration of the KOI in μM.

29 The specificity constant, k_{cat}/K_M (μM^{-1} min^{-1}), can be used to compare the catalytic efficiency of each substrate. In general, substrates with larger values of k_{cat}/K_M should be used for further assays.

Refer to the Strategic Planning section for additional details concerning interpretation of kinetic parameters for subsequent assays.

Kinase Panel Assay

30 Use the literature or sequence homology (Manning et al., 2002) to identify a panel of kinases with potential overlapping substrate specificity with the KOI.

31 Make a master mix with water, Mg^{2+} assay buffer, ATP and the optimal substrate determined above. Use a final substrate concentration that is at least 1 – 2x K_M to achieve a relatively high rate of turnover during the assay. Assume that you will add 12 μL of kinase to each well to reach a final volume of 120 μL. Assay each kinase in triplicate.

The substrate concentration can be adjusted in panel assays as well as lysate assays in order to decrease off-target phosphorylation.

32 Add 108 μL of master mix to the appropriate wells of a 96-well plate.

33 Place the plate in a fluorescence plate reader and the run protocol from step 12.

34 Dilute the recombinant KOI and panel kinases to 100 nM in enzyme dilution buffer.

35 Following the initial 20 minutes of equilibration and fluorescent readings, add 12 μL of the KOI or panel kinase to individual wells (final [enzyme] = 10 nM).

36 Mix with a multichannel pipette.

37 Place the plate back into the plate reader and continue with data collection.

38 Plot the initial linear portions of the data (<10% turnover) versus time and fit to a linear trend line.

39 Average the slopes of each kinase and plot as a bar graph with standard deviations.

Comparison of the slopes for each kinase gives insight as to any off-target activity reported by the sensor. Ideally, a CSox sensor will only act as a substrate for the KOI. However, due to the overlapping sequence specificity of certain kinases, off-target activity can be

observed. In this case, small molecule inhibitors can be added to the assay to suppress off-target kinases (see Troubleshooting).

SUPPORT PROTOCOL 2: VALIDATION OF SENSORS IN CELL LYSATES

In order to validate CSox-based kinase activity sensors, it is necessary to optimize the amount of lysate to be used in assays and demonstrate selectivity in cell lysates derived from the cell line of interest. Two methods are presented to determine activity due to off-target phosphorylation, immunodepletion and assays in the presence of selective inhibitors of the KOI.

Materials

Fluorescence plate reader (SynergyH2 or similar)

Corning 96-well half-area flat-bottom white microplates (Sigma, CLS3693)

10x Mg^{+2} assay buffer (see Reagents, the concentration of Mg^{2+} is optimized in Support Protocol 1)

10x CSox peptide sensor solution (see Reagents, the optimal concentration of CSox-based sensor is determined in Support Protocol 1)

10x ATP solution (10 mM, see Reagents)

Tissue culture media (appropriate for the cell line of interest)

Non-denaturing lysis buffer (see Reagents)

Tissue culture facility (Laminar flow hood, CO$_2$ incubator, etc)

Tissue culture plastics for cell growth

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

Ice-cold PBS

BioRad protein assay dye reagent concentrate (BioRad, 500-0006)

Anti-KOI antibody (appropriate for immunoprecipitation)

Naïve IgG antibody (isotype matched to anti-KOI antibody)

Protein A or G sepharose (GE Life Sciences, etc.)

Orbital rocker/shaker

Small molecule inhibitor of the KOI

Cell culture grade DMSO (Sigma, etc.)

Optimization of lysate amount in assay

1 Following the preparation of cell lysates (see Basic Protocol 1), thaw the lysates on ice and quantify protein content using the BioRad protein assay according to the manufacturer instructions.

2 Dilute each lysate with lysis buffer to a final concentration of 4 μg/μL.

3 Prepare serial dilutions of each lysate in lysis buffer to final concentrations of 4, 2, 1, 0.4 and 0 μg/μL (in a total of 85 μL each).

4 Prepare a master mix containing water, Mg^{2+} assay buffer, CSox peptide substrate and ATP as described in step 15 of Basic Protocol 1. Assume that you will add 25 μL of lysate to each well (i.e. 95 μL mix per well) and will evaluate each sample in triplicate.

5 Set up the plate reader protocol following Basic Protocol 1, step 14.

6 Aliquot the master mix from step 4 into a 96-well plate (95 μL in each well). Place in the plate reader and begin the kinetic protocol.

7 Following the temperature equilibration and initial read portion of the protocol, pipet 25 μL of each lysate into its designated well.

8 Mix the contents of each well with a multichannel pipette.

9 Place the plate back in the plate reader and continue the kinetic protocol.

10 Following the kinetic protocol, plot AFU versus time for each lysate.

11 Fit the linear portion of each plot, this corresponds to the relative enzyme activity for each sample.

12 Compare the amount of time each plot is linear and the average slope for each sample.

13 The amount of lysate used in subsequent assays should provide a substantial linear range (i.e. >5 min) and should allow for differences in enzyme activities in control versus stimulated samples to be observed.

