AssayQuant®
TECHNOLOGIES INC.

PhosphoSens®

ASSAY DEVELOPMENT GUIDE

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1. INTRODUCTION

Protein kinases play pivotal roles in cellular signaling and regulate many essential biological processes, including cell growth, differentiation, metabolism, proliferation, and programmed cell death. The human genome encodes for 518 protein kinases, making this one of the largest enzyme families. Many human diseases are now known to be associated with dysregulated kinase activity. The ability to accurately and precisely quantify kinase activity and define the effects of compounds that modulate this activity is essential for understanding the complex biology of protein kinases and for the development and monitoring of effective therapeutic agents against these enzymes (1-4). Precise kinase activity measurements are also critical for understanding the effects of kinase mutations on enzyme function to understand the relationships between mutations, signal transduction and cellular function (5,6).

The PhosphoSens® kinase assay platform provides a simple, one-step homogeneous, fluorescence-based assay for rapid and sensitive detection of serine/threonine and tyrosine kinase activities. PhosphoSens assays directly measure the catalytic activity of target kinases, without the need for antibody reagents, coupling steps or radioisotope labeling to develop the signal. The PhosphoSens technology uses optimized peptide substrates that provide robust assays with minimal lot-to-lot variation and high accuracy and precision. PhosphoSens assays are easily performed in continuous kinetic mode, using commonly available fluorescence plate readers in 96-, 384- and 1536-well plate formats and a wide range of sample types including recombinant enzymes, immunoprecipitated kinases, crude cell or tissue lysates (7-9) and innovative microfluidic applications extending down to single-cell analysis (10-12). Using *PhosphoSens*, kinase activity is measured under optimal conditions including pH, selected metal ion cofactors, and low to physiological (mM) ATP concentrations allowing both ATP competitive and ATP non-competitive (allosteric) kinase inhibitors to be selected and characterized. The technology is supported by >90 scientific publications with demonstrated utility across applications for studying kinase activity regulation, kinase inhibitor screening, and inhibitor mechanism of action analysis including K_i, k_{inact}, residence times and IC_{50} determinations (13,14).

2. *PHOSPHOSENS*® ASSAY PRINCIPLE

The Chelation-Enhanced Fluorescence (ChEF) method for protein kinase sensing, filed under the *PhosphoSens* trademark, was first introduced by Imperiali and coworkers (15-17). Since that time, the ChEF method has been rigorously validated and further developed (8,9,18-21) for optimum performance with a wide variety of protein kinases. The ChEF sensing mechanism exploits a synthetic α-amino acid with a side chain bearing an 8-hydroxyquinoline derivative (sulfonamido-oxine, Sox) which upon coordination to Mg(II), relays information on the phosphorylation state of proximal serine, threonine or tyrosine residues in peptide- and protein-based kinase substrates (Figure 1). In the absence of phosphorylation, the Sox shows low affinity for Mg(II); upon phosphorylation, Mg(II) affinity is enhanced due to the advantageous chelate effect, involving the Sox and the introduced phosphate group, and fluorescence is turned on.

FIGURE 1: ChEF Mechanism for Direct Protein Kinase Activity Sensing

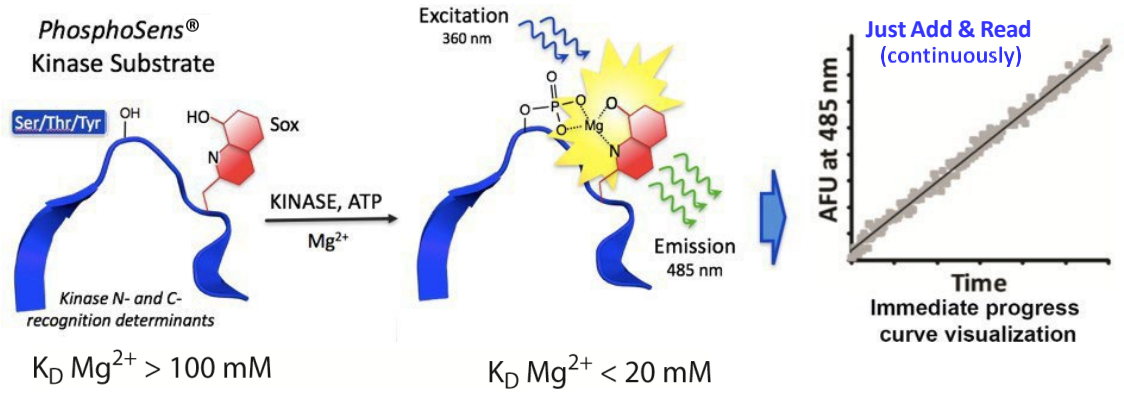
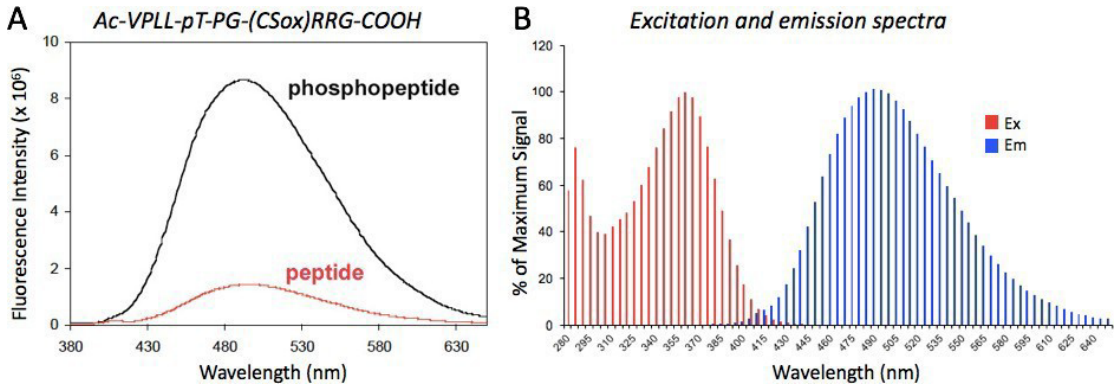


Figure 2A illustrates typical fluorescence changes upon phosphorylation of a *PhosphoSens* peptide substrate and Figure 2B shows excitation and emission spectra of a typical phosphorylated *PhosphoSens* peptide. The fluorescence properties of the Mg(II)-coordinated with the 8-hydroxyquinoline of Sox has an λExMax of ~360 nm (358-363 nm) and λEmMax of ~492 nm (485-498 nm). Since the fluorescence emission spectrum is relatively broad (see Figure 2B), fluorescence emission can be monitored between 475-508 nm with <7% loss in signal intensity.

FIGURE 2: Fluorescence Spectra of *PhosphoSens* Peptides



3. *PHOSPHOSENS* ASSAYS COVERED BY THIS INSTRUCTION MANUAL

This manual can be used as a guide for serine/threonine and tyrosine kinase assays using *PhosphoSens* or *PhosphoSens*-Lysate assay kits and stand-alone CSox peptide substrates (available as 96- or 240 assays for 96- and 384-well plates, respectively, and bulk net peptide). All kits include reaction buffer, enzyme dilution buffer, ATP, DTT and the CSox peptide substrate. With stand-alone CSox substrates, the user must provide their own stocks of buffers and other required components. All *PhosphoSens* assays are compatible with any purified recombinant protein kinase and with biological samples if the user qualifies the experimental system (see Section 4). *PhosphoSens*-Lysate assay kits contain CSox- substrates that have been optimized for the analysis of target protein kinases in unfractionated cell and tissue lysates. For a complete listing of available CSox-based kinase substrates, as configured in kits or as stand-alone substrates, please refer to our website or contact AssayQuant Technologies (hello@assayquant.com) for additional information, including

custom services for compound testing or the development of *PhosphoSens* peptide substrates and assays for new targets of interest.

The activity of protein phosphatases, either serine/threonine and tyrosine-directed, can also be measured effectively with the *PhosphoSens* platform by using CSox-based phosphopeptide substrates. This approach provides a physiologically relevant, homogeneous, continuous and highly-sensitive measure of protein phosphatase activity that is described in the *PhosphoSens* Protein Phosphatase Assay Instruction Manual. In addition to the continuous (kinetic) format, the activity of protein kinases and phosphatases can be measured in an endpoint format using Europium (III)-coordinated with the 8- hydroxyquinoline of Sox to produce a red-shifted signal with λExMax of ~360 nm (358-363 nm) and λEmMax of ~620 nm (610-630 nm) that is measured using time-resolved fluorescence with a 100-150 µsec delay and a 300-500 µsec data acquisition time, thereby eliminating any interference due to compound autofluorescence. The Europium (III) format is described in the *PhosphoSens*-Red Assay Instruction Manual (Appendix A).

4. ASSAY KIT COMPONENTS AND REQUIRED MATERIALS

4.1. MATERIALS AND EQUIPMENT NOT INCLUDED

4.1.1. Recombinant kinase: The *PhosphoSens* products are compatible with any commercially available protein kinase for which a Sox-based substrate has been developed. Kinases providers include, but are not limited to, BPS Bioscience, Carna Biosciences, EMD-Merck/Millipore, ProQinase, Proteros, SignalChem, and Thermo Fisher/Invitrogen). When choosing a commercially available kinase preparation, an assessment of purity, specific activity (in the supplier's assay format), the nature and location of co- expression and purification tags (e.g., N- or C-terminal GST, His, or FLAG tags), and the size of the construct (full-length or truncated) should be considered. The most rigorous approach is to obtain a kinase from multiple sources and compare the activity with the *PhosphoSens* platform, where the kinetic format allows a quantitative measurement allowing the most appropriate enzyme to be selected for further study.

