Prostaglandin D Synthase (hematopoietic-type) FP-Based Inhibitor Screening Assay Kit - Green

Item No. 600007

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Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. For best results, remove components and store as stated below.

Item Number	Item	384 wells Quantity	1,920 wells Quantity	Storage
600025*	H-PGDS FP Fluorescent Probe - Green	1 vial	5 vials	-20°C
600026	H-PGDS (human recombinant)	1 vial	5 vials	-80°C
600027	HQL-79 FP Positive Control	1 vial	5 vials	-20°C
600028	FP Assay Buffer Concentrate (4X)	1 vial	5 vials	-20°C
600029	H-PGDS FP Glutathione	1 vial	5 vials	-20°C
10005371	384-Well Solid Plate (black; non-binding)	1 plate	5 plates	RT
400023	Foil Plate Covers	1 cover	5 covers	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.
**Patent pending.*

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's PGDS FP-Based Inhibitor Screening Assay Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone:	888-526-5351 (USA and Canada only) or 734-975-3888
Fax:	734-971-3641
E-Mail:	techserv@caymanchem.com
Hours:	M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section, on page 3, and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring fluorescence polarization measurements using fluorescein as the fluorophore
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable
4. Microcentrifuge tubes (2 ml)

INTRODUCTION

Background

There are two distinct types of prostaglandin D synthases involved in PGD_2 production: lipocalin-type PGDS (L-PGDS) and hematopoietic PGDS (H-PGDS).[1] L-PGDS and H-PGDS are different from each other in terms of their primary amino acid sequence, cellular localization and tertiary structure.[2,3] L-PGDS, also known as β-trace, is localized in the central nervous system, male genital organs, and heart and is involved in the regulation of sleep and pain.[4] H-PGDS is associated with allergic and inflammatory reactions due to its localization in mast cells, Th2 cells, microglia, necrotic muscle fibers, and apoptotic smooth muscle cells.[4] Two well known inhibitors of H-PGDS, namely HQL-79 and tranilast, were both shown to reduce PGD_2 levels in guinea pig lung tissues chronically treated with the inhibitors.[5,6] Both inhibitors have micromolar IC_{50} values; therefore, the need to develop more potent inhibitors of the enzyme is evident. Recent patent literature describes pyrimidine amide-based compounds as H-PGDS inhibitors with low nanomolar IC_{50} values, however, to date, their effectiveness in a clinical setting has not been reported.

About This Assay

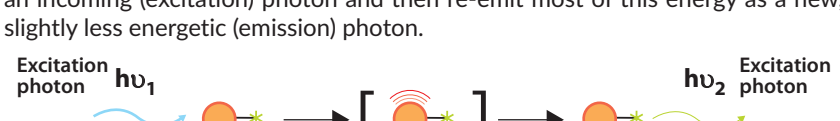
Cayman's H-PGDS FP-Based Inhibitor Screening Assay Kit - Green provides a convenient fluorescence polarization-based single step assay for screening H-PGDS inhibitors. This kit eliminates the need for the multistep assay currently used for PGD Synthase that requires the use of the unstable PGH_2 substrate. In this new assay, a potent inhibitor of H-PGDS was conjugated to fluorescein and is used as the displacement probe. Inhibitors of H-PGDS will displace the fluorescent probe leading to a decrease in fluorescence polarization (FP).[7] The PGDS FP-Based Inhibitor Screening Assay Kit is a robust assay with a Z´ of 0.75 and has a dynamic range of greater than 150 mP units. The assay has been validated using known inhibitors of H-PGDS (Tranilast, HQL-79, BSPT, etc.) with IC_{50} values ranging from nanomolar to millimolar concentrations.

It is important to note that in this assay, the binding affinity of an inhibitor is determined relative to a fluorescently-labeled inhibitor. Therefore, the inhibition value determined in this assay may not give the same value as that determined in an enzymatic assay. Nevertheless, a high correlation between assay techniques is typically observed.

