PhosphoSeek™ P I3-Kinase Assay Kit (Catalog No. K706-400; 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.[1] 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).

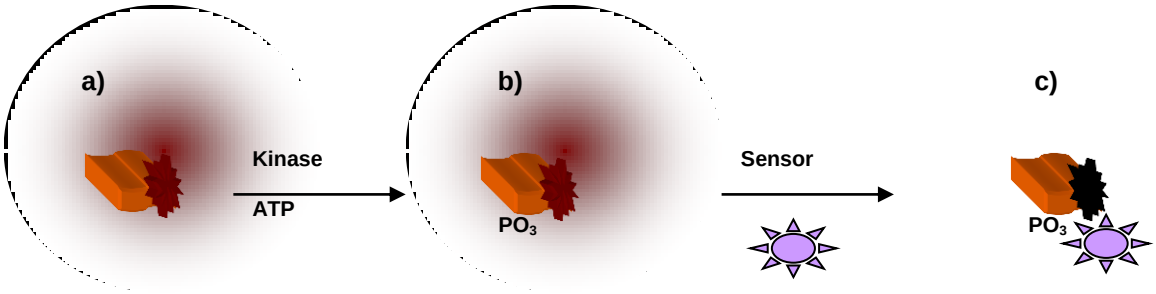


Figure 1: Schematic depiction of the PhosphoSeek PI3-Kinase assay: A substrate labeled with a fluorescent dye (a) (red starburst) is reacted with kinase resulting in phosphorylated substrate (b); the fluorescence of the substrate is quenched when the Sensor (purple star) associates to phosphorylated moieties on the substrate (c). The fluorescence intensity decreases in direct proportion to phosphorylated substrate.

II. Kit Components and Storage

Upon arrival the PI3-Kinase 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	Amount	Part#	Storage
Assay Buffer	25 mM HEPES, pH 7.0, 50 mM $MgCl_2$, 0.05 % NaN_3	10 ml	K706-400-1	2-8 °C
Lipid Substrate	Bodipy-TMR-PtdIns, C6	8 µg	K706-400-2	-20 °C
Calibrator	Bodipy-TMR-PIP, C6	2.1 µg	K706-400-3	-20 °C
Post Reaction Buffer	NaCl-based, 0.05 % NaN_3	2.5 ml	K706-400-4	2-8 °C
Sensor Stock	Stock in 1N HCl	60 µl	K706-400-5	2-8 °C
Sensor Dilution Buffer	MES/NaCl-based, 0.05 % NaN_3 , pH 6.5	18 ml	K706-400-6	2-8 °C
ATP	100 mM stock in H_2O	7 µl	K706-400-7	-20 °C
DTT	800 mM stock in H_2O	42 µl	K706-400-8	-20 °C
384 well plate	Black 384 well Cliniplate	1 EA	P400-1	RT

BODIPY-TMR is a registered trademark of Invitrogen.

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
- PI3-K Enzyme p110α/p85α (BPS Bioscience Cat# 40620)
- EDTA (BioVision Cat# 2103-100)
- Wortmannin (Biovision Cat# 1670-1)
- PI-103 (Cayman Chemicals Cat# 10009209)
- PI3-Kinase a Inhibitor 2 (Cayman Chemicals Cat# 10010177)
- LY294002 (BioVision Cat# 1667-5)

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

1. **Reconstitute Lipid Substrate and Calibrator**
   - **NOTE: Lipid 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 Lipid Substrate dissolve completely by vortexing and sonicating ~ 1 min, then spin down again.**
   - Add 14 µl 50 mM Hepes pH 7.4 to Lipid Substrate to obtain a 500 µM stock concentration.
   - Add 3.5 µl 50 mM Hepes pH 7.4 to Calibrator to obtain a 500 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**

   See 4.1 for assay sensitivity with various concentrations of substrate and Appendix A for tips on working with Lipids

4. **Prepare 6X ATP Solution (100 µM final concentration)**
   - Prepare desired amount of 600 µM ATP working solution in **cAB**.

5. **Prepare 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 3X Calibrator (1 µM final concentration)**
   - Prepare desired amount of 3 µM substrate working solution in **cAB** (Calibrator is used as a positive control only; not intended for back calculation of enzyme conversion)

7. **Prepare 3X Enzyme Working Solution**
   - To the appropriate amount of **cAB**, add enzyme that is 3X the desired final concentration (1 – 100 nM final concentration is recommended, depending on application).

8. **Combine Reagents (**Into appropriate wells, dispense):
   - 2.5 µl Inhibitor / **cAB** solution
   - 2.5 µl 6X ATP solution
   - 5 µl 3X Substrate or Calibrator solution
   - 5 µl 3X enzyme / **cAB** 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 Section IV was obtained 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 B).

10. **Prepare 1X Sensor (40 µl per well)**
    - It is important to make the sensor no more than 10 minutes before use
    - Dilute Sensor 1:300 in appropriate amount of Sensor Dilution Buffer

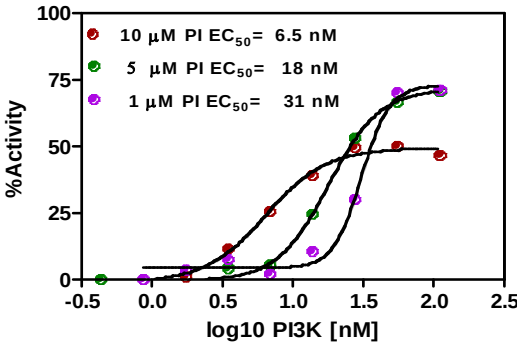
11. **Add Sensor**
    - Dispense 40 µl of 1X Sensor to each well.