Immunodepletions

14 Using the stimulated lysates from above, incubate 600 μg of lysate with 20 μL of Protein A or Protein G sepharose beads, with gentle shaking for 1 h at 4 °C in a micro-centrifuge tube.

> *The choice of Protein A or Protein G sepharose will depend on the anti-KOI antibody used* (Hage, 2006).

15 Centrifuge the sample (5 min, 1000 × g) and transfer the pre-cleared lysate to two new micro-centrifuge tubes (divide the sample in half, by volume).

16 Add 1–5 μg of anti-KOI antibody to one lysate sample and the same amount of naïve IgG to the other sample.

> The naïve antibody will serve as a negative control, since the KOI should not be depleted.

17 Incubate the immunodepletions for 1 h at 4 °C with gentle shaking.

18 Add 25 μL of Protein A or Protein G sepharose (the same type used in step 14) to each of the samples.

19 Incubate the immunodepletions for 1 h at 4 °C with gentle shaking.

20 Centrifuge the samples (5 min, 1000 × g) and transfer the supernatant to clean micro-centrifuge tubes.

> A small portion of each lysate should be saved to run western blot analysis to ensure the KOI was removed from the immunodepleted sample.

21 Run kinase activity measurements on the input (non-treated lysate), immunodepleted and naïve IgG control samples using Basic Protocol 1 with the optimal conditions identified above.

22 Determine the slopes for each of the samples. The residual activity in the immunodepleted sample will provide information regarding activity due to off-target kinases and should be compared to western blots to determine the extent of depletion of the KOI.

Inhibitor Assays

23 Following Basic Protocol 1, prepare stimulated lysates. You will require enough lysate for 7 conditions in triplicate (21 wells).

24 Prepare a stock solution of a small molecule inhibitor (10 mM in DMSO). Prepare serial dilutions in DMSO of 100x compound stocks assuming there will be 2–3 concentrations above and below the reported IC_{50} value after dilution (1x).

> For an inhibitor with an IC_{50} of 50 nM, useful concentrations would be 0.1 nM, 1 nM, 10 nM, 50 nM, 100 nM, 1000 nM. Therefore the 100x stocks will be 10 nM, 100 nM, 1 μM, 5 μM, 10 μM, and 100 μM.

25 Prepare the assay master mix with water, Mg^{2+} assay buffer, ATP and each inhibitor stock such that the final [inhibitor] in the assay represents a 100-fold dilution of the stock. Reserve the appropriate volume for the lysate to be added later to each well.

26 Set up a kinetic protocol following the guidelines in Basic Protocol 1.

27 Aliquot the master mix across the 96-well plate, place in plate reader, and begin kinetic protocol.

28 Following temperature equilibration, add the appropriate volume of lysate to each well and mix with a multichannel pipet.

29 Place the plate back in the plate reader and resume the kinetic read.

30 Plot AFU versus time for each inhibitor concentration.

31 Determine the average slope for each of the samples ran in triplicate.

32 Divide each of the average slopes by the slope from the sample with no inhibitor.

33 Convert these values to percentages and plot the resulting percent activities versus log [inhibitor].

34 Using a non-linear regression analysis software program, such as KaleidaGraph, calculate the IC$_{50}$ for the compound by fitting the data from step 33 to a 4-parameter logistic function (Eqn 3):

$$Percent\ Activity = min_{AFU} + \frac{max_{AFU} - min_{AFU}}{1 + 10^{(([inhibitor] - IC_{50}) * slope)}}$$

35 If the inhibitor is ATP-competitive and the reported IC$_{50}$ value was obtained under similar concentrations of ATP, a large deviation from the reported value could indicate sensor phosphorylation by off-target kinases.

Alternatively, if the K$_M$ of the KOI for ATP is known the K$_I$ of the inhibitor can be calculated using the Cheng-Prusoff equation and compared to the calculated K$_I$ obtained from the CSox assay.

36 In the plot from step 34, take note of the percent residual activity at the highest inhibitor concentration. If there is >10% residual activity, this could also indicate sensor phosphorylation by off-target kinases.

For approaches to mitigate off-target kinase activity see Troubleshooting.

SUPPORT PROTOCOL 3: SOX-BR SYNTHESIS AND ON-RESIN ALKYLATION

Procedures for the synthesis of 2-bromo-8-tert-butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamidoquinoline (Sox-Br, Fig. 2) and the alkylation of cysteine residues within kinase peptide substrates is given below (Lukovic et al., 2008; Pearce et al., 2001; Shults et al., 2003). Synthesis of Sox-Br will require knowledge of standard organic chemistry techniques and basic organic synthesis laboratory equipment. This procedure also assumes a general knowledge of Fmoc SPPS (Chan and White, 2000).