4.1.2. Cell and tissue lysate samples: Any *PhosphoSens* peptide substrate can be used with biological samples if the user qualifies the experimental system. For example, the generic serine/threonine or tyrosine substrates from AssayQuant can be used to measure total serine/threonine or tyrosine kinase activity in crude cell and tissue lysates or enriched fractions. Individual substrates can be qualified for use with crude lysates from appropriately stimulated cell systems. For example, Li and coworkers (7) used the Omnia® Y7 substrate for highly selective detection of the Syk tyrosine kinase in crude lysates of IgM- stimulated Ramos cells. If required, these experiments can include off-target kinase inhibitors to ensure specificity of the assay (9). Alternatively, the target kinase can be immunoprecipitated from crude lysates prior to performing an IP-kinase assay with the *PhosphoSens* technology; For examples refer to Li (7) for control experiments and plate-IP methods for the Syk tyrosine kinase, Lauchle (22) for MK2, and Chen (23) for GSK3b. Sox-based bipeptide or protein sensors, including both docking and sensing sequences, have been used to specifically and quantitatively measure the kinase activity of individual MAPK family members in crude cell and tissue lysates (9,19,20). In each of the above scenarios, the continuous-read mode of the *PhosphoSens* technology provides more information about the activation state of the isolated kinase or kinase complex in the presence of other cellular proteins. Indeed, there is considerable interest in using these more complex systems for drug development to obtain more accurate rank order of kinase inhibitor potency and for Structure-Activity Relationship (SAR) studies (Li et al., 2009). The highly quantitative and precise measure of kinase activity provided

by the *PhosphoSens* technology is an important improvement over conventional western blot analysis with phosphoprotein-specific antibodies, which is at best only semi-quantitative (1,2). Sox-based substrates, either single chain or bi- peptide formats, with improved specificity for the targets of interest will be the focus of *PhosphoSens*- Lysate sensors from AssayQuant Technologies (please inquire at hello@assayquant.com).

4.1.3. Fluorescence microplate reader: Instrument must be capable of reading fluorescence intensity in continuous (kinetic) mode with an excitation wavelength (λ_{ExMax}) of ~360 nm (358-363 nm) and an emission wavelength (λ_{EmMax}) of ~492 nm (485-498 nm). Readings can be made at the desired intervals and duration (e.g., reading every 30 seconds for 60 minutes or every 3 minutes for 150 minutes). *PhosphoSens* assays can be run on readily available microplate reader instruments including but not limited to the Tecan Safire 2™, Infinite® M1000, Infinite® F500, Molecular Device SpectraMax® M5, BMG LABTECH PHERAstar, FLUOstar OPTIMA, BioTek FLx800™, Neo, Synergy™ 2, and Synergy™ 4, and ThermoFisher Varioskan. Contact Technical Support or e-mail us directly at info@assayquant.com for instrument-specific setup guidelines.

4.1.4. Precision pipettes: Precision pipettes with disposable plastic tips to accurately deliver 2–200 µL.

4.1.5. Ultrapure deionized water: 18 Ω or higher.

4.1.6. Plastic tubes or plates: Plastic with low protein-binding properties is used for diluting and dispensing assay components. We recommend USA Scientific (1615-5500) 1.5 ml polypropylene micro- centrifuge tubes (DNase, RNase, DNA, and pyrogen free). For larger volumes, use polypropylene and not polystyrene tubes. A low protein-binding and v-bottom dilution plate is available from Greiner (651201).

4.1.7. Heat Block or Water Bath: Set at 30°C, if this is the temperature at which reactions are to be run.

4.1.8. Microtiter plates: White Microtiter plates should be used to minimize light scattering and background fluorescence and to reduce well-to-well crosstalk. These plates come in multiple configurations, where improved signal and signal/background are obtained with the white plastic no- protein binding (NBS) plates. Excellent performance has been obtained with:

Corning, half-area 96-well, white flat bottom polystyrene NBS microplates (3642)
Corning, low-volume 384-well, white flat bottom polystyrene NBS microplates (3824)
Corning, 1536-well, white flat bottom polystyrene NBS microplates (3729)

With the *PhosphoSens* Platform, we recommend using a 50 µL final reaction volume per well in a half- area 96-well plate *or* a 25 µL final reaction volume in a low-volume 384-well plate.

PhosphoSens technology also can be used with 1536-well plates, where established assays have been successfully scaled down to 5 µL for high-throughput applications. With all plate formats, the final reaction volume determines the path length, where larger volumes provide higher signals and subsequently greater assay precision. As a guide, Figure 4 illustrates the effect of assay volume on assay precision (% CVs).

4.1.9. Adhesive Seal for Microtiter Plates: To control for evaporation, especially with long kinetic reads or under low humidity conditions, plates should be sealed with an optically-clear adhesive film that still allows top-reading with minimal light scattering. We have tested many products and

recommend Perkin Elmer TopSeal-A Plus (6050185) applied with either a roller or a paddle (VWR 60941-118 or 60941-128, respectively).

4.2. MATERIALS INCLUDED

Each *PhosphoSens* kit provides the reagents listed in Table 1 allowing the user to perform 96 kinase reactions at 25 µL final reaction volume in a low-volume 384-well plate. All components are available from AssayQuant Technologies as stand-alone items that can be ordered using the Catalog numbers provided below. Components can also be ordered in bulk (please inquire at hello@assayquant.com). Bulk *PhosphoSens* Sensor Peptide is provided as lyophilized powder as mgs of net CSx-peptide (please inquire at hello@assayquant.com for bulk pricing discounts).

TABLE 1: Components provided with each *PhosphoSens*-Kinetic Kinase Assay Kit:

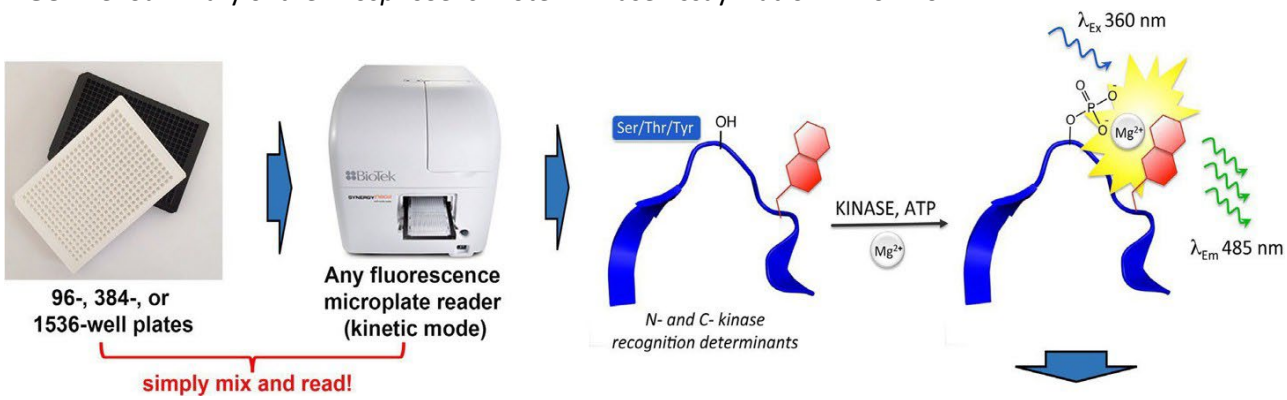
COMPONENT	DESCRIPTION	AMT	STORAGE
PhosphoSens Substrate, 1 mM	*PhosphoSens* Cysteine-Sox Kinase Sensor AQTxxxx peptide substrate, 1 mM	35 µL	-20 °C or below. Minimize repeated freeze/thaw cycles
ATP solution, 100X	100 mM ATP in nuclease-free water	150 µL	-20 °C or below. Minimize repeated freeze/thaw cycles
DTT solution, 1000X	1M DTT in nuclease-free water	150 µL	-20 °C or below. Minimize repeated freeze/thaw cycles
EGTA Solution, 1000X	550 mM Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)	150 µL	-20 °C or below.
Enzyme Reaction Buffer, 10X	500 mM HEPES, pH 7.5, 0.1% Brij-35, 100 mM $MgCl_2$	1,650 µL	-20 °C or below.
Enzyme dilution buffer, 5X	20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 1 mg/mL Bovine Serum Albumin	1,650 µL	-20 °C or below.

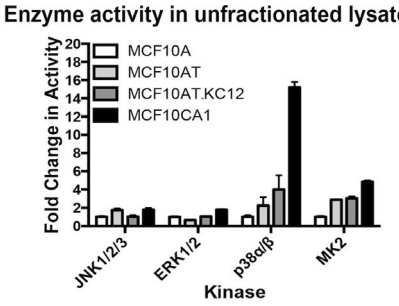
5. PERFORMING A *PHOSPHOSENS* KINETIC ASSAY

The *PhosphoSens* assay format is SIMPLE! As a one-step homogeneous assay, you just MIX and READ. There are NO washing or separation steps and NO stop solutions or additional components needed to develop the fluorescence signal. The assay reaction is initiated typically by addition of a Master Mix containing either a protein kinase or the CSox-peptide substrate to a well containing the final components needed to start the reaction. This can be done using 96-, 384- or 1536 well plate formats. Assays are commonly performed at 30 °C and fluorescence measurements are recorded in kinetic mode (e.g., readings every 30 seconds for 30-60 minutes or every 3 minutes for 150 minutes).

The λExMax of the chelated Mg(II) with the 8- hydroxyquinoline is 360 nm and the λEmMax is ~492 nm (485-498 nm). An overview of the *PhosphoSens* Platform Workflow is shown in Figure 3.

