Description

The PDE6C Assay Kit is a fluorescence polarization (FP), homogeneous, 96-well assay kit designed for the screening and profiling of PDE6C (Phosphodiesterase 6C) inhibitors. This assay takes advantage of a specific fluorescent phosphate-binding nanoparticle. The kit contains enough purified recombinant PDE6C, fluorescent probe, PDE assay buffer, Binding Agent, and diluent for 100 reactions.

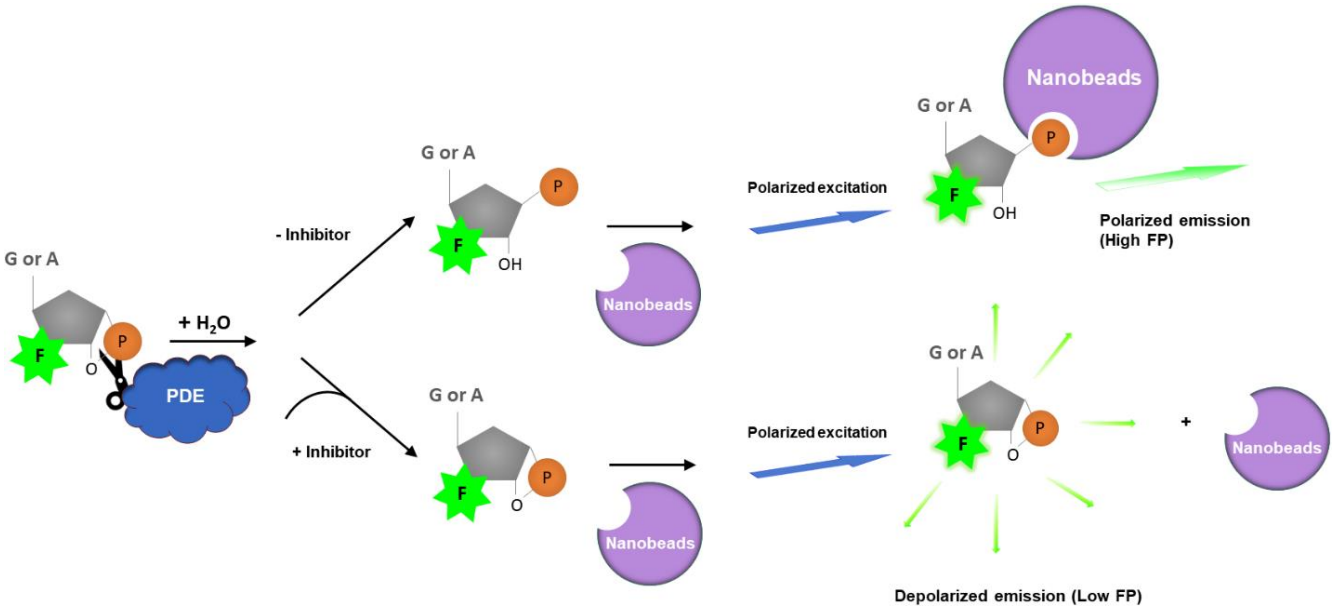

Figure 1: Illustration of the PDE6C Assay Kit principle.

The assay uses a fluorescein-labeled cyclic guanosine monophosphate (cGMP-FAM for PDE6C), in which the phosphate group is engaged within the cyclic nucleotide. This is a very small molecule that rotates fast (low FP). PDE6C catalyzes the hydrolysis of the phosphodiester bond in the cyclic nucleotide and frees the phosphate group. In a second step the free phosphate group is recognized by a specific phosphate-binding nanobead (Binding Agent) leading to the formation of a large complex, with restricted movement (high FP). FP is proportional to PDE6C activity.

This assay requires a fluorescent microplate reader capable of measuring fluorescence polarization (FP) and equipped with the required parts to read the FP signal. For more information FP technology, visit our Tech Note: FP, assay principles and applications.