Materials

8-hydroxy-2-methyl-quinoline (Sigma, H57602)

Chlorosulfonic acid (ClSO$_3$H)

Dimethylamine (NHMe$_2$)

Imidazole

Tert-butyldiphenylsilyl chloride (TBDPSiCl)

Selenium dioxide (SeO$_2$)

Sodium borohydride (NaBH$_4$)

Phosphorus pentoxide (P$_2$O$_5$)

N-bromosuccinimide (NBS)

Triphenylphosphine (PPh$_3$)

Trifluoroacetic acid (TFA)

Triisopropylsilane (TIS)

Tetramethylguanidine (TMG)

Magnesium sulfate (MgSO$_4$)

Sodium chloride (NaCl)

Potassium carbonate (K$_2$CO$_3$)

Tetrahydrofuran (THF)

Dichloromethane (CH$_2$Cl$_2$)

Ethyl acetate (EtOAc)

Hexanes

Dimethyl formamide (DMF)

#x02022; Dioxane

Ethanol (EtOH)

Diethyl ether (Et$_2$O)

Molecular sieves (4Å)

Celite (Diatomaceous earth)

Rotary evaporator

Oil bath

Silica Gel

Florisil

Fmoc-Cys(Mmt)-OH (EMD Millipore, 852031)

Syringe with frit (3 mL)

20 Gauge needles (1.5″)

Orbital shaker or vortex mixer

½ Dram vials

Synthesize 8-hydroxy-2-methylquinoline-5-sulfonyl chloride (2)

1   Dissolve 2.0 g (12.6 mmol) of 8-hydroxy-2-methyl-quinoline (**1**) in 10 mL of chlorosulfonic acid while stirring.

2   Stir reaction for 2 hr at 22 °C.

3   Transfer reaction mixture to a 2 L separatory funnel containing a slurry of brine/ice (500 mL) and CH_2Cl_2 (500 mL). Shake the separatory funnel and allow the layers to separate. Dry the organic layer over 10 g of potassium carbonate.

4   Filter the mixture and remove solvent via rotary evaporation to obtain the crude product as a yellow powder, 8-hydroxy-2-methylquinoline-5-sulfonyl chloride (**2**).

Synthesize 5-(N,N-dimethyl)sulfonamido-8-hydroxy-2-methylquinoline (3)

5   To 300 mL of THF, add a solution of dimethylamine in THF (2M, 7 mL, 14 mmol) under nitrogen.

6   Add 8-hydroxy-2-methylquinoline-5-sulfonyl chloride (**2**, 665 mg, 2.58 mmol) in portions over 3 hr with continuous stirring.

7   Allow the reaction to stir for an additional 10 min.

8   Remove the solvent via rotary evaporation. Re-dissolve the residue with 50 mL of CH_2Cl_2 and remove solvent via rotary evaporation. Repeat 2 additional times to remove residual dimethylamine.

9   Purify the residue by flash chromatography (1:2 EtOAc:Hexanes) to yield a white solid, 5-(N,N-dimethyl)sulfonamido-8-hydroxy-2-methylquinoline (**3**).

Synthesize 8-tert-butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamido-2-methylquinoline (4)

10   To 3 mL of dry DMF, add 5-(N,N-dimethyl)sulfonamido-8-hydroxy-2-methylquinoline (**3**, 515 mg, 1.93 mmol), imidazole (141 mg, 2.07 mmol), and *tert*-butyldiphenylsilyl chloride (0.55 mL, 2.11 mmol).

11   Allow the reaction to stir under N_2 for 2 hr.

12   Dilute the reaction with 250 mL of EtOAc and wash with saturated ammonium chloride (50 mL) and brine (2×50 mL). Dry the organic layer over $MgSO_4$.

13   Remove the solvent via rotary evaporation and purify by flash chromatography (1:9 EtOAc:Hexanes) to yield a white solid, 8-tert-butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamido-2-methylquinoline (**4**).

Synthesize 8-tertbutyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamido-2-formylquinoline (5)

14   To a dry flask, add 8-tert-butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamido-2-methylquinoline (**4**, 920 mg, 1.83 mmol) followed by dry dioxane (13.3 mL) and 4 Å molecular sieves (500 mg).

15   Add selenium dioxide (250 mg, 2.25 mmol).

16   Allow the reaction to stir for 17 hr at 95 °C.

17   Cool the reaction to 25 °C and filter the reaction mixture through celite. Wash the celite with additional dioxane (30 mL).

18 Remove the solvent via rotary evaporation and re-dissolve the residue in EtOAc (900 mL).

19 Wash the organic layer with brine (100 mL), water (100 mL), and saturated potassium carbonate (100 mL) and dry over $MgSO_4$.

20 Remove the solvent via rotary evaporation to yield the crude product, 8-tertbutyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamido-2-formylquinoline (**5**).

Synthesize 8-tert-butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamido-2-(hydroxymethyl)quinolone (6)

21 Dissolve sodium borohydride (70 mg, 1.80 mmol) in absolute ethanol (15 mL) and cool to 0 °C.

22 In a separate vessel, dissolve 8-tertbutyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamido-2-formylquinoline (**5**, 932 mg, 1.80 mmol) in dry CH_2Cl_2.