FIGURE 3: Summary of the *PhosphoSens* Protein Kinase Assay Platform Workflow





Enzyme kinetics
K_m, V_{max}, k_{cat}, IC_{50}, K_i, residence times etc. with recombinant enzymes

Fluorescence increase	[Km (µM)]	V_{max} (µmol.mg^{-1}.min^{-1})
3.5 - fold	0.01	1.8
3.9 - fold	0.69	2.5
4.4 - fold	1.2	1.3

An endpoint assay can also be performed using Europium (III) to produce a red-shifted signal with λExMax of ~360 nm (358-363 nm) and λEmMax of ~620 nm (610-630 nm) that is measured using time-resolved fluorescence with a 100-150 µsec delay and a 300-500 µsec data acquisition time, thereby eliminating any interference due to compound autofluorescence. The Europium (III) format is described in Appendix A.

5.1. PREPARING ASSAY REAGENTS

Prior to setting up the individual reactions, prepare the following solutions:

5.1.1. *PhosphoSens* Sox-based Substrate: The concentration of the peptide stock solution has been accurately determined by absorption spectroscopy using the extinction coefficient of sulfonamido-oxine (Sox) at 355 nm prior to being lyophilized. With *PhosphoSens* Kits, the substrate is provided as a 1 mM (100X) solution. With *PhosphoSens* Bulk peptide, this is supplied as a lyophilized powder. Resuspend the peptide substrate as indicated on the vial or in the Technical Notes section of the Certificate of Analysis to create the 1 mM (100X) stock. Gently vortex to ensure that all the substrate goes into solution. *Caution:* Avoid repeated freeze/thaw cycles by aliquoting the 1 mM stock solution into smaller volumes for storage at -20 °C or below until ready for use.

5.1.2. Peptide Substrate Solution (10X): Prepare 0.1 mM (10X) Substrate solution by thawing the 1 mM (100X) peptide substrate stock solution, mixing well by vortexing gently, removing an appropriate amount and diluting 10-fold into ultrapure deionized water. To use the entire kit, add 333 µL of water to the 37 uL of 1 mM substrate provided with the kit to make 370 µL (this is sufficient for ~148 x 25 µL reactions for low-volume 384-well plates, all at 10 µM final substrate). Gently invert tube to mix and perform a 10 second spin in a micro-centrifuge. Alternatively, only make up as as much as is needed for the current experiment.

5.1.3. ATP Solution (10X): To run final reactions at 1 mM ATP, prepare 10 mM (10X) ATP solution by adding 50 µL of 100 mM (100X) ATP to 450 µL ultrapure deionized water. Make fresh and discard after use. Note: The 100X stock should be aliquoted and stored at -20 °C or below. Minimize repeated freeze/thaw cycles.

5.1.4. DTT Solutions: Most kinase assays are run in the presence of DTT, although some protein kinases are more active in the absence of DTT, which must be determined empirically. Prepare 100 mM (100X) DTT solution by adding 5 µL of the 1 M (1000X) DTT provided to 45 µL ultrapure deionized water and use to prepare final EDB in **5.1.6** below. Prepare 10 mM (10X) DTT solution by adding 5 µL of the 1 M (1000X) DTT to 495 µL ultrapure deionized water and use to prepare final reactions per Tables 2-4. ***Caution:*** Diluted DTT is readily oxidized so use fresh dilutions.

5.1.5. EGTA Solutions: Some kinases show enhanced activity when EGTA is included in the reaction, which must be determined empirically (e.g., the activity of GRK2 is increased by 50% in the presence of 0.55 mM EGTA). Including EGTA will also chelate any Zn^{2+}, which can be present as a trace metal contaminant that can negatively affect assay performance. For these reasons, and with the exception of working with kinases activated by Ca^{2+} (EGTA can't be included because it chelates Ca^{2+}), we recommend that EGTA be added to reactions (0.55 mM final) and the EDB (0.1 mM final). We include a 0.55 M stock of EGTA in each *PhosphoSens* kit for this purpose. Prepare 20 mM EGTA solution by adding 18.2 µL of 0.55 M EGTA to 481.8 µL ultrapure deionized water and use to prepare final EDB in **5.1.6** below. Prepare 5.5 mM EGTA solution by adding 5 µL of 0.55 M EGTA to 495 µL ultrapure deionized water and use for final reactions per Tables 2-4. All data in this manual were generated using EGTA in the final reaction.

5.1.6. Enzyme Dilution Buffer (EDB, 5X): EDB is added to Blank or Background wells as a "No Enzyme" control.

5.1.7. Kinase Reaction Master Mix: Prepare as listed in Tables 2 through 4.

5.1.8. Kinase Stock: Kinases are enzymes and therefore must be maintained on ice. Following steps in Tables 2 through 4. Just before use, dilute an appropriate amount of the kinase stock to 5X (10 nM if the final desired concentration is 2 nM) in EDB (with or without DTT, depending on the enzyme) and follow steps outlined in Tables 2 through 4. The required volume of 5X kinase in EDB, then is added to assay wells to initiate the reaction (this can be done using automated reagent dispensing if timing is critical). Discard the unused portion of the diluted kinase. Aliquot the remaining undiluted kinase into individual low-protein-binding storage vials and return to the -80 °C freezer, however, indicate that these stocks have been thawed. A limited number of freeze-thaw cycles may be acceptable; however, multiple freeze- thaw cycles are likely to compromise activity.

5.1.9. Final Reaction Conditions: Typical final concentrations of each reaction component are as follows: 54 mM HEPES, pH 7.5, 1 mM ATP (or as adjusted as needed), 1.2 mM DTT (optional), 0.55 mM EGTA (optional, but recommended), 0.012% Brij-35, 10 mM MgCl2, 10 µM peptide substrate, 0.05-5 nM kinase (or adjusted as needed) and any additional co-factors or additives (as required).

5.2. KINASE TITRATION EXPERIMENT

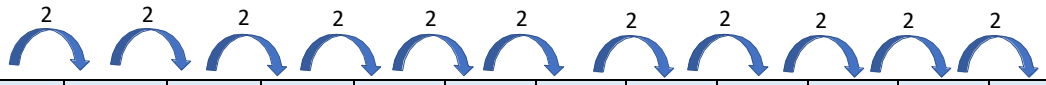
5.2.1. Additional Background: The amount of kinase used in a *PhosphoSens* assay is dependent on the specific activity of the kinase towards the optimized CSox-based substrate and must be determined empirically. Kinases obtained from commercial suppliers can vary widely with respect to their specific activity and stability, which depends on many factors. In general, 0.1-5 nM of kinase in the final reaction is a good starting concentration, however, a kinase titration experiment will allow you to choose the appropriate amount of kinase for your application (most applications are best run under conditions where only 10-15% of the substrate is phosphorylated) and to determine the amount of kinase required to completely phosphorylate the peptide substrate to create a phosphopeptide standard curve. With the latter, the fluorescence signal obtained from the fully phosphorylated peptide, in the presence of Mg(II) and other assay buffer constituents, can be used as the 100% phosphorylation control to determine the % of substrate phosphorylation achieved in an application and to convert RFU/minute to µM/minute for V_{max} and k_{cat} determinations.

5.2.2. Experimental Protocol: The steps and volumes of each component for a kinase titration experiment are outlined in Table 2 for a 96-well half-area plate. A dilution scheme is also provided. You should only plan to dilute as much kinase as you plan to use for experiments to be run that day as kinase samples are generally less stable upon dilution. Note: Protocol Tables and dilution schemes for all the experimental examples provided in this manual are available upon request for 96- and 384-well (low- volume) plate formats (please inquire at hello@assayquant.com).

TABLE 2: PROTOCOL 1 – KINASE TITRATION (50 µL assay for a half-area 96-well plate)

STEP	PROCESS			
1	Prepare Master Mix (all components except Active Kinase) by combining each of the components listed to the right. With certain kinases, additional components may be needed (e.g., cofactors, glycerol, etc.) and so the amount of water should be adjusted.	Reaction Buffer (10X) *PhosphoSens* Substrate (10X) ATP solution (10X) DTT solution (10X) EGTA solution (5.5 mM or 10X) Ultrapure deionized H2O Total volume	**For 1 RXN:** 5 µL 5 µL 5 µL 5 µL 5 µL 15 µL 40 µL	**For 40 RXNs:** 200 µL 200 µL 200 µL 200 µL 200 µL 600 µL 1.60 mL
2	Equilibrate Master Mix (all components except kinase) to assay temperature (typically 30 °C) by placing the sealed tube in a heat block or water bath set at the desired temperature for 5 minutes.			
3	Equilibrate the assay plate in the plate reader to the desired assay temperature (typically 30 °C).			
4	Aliquot 40 µL of the Master Mix into each well.			
5	Prepare serially diluted kinase as described below.			
6	Add 10 µL of each concentration of 5X kinase to designated wells to start the reaction. Mix well. Add 10 µL of 5X Enzyme Dilution Buffer (EDB) to "No-kinase" control wells.			
7	Incubate at 30 °C collecting fluorescence intensity (RFU) readings (λExMax 360 nm/λEmMax ~492 nm [485- 498 nm]) at intervals (e.g., every 30 seconds for 60 minutes or every 3 minutes for 150 minutes, etc.).			

Kinase titration instructions for Table 2: As outlined below, prepare an 11-point, 2-fold serial dilution of kinase using 5X EDB. Each dilution in the series should be at 5X the final concentration of kinase in the reaction. This dilution scheme will result in a concentration range that spans 3 log units (e.g., from 20 nM to 20 pM). To generate a titration curve that starts at 20 nM, the first 5X stock needs to be 100 nM. The 12th well in a 96-well plate row is designated as a 'No-kinase control'. Replicates (typically in triplicate) are oriented vertically in the plate. The EDB and the protein kinase stocks have glycerol, so make sure all samples are well mixed.



