SPHK1 Assay Kit

Catalog Number KA0906

400 assays

Version: 05

Intended for research use only

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Introduction

Background

The PhosphoSeek Technology for screening of kinase and phosphatase activities is a robust and homogeneous detection platform that measures the activity of a target enzyme. Assays are non-competitive with respect to substrate and do not require radioactive materials or secondary (detector) enzymes or antibodies. These biochemical assays are ideally suited for automated screening and can be read on any fluorometer. The Sensor is a proprietary fluorescent molecule that contains a trivalent metal ion, which binds to phosphorylated biological substrates. Phosphorylation is measured by the change of fluorescence of a dye-labeled and phosphorylated substrate when bound by the Sensor. The change in fluorescence directly correlates to the level of substrate conversion (Figure 1). When phospho-calibrator lipids are included in the experimental setup, the precise amount of product formation can be determined.

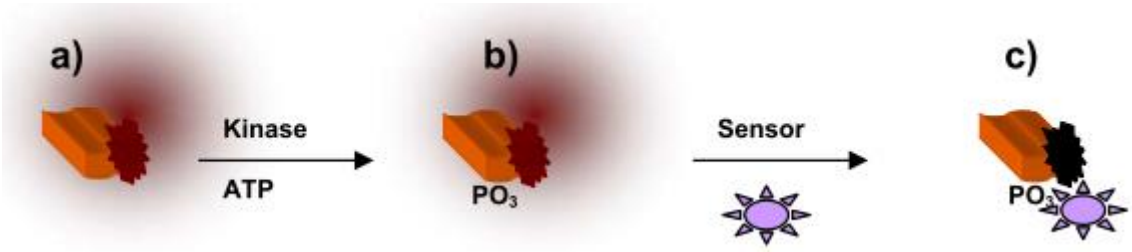


Figure 1: Schematic depiction of PhosphoSeek kinase assay: A substrate labeled with a fluorescent dye (a); (red starburst) is reacted with kinase resulting in phosphorylated substrate (b); the fluorescence of the substrate is quenched when the Sensor (purple star) associates to phosphorylated moieties on the substrate (c). The fluorescence intensity decreases in direct proportion to phosphorylated substrate.

General Information

Materials Supplied

Component	Amount	Volume
Assay Buffer	10 mM Tris, pH 7.2, 10 mM $MgCl_2$, 0.015% Triton X-100, 0.05% NaN_3	10 ml
Substrate	TAMRA-Sphingosine	4.88 µg
Calibrator	TAMRA-Sphingosine 1-phosphate	1.37 µg
Sensor Stock	Stock in 1N HCl	30 µl
Post Reaction Buffer	NaCl-based, 0.05% NaN_3	2.5 ml
Sensor Dilution Buffer	MES/NaCl-based, 0.05% NaN_3 pH 6.5	14 ml
ATP	100 mM stock in H_2O	7 µl
DTT	800 mM stock in H_2O	42 µl
384 well Plate	Black 384 well Cliniplate	1 plate

Storage Instruction

Upon arrival the SPHK1 Assay Kit should be stored as directed below. All reagents are stable for 12 months from the date of purchase, if stored and handled properly.

Component	Storage
Assay Buffer	2-8°C
Substrate	-80°C*
Calibrator	-80°C*
Sensor Stock	2-8°C
Post Reaction Buffer	2-8°C
Sensor Dilution Buffer	2-8°C
ATP	-20°C
DTT	-20°C
384 well Plate	RT

*For short-term storage, store at -20°C.

Required Materials not Provided

The materials listed in the following table were used to generate sample data shown in Calculation of Results. Materials from other suppliers may be used.

- ✓ Fluorescence Plate reader
- ✓ Sphingosine Kinase Enzyme (BPS Bioscience Cat# 40610)
- ✓ EDTA
- ✓ SPHK Inhibitor (EMD Bioscience Cat# 567731)

- ✓ DESPH Inhibitor (EMD Bioscience Cat# 310500)

Assay Protocol

Reagent Preparation

This kit contains reagents sufficient for 400 enzyme reactions (15 µl) to be performed in a 384-well plate format. Refer to Materials Supplied. Assay volumes can be modified provided the ratio of reaction volume to Sensor volume is maintained.