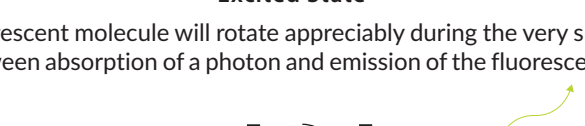
Introduction to FP

Fluorescence polarization (FP) assays are homogeneous, single-step assays ideally suited for high-throughput screening (HTS) of large numbers of samples. All FP assays employ a large molecular species, or binding partner (BP) in conjunction with a small, low molecular weight fluorescent analyte (FA).

Fluorescence is, by definition, the ability of a molecule to absorb the energy of an incoming (excitation) photon and then re-emit most of this energy as a new, slightly less energetic (emission) photon.



A small fluorescent molecule will rotate appreciably during the very small interval of time between absorption of a photon and emission of the fluorescence photon.



If the excitation light is polarized, this rotation will result in complete randomization of the plane of the emitted light. Thus, small fluorescent molecules depolarize an excitation pulse of polarized light (see well #1 in Figure 1, below).

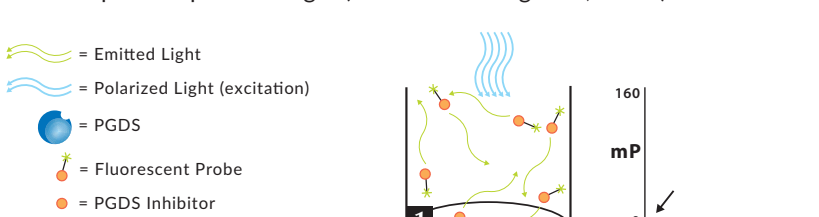


Figure 1.

Large fluorescent molecules do not rotate appreciably in the same small interval of time. They will, therefore, emit light that retains some of the polarization of the polarized excitation light. This polarization is quantified as milli-polarization units, or mP. A fluorescence polarization reader is required to make this measurement.

When a small fluorescent molecule becomes tightly bound to a large one, as in the binding of H-PGDS to the fluorescent probe, the rotational speed of the small molecule is abruptly reduced to that of the entire complex as a whole (see well #2 in Figure 2, below).

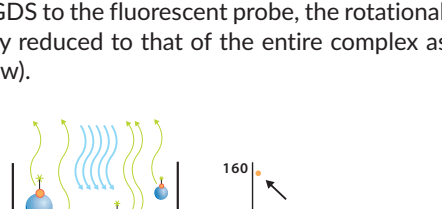


Figure 2.

Therefore, the fluorescent probe bound to H-PGDS represents a large fluorescent molecule, which exhibits a high degree of FP. A microplate well filled with the fluorescent probe: H-PGDS complex will give a high FP reading. The PGDS FP-based Inhibitor Screening Assay Kit is based on the competition of free inhibitor in the samples or standards for the high affinity binding site of H-PGDS occupied by the fluorescent probe. Addition of a small amount of H-PGDS inhibitor will result in the displacement of the fluorescent probe from the H-PGDS binding site (Figure 3, below).

Figure 3.

Some of the fluorescent probe will be released from the H-PGDS and will resume its intrinsic, rapid rate of rotation. This will cause a detectable loss of FP in the well (see well #4 in Figure 4, below).

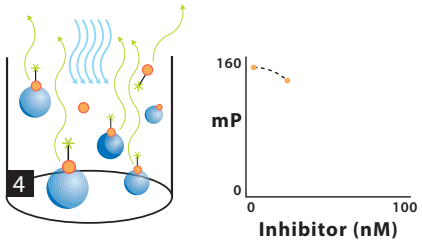


Figure 4.

The addition of large amounts of an H-PGDS inhibitor will result in a much larger reduction in the mP of the well (see well #5 in Figure 5, below). Plotting mP *versus* inhibitor concentration allows the construction of an inhibition curve with a broad dynamic range.