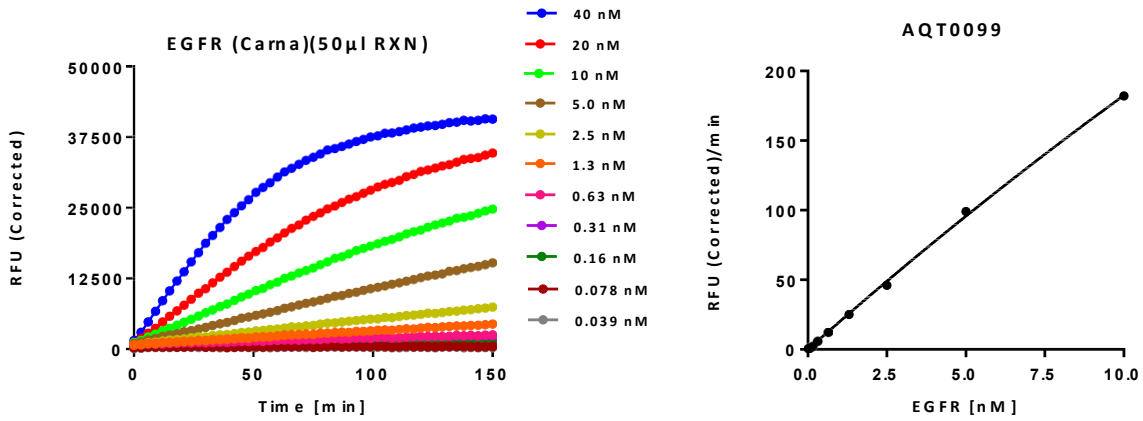
Tube #	1	2	3	4	5	6	7	8	9	10	11	12
Final [Kinase], nM	20.00	10.00	5.00	2.50	1.25	0.63	0.31	0.16	0.08	0.04	0.02	None
5X [Kinase], nM	100.00	50.00	25.00	12.50	6.25	3.13	1.56	0.78	0.39	0.20	0.10	None
Enzyme Dilution Buffer, µL	X µL as Supplied	38 µL	38 µL	38 µL	38 µL	38 µL	38 µL	38 µL	38 µL	38 µL	38 µL	76 µL
Stock Kinase	Y µL	38 µL of Tube #1	38 µL of Tube #2	38 µL of Tube #3	38 µL of Tube #4	38 µL of Tube #5	38 µL of Tube #6	38 µL of Tube #7	38 µL of Tube #8	38 µL of Tube #9	38 µL of Tube #10	None
Final Volume	76 µL	76 µL	76 µL	76 µL	76 µL	76 µL	76 µL	76 µL	76 µL	76 µL	76 µL	76 µL

Commercial preparations will vary significantly in their concentration. The first tube takes into account the required dilution of the suppliers' kinase preparation to create Tube #1 (100 nM stock). For example, if the commercial kinase preparation is provided at 1,000 nM, then add 7.6 µL to 68.4 µL of Kinase Dilution Buffer to create 76 µL of a 10-fold dilution. You need 38 µL to create each 2-fold dilution and 10 µL for each replicate at each concentration (typically triplicate wells, so 30 µL) or 68 µL of the 76 µL total. Tube #12 receives only Enzyme Dilution Buffer to use as a "No-kinase" control. In this example, with 11 kinase concentrations plus 1 control (12 conditions), each in triplicate = 36 wells (or 38% of a 96-well kit).

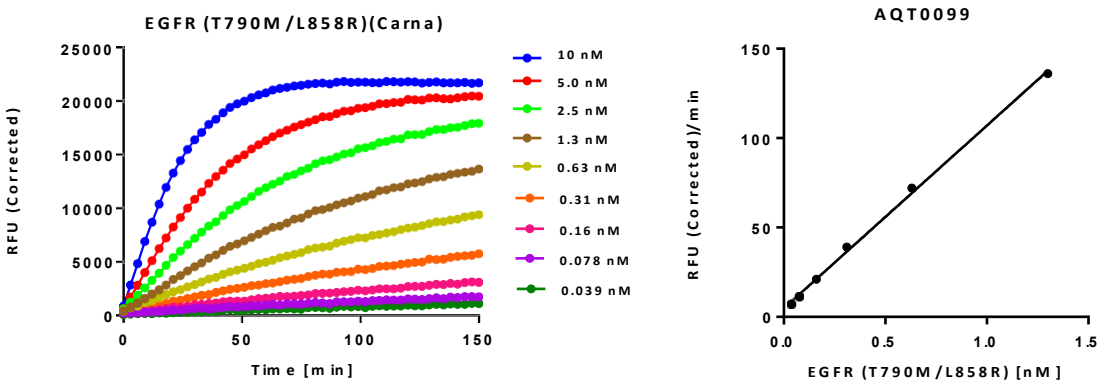
5.2.3. Data Analysis: Subtract the background fluorescence for each time point from the total fluorescence signal to obtain corrected Relative Fluorescence Units (RFU) values. Plot the corrected RFU vs. Time. Determine the slope of the initial linear portion of each curve, which is the initial reaction rate (RFU/min or convert to RFU/pmole kinase/min). Reaction rates from linear or non-linear (kinases that exhibit a lag phase) fit of the data can be generated using the Microplate instrument software or by exporting the data to another program such as DynaFit, Excel-Fit, GeneData Screener, GraphPad Prism, KinTek, Mathematica, MATLAB, or SigmaPlot.

FIGURE 4: Representative data from a kinase-titration experiment using the *PhosphoSens* Assay. A. Kinase enzyme titration for the wild-type EGFR and B. EGFR [T790M/L858R] tyrosine kinases. Kinase proteins (from Carna Biosciences) were incubated with the AQT0099 peptide substrate at 30°C for 150 minutes. RFU data were collected every 3 minutes and plotted against time. Each line represents a different kinase concentration (nM) as indicated in the legend. The data demonstrate linearity across a range of kinase concentrations from 10 nM down to 160 pM for EGFR and from 1.3 nM down to 39 pM for EGFR [T790M/L858R]. Any kinase concentration that provides linear signals with time and results in <10% of the substrate being phosphorylated can be used for subsequent experiments. C. Effect of assay volume on precision for EGFR. All incubations were performed using Corning half-area 96 well white NBS plates with experimental conditions as described above. The data demonstrate the increase in Z' values and sensitivity with increasing assay volume. AssayQuant recommends a standard final reaction volume of 50 µL in these plates, however, the final choice is based on assay requirements (i.e., can increase the volume if lower enzyme concentrations are needed).

A.



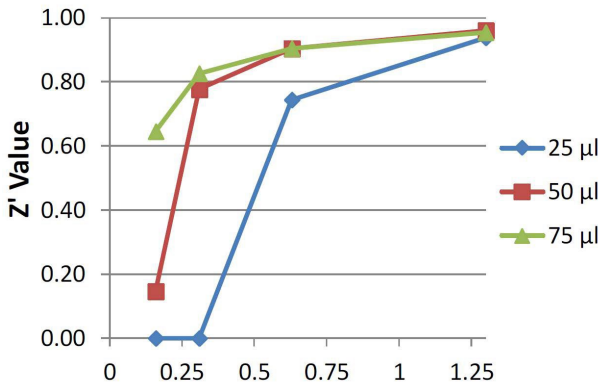
B.



C.

Z' Values for Different Final Reaction Volumes and EGFR concentrations

Conc (nM)	25 µl	50 µl	75 µl
0.039	N/A	N/A	N/A
0.078	N/A	N/A	0.47
0.16	N/A	0.15	0.65
0.31	N/A	**0.78**	**0.83**
0.63	0.74	0.90	0.90
1.3	**0.94**	0.96	0.95
2.5	0.97	0.97	0.98
5	0.97	0.98	0.98
10	0.97	0.98	0.97
20	0.96	0.98	0.96
40	0.97	0.96	0.98



Bolded Z' values corresponding to LLOQ
(lowest concentration where CV < 20%)

5.3. KINASE KINETIC PARAMETER ANALYSIS

5.3.1. Additional Background: Recombinant kinase preparations vary significantly with respect to the construct size (full-length or truncated), the nature and location of purification tags (e.g., N- or C- terminal GST, His, or FLAG tags), the presence of co-factors/activators, and the kinase activation state.

These variables can contribute to dramatic differences in kinase activity. The *PhosphoSens* assay can be used to determine the K_m (Michaelis constant) and Vmax values for any kinase preparation. With the continuous read of the *PhosphoSens* Technology, these determinations are accomplished with much less effort and with improved accuracy and precision since the chemistry of the sensor and the instrument do all the work (you can be planning the next experiment based on seeing results in real time on the screen).

To determine K_m, a fixed concentration of kinase (selected as described in Section 5.2) is combined with a serial dilution of a CSox-based peptide substrate. K_m determinations require a range of substrate concentrations, ideally from 0.1-10-fold of the estimated K_m. Note: Some substrates have high reaction rates but also high K_m values and therefore using a 10-fold higher substrate concentration, relative to the estimated K_m, is not practical. Therefore, the highest substrate concentration to achieve or approach saturation should be applied.

Many customers will only require the determination of Km and the relative turnover of substrates based on RFU readouts. V_{max} values in µmol.mg-1.min-1 can be determined as described below and presented in detail in Lukovic et al. (18).

To determine V_{max}, from the initial rates of product formation, a correction for the decrease in fluorescence intensity due to the substrate consumption is required.

The fluorescence intensity at any given time in the reaction is determined from the following equation:

(1)

$$I(t) = f_S S(t) + f_P P(t)$$

where *I(t)* is the fluorescence intensity, *S(t)* is the amount of substrate in µM, *P(t)* is the amount of product in µM, *fS* is the fluorescence intensity per µM of substrate, and *fP* is fluorescence intensity per µM of product.