Assay Procedure

1. Reconstitute Lipid Substrate and Calibrator
   - *NOTE: Substrate and Calibrator-may appear empty or have a pink film-this is normal. Must spin down the vials before opening and in the case of the Substrate dissolve completely by vortexing and sonicating ~ 1 min, then spin down again.*
   - Add 67 µl methanol to Lipid Substrate to obtain a 100 µM stock concentration.
   - Add 17 µl methanol to Calibrator to obtain a 100 µM stock concentration.
   - ✓ Store on ice for immediate use (-80°C long term)
2. Prepare Complete Assay Buffer (cAB)
   - Add DTT to Assay Buffer to obtain a 5 mM final concentration
   - ✓ Equilibrate to room temperature and use within 8 hours of preparation.
3. Prepare 3X Substrate (1 µM final concentration)
   - Prepare desired amount of 3 µM substrate working solution in cAB
4. Prepare 6X ATP Solution (100 µM final concentration)
   - Prepare desired amount of 600 µM ATP working solution in cAB.
5. 6X Inhibitor Solutions
   - Prepare 6X desired inhibitor concentration in an appropriate amount of cAB. If no inhibitor is used, adjust volume with cAB.
6. Prepare Calibrator Solutions
   (% Phospholipid = 100, 50, 25, 12.5, 6.25, 0)
   - Prepare 300 µl of 1 µM substrate solution and 100 µl calibrator solution in cAB.
   - Prepare 6 serial dilutions in vials: Dispense 50 µl of substrate solution into vials 2-6. Add 50 µl calibrator solution to vial 2 and transfer 50 µl from vials 2-5. Vial 1 contains 100% calibrator and vial 6, substrate.
7. Prepare 3X Enzyme Working Solution
   - To the appropriate amount of cAB, add enzyme that is 3X the desired final concentration (1-200 nM final concentration is recommended, depending on application).

8. Combine Reagents into appropriate wells
   - 2.5 µl Inhibitor solution
   - 2.5 µl 6X ATP solution
   - 5 µl 3X Substrate solution
   - 5 µl 3X enzyme solution

   = 15 µl Reaction Volume

   Reagents can be combined prior to dispensing.
   - Dispense 15 µl Calibrator solutions
   - Include appropriate controls

   Substrate – Enzyme – Inhibitor

   Substrate + Enzyme – Inhibitor

   Calibrator – Enzyme – Inhibitor

   ✓ Cover plate and incubate for 30–90 minutes (Data shown in Sample Data was abtained after 1 hour incubation)
9. Add Post Reaction Buffer
   - Dispense 5 µl Post Reaction Buffer to each well.

   EDTA can be added fresh to the Post Reaction Buffer (see Appendix A).
10. Prepare 1X Sensor (30 µl per well)
    - It is important to make the sensor no more than 10 minutes before use
    - Dilute Sensor 1:500 in appropriate amount of Sensor Dilution Buffer.
11. Add Sensor
    - Dispense 30 µl of 1X Sensor to each well.

    ✓ Cover plate and incubate for 60 minutes at room temperature
12. Measure Fluorescence
    - Shake plate
    - Monitor fluorescence using 540nm excitation and 580nm emission.

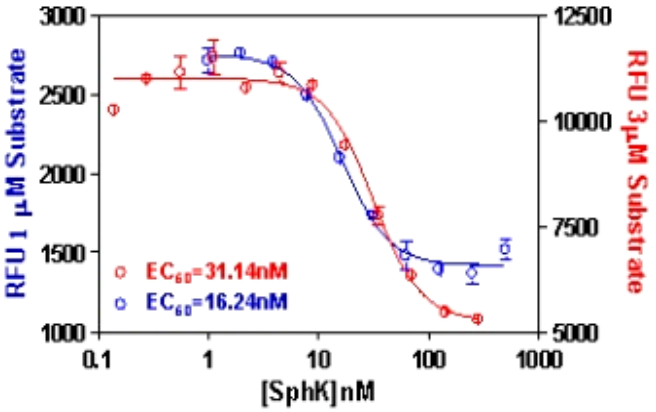
    Plates may be read for up to 5 hours without loss of signal. Increasing the time of incubation with Sensor decreases the raw RFU, however the S/B remains constant.