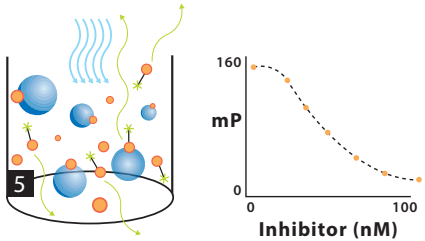


Figure 5.

Cayman's H-PGDS FP-Based Inhibitor Screening Assay Kit allows for the rapid identification of H-PGDS inhibitors with a wide range IC_{50} values.

PRE-ASSAY PREPARATION

NOTE: Water used to prepare all FP reagents and buffers must be deionized. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are adequate for this kit. UltraPure water may be purchased from Cayman (Item No. 400000).

Buffer Preparation

Store all diluted buffers at 4°C; they will be stable for about six months.

FP Assay Buffer Concentrate (4X)

Dilute the contents of one vial of FP Assay Buffer Concentrate (4X) (Item No. 600028) with 18 ml of deionized water to a final volume of 24 ml to yeild a 1X FP Buffer. Be certain to mix the vial to resuspend any salts that may have precipitated. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.*

Inhibitor Preparation

Inhibitors can be dissolved in DMSO, ethanol, or methanol. A final volume of 2.5 µl should be added to each inhibitor well. We recommend that several concentrations of inhibitor be used when effective inhibition is unknown. In addition, it is important to add 2.5 µl of the same solvent used to prepare inhibitor solutions to the 100% activity wells.

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

H-PGDS FP Fluorescent Probe - Green

Dilute the H-PGDS FP Fluorescent Probe (Item No. 600025) with 180 µl of 1X FP Buffer.

H-PGDS (human recombinant)

Dilute H-PGDS (human recombinant) (Item No. 600026) with 900 µl of 1X FP Buffer.

HQL-79 FP Positive Control

To prepare the Positive Control for use in the FP: Obtain 12 clean microfuge tubes and label them 1 through 12. Add 100 µl of HQL-79 FP Positive Control (Item No. 600027) to tube A12 and 50 µl DMSO to tubes A1-A11. Serially dilute the control by removing 50 µl from tube A12 and place it in tube A11; mix thoroughly. Next, remove 50 µl from tube A11 and place it into tube A10; mix thoroughly. Repeat this process for tubes A9-A2. The diluted controls should be used immediately.

H-PGDS FP Glutathione (GSH)

Each vial of H-PGDS FP GSH (Item No. 600029) contains 1,500 µl of 100 mM solution. It is ready to use as supplied for use in the Assay Cocktail.

Assay Cocktail

NOTE: 19 ml of reconstituted assay cocktail is enough for either a standard 96-well, 384-well, or higher density plate. Store any unused reagents at -20°C and use within 30 days.

To prepare the assay cocktail combine the volumes shown in table 1 into a 50 ml conical tube and mix thoroughly.

Component	Volume
FP Assay Buffer	18.65 ml
H-PGDS FP Fluorescent Probe	138 µl
H-PGDS FP GSH	1,250 µl
H-PGDS (human recombinant)	880 µl

Table 1. Assay cocktail preparation

Performing the Assay

Pipetting Hints

- Use different tips to pipette the buffer, probe, reagent, and protein.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Follow the steps below to accurately measure mP in the assay. *NOTE: Volumes shown are for a 384-well plate format. For a 96-well plate format, use 190 µl of Assay Cocktail and 10 µl inhibitor standard or inhibitor of interest.*

1. **Assay Cocktail**

   Add 47.5 µl of the Assay Cocktail to each well of a 384-well plate.

2. **Maximum Binding Wells/100% Activity Wells**

   Add 2.5 µl of tube 1 to wells A1 and B1.

3. **HQL-79 FP Positive Control**

   Add 2.5 µl of tube 2 to wells A2 and B2 of a 384-well plate. To wells A3 and B3 add 2.5 µl from tube 3. Continue with this procedure until all the standards are aliquoted.