Note: As of July 2024, this protocol has been re-optimized for performance. Previous versions of this kit are available upon request.

Background

Phosphodiesterases (PDEs) play an important role in the dynamic regulation of the second messengers cAMP (cyclic adenosine monophosphate) and cGMP (cyclic guanosine monophosphate) signaling, by hydrolyzing them. The PDE superfamily is composed of 11 families, with PDE4, 7 and 8 being cAMP-specific hydrolases, and thus regulating positive and negative responses to it. PDE6C, also known as cGMP-inhibited phosphodiesterase, is involved in light detection and cone phototransduction. PDE6C encodes the alpha-prime subunit of cone-specific phosphodiesterase, an enzyme found exclusively in the light-detecting (photoreceptor) cells called cones. Cone cells are found in a specialized tissue at the back of the eye known as the retina, and mutations in PDE6c are

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associated with eye diseases including Cone Dystrophy 4 and Achromatopsia. Continuing to understand PDE6C roles and the development of newer specific therapeutic tools will open new treatment avenues in PDE-related disorders.

Applications

Study enzyme kinetics and screen small molecule inhibitors for drug discovery in high throughput screening (HTS) applications.

Supplied Materials

Catalog #	Name	Amount	Storage
60062	PDE6C, GST-Tag*	>1 µg	-80°C
60201	FAM-Cyclic-3', 5'-GMP**	2 x 1.2 nmoles**	-80°C
60393	PDE Assay Buffer (Incomplete)	25 ml	-20°C
60390	PDE Binding Agent	100 µl	4°C
60392	Binding Agent Diluent (cGMP)	10 ml	4°C
	0.5 M DTT	200 µl	-20°C
79685	Low binding, black 96-well plate	1	Room Temp.

** The concentration of protein is lot-specific and will be indicated on the tube containing the protein.*
*** FAM-Cyclic-3', 5'-GMP is provided as a powder. Each vial will need to be resuspended in 400 µl of Complete PDE Assay Buffer before use.*

Materials Required but Not Supplied

- Adjustable micropipettor and sterile tips
- Rotating or rocker platform
- Fluorescent microplate reader capable of measuring fluorescence polarization (λex=470 (5 nm bandwidth) and detection at λem=528 (10 nm bandwidth)

Stability

This assay kit will perform optimally for up to **6 months** from date of receipt when the materials are stored as directed.

Safety

This product is for research purposes only and not for human or therapeutic use. This product should be considered hazardous and is harmful by inhalation, in contact with skin, eyes, clothing, and if swallowed. If contact occurs, wash thoroughly.

Contraindications

- The final concentration of DMSO in the assay should not exceed 1%.
- Fluorescent compounds that have λex=470 nm and detection at λem=528 nm can interfere with the readouts.

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- It is recommended that the compound alone is tested to determine any potential interference of the compound on the assay results.

Assay Protocol

- All samples should be run in duplicate while controls should be performed in quadruplicate.
- The assay should include "Blank", "Reference Control" (minimum FP), "Positive Control" (maximum FP), and "Test Inhibitor" conditions.
- It is recommended all controls are run side by side as they may be necessary for result calculation.
- We recommend using Sildenafil Citrate (#27105) as an internal control for the assay. If not running a dose response curve for the control inhibitor, run at 0.1X, 1X and 10X the IC_{50} value shown in the validation data below.
- We recommend maintaining the diluted protein on ice during use.
- For detailed information on protein handling please refer to Protein FAQs (bpsbioscience.com).
- For instructions on how to prepare reagent dilutions please refer to Serial Dilution Protocol (bpsbioscience.com).

1. Prepare Complete PDE Assay Buffer by adding 20 µl of 0.5M DTT to 10 ml of PDE Assay Buffer (Incomplete).

2. Thaw **PDE6C** on ice. Briefly spin the tube containing the enzyme to recover its full content.

3. Dilute PDE6C with Complete PDE Assay Buffer to 13 pg/µl (40 µl/well), by performing a serial dilution. For instructions on how to prepare reagent dilutions please refer to Serial Dilution Protocol (bpsbioscience.com).