23 Add the solution of from step 22 drop-wise to the solution from step 21.

24 Allow the reaction to stir for 15 min.

25 Dilute the reaction mixture with diethyl ether (800 mL) and wash with saturated ammonium chloride (200 mL), water (2 × 100 mL) and brine (100 mL). Dry the organic layer over $MgSO_4$.

26 Remove the solvent by rotary evaporation to yield the crude product as a pale yellow residue, 8-tert-butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamido-2-(hydroxymethyl)quinolone (**6**).

Synthesize 2-bromo-8-tert-butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamidoquinoline (7, Sox-Br)

27 Dry 8-tert-butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamido-2-(hydroxymethyl)quinoline (**6**) over P_2O_5 for 15 hr in a dessicator.

28 Dissolve the dried 8-tert-butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamido-2-(hydroxymethyl)quinoline (**6**, 804 mg, 1.54 mmol) in dry CH_2Cl_2 (8 mL) under nitrogen.

29 Cool the solution to 0 °C and add N-bromosuccinimide (275 mg, 1.54 mmol) and triphenylphosphine (445 mg, 1.69 mmol).

30 Stir the reaction for 4 hours at 0 °C.

31 Dilute the reaction mixture with diethyl ether (1200 mL) and wash the organic layer with water (150 mL) and brine (100 mL). Then dry the organic layer over $MgSO_4$.

32 Remove the solvent via rotary evaporation and purify the crude material by flash column chromatography (Florisil, 100–200 mesh, 9:1 Hexanes:EtOAc) to yield

Sox-Br (**7**). Product identity and purity should be determined by MS and NMR, referring to previously published spectra (Shults et al., 2003).

> The final product is a yellow oil that generally contains an appreciable amount of residual (**4**). However, (**4**) does not interfere with the alkylation reaction and can be easily washed away after resin-based coupling of (**7**) to the peptide.

On-Resin Alkylation of Peptides with Sox-Br

33   Following SPPS of the designed substrate sequence with Cys(Mmt) at the site of Sox incorporation (Support Protocol 1), weigh 20 μmol of the dried peptide on resin and transfer to a 3 mL syringe with frit. The resin should be between the frit and plunger.

34   Swell the resin in CH_2Cl_2 (5×5 min $\times$ 2 mL) with agitation.

35   Expunge the CH_2Cl_2 and introduce the resin-bound peptide to a solution of 1% TFA and 5% TIS in CH_2Cl_2. Agitate the syringe filled with deprotection solution/resin for 20 min.

> This dilute acid solution will selectively remove the Mmt protecting group on Cys. The Mmt cation has a bright yellow color, accordingly, the TFA solution should turn yellow as the Mmt group is cleaved.

36   Repeat step 35 until all yellow color has disappeared in subsequent deprotection steps. Typically this process will take about 2 hours (6×20 min).

37   Following successful deprotection, wash the resin ($5 \times CH_2Cl_2$ then $5 \times DMF$) and expunge all remaining DMF from the last wash.

38   Dissolve 12.5 μL (100 μmol) of freshly distilled TMG in 430 μL of anhydrous DMF in a ½ dram vial.

39   In a separate ½ dram vial, dissolve Sox-Br (35.0 mg, 60 μmol) in 320 μL of anhydrous DMF.

40   Introduce the solution from step 38 to the resin-filled syringe and allow to mix for 2 mins.

41   Introduce the solution from step 39 to the resin/TMG/DMF-filled syringe and mix on an orbital shaker or low-speed vortexer for 12 hrs.

42   Following alkylation, wash the resin ($5 \times DMF$, $5 \times CH_2Cl_2$, $5 \times MeOH$, $5 \times CH_2Cl_2$) and proceed with peptide purification as normal.

> Labeled peptides can be visualized on HPLC by monitoring the absorbance of Sox at 360 nm.

REAGENTS AND SOLUTIONS

Use deionized, distilled water throughout.

Non-denaturing Lysis Buffer

50 mM Tris-HCl (pH = 7.5 at 22° C)

150 mM NaCl

100 μM Na_3VO_4

1% Triton X-100

1% Protease Inhibitor Cocktail III (EMD Millipore, 539134-1SET)

1% Phosphatase Inhibitor Cocktail III (Sigma, P0044)

1 mM DTT

2 mM EGTA

50 mM β-glycerophosphate

10 mM sodium pyrophosphate

30 mM NaF

Store at −20 °C for up 6 months

Mg^{2+} Assay Buffer, 10x

500 mM Tris-HCl (pH = 7.5 at 22 °C)