4. **Inhibitors**

   Add 2.5 µl of inhibitor per well. It is recommended that each concentration of inhibitor be assayed at least in duplicate (triplicate recommended). To determine IC_{50}s for a particular inhibitor, a full concentration titration should be performed as described for the HQL-79 FP Positive Control. Comparison of a single concentration of inhibitor to the maximum binding well will provide an assessment of the relative affinity of the inhibitor for H-PGDS.

5. **Incubation of the Plate**

   Cover the plate with the plate cover supplied in the kit and incubate for 60-90 minutes at room temperature. The FP signal is stable for at least two hours.

6. **Reading the Plate**

   Read the plate(s) with excitation and emission wavelengths of 470 nm and 530 nm, respectively. The measurements are taken in the fluorescent polarization mode with the z-height set to the middle of the well and the G-factor set to 1.13 on a Tecan Safire 2 reader.

Effect of Solvents

The type of solvent used to prepare/dilute the inhibitor(s) of interest can affect the mP values. If a solvent other than DMSO is used to dissolve the inhibitor of interest, the Maximum Binding/100% Activity Wells should also have 5% of the specific solvent added in place of DMSO. For example, 2.5 µl of solvent should be added to 47.5 µl of Assay Cocktail.

ANALYSIS

Calculations

Fluorescence polarization of a molecule is defined as:

$$\text{Polarization (mP)} = 1{,}000 \times \frac{(I_{\parallel} - I_{\perp})}{(I_{\parallel} + I_{\perp})}$$

A plot of mP *versus* inhibitor concentration on semi-log axes results in a sigmoidal dose-response curve typical of competitive binding assays. This data can be fit to a 4-parameter logistic equation as shown in Figure 6 (see page 20).

When full titration curves have been performed, the concentration of inhibitor that reduces the mP signal by 50% (IC_{50}) can be estimated from a graph for each inhibitor tested.

If a particular inhibitor is tested at only one or two concentrations, an estimate of relative efficacy can be determined using the following equation:

$$\text{\% Signal Reduction} = \frac{\text{(mP 100\% Activity - mP Sample)}}{\text{(mP 100\% Activity)}} \times 100$$

Performance Characteristics

Z´-Factor:

Z´-factor is a term used to describe the quality of an assay,[7] which is calculated using the following equation:

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{\left| \mu_{c+} - \mu_{c-} \right|}$$

The theoretical upper limit for the Z´-factor is 1.0. A robust assay has a Z´-factor >0.5.[8] The Z´-factor for Cayman's H-PGDS FP-Based Inhibitor Screening Kit - Green was determined to be 0.75.

Sample Data

The curve presented here is an example of the data typically produced by HQL-79 with this kit; however, your results will not be identical to these.

HQL-79 (µM)	mP		Average mP
250	84	85	84.5
125	87	91	89
62.5	103	102	102.5
31.25	122	114	118
15.6	146	140	143
7.8	168	157	162.5
3.9	186	182	184
1.95	199	190	194.5
0.98	206	203	204.5
0.49	207	208	207.5
0.24	210	212	211
0	224	215	219.5