4. Add 40 µl of diluted PDE6C to the "Positive Control" and "Test Inhibitor" wells.

5. Add 45 µl of Complete PDE Assay Buffer to the "Blank" wells.

6. Add 40 µl of Complete PDE Assay Buffer to the "Reference Control" wells.

7. Prepare the Test Inhibitor (5 µl/well): for a titration prepare serial dilutions at concentrations 10-fold higher than the desired final concentrations. The final volume of the reaction is 50 µl.

   7.1 If the Test Inhibitor is water-soluble, prepare serial dilutions 10-fold more concentrated than the desired final concentrations in Complete PDE Assay Buffer.

   For the positive and negative controls, use Complete PDE Assay Buffer as Diluent Solution.

   OR

   7.2 If the Test inhibitor is soluble in DMSO, prepare the inhibitor in 100% DMSO at a concentration 100-fold higher than the highest desired concentration, then dilute the inhibitor 10-fold in Complete PDE Assay Buffer to prepare the highest concentration of the 10-fold intermediate dilutions. The concentration of DMSO is now 10%.

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Use 10% DMSO in Complete PDE Assay Buffer (vol/vol) for the serial dilution to keep the concentration of DMSO constant.

For positive and negative controls, prepare 10% DMSO in Complete PDE Assay Buffer (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).

Note: The final concentration of DMSO should not exceed 1%.

8. Add 5 µl of the inhibitor serial dilution to the "Test Inhibitor" wells.

9. Add 5 µl of the Diluent Solution to the "Blank", "Reference Control", and "Positive Control" wells.

10. Resuspend one vial of FAM-Cyclic-3', 5'-GMP in 400 µl of Complete PDE Assay Buffer to make a 3 µM solution.

11. Initiate the reaction by adding 5 µl of FAM-Cyclic-3', 5'-GMP (3 µM) to each well designated for the "Reference Control," "Positive Control," and "Test Inhibitor."

12. Incubate at Room Temperature (RT) for 1 hour.

Component	Blank	Reference Control	Positive Control	Test Inhibitor
FAM-Cyclic-3', 5'-GMP	-	5 µl	5 µl	5 µl
Complete PDE Assay Buffer	45 µl	40 µl	-	-
Test Inhibitor	-	-	-	5 µl
Diluent Solution	5 µl	5 µl	5 µl	-
Diluted PDE6C (13 pg/µl)	-	-	40 µl	40 µl
Total	**50 µl**	**50 µl**	**50 µl**	**50 µl**

13. Gently mix the tube containing the **PDE Binding Agent** and dilute 100-fold with **Binding Agent Diluent (cGMP)** (100 µl/well).

14. Add 100 µl of diluted PDE Binding Agent to each well.

15. Incubate at RT for 30 minutes with gentle agitation (*Note: The signal is stable from 20 to 60 minutes*).

16. Read FP in a fluorescence plate reader capable of measuring fluorescence polarization (λex = 470 (5 nm bandwidth); λem = 528 (10 nm bandwidth)) **and set to FP**.

17. Subtract the "Blank" value from all other values.

Calculating Results

Fluorescence polarization is a measure of the amount of molecular rotation that takes place in the time between excitation and emission of the fluorescence probe. It can be determined from the measurements of perpendicular ($I_{\perp}$) and parallel ($I_{\parallel}$) fluorescence intensity values emitted by the probe relative to the direction of the polarized excitation light (Figure 2).