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

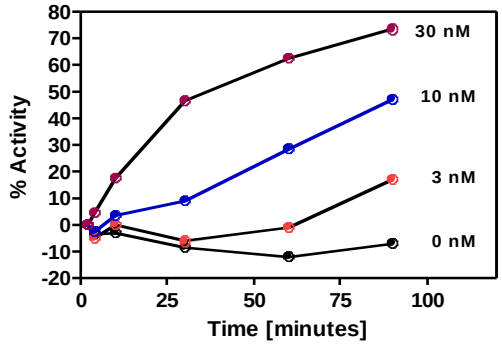
12. **Measure Fluorescence**
    - Shake plate
    - Monitor fluorescence using 540 nm excitation and 580 nm emission.

IV. SAMPLE DATA

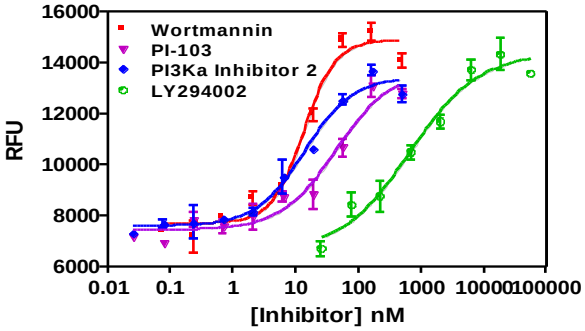
Graphs were generated using GraphPad Prism™ Software[§]. Curve fit was performed using sigmoidal dose response (variable slope). Error bars represent one standard deviation from the mean of two replicates



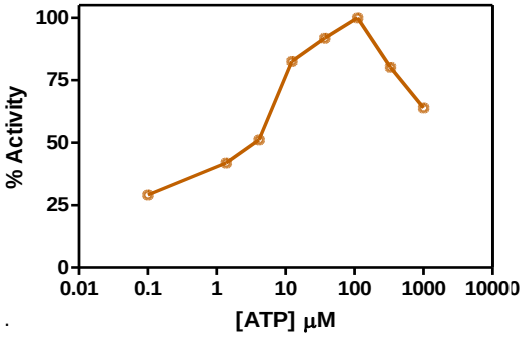
Enzyme Dose Response Curve: Decreasing concentrations of enzyme were mixed withvarious concentrations of substrate in **cAB** containing 100 µM ATP.



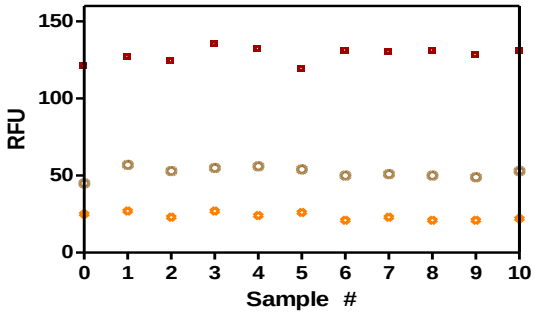
Progress Curves: Various amounts of enzyme were added to wells containing substrate (5 µM) and reactions started by addition of enzyme/ATP (100 µM ATP). Reactions were terminated at various time points by addition of 5 µl post-reaction buffer containing EDTA (5 mM final concentration).



Inhibition Curve: Varying amounts of Inhibitors were added to enzyme (30 nM) and substrate (5 µM) in cAB containing 75 µM ATP.



ATP Tolerance curve: Varying amounts of ATP were used in the absence or presence of enzyme. Percent activity was calculated based on the delta RFU



between the samples

Statistics: Statistical data were produced using 0% (top) and 100% phospho-calibrators (bottom) or 27 nM PI3-Kα (middle). Z'-factors of 0.8 and 0.7 were obtained using phospho-calibrators or reacted samples, respectively. A Z'-factor of > 0.5 indicates a robust assay [1].

Table 1: IC_{50} values comparison between the obtained and reported values.

Compound	IC_{50} (nM): Observed	Reported[2-5]
Wortmannin	13	10
PI-103	45	8
PI-3Kα inh.2	13	2
LY294002	680	1400

V. 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

VI. References

1. Zhang, JH *et al, J. Biomol. Screen.* (1999) **4**, 67.
2. Fruman, DA *et al, Annu. Rev. Biochem* (2008) **67**, 481.
3. Knight, ZC *et al, Cell* (2006) **125**, 733.
4. Hayakawa M *et al, Bioorg. Med. Chem*. (2006) **14**, 3847.
5. Vlahos CJ *et al, J. Biol. Chem*. (1994) **269**, 5241.

§Prism is a registered trademark of GraphPad.

VII. Purchaser Notification

Warranty: BioVision 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 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.

Appendix A – Working with Lipids

Due to their hydrophobic nature, lipids tend to adhere to plastic surfaces and can be challenging to work with. The following recommendations may help in working with these substrates:

- Use siliconized tips and tubes to reduce loss of lipid.
- Sonicate working solution of lipids before dispensing into wells.
- Phosphatidylinositol performs best when prepared 10-30 minutes before use.

Appendix B – 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 1:150; 1:300; 1:450 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 10 µl of the substrate/enzyme mix to all wells, incubate in wells for ~10 minutes
2. Add 5 µl EDTA to t = 0 wells (Pre-dilute 500 mM EDTA stock to 50 mM; then 5 µl addition results in ~ 12.5 mM final)
3. Add 5 µl 3X ATP to all wells starting with the those in which reactions will proceed the longest
4. Stop the reaction with EDTA at desired time points
5. Incubate the plate for 10 minutes at room temperature
6. Add 5 µl Post Reaction Buffer to all wells, incubate for 10 minutes at room temperature
7. Add 40 µl Sensor and incubate for 60 minutes at room temperature