10 mM EGTA

20 mM DTT

0.1% Brij-35

10x $[MgCl_2]$ (Alfa Aesar, 10797) as determined from Support Protocol 1

Store at −20 °C for up to 6 months

CSox peptide sensor solution, 10x

10x [substrate] in water as determined from Support Protocol 1

DMSO can be added to aid dissolution

Store at 4 °C for 6 – 12 months or lyophilized at −20 °C indefinitely

ATP Solution, 10x

10 mM ATP (EMD Millipore, 119125) in water

Store in aliquots at −20 °C for up to 6 months, avoid freeze-thaw cycles

Enzyme Dilution Buffer

50 mM Tris-HCl (pH = 7.5 at 22 °C)

1 mM EGTA

2 mM DTT

0.01% Brij-35

10% Glycerol

COMMENTARY

Background Information

The approach presented in the above protocol represents our optimized conditions for conducting assays with CSox-based activity sensors. Our original sensor design involved the asymmetric synthesis of an amino acid analog, which included the Sox fluorophore as a β-alanyl derivative (Shults and Imperiali, 2003). While selective substrates that reported on kinase activity in cell lysates could be obtained using this approach (Shults et al., 2005), the relatively short nature of the linkage of the peptide backbone to the Sox fluorophore necessitated preorganization of the Mg^{2+} binding site using a β-turn motif. Consequently, this first generation design resulted in the deletion of either the N- or C-terminal kinase substrate recognition sequence. To circumvent the necessity for this constraint, second generation sensors were developed in which the Sox fluorophore was covalently linked to the side chain of cysteine (Lukovic et al., 2008). This second generation design, utilizing the unnatural amino acid termed CSox, introduces greater flexibility into the Mg^{2+} binding site. Indeed, this design enabled substrates to be generated by mutation of a single amino acid to CSox leading to improved substrate performance and greater ease of synthesis.

Perhaps the most widely used *in vitro* kinase activity assay involves monitoring the transfer of ^{32}P from $[\gamma\text{-}^{32}P]ATP$ to a peptide or protein substrate. Although sensitivity remains a strength of this approach, the necessity of specialized protocols for handling radioactivity can present a barrier to the implementation of this assay. Moreover, a separation step is required to parse signal from radiolabeled ATP and phosphorylated product, making this assay inherently discontinuous. In addition, ATP is not a selective substrate for protein kinases, rendering this approach incompatible with complex cell lysates. In contrast, CSox-based sensors can be used on standard fluorescence equipment without specialized training and can be tuned to selectively report on the activity of a KOI in unfractionated cell lysates (Lukovic et al., 2009; Stains et al., 2011; Stains et al., 2012). Moreover, phosphoryl transfer can be monitored in real-time, allowing for straightforward kinetic analysis. In addition to CSox-based sensors, a variety of other fluorescence-based assay formats now exist for the analysis of protein kinase activity in real-time (Gonzalez-Vera, 2012; Rothman et al., 2005; Sharma et al., 2008). However, relatively few of these assay formats has been demonstrated to provide selective readouts of kinase activity in unfractionated biological samples. Currently, the most widely accepted kinase activity assay in biological samples involves the use of phospho-specific antibodies, such as western blot analysis, Luminex, LanthaScreen, and other types of immunohistochemistry. These assays generally infer kinase activity through quantitative analysis of the phosphorylation status of residues on the KOI known to influence activity. The advantage of these approaches include the sensitivity and specificity associated with antibody-based assays. However, it should be noted that assays utilizing phospho-specific antibodies only provide a proxy for kinase activity and should not be thought of as equivalent to an enzymatic activity measurement. For example, many kinases contain multiple sites of phosphorylation that differentially regulate enzymatic activity

(Walsh et al., 2005). In addition, increasing evidence is accumulating that suggests that post-translational modifications (PTMs) other than phosphorylation can also control kinase activity (Paulsen et al., 2012). Finally, the enzymatic activity of kinases can be controlled by mechanisms other than posttranslational modification, such as protein-protein interactions (Ishizaki et al., 1996). In contrast, fluorescence-based kinase activity sensors can provide a direct measurement of enzymatic activity in biological samples. One such approach involves appending two fluorescent proteins (FPs) to either termini of a kinase substrate that is capable of undergoing a conformational change in response to phosphorylation (Ting et al., 2001). If the FPs are chosen appropriately, a change in fluorescence resonance energy transfer (FRET) can be observed upon phosphorylation of the substrate. An important advantage of this sensing modality is the ease of delivery, since these protein-based probes can be genetically encoded and produced within a living cell in culture. Despite their clear utility, the change in FRET signal (increase or decrease) observed in FP-based sensors can be difficult to rationally design. Furthermore, the typical response of these sensors is modest (~20 – 50%) upon phosphorylation (Rothman et al., 2005). Alternatively, CSox-based probes, which utilize CHEF to report on fluorescence, are capable of producing ~2 – 10-fold increases in signal upon phosphorylation (Lukovic et al., 2008; Shults et al., 2006). CSox-based probes are also generalizable to both Ser/Thr and Try kinases. Lastly, it is important to note that CSox-based kinase activity sensors provide direct enzymatic measurements of protein kinase activity that reflect the ensemble state of all PTMs present on the KOI.

