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PhosphoSeek™ PTP1B Assay Kit (Catalog No. K707-400; 400 assays)

I. Assay Theory

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).

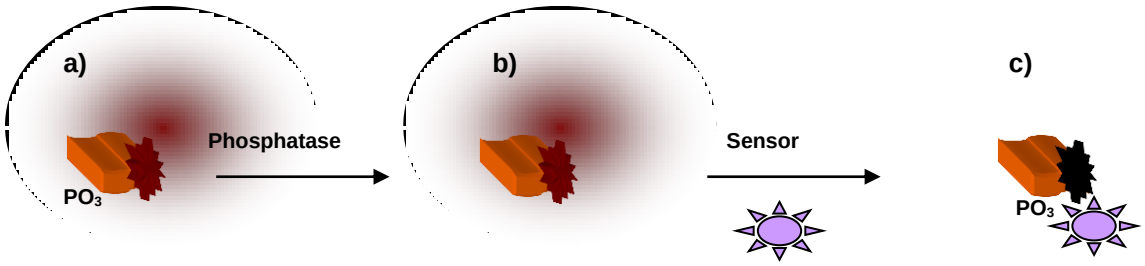


Figure 1: Schematic depiction of PhosphoSeek phosphatase assay: A phosphorylated substrate labeled with a fluorescent dye (red starburst) (a) is reacted with phosphatase resulting in de-phosphorylated substrate (b). Unreacted phosphorylated substrate is quenched when the Sensor (purple star) associates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

II. Kit Components and Storage

Upon arrival, the PhosphoSeek PTP1B phosphatase 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.

Reagent	Description	Volume	Part#	Storage
Assay Buffer	40 mM Tris Base, pH 7.2, 20 mM $MgCl_2$, 0.02 % Brij, 0.05 % NaN_3	10 ml	K707-400-1	2-8 °C
Sensor Stock	Stock in 1 N HCl	30 µl	K707-400-2	2-8 °C
Post Reaction Buffer	Na Acetate-based, 0.05 % NaN_3	2.5 ml	K707-400-3	2-8 °C
Sensor Dilution Buffer	MES/NaCl-based, 0.05 % NaN_3 , pH 6.5	14 ml	K707-400-4	2-8 °C
PTP1B Substrate	5-TMR-KVEKIGEGT(pY)GVVYK-OH 200 µM in H_2O	100 µl	K707-400-5	-20 °C
PTP1B Calibrator	5-TMR-KVEKIGEGTYGVVYK-OH 200 µM in H_2O	2.5 ml	K707-400-6	-20 °C
384 well Cliniplate	Black 384 well Cliniplate	1 EA	P400-1	RT

Required Materials not Provided

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

- Fluorescence Plate reader
- PTP1B Enzyme (Enzo Lifesciences Cat# Se-332)
- RK-682 (Enzo Lifesciences Cat# PR-112)

III. REAGENT PREPARATION AND ASSAY PROTOCOL

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

Equilibrate Kit Components to room temperature and use within 8 hours of preparation or store according to section II.

1. Prepare 3X Substrate (3 µM final concentration)

- Prepare desired amount of 9 µM substrate working solution in Assay Buffer (**AB**).

2. Prepare 3X Inhibitor Solutions

- Prepare 3X desired inhibitor concentration in an appropriate amount of **AB**. If no inhibitor is used, adjust volume with **AB**.

3. Prepare 3X Calibrator (3 µM final concentration)

- Prepare desired amount of 9 µM substrate working solution in **AB**

4. Prepare 3X Enzyme Working Solution

- To the appropriate amount of **AB**, add enzyme that is 3X the desired final concentration (0.05 nM – 10 nM final concentration is recommended, depending on application).

5. Combine Reagents

Into appropriate wells, dispense:

- 5 µl 3X Inhibitor solution
- 5 µl 3X Substrate or Calibrator solution
- 5 µl 3X Enzyme solution

= 15 µl Reaction Volume

Include appropriate controls

Substrate – Enzyme – Inhibitor

Substrate + Enzyme – Inhibitor

Calibrator – Enzyme – Inhibitor

➲ ***Cover plate and incubate for 30–90 minutes*** *(Data shown in V was obtained after 30 minute incubation).*

6. Add Post Reaction Buffer

- 5 µl Post Reaction Buffer to each well.

