COX Fluorescent Inhibitor Screening Assay Kit

Item No. 700100

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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	Quantity	Storage
700101	COX-FIS Assay Buffer (10X)	1 vial	-20°C
700102	COX-FIS Heme	1 vial	-20°C
700103	COX-1 (ovine) COX-FIS Assay Reagent	1 vial	-80°C
700104	COX-2 (human recombinant) COX-FIS Assay Reagent	1 vial	-80°C
700105	COX-FIS Arachidonic Acid	1 vial	-20°C
700106	COX-FIS Potassium Hydroxide	1 vial	-20°C
700001	DMSO Assay Reagent	1 vial/3 ml	-20°C
700002	ADHP Assay Reagent	4 vials	-20°C
760158	DuP-697 Assay Reagent	1 vial	-20°C
760159	SC-560 Assay Reagent	1 vial	-20°C
400017	96-Well Plate (black)	1 plate	Room temperature
400012	96-Well Cover Sheet	1 cover	Room temperature

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 975-3999. We cannot accept any returns without prior authorization.

WARNING: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: 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 fluorometer with the capacity to measure fluorescence using an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm
2. Adjustable pipettes and a repeat pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Cyclooxygenase (COX, also called Prostaglandin H Synthase or PGHS) is a bifunctional enzyme exhibiting both COX and peroxidase activities. The COX component converts arachidonic acid to a hydroperoxy endoperoxide (PGG_2), and the peroxidase component reduces the endoperoxide to the corresponding alcohol (PGH_2), the precursor of PGs, thromboxanes, and prostacyclins.[1,2]

It is now well established that there are two distinct isoforms of COX. COX-1 is constitutively expressed in a variety of cell types and is involved in normal cellular homeostasis. A variety of stimuli, such as phorbol esters, lipopolysaccharides, and cytokines, lead to the induced expression of a second isoform of COX, COX-2. COX-2 is responsible for the biosynthesis of PGs under acute inflammatory conditions.[3,4] This inducible COX-2 is believed to be the target enzyme for the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs.[4]

About This Assay

Cayman's COX Fluorescent Inhibitor Screening Assay provides a convenient fluorescence-based method for screening both ovine COX-1 and human recombinant COX-2 for isozyme-specific inhibitors. The assay utilizes the peroxidase component of COXs. The reaction between PGG_2 and ADHP (10-acetyl-3,7-dihydroxyphenoxazine) produces the highly fluorescent compound resorufin. Resorufin fluorescence can be easily analyzed with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm (see Figure 1 on page 6).

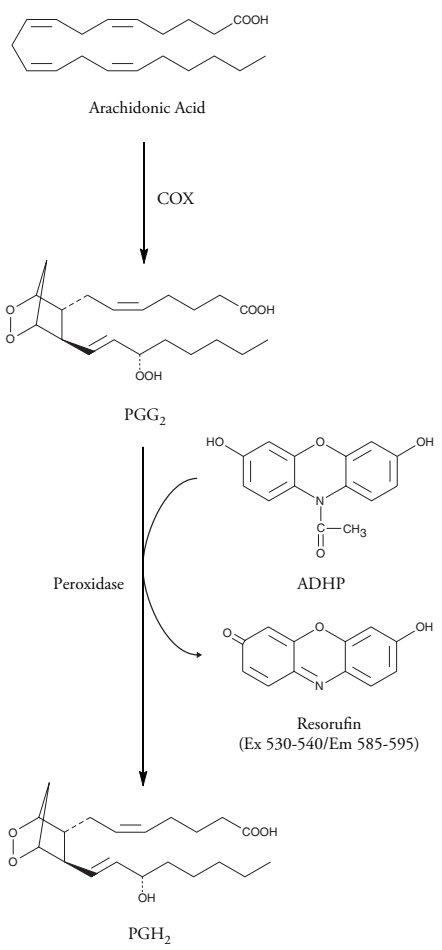


Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. Assay Buffer (10X) - (Item No. 700101)

Dilute 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water. This final Buffer (100 mM Tris-HCl, pH 8.0) should be used in the assay and for diluting reagents. When stored at 4°C, this diluted buffer is stable for at least six months.

2. Heme - (Item No. 700102)

The vial contains 150 µl of heme in dimethylsulfoxide (DMSO). Dilute 40 µl of Heme with 960 µl of diluted Assay Buffer. The diluted Heme is stable for 12 hours at room temperature

3. COX-1 (ovine) - (Item No. 700103)

The vial contains a solution of ovine COX-1 and should be kept on ice when thawed. Dilute 50 µl of enzyme with 550 µl of diluted Assay Buffer and store on ice. This is enough enzyme to use in 60 wells. Adjust the volume accordingly if assaying more wells. The diluted enzyme is stable for one hour. There is enough COX-1 supplied to assay 96 wells.