The amount of substrate and product at any given point are related by:

(2)

$$S(t) + P(t) = S_0$$

where *S0* is the initial amount of substrate.
Substitution of eq. (2) into eq. (1) followed by rearrangement yields:

(3)

$$P(t) = \frac{I(t) - f_S S_0}{f_P - f_S}$$

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The initial velocity of the reaction is the change in the amount of product over time, so taking the derivative of eq. (3) with respect to time gives:

(4)

$$v = \frac{dP(t)}{dt} = \frac{\frac{dI(t)}{dt}}{f_P - f_S}$$

The initial slope of the reaction, dI(t)/dt, should be measured within the first 10% of substrate turnover to ensure initial rate analysis. The constants f_P and f_S are calculated from the standard cures of RFU versus concentration of P and S, respectively. These values will depend on the concentration of Mg^{2+} and the Mg^{2+} dissociation constant of each peptide and can be determined empirically under the desired assay conditions. The K_m and V_{max} are determined from a direct, non-linear fit of v *vs.* [S] plots using the Briggs-Haldane equation:

(5)

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

NOTE: A phosphopeptide standard curve to convert the RFU signal into an absolute product concentration is necessary for this analysis. As required, the user can request a custom synthesis of the target phosphopeptide standard corresponding to the CSox peptide substrate in which they are interested (please inquire at hello@assayquant.com). Alternatively, the phosphopeptide can be prepared via kinase-mediated phosphorylation, which generally provides suitable samples for standard curve analysis.

The phosphopeptide standard curve based on enzymatic synthesis of the product is generated by combining a fixed concentration of kinase with a serial dilution of CSox peptide substrate at the same concentrations used in the kinetic assay. Typically, the amount of kinase used to generate the phosphopeptide standard curve is higher than the concentration used in the kinetic analysis to ensure complete phosphorylation of the peptide substrate. The kinase concentration required to reach signal saturation will vary between kinases. A titration of kinase (as outlined in Section 5.2) can be performed with the highest concentration of peptide substrate to determine the amount of kinase required to achieve complete phosphorylation.

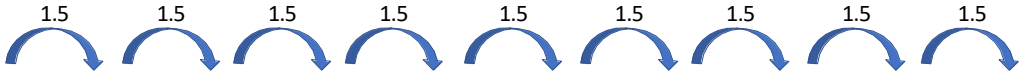
5.3.2. Experimental Protocol: In this experiment, it is best to first do the serial dilutions of the substrate (see below) so that these dilutions are ready to add at the appropriate time as outlined in Table 3.

TABLE 3: PROTOCOL 2 - K_m and V_{max} - Determination (50 µL assay for a half-area 96-well plate)

STEP	PROCESS			
1	Prepare Master Mix for **Peptide Substrate Control ('No Kinase')** by combining the components listed to the right. Include other components as required.	Enzyme Dilution Buffer (EDB, 5X) Reaction Buffer (10X) ATP solution (10X) DTT solution (10X) Ultrapure deionized H_2O Total volume	**For 1 RXN:** 10 µL 5 µL 5 µL 5 µL 15 µL 40 µL	**For 100 RXNs:** 1000 µL 500 µL 500 µL 500 µL 1500 µL 4.0 mL
2	Prepare Master Mix for **Phosphopeptide Standard Curve** by combining each of the components listed to the right. Include other components as required.	Reaction Buffer (10X) Active Kinase (5X, in 5X Enzyme Dilution Buffer, where 1X concentration completely phosphorylates the highest peptide concentration) ATP solution (10X) DTT solution (10X) Ultrapure deionized H_2O Total volume	5 µL 10 µL 5 µL 5 µL 15 µL 40 µL	500 µL 1000 µL 500 µL 500 µL 1500 µL 4.0 mL
3	Prepare Master Mix for **Kinetic Reactions** by combining each of the components listed to the right. Include other components as required.	Reaction Buffer (10X) Active Kinase (5X, in 5X Enzyme Dilution Buffer, where 1X concentration results in linear rate & <15% phosphorylation) ATP solution (10X) DTT solution (10X) Ultrapure deionized H_2O Total volume	5 µL 10 µL 5 µL 5 µL 15 µL 40 µL	500 µL 1000 µL 500 µL 500 µL 1500 µL 4.0 mL
4	Equilibrate Master Mixes to assay temperature (typically 30 °C) by placing the sealed tube in a heat block or water bath set at the desired temperature for 5 minutes.			
5	Equilibrate assay plate in the plate reader to the desired assay temperature (typically 30 °C).			
6	Prepare serial dilutions of the *PhosphoSens* peptide substrate as outline below. Aliquot 10 µL of each serial dilution concentration to three sets of wells (i.e., **i.** Serial dilution for peptide control; **ii.** Phosphopeptide generation reaction; **iii.** Kinetic reaction).			
7	Aliquot 40 µL of the Peptide Control Master Mix into one of the peptide serial dilution sets (i), then aliquot 40 µL of the Phosphopeptide Generation Master Mix into another peptide serial dilution set (ii) and finally aliquot 40 µL of the Kinase Reaction Master Mix into the final peptide serial dilution set (iii). Mix well.			
8	Incubate at 30 °C collecting fluorescence intensity (RFU) readings (λ_{ExMax} 360 nm/λ_{EmMax} ~492 nm [485-498 nm]) at defined intervals (e.g., every 30 seconds for 60 minutes or every 3 minutes for 150 minutes, etc.).			

Substrate titration instructions for Table 4: As outlined below, prepare a 10-point, 1.5-fold serial dilution of substrate using ultrapure deionized water. Each dilution in the series should be at 5X the final concentration of substrate in the reaction. This dilution scheme will result in a concentration range of 38- fold (e.g., from 100 µM to 2.6 µM). To generate a titration curve that starts at 100 µM, your first 5X stock needs to be 500 µM. Replicates (typically in triplicate) are oriented vertically in the plate.

Substrate Titration
96-well plate serial 1.5-fold dilutions:



Tube #	1	2	3	4	5	6	7	8	9	10
Final [Substrate], µM	100.00	66.67	44.44	29.63	19.75	13.17	8.78	5.85	3.90	2.60
5X [Substrate], µM	500.00	333.33	222.22	148.15	98.77	65.84	43.90	29.26	19.51	13.01
Ultrapure Water, µL	150 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
Stock Substrate, 1 mM	150 µL of 1 mM	200 µL of Tube #1	200 µL of Tube #2	200 µL of Tube #3	200 µL of Tube #4	200 µL of Tube #5	200 µL of Tube #6	200 µL of Tube #7	200 µL of Tube #8	200 µL of Tube #9
Final Volume	300 µL	300 µL	300 µL	300 µL	300 µL	300 µL	300 µL	300 µL	300 µL	300 µL

The first tube takes into account the required dilution of the 1 mM Sox-Substrate stock provided in a kit, to create Tube #1 (a 2- fold dilution generates a 500 µM stock). The starting concentration should be adjusted so that you bracket the estimated Km value (in this case, ~16 µM with 5 points above and below to span from 100 µM down to 2.6 µM).

This requires 200 µL to create each 1.5-fold dilution and 10 µL for each replicate well at each concentration x 3 conditions (typically triplicate wells, so 90 µL) or 290 µL out of the 300 µL total. With this example, the 10 substrate concentrations, each in triplicate and 3 conditions = 90 wells (almost 1 kit), however, since the titration starts at 100 µM, it requires 150 µL of the 1 mM stock substrate or 2.5 vials as provided in a *PhosphoSens* kit (CSox substrates are available as standalone and as bulk powder). If the expected Km is lower, the starting concentration can be lower.

5.3.3 K_m and V_{max} Data Analysis

Step 1. Subtract background
Subtract the RFU value of the peptide control (i.e., no kinase control) from the RFU value of the kinase reaction at each time point. The RFU value that remains represents the signal from phosphorylated peptide. Since the background fluorescence from the peptide control intensifies at increasing concentrations, the RFU value used for the background subtraction should be determined from the control peptide at the same concentration as the peptide in the kinase reaction.

Step 2. Determine reaction velocities (v)
Plot the background subtracted RFU values from Step 1 from the kinase reaction versus time and calculate the initial reaction velocities (slope of line; RFU/second) from the linear portion of curve.

Step 3. Calculate the slope of the phosphopeptide standard curve
Construct the phosphopeptide standard curve as described in Section 5.3 by plotting the saturating RFU values of each standard curve reaction versus the concentration of peptide substrate in the reaction. Calculate the slope of this standard curve (RFU/µM).