Data Analysis

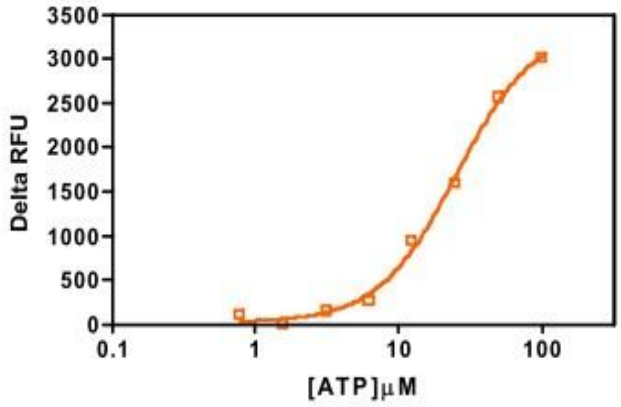
Calculation of Results

- ✓ Sample Data

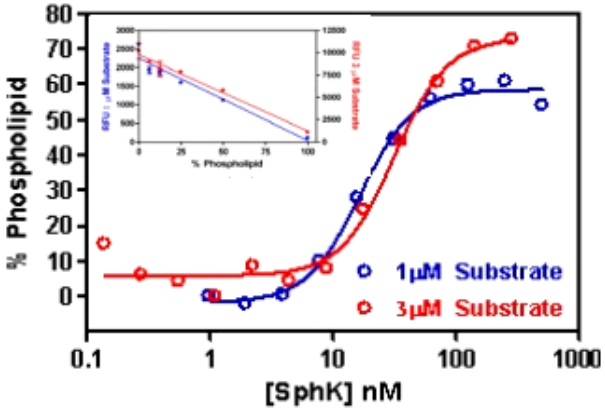
  Graphs were generated using GraphPad Prism Software. Curve fit was performed using sigmoidal dose response (variable slope). Error bars represent one standard deviation from the mean of two replicates.



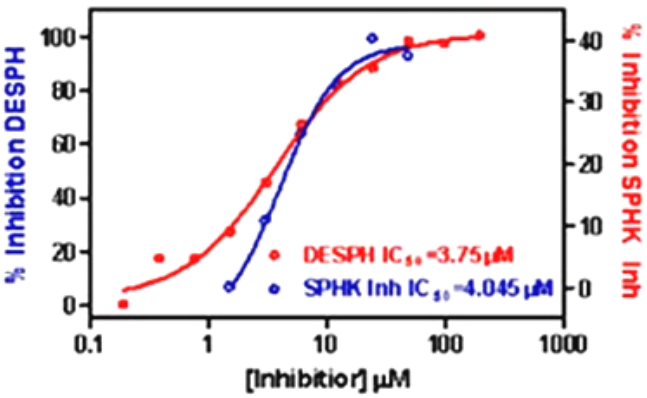
Reactions were terminated at various time points by addition of 5 µl postreaction buffer (See Appendix A)



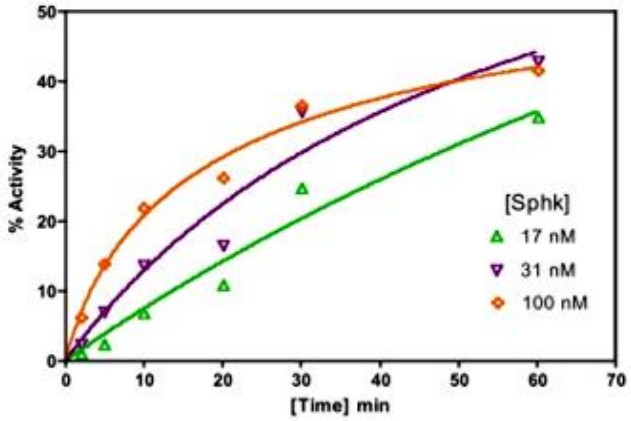
ATP Tolerance curve: Varying amounts of ATP were used in the absence or presence of enzyme and 3 µM Substrate.



