COX (ovine) Colorimetric Inhibitor Screening Assay Kit

Item No. 760111

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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
760114	Assay Buffer (10X)	2 vials	-20°C
760116	Hemin	2 vials	-20°C
760110	COX-1 (ovine)	1 vial	-80°C
760108	COX-2 (ovine recombinant)	1 vial	-80°C
760113	Arachidonic Acid (substrate)	2 vials	-80°C
760115	Potassium Hydroxide	2 vials	-20°C
760117	Colorimetric Substrate	2 vials	-20°C
400014	96-Well Plate (Colorimetric Assay)	2 plates	RT
400012	96-Well Cover Sheet	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 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 absorbances between 590-611 nm
2. Adjustable pipettes and multichannel or repeating pipettes
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 mitogenic 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] This inducible COX-2 is believed to be the target enzyme for the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs.

About This Assay

Cayman's COX (ovine) Colorimetric Inhibitor Screening Assay measures the peroxidase component of COXs. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm.[4] Inhibition of COX activity, by a variety of selective and nonselective inhibitors, showed potencies similar to those observed with other *in vitro* methods.

The COX (ovine) Colorimetric Inhibitor Screening Assay Kit includes both ovine COX-1 and COX-2 enzymes in order to screen isozyme-specific inhibitors. This COX assay is a time saving tool for screening vast numbers of inhibitors.

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 Assay Buffer (0.1 M Tris-HCl, pH 8) should be used for dilution of Hemin and COX enzymes prior to assaying. When stored at 4°C, this diluted Assay Buffer is stable for at least two months.

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

   Each vial contains 300 µl of Hemin in DMSO. Dilute 88 µl of Hemin with 1.912 ml of diluted Assay Buffer prior to use. This diluted Hemin is stable for 12 hours at room temperature.

3. **COX-1 (ovine) - (Item No. 760110)**

   This vial contains ovine COX-1 and should be kept on ice when thawed. Dilute 120 µl of enzyme with 360 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 (ovine recombinant) - (Item No. 760108)**

   This vial contains recombinant ovine COX-2 and should be kept on ice when thawed. Dilute 120 µl of enzyme with 360 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)**

   This vial contains a solution of arachidonic acid in ethanol. Transfer 100 µl of the supplied substrate to another vial, add 100 µl of Potassium Hydroxide (Item No. 760115), vortex, and dilute with 1.8 ml of HPLC-grade water to achieve a final concentration of 1.1 mM. Use the prepared Arachidonic Acid solution within 30 minutes. A 20 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)**

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

7. **Colorimetric Substrate - (Item No. 760117)**

   This vial contains a solution of TMPD. The reagent is ready to use as supplied.

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 absorbance of these wells must be subtracted from the absorbance measured in the COX wells. We suggest that each COX sample be assayed in triplicate. We suggest you record the contents of each well on the template sheet provided on page 14.

	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 1. 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 220 µl in all the wells.
- If the appropriate inhibitor dilution is not known, it may be necessary to assay at several dilutions.
- Use the diluted Assay Buffer in the assay and pre-equilibrate to 25°C.
- We recommend assaying samples in triplicate, but it is the user's discretion.
- Initiate the reactions with Arachidonic Acid while in close proximity to the plate reader as it is necessary to read the plate precisely two minutes after initiation of the reaction.

Performing the Assay

1. **Background Wells -** add 160 µl of Assay Buffer and 10 µl of Hemin to three wells.
2. **100% Initial Activity Wells -** add 150 µl of Assay Buffer, 10 µl of Hemin, and 10 µl of enzyme (either COX-1 or COX-2) to three wells.
3. **Inhibitor Wells -** add 150 µl of Assay Buffer, 10 µl of Hemin, and 10 µl of enzyme (either COX-1 or COX-2) to three wells.
4. Add 10 µl of inhibitor* to the Inhibitor wells and 10 µl of solvent (same solvent used for dissolving inhibitor) to the 100% Initial Activity wells and background wells.
5. Carefully shake the plate for a few seconds and incubate for five minutes at 25°C. *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 inhibitors is suggested.*
6. Add 20 µl of the Colorimetric Substrate solution to all the wells that you are using.
7. Quickly add 20 µl of Arachidonic Acid to all the wells you are using.
8. Carefully shake the plate for a few seconds and incubate for precisely two minutes at 25°C**.
9. Read the absorbance at 590 nm.