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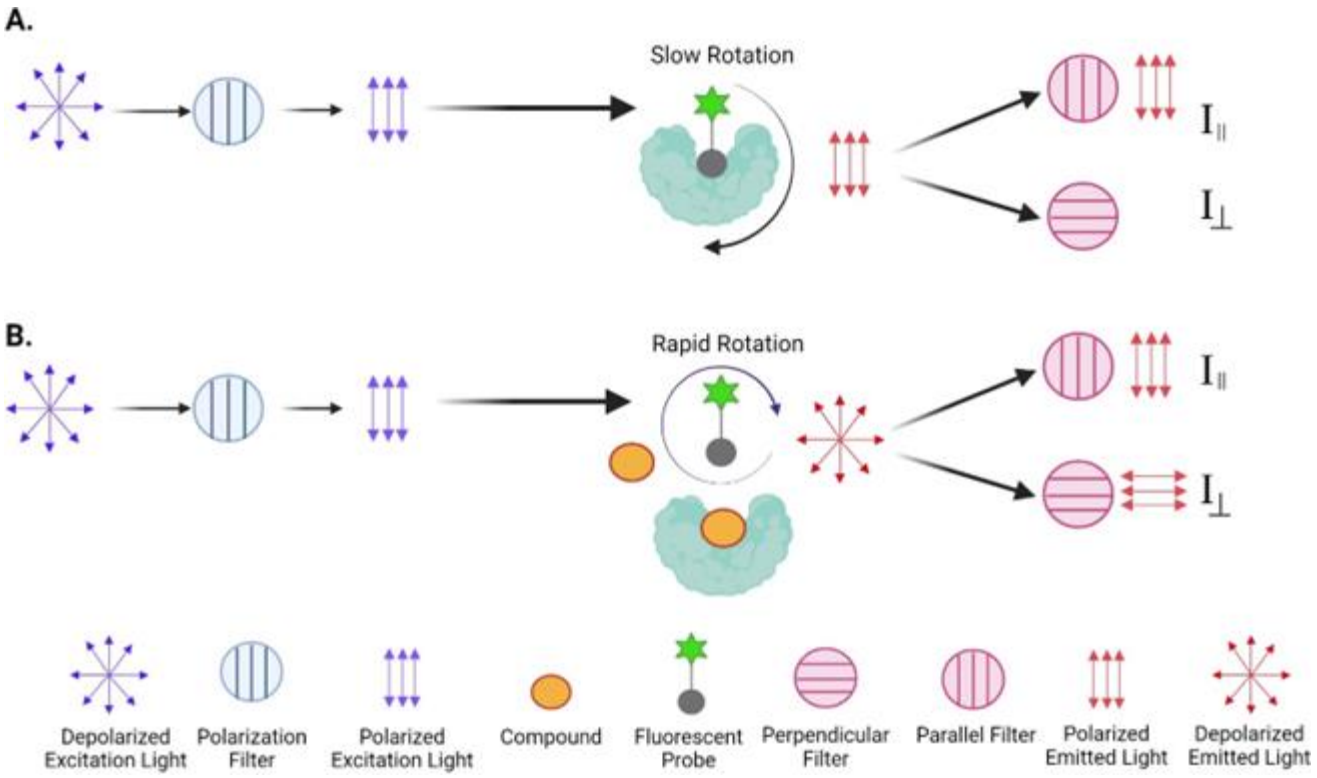

Figure 2: Fluorescence polarization principle.
A. When the fluorescently labeled probe binds to a larger protein it creates a complex of a big molecular weight that has a slow rotation ability. In this state the probe has a reduced rotational diffusion so when it is excited by polarized light, it still emits highly polarized light with a degree of polarization that is inversely proportional to the rate of molecular rotation.
B. In the presence of a compound that has affinity for the protein, the fluorescent probe remains in solution and can rotate rapidly. Unbound probe has a high rotational diffusion so when it is excited by the polarized light it emits light in orientations that can be detected by both the perpendicular and parallel filters.

