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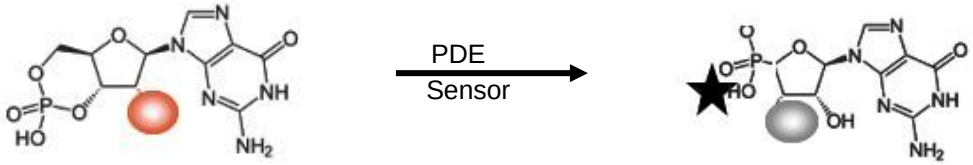
PhosphoSeek™ PDE5A Assay Kit (Catalog No. K705-400; 400 assays)

I. Assay Theory

The PhosphoSeek™ Technology for screening of phosphodiesterase 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. Assays can be performed using defined reaction components (biochemical assay) or for detection of endogenous enzyme activities using cellular lysates. Enzyme progress can be monitored after reactions have occurred or in continuous mode as substrate conversion occurs in real time. The assays are ideally suited for automated screening and can be read on any fluorometer. The Sensor is a proprietary molecule that contains a trivalent metal ion, which binds to phosphoryl groups on biological substrates. Activity is measured by the change of fluorescence of a dye-labeled cyclic guanosine monophosphate (cGMP) upon hydrolysis and binding by Sensor. The change in fluorescence directly correlates to the level of substrate conversion (Figure 1).



Fluor-labeled cGMP Quenched GMP

Figure 1: Schematic depiction of PhosphoSeek Phosphodiesterase (PDE5A) assay: Fluor-labeled cGMP (left, with orange circle) is reacted with a phosphodiesterase resulting in the cleavage at the 3' cyclic phosphate bond. As a result of hydrolysis the Sensor (black star) can associate to the phosphoryl group and quench fluorescence.

II. Kit Components and Storage

Upon arrival, the PhosphoSeek PDE5A 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	10 mM Tris, pH 7.2, 10 mM $MgCl_2$, 4 mM $MnCl_2$, 0.015 % Triton X-100, 0.05 % NaN_3	10 ml	K705-400-1	2-8 °C
Sensor Stock	Stock in 1N HCl	30 µl	K705-400-2	2-8 °C
Sensor Dilution Buffer	MES/NaCl-based, 0.05% NaN_3 pH 6.5	14 ml	K705-400-3	2-8 °C
cGMP Substrate	TAMRA-cGMP 500 µM in DMSO	14 µl	K705-400-4	-20 °C
GMP Calibrator	TAMRA-GMP 500 µM in H_2O	1 µl	K705-400-5	-20 °C
384 well plate	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
- PDE5A Enzyme
- Zaprinast (Biovision Cat# 1715-25)
- IBMX (Biovision Cat# 1714-100)

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. Optimal dilutions of assay components vary between biochemical and lysate-based assays and between endpoint and kinetic monitoring modes. Refer to section III.2 for concentrations of working solutions appropriate for a given application.

Equilibrate Kit Components to room temperature and use within 8 hours of preparation or store according to section 2.0.

III.1 REAGENT PREPARATION

1. **Prepare cGMP Substrate** (1 μM or 3 μM final concentrations for biochemical or lysate based assays, respectively.)
   - Prepare desired amount of substrate working solution in Assay Buffer (**AB**).
2. **Prepare Inhibitor Solutions**
   - Prepare desired inhibitor concentration in an appropriate amount of **AB**. If no inhibitor is used, adjust volume with AB.
3. **Prepare GMP Calibrator** (1 μM or 3 μM final concentrations for biochemical or lysate based assays, respectively.)
   - Prepare desired amount of Calibrator working solution in **AB**. (Calibrator is used as a positive control only; not intended for back calculation of enzyme conversion)
4. **Prepare Enzyme Working Solution**
   - To the appropriate amount of **AB**, add enzyme. Recommended final concentrations are between 0.01 nM – 10 nM (depending on application).

OR

Prepare Lysate Solution

a) From Tissue

- Add 2.5 volumes of complete Lysis Buffer (see ***Appendix A***) to fresh or flash-frozen tissue sample (v/w).
- Process in Dounce homogenizer
- Add 3 volumes of complete Lysis Buffer

b) From Cultured Cells

- Wash cells in TBS and pellet; Add 100 μl complete Lysis Buffer (see ***Appendix A***) per 1 x 10^6 cells.
- Draw cell solution through 20G needle
- Add 3 volumes of complete Lysis Buffer per volume of lysate

➲ ***Incubate for 3 hours at room temperature with gentle shaking***

- Centrifuge at 13,000 rpm for 20 minutes
- Transfer lysate supernatant to fresh vials and discard pellet
- Measure protein concentration using BCA following manufacturers recommendations.
- Dilute sample to 4 μg/μl in Cytobuster reagent (***Appendix A)***
- aliquot and store at -20°C

5. **Prepare 1X Sensor**
   - Prepare 1X Sensor no more than 10 minutes before use.
   - For Endpoint Assays: dilute Sensor in appropriate amount of Sensor Dilution Buffer.
   - For Kinetic Assays: dilute Sensor in appropriate amount of Assay Buffer.
6. **Combine Reagents (R**efer to Section III.2)

   Include appropriate controls

   Substrate – Enzyme – Inhibitor
   Substrate + Enzyme – Inhibitor
   Calibrator – Enzyme – Inhibitor
7. **Measure Fluorescence**
   - Shake plate.
   - Monitor fluorescence (Ex 540 nm/Em 580 nm)
   - 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 the same.

