PhosphoSens®-Red Time Resolved Fluorescence (TRF) Kinase Assay – High Throughput Screen (HTS) and Determination of Kinase Inhibitor IC_{50} Values

INTRODUCTION:

Because many compound libraries contain compounds that may be capable of autofluorescence, AssayQuant offers PhosphoSens Red assays that allow detection at a longer (>600 nm) wavelength, with the same excitation maximum that uses time-resolved fluorescence (TRF). Our end-point assays utilize the same reaction set up as our continuous assays, and once the reaction is run, a Europium ion (Eu^{3+}) based reagent is added to each well, which displaces Mg^{2+} and replaces it forming a chelation complex. Excitation of the Sox fluorophore results in time resolved fluorescence detectible at 620 nm (red) that can be measured using standard filters. We recommend using a 100 µsecond delay between excitation and detection at the emission wavelength, with a 300 µsecond acquisition time, to avoid signal contamination from fluorescent materials in the reaction mixture, which have a very short (nanosecond) lifetime.

MATERIALS INCLUDED:

Each PhosphoSens Red-Shift Kinase 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
ATP Solution, 100 mM	100 mM ATP in nuclease-free water	150 µL	-20°C or below. Minimize repeated freeze/thaw cycles
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
Europium (III), 30 mM	30mM Europium (III) in 0.6 mM HCl	1,200 µL	Room temperature 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:

Materials and equipment required to run the PhosphoSens-Kinetic Kinase Assay Kit are also required to set up and perform a PhosphoSens-Red Assay. The only exceptions are when working with Tyrosine kinases as their substrates tend to be more acidic and/or when the plates will not be read within an hour, we recommend adding base or stopping the reaction using the methods detailed below.

Recombinant Kinase:

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

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) to monitor the reaction over time and select a time point within the linear range for the Red Shift Endpoint Assay. 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) if desired. The instrument must also have the capacity to read time resolved fluorescence with an excitation wavelength (λExMax) of ~360 nm (358-363 nm) and an emission wavelength (λEmMax) of 620 nm. We recommend using a 100 µsecond delay between excitation and detection at the emission wavelength, with a 300 µsecond acquisition time. 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 prevent 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). After Europium has been added, the plate should be read without a plate seal.

0.5 M NaOH Solution:

0.5 M NaOH can be added after the kinase reaction is nearly complete to increase the pH of the reaction solution before adding the 30 mM Europium (III) stock solution to each well to a final concentration of 5 mM.

NOTE: This pH correction step is advised when working with tyrosine kinases as their substrates include more acidic residues.

Stop Solution (1 M HCl and 1 M NaOH):

1 M HCl and 1 M NaOH may be needed to stop the reaction. Stopping the reaction allows for plates to be read overnight in batch mode.

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 a stopped reaction should be negligible following Europium addition over 60 minutes.

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.

Adenosine 5′-triphosphate disodium salt hydrate (ATP) Solution (10 mM):

ATP is supplied as a 100mM stock solution.

Prepare 10 mM ATP solution by adding 50 µL of 100 mM ATP to 450 µL ultrapure deionized water.*

- Storage: Discard the remaining 10 mM ATP solution after use. Bulk solutions of the 100 mM ATP solution can be aliquoted and stored at -20 °C or below for future use.
- **NOTE: These instructions are for running the assay at 1 mM ATP. If running the assay at ATP K_m, please prepare 500 µL of a stock of ATP at 10X the ATP K_m.*

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

DTT is supplied as 1 M 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 metal^{+2}, which can be present as a trace contaminant 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} (EGTA 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.

Kinase Stock:

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). 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).

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

Europium (III) Solution:

Europium is supplied as 30 mM stock solution.

The 30 mM Europium (III) stock solution can be added directly to each well to a final concentration of 5 mM.

- Storage: Bulk solutions of the Europium can be aliquoted and stored at RT or below for future use.

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 kinase (or adjusted as needed) and any additional co-factors or additives (as required). After the reaction has completed, Eu^{+3} is added to a final concentration of 5 mM.