4. COX-2 (human recombinant) - (Item No. 700104)

The vial contains a solution of human recombinant COX-2 and should be kept on ice when thawed. Dilute 50 µl of enzyme with 550 µl of diluted Assay Buffer and store on ice. This is enough enzyme to use in 60 wells. Adjust the volume accordingly if assaying more wells. The diluted enzyme is stable for one hour. There is enough COX-2 supplied to assay 96 wells.

5. Arachidonic Acid - (Item No. 700105)

The vial contains a solution of arachidonic acid in ethanol. Transfer 100 µl of the supplied solution to another vial, add 100 µl of Potassium Hydroxide (KOH) (Item No. 700106), vortex, and dilute with 800 µl of HPLC-grade water to achieve a final concentration of 2 mM. Use the prepared Arachidonic Acid solution within 30 minutes. A 10 µl aliquot will yield a final concentration of 100 µM in the wells. If a lower concentration is desired, dilute further with HPLC-grade water and use within 30 minutes.

6. Potassium Hydroxide - (Item No. 700106)

The vial contains 0.1 M potassium hydroxide (KOH). The reagent is ready to use as supplied.

7. DMSO Assay Reagent - (Item No. 700001)

The vial contains 3 ml of dimethylsulfoxide (DMSO). The reagent is ready to use as supplied.

8. ADHP Assay Reagent - (Item No. 700002)

The vials contain a clear lyophilized powder of ADHP (10-acetyl-3,7-dihydroxyphenoxazine). Immediately prior to assaying, dissolve the contents of one vial with 100 µl DMSO Assay Reagent (Item No. 700001) and then add 900 µl of diluted Assay Buffer. The reconstituted substrate is stable for 30 minutes. After 30 minutes, increased background fluorescence will occur.

9. DuP-697 - (Item No. 760158) - *optional*

The vial contains 6 µM DuP-697 in DMSO. DuP-697 is a potent inhibitor of COX-2.[5] DuP-697 can be used as a control for screening COX-2 inhibitors. Dilute 50 µl of DuP-697 with 550 µl of DMSO Assay Reagent (Item No. 700001). Assaying 10 µl with COX-2 will yield approximately 50% inhibition (see Figure 4 on page 14).

10. SC-560 - (Item No. 760159) - *optional*

The vial contains 6.6 µM SC-560 in DMSO. SC-560 is a potent inhibitor of COX-1.[6] SC-560 can be used as a control for screening COX-1 inhibitors. Dilute 15 µl of SC-560 with 985 µl of DMSO Assay Reagent (Item No. 700001). Assaying 10 µl with COX-1 will yield approximately 50% inhibition (see Figure 3 on page 13).

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% initial activity and three wells designated as background wells. We suggest that each inhibitor sample be assayed in triplicate and that you record the contents of each well on the template sheet provided on page 19. A typical layout of samples and compounds to be measured in triplicate is given below (see Figure 2).

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	BW	7	7	7	15	15	15	23	23	23
B	A	A	A	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

BW - Background Wells
A - 100% Initial Activity Wells
1-30 - Inhibitor Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- 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.

General Information

- The final volume of the assay is 200 µl in all the wells.
- Use the diluted Assay Buffer in the assay.
- All reagents except the enzymes must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- You do not have to use both enzymes. You can use either COX-1 or COX-2 for the study.
- We recommend assaying samples in triplicate, but it is the user's discretion to do so.
- Thirty inhibitor samples can be assayed in triplicate or forty-six in duplicate.
- The assay is performed at room temperature.
- Monitor the fluorescence with an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm.
- Initiate the reactions with arachidonic acid while in close proximity to the fluorometer as it is necessary to read the plate two minutes after initiation of the reaction.

Performing the Assay

1. **100% Initial Activity Wells** - add 150 µl of Assay Buffer, 10 µl of Heme, 10 µl of ADHP, 10 µl of enzyme (either COX-1 or COX-2), and 10 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
2. **Background Wells** - add 160 µl of Assay Buffer, 10 µl of Heme, 10 µl of ADHP, and 10 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
3. **Inhibitor Wells** - add 150 µl of Assay Buffer, 10 µl of Heme, 10 µl of ADHP, 10 µl of enzyme (either COX-1 or COX-2), and 10 µl of inhibitor* to three wells.
4. Initiate the reactions by **quickly** adding 10 µl of Arachidonic Acid solution to all the wells being used.
5. Incubate for two minutes at room temperature.
6. Read the plate using an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples.

