COX Fluorescent Inhibitor Screening Assay Kit

Item No. 700100

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GENERAL INFORMATION

Materials Supplied

Kit components may be stored at -80°C prior to use. After opening the kit, we recommend each kit component be stored according to the temperature listed below.

Item Number	Item	Quantity	Storage
760114	Assay Buffer (10X)	2 vials	-20°C
760116	Hemin	2 vials	-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
760113	Arachidonic Acid (substrate)	2 vials	-80°C
760115	Potassium Hydroxide	2 vials	-20°C
700001	DMSO Assay Reagent	1 vial/3 ml	-20°C
700002	ADHP Assay Reagent	6 vials	-20°C
760158	DuP-697 Assay Reagent	1 vial	-20°C
760159	SC-560 Assay Reagent	1 vial	-20°C
400017	96-Well Solid Plate (black)	2 plates	RT
400012	Plate Cover	2 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.

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.

If You Have Problems

Technical Service Contact Information

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

Fax: 734-971-3640

Email: techserv@caymanchem.com

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 or multichannel 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 7).

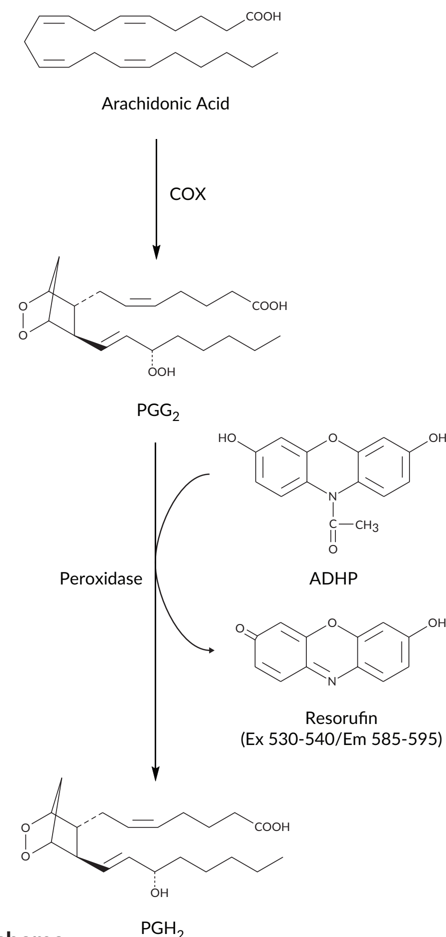

Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Assay Buffer (10X) - (Item No. 760114)**

   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. **Hemin - (Item No. 760116)**

   The vial contains 150 µl of Hemin in dimethylsulfoxide (DMSO). Dilute 40 µl of Hemin with 960 µl of diluted Assay Buffer. The diluted Hemin 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 40 µl of enzyme with 440 µl of diluted Assay Buffer and store on ice. This is enough enzyme to assay approximately 48 wells. Scale the amounts accordingly if assaying a different number of 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 40 µl of enzyme with 440 µl of diluted Assay Buffer and store on ice. This is enough enzyme to assay approximately 48 wells. Scale the amounts accordingly if assaying a different number of wells. The diluted enzyme is stable for one hour. There is enough COX-2 supplied to assay 96 wells.

5. **Arachidonic Acid (substrate) - (Item No. 760113)**

   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. 760115)**

   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 60 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. Assaying 10 µl with COX-2 will yield greater than 90% inhibition.

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

    The vial contains 66 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. Assaying 10 µl with COX-1 will yield greater than 90% inhibition.

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 non-enzymatic or background wells. The fluorescence of these wells must be subtracted from the fluorescence measured in the COX wells. We suggest that each COX sample be assayed in triplicate. Record the contents of each well on the template sheet provided on page 18. A typical layout of samples to be measured in triplicate is shown below.

	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

Read all of these instructions before setting up your plate!

1. **100% Initial Activity Wells** - add 150 µl of Assay Buffer, 10 µl of Hemin, 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 Hemin, 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 Hemin, 10 µl of enzyme (either COX-1 or COX-2), and 10 µl of inhibitor* to three wells.
4. Incubate the plate for five minutes at room temperature. NOTE: Most inhibitors exhibit time-dependent inhibition of COX activity. Altering incubation times with the inhibitor can significantly change the apparent IC_{50} value of the compound. Determining the optimal pre-incubation times for unknown inhibitors is suggested.
5. Add 10 µl of ADHP to sample and control wells.
6. Initiate the reactions by **quickly** adding 10 µl of Arachidonic Acid solution to sample and control wells.
7. Incubate for two minutes at room temperature.
8. 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 14 and 15, respectively).

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

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%.

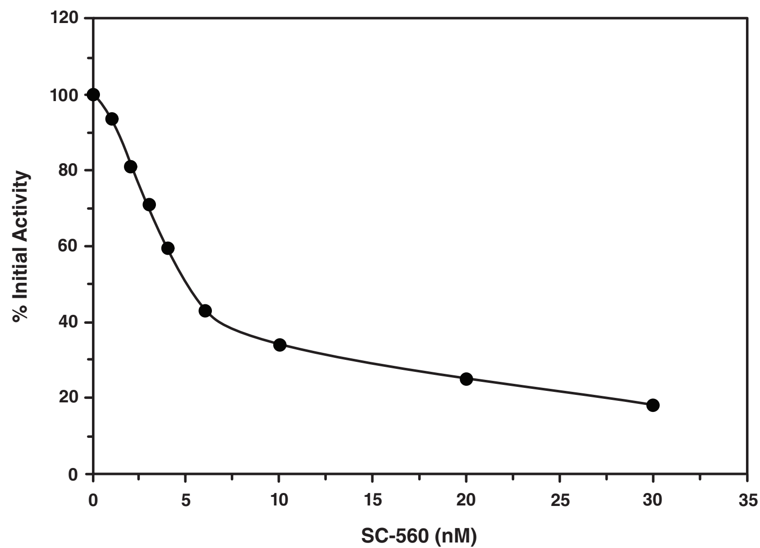

Figure 3. Inhibition of ovine COX-1 by SC-560

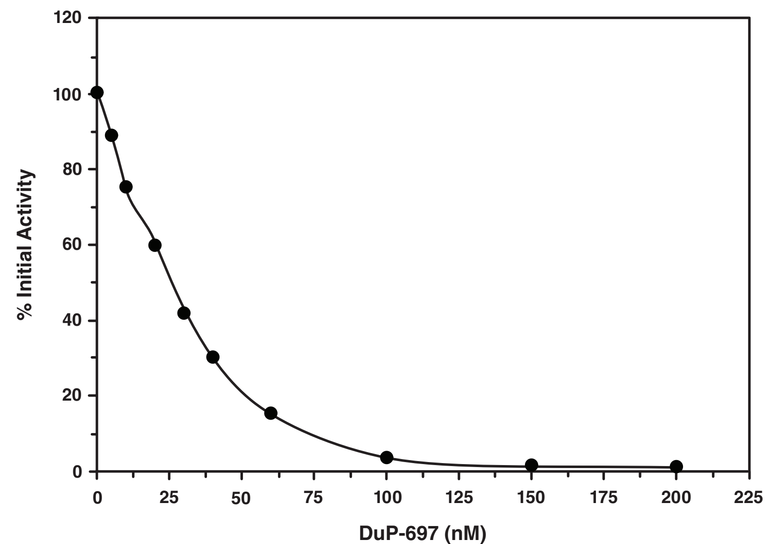

Figure 4. Inhibition of human recombinant COX-2 by DuP-697

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

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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).
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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).

	A	B	C	D	E	F	G	H
12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								

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.

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