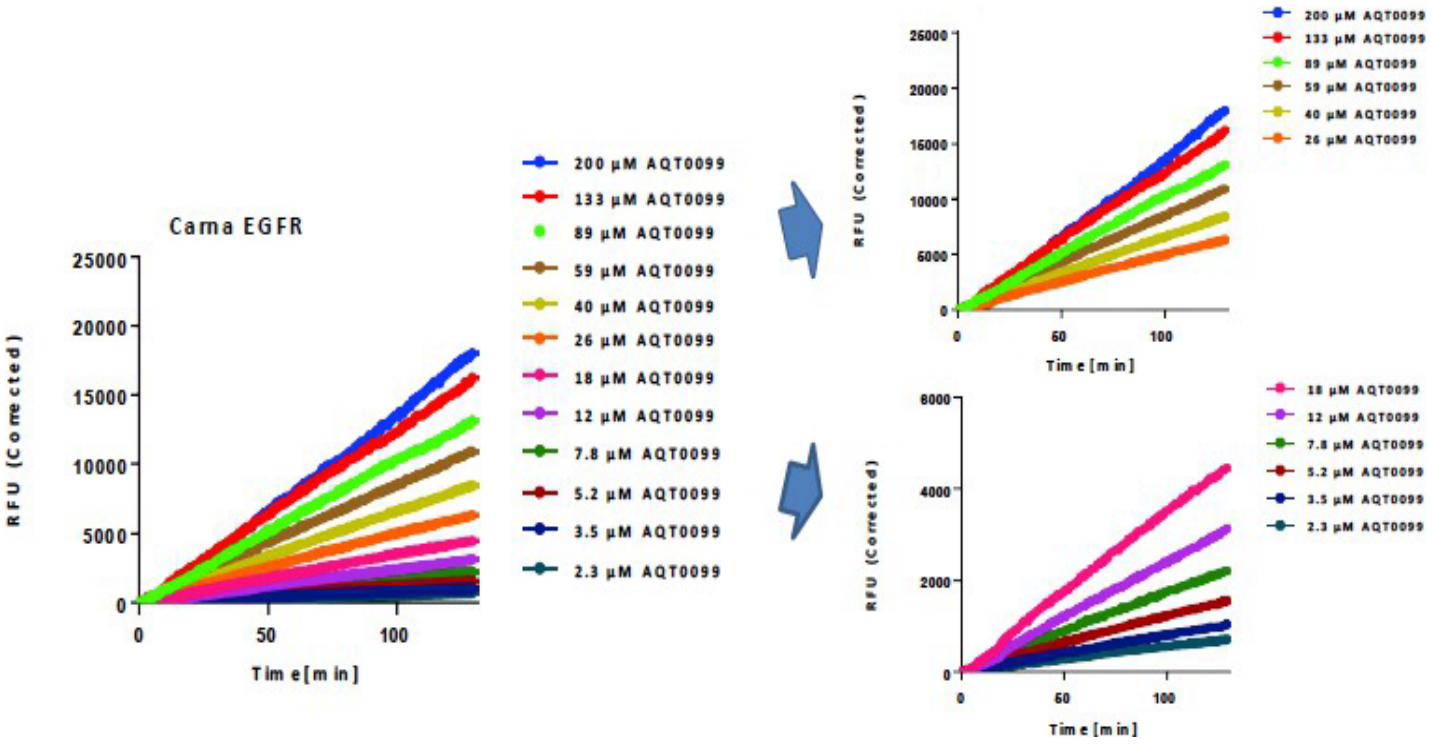
Step 4. Convert reaction velocities to µM/second
Convert the reaction velocities to µM/second by dividing the reaction velocities from Step 2 (RFU/second) by the slope from the phosphopeptide standard curve (RFU/µM) from Step 3.

Step 5. Calculate the Km and Vmax
Using data analysis software, calculate the Km and Vmax. Data can be manually fitted to either Michaelis-Menten or Hanes plots using traditional analyses presented in a standard enzyme kinetics textbook (24). Alternatively, we recommend applying a linear regression analysis, for example using the “Enzyme Kinetics Module" in the SigmaPlot software package (25). Other software packages include: DynaFit (Biokin), GraphPad Prism, KinTek, Mathematica and MATLAB.

FIGURE 5: Representative data from a titration of the *PhosphoSens* peptide substrate CSKS-AQT0099 with the EGFR tyrosine Kinase:

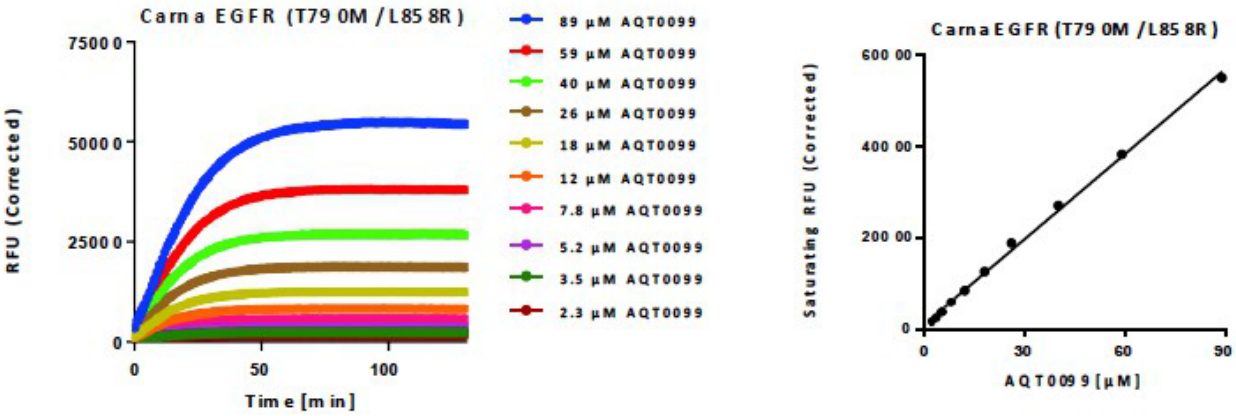
A. Serially diluted substrate was incubated with 8 nM EGFR (Carna Biosciences) for 120 minutes at 30 C to determine reaction velocities (RFU Corrected/min) at each substrate concentration. Serially diluted substrate was also incubated with 1 nM EGFR (T790M/L858R) for 112 minutes at 30 C to determine reaction velocities (RFU Corrected/min) at each substrate concentration (data not shown).
B. Phosphopeptide standard curve: 20 nM EGFR (T790M/L858R) (Carna Biosciences) was used to completely phosphorylate all substrate and determine saturating RFU values at each substrate concentration to generate a phosphopeptide standard curve. The slope for this curve was determined to be 621 ± 11 RFU Corrected/µM.
C. Km and Vmax Determination: Reaction Velocities (µM/min) were determined by dividing the reaction velocities from the kinetic progress curves (RFU Corrected/min) by the slope from the phosphopeptide standard curve (621 ± 11 RFU Corrected/µM). Data were fit with the Michaelis-Menten equation to determine the Km and Vmax for the EGFR and EGFR (T790M/L858R).
    - EGFR: Km (79 ± 1.9 µM) and Vmax (0.32 ± 0.004 µM/min)
    - EGFR (T790M/L858R): Km (92 ± 5.0 µM) and Vmax (0.55 ± 0.017 µM/min).

A.



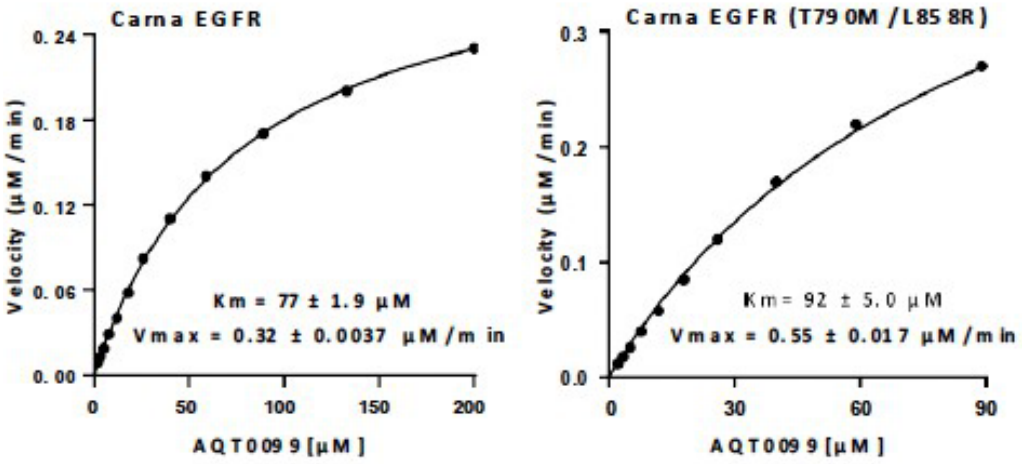
Conc (µM)	Reaction Velocity (Corrected RFU/min)	StDev (Corrected RFU/min)
2.3	5.8	0.030
3.5	8.3	0.043
5.2	12	0.059
7.8	18	0.085
12	25	0.094
18	36	0.13
26	51	0.17
40	67	0.17
59	86	0.21
89	106	0.21
133	127	0.48
200	142	0.40

B.



Conc (µM)	Maximum RFU Corrected
2.3	1,689
3.5	2,436
5.2	3,665
7.8	5,678
12	8,377
18	12,525
26	18,802
40	26,953
59	38,198
89	54,976

C.



Conc (µM)	EGFR Reaction Velocity (µM/min)	EGFR (T790M/L858R) Reaction Velocity (µM/min)
2.3	0.0093	0.0120
3.5	0.013	0.018
5.2	0.019	0.026
7.8	0.029	0.040
12	0.040	0.058
18	0.058	0.085
26	0.082	0.120
40	0.11	0.17
59	0.14	0.22
89	0.17	0.27
133	0.20	0.32
200	0.23	ND

5.4. DETERMINATION OF KINASE INHIBITOR IC_{50} VALUES

5.4.1. Additional Background: Kinase inhibitors are typically organic structures that need to be diluted in 100% DMSO to 100X and then diluted 1:10 in water to generate a 10X stock, from which aliquots are added to each reaction. The dilution scheme provided will result in a concentration range that spans nearly 5 log units (e.g., from 1 µM to 17 pM). To generate a titration curve that starts at 1 µM, your first 100X stock needs to be 100 µM. The 12th well in a 96-well plate row is designated as a 'No- Inhibitor control'. Replicates (typically in triplicate) are oriented vertically in the plate. A concentration of protein kinase should be selected so that the initial reaction rate can be determined under conditions where less than 15% of the substrate becomes phosphorylated.

5.4.2. Experimental Protocol: The steps and volumes of each component for a kinase inhibitor titration experiment are outlined in Table 4 for a 96-well half-area plate. You should only plan to dilute as much inhibitor as you plan to use in a set of experiments as inhibitors are generally less stable upon dilution and in aqueous solutions.