- Dispense 30 µl of 1X Sensor to each well.

➲ ***Cover plate and incubate for 60 minutes at room temperature***

7. 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 an appropriate amount of Sensor Dilution Buffer

8. Add Sensor

9. Measure Fluorescence

- Shake plate
- Monitor fluorescence using 540 nm excitation and 580 nm emission.

IV. COMPATIBLE SUBSTANCES

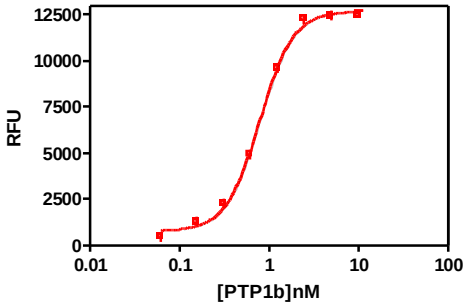
To determine the tolerance of the Sensor to substances commonly used for screening (see below) the various substances were added to samples containing either 0 % or 100 % of control concentration of phosphosubstrate in AB. Following addition of Sensor, the S/B and Δ RFU between the controls were determined. 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

V. 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.

IV.1 Enzyme Dose Response Curve



Enzyme Dose Response Curve: Decreasing concentrations of enzyme were mixed with substrate (3 μM) in Assay Buffer. Relative fluorescence units (RFU) increase as a function of enzyme activity. The EC_{50} is 0.8 nM

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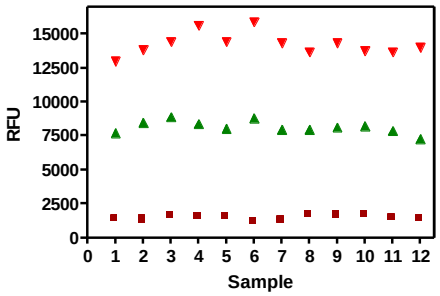
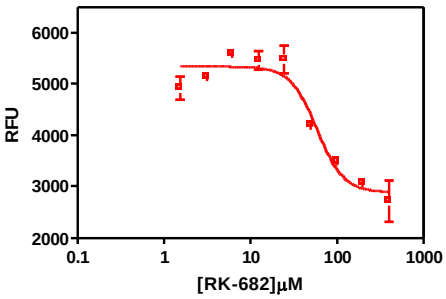
IV.2 Statistics



Statistics: Statistical data were produced using 0 nM (bottom), 0.9 nM (middle), and 7.2 nM (top) PTP1b. Z'-factors of 0.62 and 0.77 were obtained respectively for the samples with enzyme. A Z'-factor of > 0.5 indicates a robust assay [2].

IV.3 Inhibitor Concentration Curve



Inhibition Curve: Decreasing amounts of RK-682 in DMSO were prepared and added to enzyme (2.5 nM) and substrate (3 μM) in AB. The obtained IC_{50} of 59.5 μM is close to the reported literature value of 54 μM [1].

VI. REFERENCES

1. Hamaguchi *et al. FEBS Lett.* (1995) **372,** 54.
2. Zhang, JH *et al, J. Biomol. Screen.* (1999) **4**, 67.

[§]Prism is a registered trademark of GraphPad.

VII. PURCHASER NOTIFICATION

Warranty: BioVision's products are warranted to meet standard product specifications and to conform to label description when used and stored properly. Unless otherwise stated this warranty is limited to 12 months from date of sale for products stored, used and handled according to BioVision's instructions. BioVision's sole liability for the product is limited to replacement of the product or refund of the purchase price. BioVision's products are supplied for laboratory applications only. They are not intended for medical, diagnostic or therapeutic use. BioVision's products may not be resold, modified for resale or used to manufacture commercial products without prior written consent from BioVision.