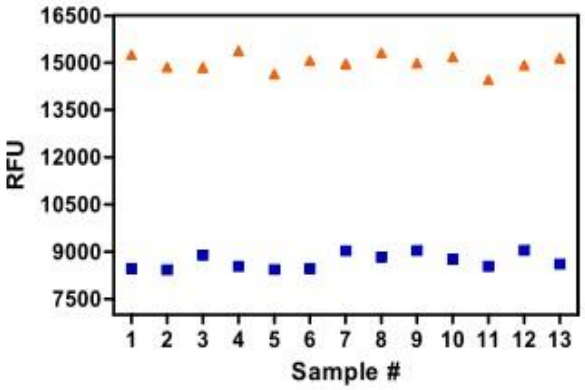
Enzyme Dose Response Curve: Decreasing concentrations of enzyme were mixed with various concentrations of substrate in cAB containing 100 µM ATP (top). Product conversion was determinate (bottom) by back calculation using phosphor-lipid calibrators (bottom insert).



Inhibition Curve: Varing amounts of inhibitors were added to substrate (3 µM) in cAB containing 75 µM ATP and samples reacted in the presence or absence of 70 nM enzyme. The % inhibition was computed based on delta RFU. Sphk inhibitor precipitates at high concentrations and inhibition of more than 40% could not be achieved. IC_{50} values correspond closely to literature values [2-3].



Enzyme Progress Curve: Various amounts of enzyme were added to wells containing substrate (3 µM) and reactions started by addition of enzyme/ATP (100 µM ATP).



Statistics: A Z' factor of 0.8 and CV , 3% were obtained using 70 nM enzyme and 3 µM Substrate in the presence (triangles) or absence (squares) of 200 µM of the inhibitor DESPH. A Z'-factor of > 0.5 indicates a robust assay.

✓ Compatible Substances

To determine the tolerance of the Sensor to substances commonly used for screening (see below) the various substances were added to samples containing either 0% or 100% of control concentration of phosphosubstrate in cAB. Following addition of Sensor, the S/B and Δ RFU between the controls were determined. Compatible substance concentrations listed are those that resulted in < 15% loss of Δ RFU and <15% loss of S/B.

Substance	Compatible Concentration
MeOH	10%
DMSO	10%
BSA	0.5%
EDTA	10 mM
Sodium Orthovanadate	2 mM
Sodium Tartrate	2 mM

Resources

References

1. Zhang, JH et al, J. Biomol. Screen. (1999) 4, 67.
2. Igarashi, Y., et al. J. Biol. Chem. (1990) 265, 5385.
3. French, K.J., et al. Cancer Res. (2003) 63, 5962.

- ✓ Appendix A

Performing Progress Curves

When performing progress curves, enzyme activity must be terminated at various time points. While the addition of Sensor does terminate enzyme activity, a shift in signal to background is observed with prolonged incubation of Sensor with substrate, which causes an offset of RFU acquired at different time points. Therefore, Sensor cannot be used as a stop reagent when performing progress curves. Instead, it is recommended to terminate enzyme activity with EDTA (in final concentrations between 5 mM – 10 mM) in water. Since EDTA may deplete Sensor, a reduction of signal to background may be observed. It is therefore recommended to establish the optimal concentration of diluted Sensor (recommended dilutions are 1:200;1:300;1:400 and1:500 in sensor dilution buffer) using 0% and 100% phospholipids. If only substrate is available, this experiment can be performed with substrate in the presence and absence of enzyme. Once the ideal concentration of Sensor has been established, a time course can be performed using the following procedure:

1. Add 5 µl EDTA to all wells (Pre-dilute 500 mM EDTA stock to 50 mM; then 5 µl addition results in ~ 12.5 mM final)
2. Prepare sufficient amounts of 3X substrate, 3X ATP and 3X enzyme in separate vials
3. Add 5 µl of 3X substrate, 3X ATP and 3X enzyme into wells for the t=0 time point
4. Combine remaining 3X substrate, 3X ATP and 3X enzyme in vials
5. Add 15 µl of the substrate/enzyme/ATP mix to remaining wells at given time points
6. Add 5 µl Post Reaction Buffer to all wells
7. Add 30 µl Sensor and incubate for 60 minutes at room temperature