Polarization is defined as the difference between the emission intensities of parallel fluorescence ($I_{||}$) and perpendicular fluorescence ($I_{\perp}$), divided by the total fluorescence emission intensity. The polarization value (P) being a ratio of light intensities, is a dimensionless number, often expressed in milli P units where 1 P unit = 1000 mP units. To calculate P one has to take into consideration that light is not transmitted equally well through both parallel and perpendicular channels and therefore a correction must be made. This correction factor is called the "G Factor" (G) and it is specific to the instrument used. mP can thus be calculated using the following formula:

$$mP = \left(\frac{I_{II} - G(I_{\perp})}{I_{II} + G(I_{\perp})}\right) x\ 1000$$

Modern instruments usually have the G factor pre-calculated and can automatically calculate fluorescence polarization for your experiments. If you need to determine, set up or calculate the G factor please refer to your instrument manual (the instrument manual should contain information about how to establish the G-factor) or check our FAQ section (FAQs (bpsbioscience.com).

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For accurate calculations it is necessary to provide the correct plate schematic when setting up your instrument, with defined positions for the "Blank" and "Reference" (also known as Substrate Control) wells, and to ensure that the emission intensities from the "Blank" wells are subtracted from all other wells prior to further data analysis.

We encourage you to analyze raw data and if appropriate to exclude those "Blank" or "Reference" wells that show aberrant readouts prior to mP determination.

The % of Activity can be calculated as follows:

$$\%\ of\ Activity = \frac{(mP\ value\ from\ Test\ Inhibitor - mP\ value\ from\ Reference\ Control)}{(mP\ value\ from\ Positive\ Control - mP\ value\ from\ Reference\ Control)} x\ 100$$

Example of Assay Results

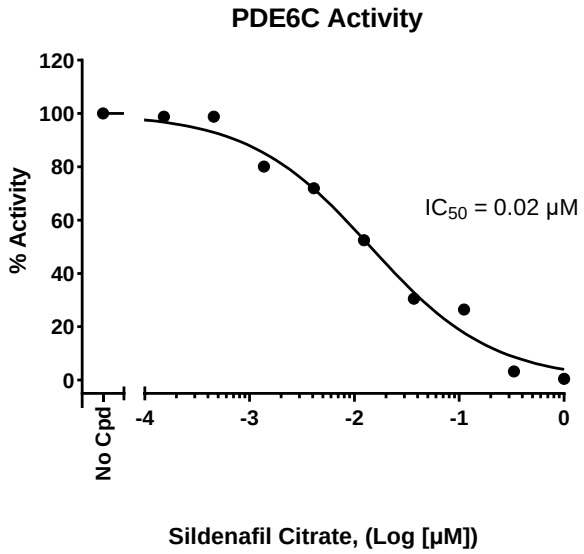

Figure 3: Inhibition of PDE6C by Sildenafil Citrate.
PDE6C was incubated with increasing concentrations of Sildenafil Citrate (#27105) in the presence of 300 nM FAM-Cyclic-3', 5'-GMP substrate. Fluorescence Polarization was measured using a Tecan M1000 fluorescent microplate reader. Results are expressed in % activity, in which FP in the absence of inhibitor (positive control) is set to 100%.

Data shown is representative. For lot-specific information, contact BPS Bioscience, Inc. at support@bpsbioscience.com.

Troubleshooting Guide
Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For further questions, email support@bpsbioscience.com

References
Ahmed W., *et al.*, 2021 *Biomedicine & Pharmacotherapy* 134:111128.
Huang W., *et al.*, 2020 *Nat Commun* 11:3191.

Related Products

Products	*Catalog #*	*Size*
PDE5A1 Assay Kit	60351	384 reactions
PDE5A1, GST-Tag Recombinant	60050	10 µg
Taladafil	27657	50 mg/100 mg/500 mg
Vardenafil HCl Trihydrate	27776	10 mg/100 mg
PDE7A/CRE Reporter – HEK293 Recombinant Cell Line	60413	2 vials
PDE10A – HEK293 Recombinant Cell Line	60410	2 vials

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