PhosphoSens®-Kinetic Continuous Fluorescent Intensity (FI) Phosphatase Assay and Determination of Phosphatase Inhibitor IC_{50} Values

INTRODUCTION:

PhosphoSens-Kinetic Continuous assays utilize a simple add-and-read protocol, providing the capability for real-time, kinetic measurements, with a straight-forward, fluorescence intensity (FI) measurement. These assays provide high accuracy and precision by allowing measurement of the initial reaction rate (measured as the slope of the initial linear region), typically during the first 30-60 minutes and include many data points, with readings performed every 30 seconds to two minutes. This continuous assay format also allows for correction of compound autofluorescence because this background signal does not change over time and can be corrected for by subtracting background signals (includes compound and all components of the reaction besides the enzyme) from the total fluorescence (all components including enzyme) to determine the net signal at each time point. The continuous format is compatible with essentially any commercially available fluorescence microplate reader capable of kinetic readings, and associated laboratory automation equipment. No stop solution or quench/develop step is needed, minimizing time, while maximizing throughput.

MATERIALS INCLUDED:

Each PhosphoSens-Kinetic Phosphatase Kit includes all required reagents to perform 96 reactions in a low-volume 384-well plate, using 25 µL as a final reaction volume and a Sensor Peptide Substrate concentration of 10 µM.

COMPONENT	DESCRIPTION	VOLUME	STORAGE
PhosphoSens Substrate, 1 mM	PhosphoSens Sensor Peptide Substrate, 1 mM	35 µL	-20°C or below
DTT Solution, 1 M	1 M DTT 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 MgCl2	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 BSA	1,650 µL	-20°C or below. Minimize repeated freeze/thaw cycles
EGTA Solution, 550 mM	550 mM EGTA in 2 M NaOH	150 µL	-20°C or below

Components can also be ordered in bulk. Bulk PhosphoSens Sensor Peptide Substrate is provided as lyophilized powder and can be paired with PhosphoSens Reagent Packs, which include sufficient volumes of required reagents to pair with 1mg of Sensor Peptide. Please inquire at orders@assayquant.com for bulk pricing.

MATERIALS NOT INCLUDED:

Recombinant Phosphatase:

PhosphoSens products are compatible with any commercially available protein phosphatase for which a Sox-based Sensor Peptide Substrate has been developed. When choosing a commercially available phosphatase 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 phosphatase 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.

Fluorescence microplate reader:

Instrument must be capable of reading fluorescence intensity in continuous (kinetic) mode with an excitation wavelength (λExMax) of ~360 nm (358-363 nm) and an emission wavelength (λEmMax) 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). Contact Technical Support or e-mail us directly at support@assayquant.com for instrument-specific setup guidelines.

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.

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).

PREPARING ASSAY REAGENTS:

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

PhosphoSens Sensor Peptide Substrate (1 mM):

Bulk PhosphoSens sensor peptides are supplied as 1mg lyophilized powder. Dissolve the sensor peptide substrate as indicated on the vial or in the Technical Notes section of the Certificate of Analysis to create the 1 mM stock. If the sensor peptide requires the addition of dimethyl sulphoxide (DMSO), add the DMSO first to ensure solubility followed by the aqueous component, gently vortexing the solution between additions to ensure complete dissolution.

Sensor peptides that are provided in the PhosphoSens Evaluation Kits, are already solubilized as a 1 mM solution in the solvents defined in the Technical Notes section of the Certificate of Analysis.

- Storage: Store unused aliquoted 1 mM sensor peptide stocks at -20 °C or below until ready for use.

PhosphoSens Sox-based Substrate Solution (100 µM):

Sensor peptides that are provided in the PhosphoSens Evaluation Kits, are already solubilized as a 1 mM solution in the solvents defined in the Technical Notes section of the Certificate of Analysis.

Prepare 0.1 mM (100 µM) substrate solution by thawing the 1 mM peptide substrate stock solution, mixing well by vortexing gently, removing an appropriate amount and diluting 10-fold into ultrapure deionized water.

- Storage: Discard excess.

DL-Dithiothreitol (DTT) Solutions (10 mM):

DTT is supplied as 1M stock solution.

Prepare 10 mM DTT solution by adding 5 µL of the 1 M DTT to 495 µL ultrapure deionized water.

- Storage: Discard after use. Bulk solutions of the 1M DTT solution can be aliquoted and stored at -20 °C or below for future use.
- *CAUTION: Diluted DTT is readily oxidized so use fresh dilutions.*

Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) Solution (5.5 mM):

EGTA is supplied as 550mM stock solution.

Prepare 5.5 mM EGTA solution by adding 5 µL of 550mM EGTA to 495 µL ultrapure deionized water.