Critical Parameters

When the goal is using CSox-based sensors to probe kinase activity in biological samples, the selectivity of the substrate sequence, and the corresponding sensor, are of critical importance. If the selectivity of the chosen substrate is not defined, sources of off-target activity can be identified by employing a panel of recombinant enzymes (Support Protocol 1) as well as immunodepletions and inhibitor assays in cell lysates (Support Protocol 2). Sources of off-target activity identified *in vitro* can be addressed by employing known inhibitors for the identified off-target kinase in order to suppress background signal from off-target kinase activity. The suppression of unknown off-target activity in cell lysates requires more careful optimization. In this case two strategies can be pursued. If a selective inhibitor for the target kinase is known this can be used to background subtract the effects of off-target kinases (i.e. one can run assays in the presence or absence of inhibitor and subtract off-target signal remaining in the inhibitor treated samples). Alternatively, the known promiscuity of kinase inhibitors (Davis et al., 2011) can be exploited to suppress the activity of entire families of potential off-target enzymes.

As a complementary approach, methods have been described for the high-throughput optimization of peptide substrates for a KOI (Gonzalez-Vera et al., 2009). These approaches can be employed to afford catalytically efficient peptide substrates that can be translated into CSox-based probes as described in the protocol above. In addition, if KOI-specific targeting domains are known, these can be appended to CSox-based peptides to further increase KOI selectively (Lukovic et al., 2009; Stains et al., 2011).

Troubleshooting

An overall flowchart of the protocols described in this unit is shown in Figure 3. A troubleshooting guide is presented in Table 1.

Anticipated Results

The successful application of these protocols should afford a selective CSox-based kinase activity sensor that can be used to assess perturbations in kinase activity in biological samples. Lysates prepared using the conditions outlined in Basic Protocol 1 should produce differing rates of phosphorylation of the CSox-based sensor that reflect endogenous activity of the KOI (Fig. 4). Using these procedures we have generated several CSox-based activity probes capable of detecting endogenous kinase activity in cell lysates and tissue homogenates (Lukovic et al., 2009; Stains et al., 2011; Stains et al., 2012).

Time Considerations

Sox- Br and CSox-containing peptide sequences can be generated in 3–4 weeks (Support Protocol 3). Initial validation of the substrate peptide, determination of fluorescent properties, kinetic parameters of phosphorylation, and *in vitro* kinase panel screens can be performed in 1–2 weeks (Support Protocol 1). The individual kinase activity measurement can be performed in a matter of hours. Further validation of the sensors in cell lysates can be more time consuming, given the requirement to culture cells, but can be achieved in 3–4 weeks provided a suitable cell line and stimulation condition for the KOI is known (Support Protocol 2).