- *NOTE: When running a high throughput screen in the Red-Shift Assay format, it is recommended that signal:background and Z' values be determined prior to starting the screen to ensure that they are within acceptable range. It is also recommended that separate blanks be run for each compound. If this is not possible, separate blanks should at least be run for compounds that are predicted to have absorbance or fluorescence to avoid false negatives or positives.*

HIGH THROUGHPUT SCREEN (HTS) and IC_{50} PROTOCOL:

NOTE: The PhosphoSens-Red assay is an endpoint assay with throughput and scalability ideal for high throughput screens. Although IC_{50} values can be determined using Red Shift Assay, it is highly recommended that IC_{50} values be determined by utilizing the slopes of the linear portion of the complete progress curves generated using PhosphoSens-Kinetic (continuous) assay format. This assay format yields much more data for the determination and increases the accuracy of the value determined. To use this endpoint format to determine compound potencies, you should collect TRF signals at multiple time points.

This protocol outlines assay conditions when working in a low-volume 384-well plate with 20 µL final well volume (before NaOH and Europium addition) and 10 µM Sensor peptide at 1 mM ATP. It assumes that an optimal concentration of kinase and DMSO in the reaction has been previously determined or otherwise chosen.

- If performing an HTS screen, compounds can be added from 50X stocks in 100% DMSO directly to the assay to avoid intermediate compound precipitation. Blanks and enzyme controls should also contain an equal amount of 100% DMSO. It is recommended to run separate compound blanks for compounds that are predicted to have absorbance or fluorescence to avoid false negatives or positives. For an IC_{50} determination, 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. Each compound concentration should have a minimum of 8 timepoints collected to most accurately access initial reaction rate which is why the kinetic assay format is recommended for IC_{50} value determinations. 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="7">1</td><td rowspan="7">Prepare 'Master Mix' by combining each of the components listed to the right.
Include other components as required.</td><td><u>Component:</u></td><td><u>For 1 Well</u>:</td></tr>
<tr><td>Enzyme Reaction Buffer (10X)</td><td>2 µL</td></tr>
<tr><td>*ATP solution (10 mM)</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><u>Ultrapure deionized H2O</u></td><td><u>5.6 µL</u></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 or inhibitor dilution in 100% DMSO or 100% DMSO vehicle to each well.
*When performing a HTS, it is important to have blank and control wells with 0.4 µL of 100% DMSO on each plate.</td></tr>
<tr><td>4</td><td colspan="3">Add 13.6 µL 'Master Mix' with Reaction Buffer, ATP, 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>
<tr><td>8</td><td colspan="3">Remove plate from reader and remove plate seal.</td></tr>
<tr><td>NOTE</td><td colspan="3">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. The final amount of Europium added per well should be adjusted for a final concentration of 5 mM Europium. If this is not the case, proceed to step 11.</td></tr>
<tr><td>9</td><td colspan="3">Transfer 2.5 µL 1M HCl into each well. Incubate for 5 minutes to inactivate the kinase.</td></tr>
<tr><td>10</td><td colspan="3">Add 2.5 µL 1M NaOH to each well. Incubate for 5 minutes to neutralize HCl.</td></tr>
<tr><td>NOTE</td><td colspan="3">If running a screen for a tyrosine kinase, due to the acidic nature of these substrate sequences, it is suggested to adjust the pH by proceeding to step 11. If this is not the case, proceed to step 12.</td></tr>
<tr><td>11</td><td colspan="3">Add 5 µL of 0.5 M NaOH to each well and incubate for 5 minutes at room temperature to adjust the pH. This will yield a higher signal:background for acidic substrates.</td></tr>
<tr><td>12</td><td colspan="3">Add 5 µL of the 30 mM Europium solution to each well, for a final Europium concentration of 5 mM, and incubate for 5 minutes at room temperature.
*If following Step 11, add 6 µL of the 30 mM Europium solution to each well, for a final Europium concentration of 5 mM.</td></tr>
<tr><td>13</td><td colspan="3">Return the plate to the reader and take an endpoint time-resolved fluorescence (RFU) reading (lExMax 360 nm/lEmMax ~620 nm) with the plate unsealed. The gain will need to be adjusted based on the reader and substrate. For the Biotek Neo2 reader, a gain of 70 works for most substrates.</td></tr>
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DATA ANALYSIS:

Subtract the background determined with the "No kinase" buffer only blank for each time point from the total signals at each time point to obtain corrected Relative Fluorescence Units (RFU) values. It is highly recommended to run "No kinase" buffer only blanks at each compound concentration to correct for compound absorbance or fluorescence.
Plot corrected RFU vs [inhibitor] 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.