III.2 ASSAY PROTOCOL

Concentrations of working solutions and order of addition of reagents vary depending on application. Biochemical Assays were performed in a 384 well plates while assays with lysate were performed in 96 well plate.

III.2.1 Biochemical Endpoint Assay

Combine

- 5 μl 3X cGMP Substrate (or Calibrator) (1 μM final conc.)
- 5 μl 3X Inhibitor Solution
- 5 μl 3X Enzyme Solution

= 15 μl Reaction Volume

➲ ***Cover plate and incubate for 30–90 minutes***

- 30 μl 1:667 1X Sensor, diluted in Sensor Dilution Buffer

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

Addition of Sensor in Sensor Dilution Buffer will terminate PDE5A activity.

III.2.2 Biochemical Kinetic Assay

Combine

- 15 μl 3X cGMP Substrate (or Calibrator) (1 μM final conc.)
- 15 μl 1:333 1X Sensor, diluted in **AB**

➲ ***Incubate for 5 minutes***

- 7.5 μl 6X Inhibitor Solution
- 7.5 μl 6X Enzyme Solution

= 45 μl Reaction Volume

➲ Read immediately following addition of Enzyme Solution in one-minute intervals for 60-90 minutes.

III.2.3 Endpoint Assay using Lysate

Combine

- 15 μl 4X cGMP Substrate (or Calibrator) (3 μM final conc.)
- 15 μl 4X Inhibitor Solution
- 30 μl Lysate Soultion

= 60 μl Reaction Volume

➲ ***Cover plate and incubate for 30–90 minutes***

- 60 μl 1:667 diluted Sensor

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

Addition of Sensor in Sensor Dilution Buffer will terminate enzyme activity.

III.2.4 Kinetic Assay using Lysate

Combine

- 10 μl 6X cGMP Substrate (or Calibrator) (3 μM final conc.)
- 15 μl 1:333 Sensor, diluted in **AB**

➲ ***Incubate for 5 minutes***

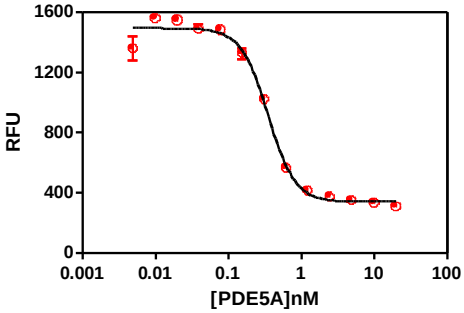
- 5 μl 12X Inhibitor Solution
- 30 μl Lysate Solution

= 60 μl Reaction Volume

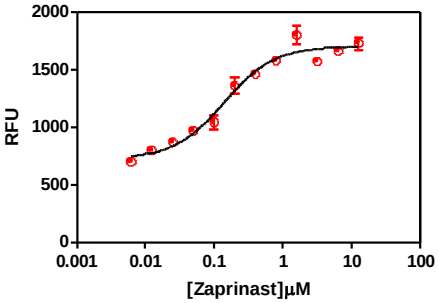
➲ Read immediately following addition of lysate in one-minute intervals for 60-90 minutes.

IV. SAMPLE: ENDPOINT DATA

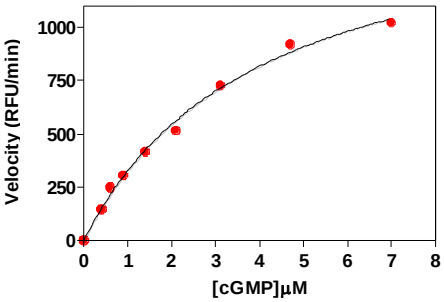
Graphs were generated using GraphPad Prism™ Software[§]. Curve fit was performed using sigmoidal dose response (variable slope). Error bars represent one standard deviation from the mean of two replicates.