*Inhibitors can be dissolved in Assay Buffer, methanol, ethanol, or DMSO and should be added to the assay in a final volume of 10 µl. In the event that the appropriate concentration of inhibitor needed for COX inhibition is completely unknown, we recommend that several dilutions of the inhibitor be assayed.

ANALYSIS

Calculations

1. Determine the average fluorescence of each sample.
2. Subtract the fluorescence of the background wells from the fluorescence of the 100% initial activity and the inhibitor wells.
3. Determine the percent inhibition for each sample. To do this, subtract each inhibitor sample value from the 100% initial activity sample value. Divide the result by the 100% initial activity value and then multiply by 100 to give the percent inhibition.
4. Graph either the Percent Inhibition or Percent Initial Activity as a function of the inhibitor concentration to determine the IC_{50} value (concentration at which there was 50% inhibition). Examples of COX-1 and COX-2 inhibition by SC-560 and DuP-697, respectively are shown in Figures 3 and 4 (see pages 13 and 14, respectively).

$$\% \text{ Inhibition} = \frac{[\text{Initial Activity} - \text{Sample Activity}]}{\text{Initial Activity}} \times 100$$

Performance Characteristics

Precision:

When a series of ten COX measurements were performed on the same day, the intra-assay coefficient of variation was 2.6%. When a series of ten COX measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 2.8%.

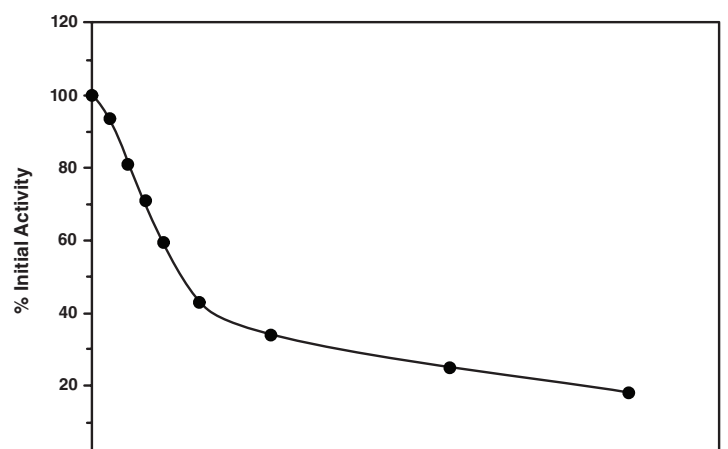


Figure 3. Inhibition of ovine COX-1 by SC-560 (IC_{50} = 5 nM)

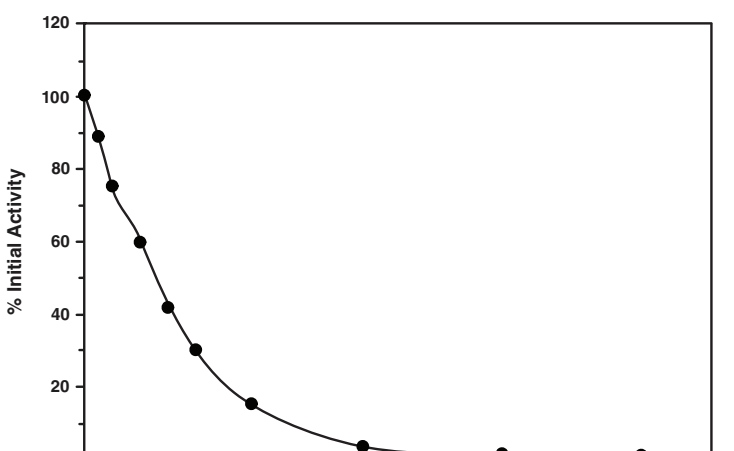


Figure 4. Inhibition of human recombinant COX-2 by DuP-697 (IC_{50} = 25 nM)

RESOURCES

Interferences

Any antioxidant will interfere with the assay and will appear to be a COX inhibitor. Resveratrol is an antioxidant, as well as, a selective inhibitor of COX-1.[7] Using this assay, Resveratrol will also appear to be a COX-2 inhibitor. If the inhibitor being assayed is also an antioxidant, it is recommended that one of Cayman's COX Inhibitor Screening Assays, which utilizes an EIA detection (Item Nos. 560101 or 560131) be used for the inhibition analysis.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No fluorescence detected above background in the inhibitor wells	A. Enzymes were not added to the wells B. Inhibitor concentration was high enough to knock out all of the COX activity	A. Make sure to add all the components to the wells and re-assay B. Reduce the inhibitor concentration and re-assay
The fluorometer exhibited 'MAX' values for the wells	The GAIN setting is too high	Reduce the GAIN and re-read
No inhibition seen with compound	A. The compound concentration is not high enough B. The compound is not an inhibitor of the enzyme	Increase the compound concentration and re-assay