Acknowledgments

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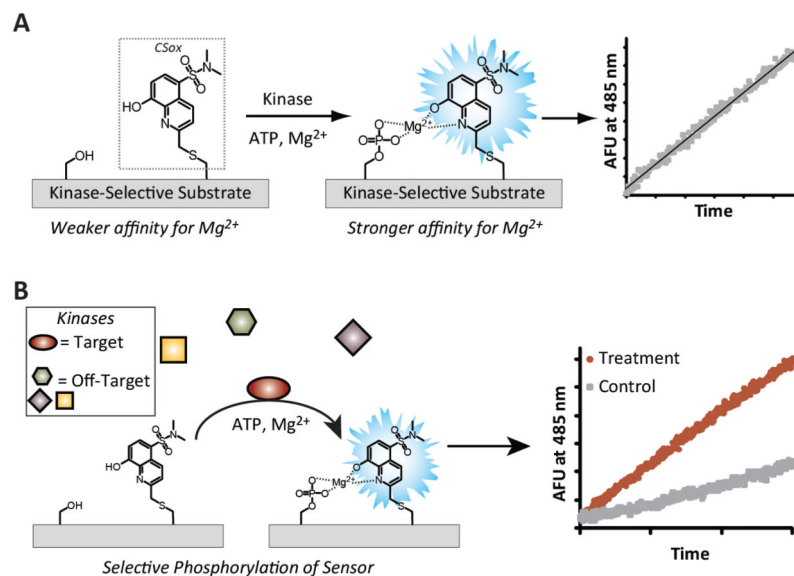
Figure 1.
Monitoring kinase enzymatic activity using CSox-based activity sensors. (**A**) A known peptide substrate for a KOI is synthesized (grey box, left) and the Sox fluorophore is placed adjacent to the hydroxyl-containing amino acid, that is the site of phosphorylation, via alkylation of a cysteine residue engineered into the substrate (CSox). Addition of the target kinase causes phosphorylation of the synthetic substrate, leading to chelation of Mg^{2+}, and a concurrent increase in the fluorescence of CSox (middle). The change in fluorescence can be monitored in real-time (excitation = 360 nm, emission = 485 nm) and is directly proportional to the enzymatic activity of the target kinase (right, slope of black trend line). (**B**) Selective phosphorylation of a validated CSox-based activity probe in cell lysates containing endogenous off-target kinases. Cell lysates can be derived from treated and control samples, allowing for the direct quantification of KOI activity under biologically relevant conditions.
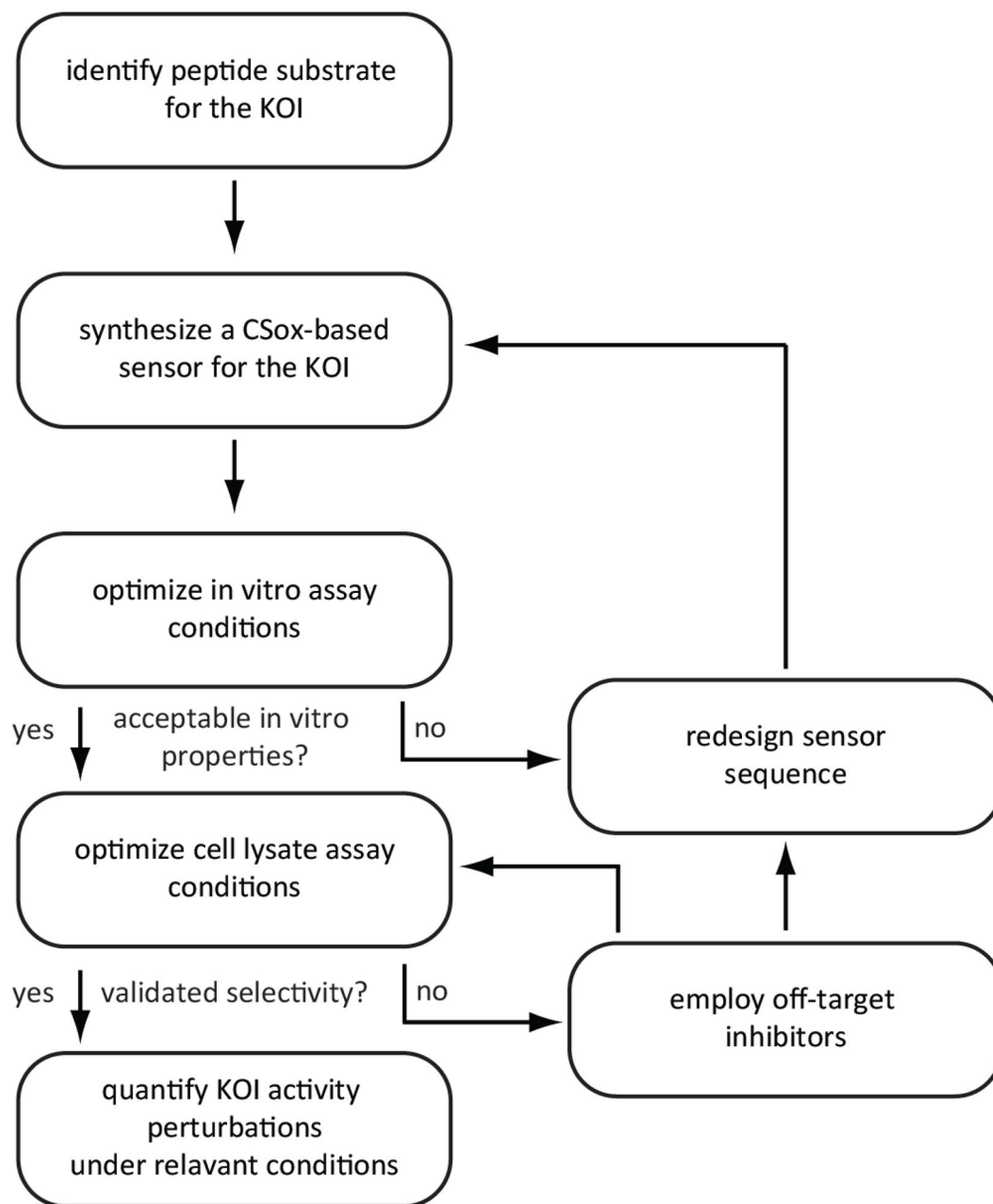
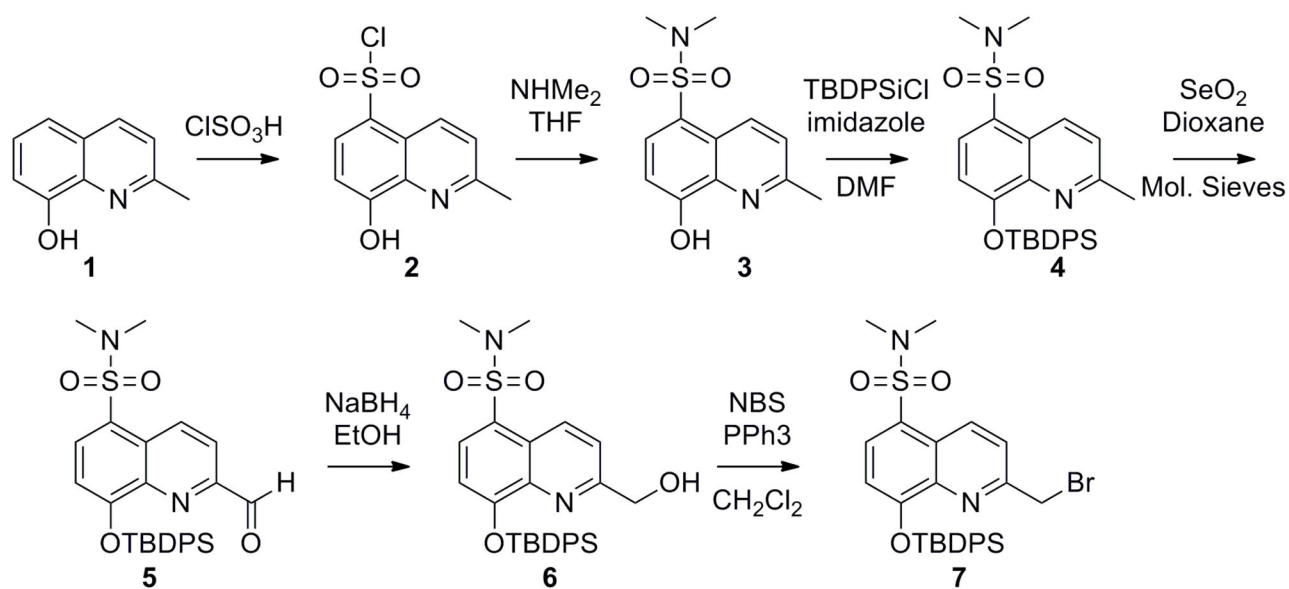**Figure 2.**
Synthetic scheme to afford Sox-Br.