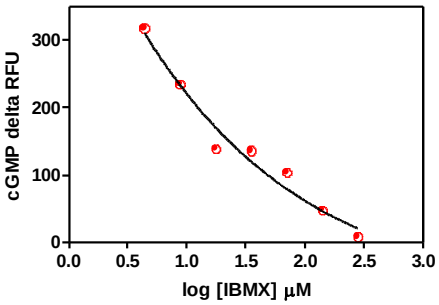
Biochemical Endpoint Enzyme Dose Response Curve: Decreasing concentrations of enzyme were mixed with substrate (1 μM) in Assay Buffer. Relative fluorescence units (RFU) increase as a function of enzyme activity. The EC_{50} is 357 pM



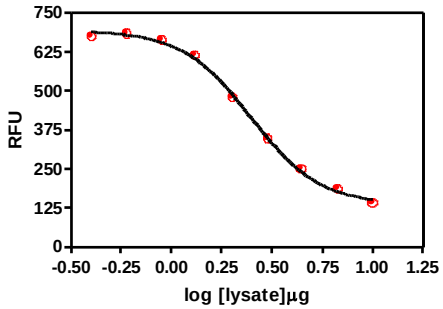
Biochemical Endpoint Inhibition Curve: Decreasing amounts of Zaprinast in DMSO were prepared and added to wells containing enzyme (1.35 nM) and substrate (1 μM) in Assay Buffer. The obtained IC_{50} value of 138 nM is close to the reported literature value [1].



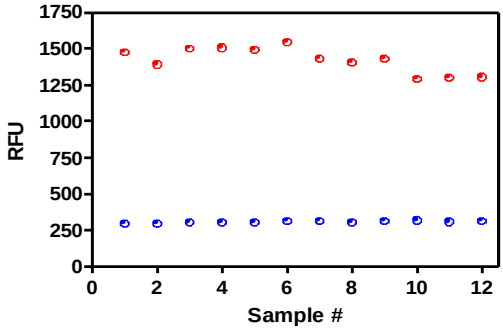
Biochemical Endpoint Substrate K_m: Various concentrations of substrate were reacted with 4 nM PDE5A and the reaction was monitored in kinetic mode over time. Slopes within the linear range of reaction the reaction were plotted as a function of substrate concentration. A V_{max} of 1636 RFU/min and a K_m of 4.0 μM were calculated using using the Michaelis-Menten equation (Graph Pad Prism)



Lysate Enpoint Inhibition Curve: Decreasing amounts of IBMX in DMSO were prepared and added to wells containing lysate (4μg) and substrate (3 μM) in Assay Buffer.

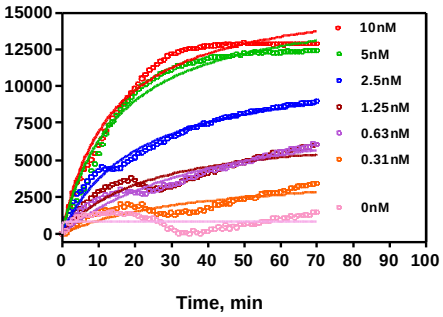


Endpoint Lysate Tolerance Curve. Rat Brain cell lysates were titrated by adding decreasing amounts of cell lysate from whole rat brain to wells containing substrate (3 μM) in Assay Buffer.

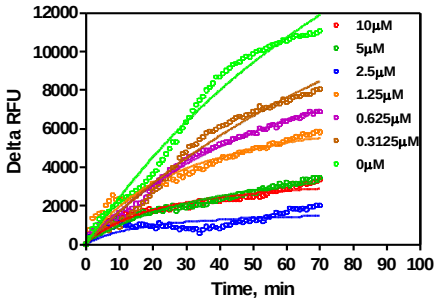


Biochemical Enpoint Statistics: Statistical data were produced using 0 nM (top) and 3.6 nM (bottom) PDE5A. A Z'-factor of 0.75 was obtained. A Z'-factor of >0.5 indicates a robust assay [2].

V. SAMPLE: KINETIC DATA



Kinetic Mode: Various amounts of enzyme were added to wells containing substrate (1 μM) and sensor diluted in Assay Buffer. Fluorescence was monitored every minute over a period of 70 minutes.



Zaprinast Dose Response Curve in Kinetic Mode: Various amounts of inhibitor were added to wells containing substrate (1 μM) and Sensor diluted in Assay Buffer. Reactions were initiated with the addition of enzyme (4 nM) and fluorescence was monitored every minute over a period of 70 minutes.

§Prism is a registered trademark of GraphPad.

VI. 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	0.7 %
EDTA	3 mM
Sodium Orthovanadate	2 mM
Sodium Tartrate	2 mM
Tris	50 mM

VII. REFERENCES

1. Gillespie, P.G. *et al. Mol. Pharmacol.* (1989) **36,** 773.
2. Zhang, JH *et al, J. Biomol. Screen.* (1999) **4**, 67.

VIII. 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.

Appendix A: Composition of Complete Lysis Buffer

- 5 ml Cytobuster Protein Extraction Reagent (Novagen Cat# 71009)
- 33 μl Phosphatase Inhibitor Cocktail 1 (Sigma Cat# P2850)
- 25 μl Phosphatase Inhibitor Cocktail 2 (Sigma Cat# P5726)
- 50 μl 500 mM DTT
- 1 Complete Mini Tablet (Roche Cat# 14791200)
- 5 μl 500 mM Imidazole
- 3 μl 500 mM Sodium Molybdate
- 3 μl 500 mM Sodium Tartrate