Table 2. Typical results

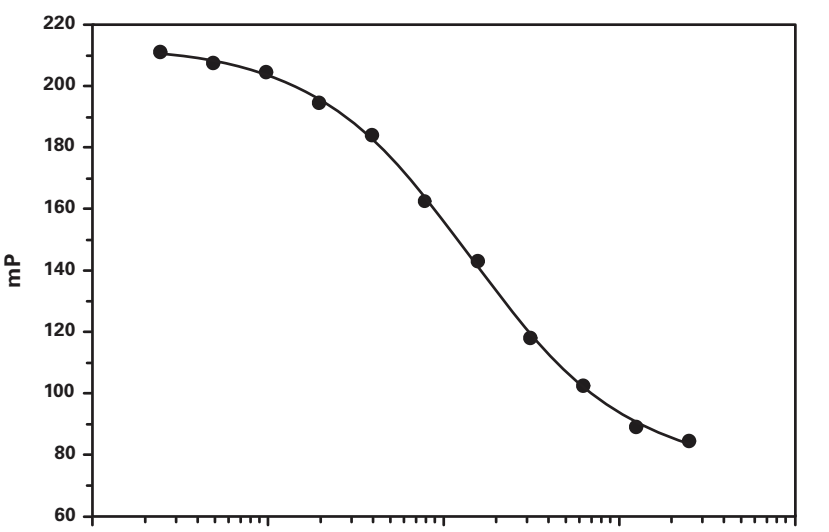


Figure 6. Representative inhibition curve using HQL-79

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
A. Erratic values B. Dispersion of duplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells or try more replicates of inhibitor standard to achieve consistency B. Carefully tap the side of the plate with your finger to remove the bubbles
High background mP	Dilution error	Check the dilution of each component

References

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2. Pinzar, E., Miyano, M., Kanaoka, Y., *et al. J. Biol. Chem.* **275(40)**, 31239-31244 (2000).
3. Inoue, T., Okano, Y., Kado, Y., *et al. J. Biochem.* **135**, 279-283 (2004).
4. Aritake, K., Kado, Y., Inoue, T., *et al. J. Biol. Chem.* **281(22)**, 15277-15286 (2006).
5. Matsushita, K., Nobutoshi, A., and Hizue, A. *Jpn. J. Pharmacol.* **78(1)**, 1-10 (1998).
6. Matsushita, K., Nobutoshi, A., and Aritake, K. *Jpn. J. Pharmacol.* **78(1)**, 11-22 (1998).
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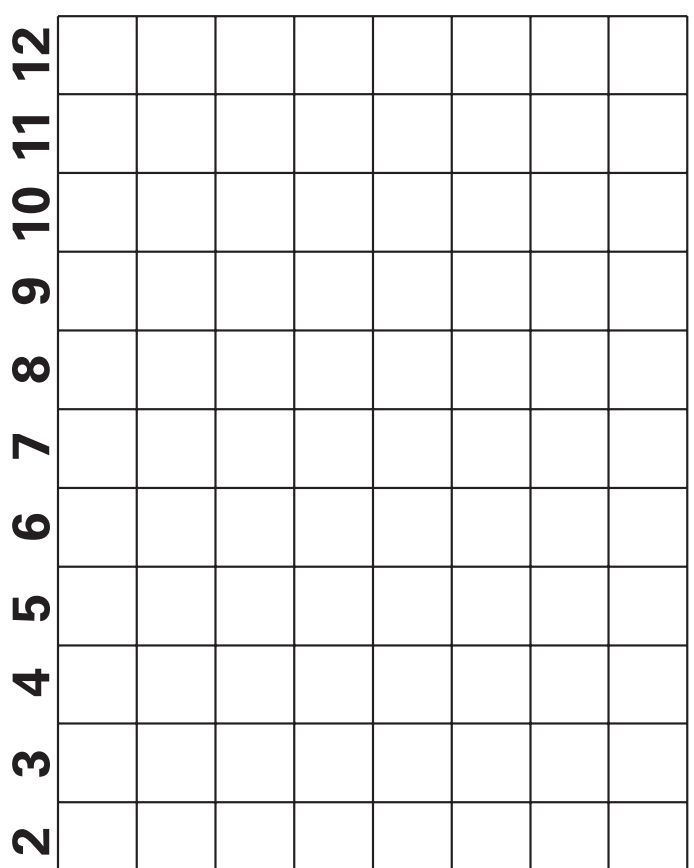
8. Zhang, J.-H., Chung, T.D.Y., and Oldenburg, K.R. *Journal of Biomolecular Screening* **4(2)**, 67-73 (1999).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.