- Storage: Discard the remaining 5.5 mM EGTA solution after use. Bulk solutions of the 550mM EGTA solution can be aliquoted and stored at -20 °C or below for future use.
- **NOTE: EGTA will chelate most metals^{+2}, which can be present as a trace contaminants and can negatively affect assay performance. For this reason, EGTA should be added to reactions (0.55 mM final) unless the kinase requires activation by Ca^{+2} (Ca^{+2} will chelate Ca^{+2}). Please refer to the spec sheets of the specific kinases for more information regarding additives such as EGTA.*

Enzyme Dilution Buffer (EDB, 5X):

EDB is provided as a 5X stock solution.

EDB is added to Blank or Background wells as a "No Enzyme" control and is used to dilute enzymes.

- Storage: Bulk solutions of the EDB can be aliquoted and stored at -20 °C or below for future use.

Enzyme Reaction Buffer (ERB, 10X):

ERB is provided as a 10X stock solution.

ERB is added directly to the 'Master Mix'.

- Storage: Bulk solutions of the EDB can be aliquoted and stored at -20 °C or below for future use.

Phosphatase Stock:

Dilute an appropriate amount of the phosphatase stock to 5X (10 nM if the final desired concentration is 2 nM) in EDB (with or without DTT, depending on the enzyme). The required volume of 5X phosphatase in EDB, then is added to assay wells to initiate the reaction (this can be done using automated reagent dispensing if timing is critical).

- Storage: Discard the unused portion of the diluted kinase. Please refer to the phosphatase suppliers handling and storage for long-term storage conditions.

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, but recommended), 0.012% Brij- 35, 10 mM MgCl2, 10 µM sensor peptide substrate, 0.05 - 10 nM phosphatase (or adjusted as needed) and any additional co-factors or additives (as required).

IC_{50} PROTOCOL:

This protocol outlines assay conditions when working in a low-volume 384-well plate with 20 µL final well volume and 10 µM Sensor peptide at 1 mM ATP. It assumes that an optimal concentration of phosphatase and DMSO in the reaction has been previously determined or otherwise chosen.
A 12-point compound titration (including a "0" compound concentration) should be run in duplicate using a 3-fold dilution scheme for each compound with a separate blank at each compound concentration. Final assay concentrations will be the same as above except for the additional of 2% DMSO final with or without compound.

<table>
<tr><th>STEP</th><th colspan="3">PROCESS</th></tr>
<tr><td>0</td><td colspan="3">A Kinase titration and DMSO titration should be performed beforehand to determine optimal concentrations.</td></tr>
<tr><td rowspan="6">1</td><td rowspan="6">Prepare 'Master Mix' by combining each of the components listed to the right.
Include other components as required.</td><td>Component:</td><td>For 1 Well:</td></tr>
<tr><td>Enzyme Reaction Buffer (10X)</td><td>2 µL</td></tr>
<tr><td>DTT solution (10 mM)</td><td>2 µL</td></tr>
<tr><td>EGTA Solution (5.5 mM)</td><td>2 µL</td></tr>
<tr><td>Ultrapure deionized H2O</td><td>7.6 µL</td></tr>
<tr><td>Total volume</td><td>13.6 µL</td></tr>
<tr><td>2</td><td colspan="3">Add 2 µL 100 µM Sensor Peptide to each well. (can be added to the Master Mix if the same for all wells)</td></tr>
<tr><td>3</td><td colspan="3">Add 0.4 µL 50X inhibitor dilutions in 100% DMSO or 100% DMSO vehicle to each well.</td></tr>
<tr><td>4</td><td colspan="3">Add 13.6 µL 'Master Mix' with Reaction Buffer, DTT, and EGTA to each well.</td></tr>
<tr><td>5</td><td colspan="3">Seal the plate and incubate at 30 °C for 5 minutes to equilibrate the plate</td></tr>
<tr><td>6</td><td colspan="3">*Add 4 µL of EDB or EDB with enzyme at 5X the final concentration</td></tr>
<tr><td>7</td><td colspan="3">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>
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IC_{50} DATA ANALYSIS:

If you have an unphosphorylated control peptide, subtract the background determined for each time point from the total signals at each time point to obtain corrected Relative Fluorescence Units (RFU) values for both the peptide alone and peptide plus enzyme wells. If you do not have an unphosphorylated control, proceed to data processing. It is highly recommended to run blanks at each compound concentration to correct for compound absorbance or fluorescence. Plot (corrected) RFU/min vs [inhibitor conc] and determine the IC_{50} 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. *Slopes will be negative, with 0% inhibition being the slope of the control and 100% being the slope of the substrate only control.