References

1. Nugteren, D.H. and Hazelhof, E. Isolation and properties of intermediates in prostaglandin biosynthesis. *Biochim. Biophys. Acta* **326**, 448-461 (1973).
2. Hamberg, M. and Samuelsson, B. Detection and isolation of an endoperoxide intermediate in prostaglandin biosynthesis. *Proc. Natl. Acad. Sci. USA* **70**, 899-903 (1973).
3. Xie, W., Chipman, J.G., Robertson, D.L., *et al.* Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. USA* **88**, 2692-2696 (1991).
4. Blobaum, A.L. and Marnett, L.J. Structural and functional basis of cyclooxygenase inhibition. *J. Med. Chem.* **50(7)**, 1425-1441 (2007).
5. Kargman, S., Wong, E., Greig, G.M., *et al.* Mechanism of selective inhibition of human prostaglandin G/H synthase-1 and -2 in intact cells. *Biochem. Pharmacol.* **52**, 1113-1125 (1996).
6. Smith, C.J., Zhang, Y., Koboldt, C.M., *et al.* Pharmacological analysis of cyclooxygenase-1 in inflammation. *Proc. Natl. Acad. Sci. USA* **95**, 13313-13318 (1998).
7. Jang, M., Cai, L., Udeani, G.O., *et al.* Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **275**, 218-220 (1997).

Related Products

COX Activity Assay Kit - Item No. 760151
COX Fluorescent Activity Assay Kit - Item No. 700200
COX Inhibitor Screening Assay - Item No. 560131
COX (ovine) Inhibitor Screening Assay - Item No. 560101
Colorimetric COX (ovine) Inhibitor Screening Assay - Item No. 760111
COX-1 (ovine) - Item No. 60100
COX-2 (human recombinant) - Item No. 60122
COX-2 (ovine) - Item No. 60120

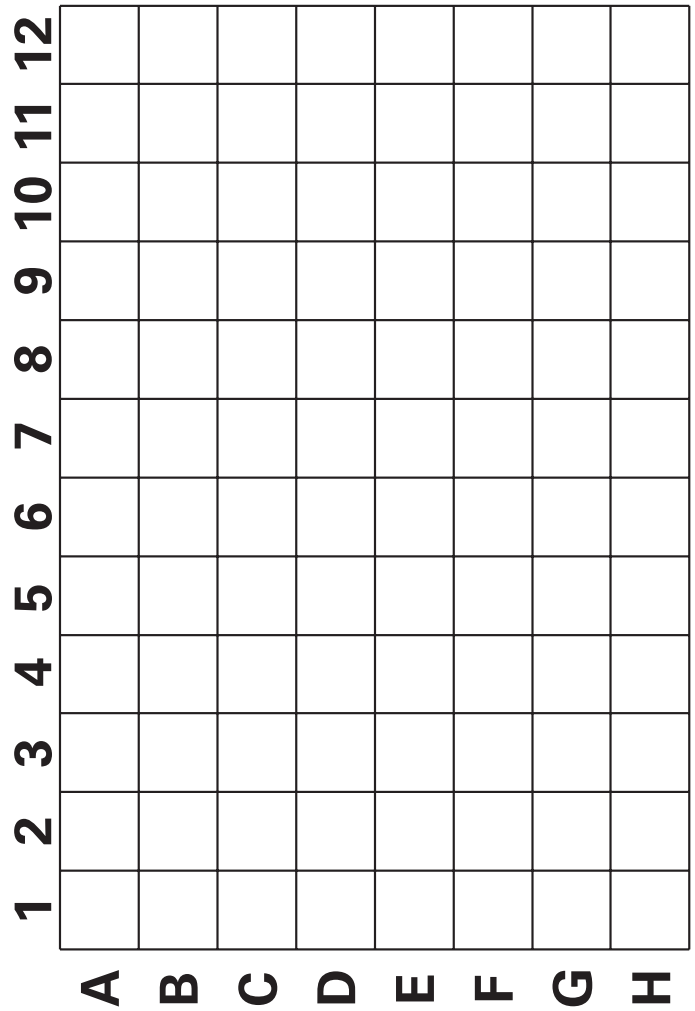
Warranty and Limitation of Remedy

Cayman Chemical Company makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. Cayman **warrants only** to the original customer that the material will meet our specifications at the time of delivery. Cayman will carry out its delivery obligations with due care and skill. Thus, in no event will Cayman have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if Cayman is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of Cayman, its directors or its employees.

Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.



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