Figure 3.
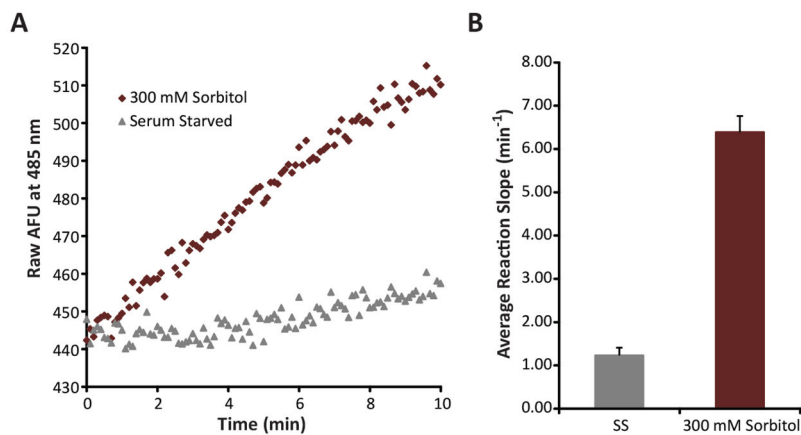Flowchart describing the development and validation of a CSox-based activity sensor.

Figure 4.
Representative data using a CSox-based MK2 sensor to quantify MK2 activity in HeLa cell lysates. (**A**) The increase in the fluorescence of the MK2 sensor is monitored over time in the presence of stimulated cell lysates (300 mM Sorbitol) or serum starved lysates. A clear increase in the rate of phosphorylation of the MK2 sensor is observed in the presence of stimulated lysates. (**B**) The average reaction slopes of triplicate assays with the MK2 biosensor in the presence of the indicated lysates.

Table 1

Troubleshooting Guide for CSox-Based Activity Sensors

Problem	Cause	Solution
Initial sequences do not produce desirable kinetic parameters	The position of CSox may be negatively effecting enzyme recognition and catalysis	Redesign sensor constructs incorporating CSox at the (+)3 or (−)3 position relative to the site of phosphorylation
A small (<2-fold) change in fluorescence is observed between the substrate and product peptide	Acidic residues in the peptide sequence can increase the affinity of the substrate peptide for Mg^{2+}	Choose an alternative substrate with fewer acidic residues Modulate the $[Mg^{2+}]$ to bias binding to the phosphorylated peptide (Support Protocol 1)
Little or no difference in phosphorylation of the sensor is observed in stimulated versus non-stimulated lysates	Buffer components, such as phosphatase and protease inhibitors, may have expired	Confirm the presence of full-length, phosphorylated kinase using an alternative method, such as phosphor-specific western blotting
	The sensor may be phosphorylated by off-target enzymes	Reduce the concentration of the CSox-based activity probe
		Employ broad specificity inhibitors (Davis et al., 2011) to reduce assay background
		Redesign the substrate sequence to reduce off-target phosphorylation (Gonzalez-Vera et al., 2009)
	Large amounts of lysis buffer in the assay may reduce the activity of the KOI	Perform assays with the recombinant KOI and varying amounts of lysis buffer; adjust lysate assays accordingly