TABLE 4: PROTOCOL 3 – KINASE INHIBITOR IC50 (50 µL assay for a half-area 96-well plate)

STEP	PROCESS			
1	Prepare an 11-point, 3-fold serial dilution of the kinase inhibitor in 100% DMSO to create 100X stocks of the intended final concentrations (a serial dilution scheme is provided below) or a "No Inhibitor" DMSO control. This is followed by a 10-fold dilution in water. Add 5 µL to each well.			
2	Prepare Master Mix (all components except Active Kinase) by combining each of the components listed to the right. Include other components as required.	Reaction Buffer (10X) PhosphoSens Substrate (10X) ATP solution (10X) DTT solution (10X) Ultrapure deionized H2O Total volume	For 1 RXN: 5 µL 5 µL 5 µL 5 µL 15 µL 35 µL	For 40 RXNs: 200 µL 200 µL 200 µL 200 µL 600 µL 1.40 mL
3	Prepare 5X Kinase in 5X Enzyme Dilution Buffer. Include a "Buffer Only" control. Add 10 µL per well.			
4	Equilibrate Master Mix to assay temperature (typically 30 °C) by placing the sealed tube in a heat block or water bath set at the desired temperature for 5 minutes.			
5	Equilibrate the assay plate with kinase and inhibitor in the plate reader to the desired assay temperature (typically 30 °C) for 1 minute. Longer times can be used to assess the effect of pre-incubation on inhibitor potency.			
6	Aliquot 35 µL of the Master Mix into each well to start the reaction. Mix well.			

Inhibitor titration instructions for Table 4: As outlined below, prepare an 11-point, 3-fold serial dilution of the inhibitor.

Serial 3-fold Dilutions: 

Tube #	1	2	3	4	5	6	7	8	9	10	11	12
Final [Inhibitor], nM	1000.000	333.333	111.111	37.037	12.346	4.115	1.372	0.457	0.152	0.051	0.017	None
100X [Inhibitor], µM	100.000	33.333	11.111	3.704	1.235	0.412	0.137	0.046	0.015	0.005	0.002	None
100% DMSO, µL	X µL	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL	15 µL
Stock Inhibitor in 100% DMSO, µL	Y µL as Prepared	5 µL of Tube #1	5 µL of Tube #2	5 µL of Tube #3	5 µL of Tube #4	5 µL of Tube #5	5 µL of Tube #6	5 µL of Tube #7	5 µL of Tube #8	5 µL of Tube #9	5 µL of Tube #10	None
Final Volume, µL, of 100X Inhibitor	15 µL	15 µL	15 µL	15 µL	15 µL	15 µL	15 µL	15 µL	15 µL	15 µL	15 µL	15 µL
Add µL water for 1:10	135 µL	135 µL	135 µL	135 µL	135 µL	135 µL	135 µL	135 µL	135 µL	135 µL	135 µL	135 µL
Final Volume, µL, of 10X Inhibitor	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL

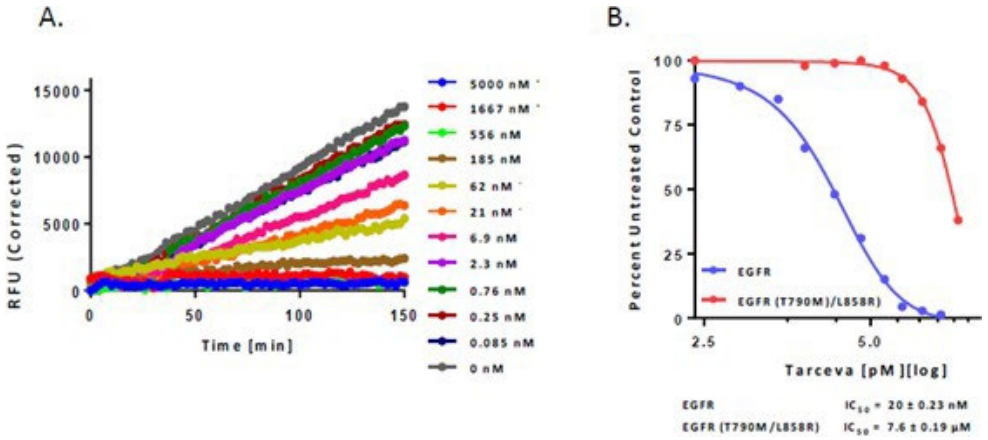
Inhibitors should be serial diluted in 100% DMSO to 100X and then diluted 1:10 with water to generate a 10X stock. You will need 5 µL to create each 3-fold dilution and 5 µL for each 96-well plate well replicate well and and 2.5 µL for each 384-well plate well replicate at each concentration (typically triplicate wells, so 15 µL for 96 well and 7.5 µL for 384 well). In this example, with 11 inhibitor concentrations plus 1 control (12 conditions), each in triplicate = 36 wells (or 38% of a 96-well kit).

5.4.3. Data Analysis: Subtract the background fluorescence determined with the "No kinase" control for each time point from the total signal to obtain corrected Relative Fluorescence Units (RFU) values. Plot the corrected RFU vs. Time for each inhibitor concentration and determine the initial reaction rates (slope of the linear portion) for each progress curve for each inhibitor concentration. Plot velocity (RFU/minute or µM/second) vs [inhibitor] and determine the IC50 using a 4-parameter logistic fit, which can be performed using the instrument software or by exporting the data to another program such as DynaFit, KinTek, SigmaPlot or GraphPad Prism.

FIGURE 6: Representative data from an inhibitor dose-response titration using the *PhosphoSens* assay.

A. Progress Curves: 3 nM EGFR or 1 nM EGFR (T790M/L858R), both from Carna Biosciences, were incubated with 20 µM AQT0099 in the presence of Tarceva (Erlotinib) from 0 - 9.0 µM for 150 minutes at 30 C to obtain RFU signals over time, which were then corrected by subtracting background fluorescence determined in control (no kinase) reactions.

B. The IC50 value was determined from a plot of Velocity (RFU Corrected/pmole/min) versus Tarceva (Erlotinib) concentration using a 4-parameter logistic curve fit. For the EGFR, the IC50 value was determined to be 20 ± 0.29 nM, which is in the same range as the IC50 value (40 nM) reported by Kitagawa, et al. (2013) determined under similar conditions using 1 mM ATP. The IC50 value determined for the EGFR (T790M/L858R) was 7.6 ± 0.19 µM or 380-fold less sensitive to Tarceva (Erlotinib), illustrating the resistance to drug that develops in patients. Note the high precision of the *PhosphoSens* assay format (each data point is the mean ± std dev) that results from determining reaction rates at each inhibitor concentration.



6. TROUBLESHOOTING

TABLE 5: Troubleshooting and Frequently Asked Questions (FAQs)

OBSERVATION	CATEGORY	POTENTIAL EXPLANATION
Plateau observed where RFU is constant	Maximum signal	Sufficient enzyme and time have elapsed to completely phosphorylate all the substrate. Alternatively, the kinase being used is not stable under the conditions being tested. Need to optimize conditions, which may require testing kinase preparations from different suppliers. Using a chemically synthesized phosphopeptide corresponding to the substrate provides a control to show the maximum RFU that should be achieved.
Different Sox-based substrates have different RFU maxima	Maximum signal	Each Sox-based substrate will have a somewhat different maximum RFU as this is influenced by the sequence of the peptide
Maximum RFU for the same substrate varies by kinase	Maximum signal	The maximum RFU for a given Sox-substrate is constant, therefore kinase may be unstable under conditions tested. Do a kinase titration and systematically test buffer components (e.g., add BSA or glycerol). May also need to rule out product inhibition.
Low Signal to Background	Minimum signal	1) The concentration of kinase used may be too low or the kinase is unstable or insufficiently activated. Kinase may need to be pre-activated and/or additional co-factors may be required for the kinase to achieve full activity, e.g., Ca(II) or lipid for PKCs, Ca(II)/Calmodulin for some CAMKs. An activating kinase may also be used if it doesn't phosphorylate the Sox-based substrate. 2) It may be necessary to titrate the ATP and/or Mg(II) to determine the concentration that results in the maximum fluorescence increase, which can be peptide specific. Generally, a 1.5-fold increase in fluorescence upon phosphorylation is all that is required to achieve a robust Z' value of >0.8). 3) Some samples may contain compounds that interfere with fluorescence and/or activity measurements in this assay. It is advisable to run a background fluorescence scan prior to kinetic data acquisition. Below is a list of known compounds for which the indicated concentration results in < 10% inhibition of the *PhosphoSens*® signal (higher concentrations should be avoided): • $CaCl_2$, 2.5 mM • Detergents (0.01% SDS, 1% Triton X-100) • DMSO, 10% • DTT, 5 mM • EDTA, 1 mM or EGTA, 2 mM • $MnCl_2$, 250 µM can be used for Mn(II)-dependent kinases (this is 250x physiological levels) • NaCl, 150 mM • Na_3VO_4, 40 µM • β-glycerophosphate, 10 mM
Background (all reaction components but without enzyme) fluorescence increases over time	Stable Background	Evaporation from wells, especially with long kinetic reads or under low humidity conditions, can be significant. Plates should be sealed with an optically-clear adhesive film that still allows top-reading with minimal light scattering. We recommend Perkin Elmer TopSeal-A Plus (6050185) applied with either a roller or a paddle (VWR 60941-118 or 60941-128, respectively).

For additional assistance, please contact us at support@assayquant.com

7. TECHNOLOGY LICENSING

Use of the sulfonamido-oxine (Sox) fluorophore to report peptide phosphorylation and kinase activity via chelation-enhanced fluorescence (CHEF) was developed by the Imperiali laboratory at the Massachusetts Institute of Technology (MIT) (15-17).

These products are sold under an exclusive license from MIT to AssayQuant Technologies, Inc. and are covered by patents 10/681,427, 10/682,427, 07872278.2 (issued 2016) and 62,331,903 (pending). The first-generation kinetic-based sensors were originally commercialized under the Omnia® brand by Invitrogen/Life Technologies/Thermo Fisher. Starting in February 2016, sensors based on the original Sox-technology and second-generation improvements developed by the Imperiali lab, have been exclusively offered by AssayQuant and are marketed under the brand name of *PhosphoSens*. These sensors provide a powerful yet simple one-step format that is direct, continuous (kinetic), homogeneous, quantitative and can be used with mM ATP, purified enzymes and crude cell or tissue lysates (9,18,19).

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Appendix A – Performing a *PhosphoSens* -Red Assay

A.1 ASSAY KIT COMPONENTS AND REQUIRED MATERIALS

A.1.1. MATERIALS AND EQUIPMENT NOT INCLUDED

Unless specified below, any materials and equipment required to use the *PhosphoSens* Kinase Assay Kit are also required in order to set up and perform a *PhosphoSens* Red Assay.

A.1.1.1. Fluorescence microplate reader: Instrument must be capable of reading time-resolved fluorescence in endpoint mode with an excitation wavelength (λExMax) of ~360 nm and an emission wavelength (λEmMax) of ~620 nm. Adjust the gain appropriately. *PhosphoSens* assays can be run on readily available microplate reader instruments including but not limited to the BioTek FLx800™, Neo, Synergy™ 2, and Synergy™ 4, Tecan Safire 2™, Infinite® M1000, Infinite® F500, Molecular Device SpectraMax® M5, BMG LABTECH PHERAstar, FLUOstar OPTIMA, and ThermoFisher Varioskan.

A.1.1.2. Stop Solution: 1M HCl and 1M NaOH are needed to stop the reaction.

*Of note: You don't need to stop the reaction when you are reading the plate within 60 minutes after Europium addition. The difference in signal between an active reaction and stopped reaction should be negligible. Stopping the reaction allows for plates to be read in batch mode.

A.1.2. MATERIALS INCLUDED

Each *PhosphoSens*-Red kit includes all required reagents to perform 96 reactions, using 25 µL as a final reaction volume in 384-well plates, with a final substrate concentration of 10 µM.

Components can also be ordered in bulk. Bulk *PhosphoSens* peptide substrate is provided as lyophilized powder as mgs of net CSx-peptide (please inquire at orders@assayquant.com for bulk pricing).

COMPONENT	DESCRIPTION	AMT	STORAGE
PhophoSens Substrate, 100x	*PhosphoSens* Cysteine-Sox Kinase Sensor AQTxxxx peptide substrate, 1 mM	35 µL	-20 °C or below. Minimize repeated freeze/thaw cycles
ATP solution, 100x	100 mM ATP in nuclease-free water	150 µL	-20 °C or below. Minimize repeated freeze/thaw cycles
DTT solution, 1000x	1M DTT in in nuclease-free water	150 µL	-20 °C or below. Minimize repeated freeze/thaw cycles
Enzyme reaction buffer, 10x	500 mM HEPES, pH 7.5, 0.1% Brij-35, 100 mM $MgCl_2$	1,650 µL	-20 °C or below.
Enzyme dilution buffer, 5x	20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 1 mg/mL Bovine Serum Albumin	1,650 µL	-20 °C or below.
EGTA Solution, 1000X	550 mM Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)	150 µL	-20 °C or below.
Europium, 6x	30mM Europium (III) in 0.6 mM HCl	1,200 µL	Room temperature or below.

A.2. PERFORMING A *PHOSPHOSENS* -RED ENDPOINT ASSAY

A.2.1. PREPARING ASSAY REAGENTS

Prior to setting up the individual reactions, prepare the following solutions:

A.2.1.1. ***PhosphoSens*** **Sox-based Substrate:** Prepare as instructed in section 5.1.1.

A.2.1.2. **Peptide Substrate Solution (10X):** Prepare as instructed in Section 5.1.2.

A.2.1.3. **ATP Solution (10X):** Prepare as instructed in Section 5.1.3.

A.2.1.4. **DTT Solutions:** Prepare as instructed in Section 5.1.4.

A.2.1.5. **EGTA Solution:** Prepare as instructed in Section 5.1.5.

A.2.1.6. **Enzyme Dilution Buffer (EDB, 5X) with DTT:** Prepare as instructed in Section 5.1.6.

A.2.1.7. **Kinase Reaction Master Mix:** Prepare as instructed in Table 2

A.2.1.8. **Kinase Stock:** Prepare as instructed in Section 5.1.8.

A.2.1.9. **Europium (III) Solution:** Add the 30 mM (6x) Europium (III) stock solution directly to each well to a final concentration of 5 mM.

A.2.1.10. **Stop Solution:** 5µl 1M HCl and 5µl 1M NaOH.

A.2.1.11. **Final Reaction Conditions:** Typical final concentrations of each reaction component are as follows: 54 mM HEPES, pH 7.5, 1 mM ATP (or as adjusted as needed), 1.2 mM DTT (optional if needed), 0.55 mM EGTA (optional if needed), 0.012% Brij- 35, 10 mM MgCl2, 10 µM peptide substrate, 0.05 - 5 nM kinase (or adjusted as needed) and any additional co-factors or additives (as required). After the reaction has completed, Eu3+ is added to a final concentration of 5 mM.

A.2.2. DETERMINATION OF KINASE INHIBITOR IC_{50} VALUES

NOTE: This protocol assumes that an optimal concentration of kinase in the reaction has been previously determined or otherwise chosen. If this is not the case, and kinase concentration cannot be determined via a kinase titration assay in the *PhosphoSens* kinetic format (see section 5.2), please contact Technical Support at support@assayquant.com for assistance with adapting a kinase titration experiment for use in the *PhosphoSens*-Red format.

A.2.2.1. Experimental Protocol: The steps and volumes of each component for a kinase inhibitor titration experiment are outlined in Table A3 for a 96-well half-area plate. Volumes can be reduced by half to accommodate a 384-well plate format. You should only plan to dilute as much inhibitor as you plan to use in a set of experiments as inhibitors are generally less stable upon dilution and in aqueous solutions.

Table A3: PROTOCOL – KINASE INHIBITOR IC_{50} (50 µL assay for a half-area 96-well plate)

<table>
<tr><th>STEP</th><th colspan="4">PROCESS</th></tr>
<tr><td>1</td><td colspan="4">An Enzyme / DMSO titration test should be performed before hand to determine DMSO tolerance.</td></tr>
<tr><td rowspan="8">2</td><td rowspan="8">Prepare Master Mix (all components except Active Kinase) by combining each of the components listed to the right. Include other components as required.</td><td></td><td><u>For 1 RXN</u>:</td><td><u>For 40 RXNs</u>:</td></tr>
<tr><td>Reaction Buffer (10X)</td><td>5 µL</td><td>200 µL</td></tr>
<tr><td>PhosphoSens Substrate (10X)</td><td>5 µL</td><td>200 µL</td></tr>
<tr><td>ATP solution (10X)</td><td>5 µL</td><td>200 µL</td></tr>
<tr><td>DTT solution (10X)</td><td>5 µL</td><td>200 µL</td></tr>
<tr><td>EGTA solution (10X)</td><td>5 µL</td><td>200 µL</td></tr>
<tr><td><u>Ultrapure deionized H2O</u></td><td><u>10 µL</u></td><td><u>400 µL</u></td></tr>
<tr><td>Total volume</td><td>35 µL</td><td>1.4 mL</td></tr>
<tr><td>3</td><td colspan="4">Add 5 µL of 10X inhibitor in 10% DMSO per well.</td></tr>
<tr><td>4</td><td colspan="4">Add 35 µL of Master Mix to each well.</td></tr>
<tr><td>5</td><td colspan="4">Incubate at 30°C for 5 minutes to equilibrate the plate.</td></tr>
<tr><td>6</td><td colspan="4">Add 10 µL of 5X Kinase in Enzyme Dilution Buffer to each well. Include a no Kinase, EDB only control.</td></tr>
<tr><td>7</td><td colspan="4">Add plate to reader and monitor kinase activity by collecting fluorescence intensity (RFU) readings (lExMax 360 nm/lEmMax ~492 nm [485-498 nm]) every 0.5-2.0 minutes at 30°C until the progress curve of the no-inhibitor control reaches very close to the top the linear range.</td></tr>
<tr><td>8</td><td colspan="4">Add 10 µL of the 30 mM Europium (6X) solution to each well and incubate for at least 5 minutes at room temperature.</td></tr>
<tr><td>NOTE</td><td colspan="4">If running a screen where the plates will be at room temperature for longer than 60 minutes, it is suggested to stop the reaction by following steps 9 and 10. If this is not the case, proceed to step 11.</td></tr>
<tr><td>9</td><td colspan="4">Transfer 5µL 1M HCl into each well. Incubate for 5 minutes to inactivate the kinase.</td></tr>
<tr><td>10</td><td colspan="4">Add 5µL 1M NaOH to each well to neutralize HCl.</td></tr>
<tr><td>11</td><td colspan="4">Return the plate to the reader and take an endpoint time-resolved fluorescence (TRF) reading (lExMax 360 nm/lEmMax ~620 nm) with the plate unsealed.</td></tr>
</table>

A.2.2.2. Data Analysis:

NOTE: Before determining an IC50 from the collected data, it is advised to evaluate the signal-to-background ratio (S/B) and/or Z' score of the raw RFUs of the non-treated kinase control.

Subtract the background fluorescence determined with the "No kinase" buffer only control for each time point from the total signal to obtain corrected Relative Fluorescence Units (RFU) values. Plot corrected RFU vs [inhibitor] and determine the IC50 using a 4-parameter logistic fit, which can be performed using the instrument software or by exporting the data to another program such as DynaFit, KinTek, SigmaPlot or GraphPad Prism.