*Inhibitors can be dissolved in methanol, dimethylsulfoxide, or ethanol and should be added to the assay in a final volume of 10 µl. In the event that the appropriate concentration of inhibitor is completely unknown, we recommend that several dilutions of the inhibitor be made.

**For a more accurate determination of reaction rates and apparent IC_{50} values, we recommend reading the samples kinetically, collecting as many time points as possible for the two minute assay read time. Determine the initial rate based on the linear portion of the kinetic curve.Calculations can be performed as shown below substituting initial rates for absorbance.

ANALYSIS

Calculations

1. Determine the average absorbance of all the samples.
2. Subtract the absorbance of the background wells from absorbances of the 100% Initial Activity and the Inhibitor wells.
3. Subtract each Inhibitor Sample from the 100% Initial Activity Sample, then divide by the 100% Initial Activity Sample, and multiply by 100 to give the percent inhibition.
4. Either graph the Percent Inhibition or Percent Initial Activity by the Inhibitor Concentration to determine the IC_{50} value (concentration at which there was 50% inhibition).

RESOURCES

Interferences

Antioxidants

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.[5] Using this assay, Resveratrol will also appear to be a COX-2 inhibitor. If the inhibitor you are assaying is also an antioxidant, it is recommended that you use one of Cayman's COX Inhibitor Screening Assays which utilizes an EIA detection (Item Nos. 560101 or 560131).

Solvents

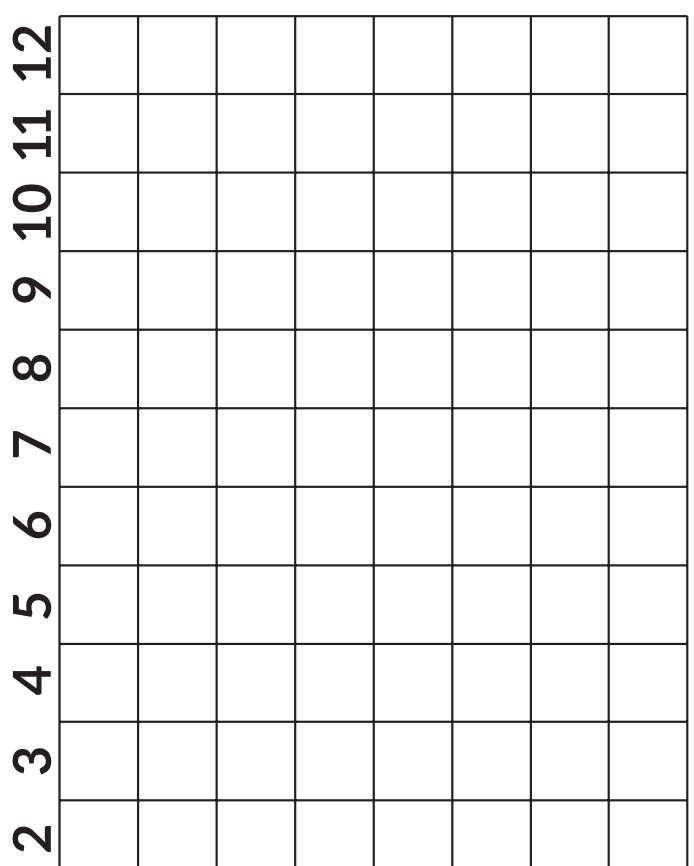
Methanol, dimethyl sulfoxide, and ethanol have no effect on COX activity. COX inhibitors can be dissolved in any of the above solvents and should be added to the assay in 10 µl.

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 absorbance above 0.1 is seen in the Inhibitor wells	A. Enzyme, Arachidonic Acid, or Colorimetric substrate was not added to the well(s) B. Inhibitor concentration is too high and inhibited all of the enzyme activity C. Something is interfering with the assay	A. Make sure to add all components to the wells B. Reduce the concentration of the inhibitor and re-assay C. Make sure you are not adding an antioxidant to the assay
No inhibition seen with inhibitor	The inhibitor concentration is not high enough or the compound is not an inhibitor of that enzyme	Increase the inhibitor concentration and re-assay

References

1. Nugteren, D.H. and Hazelhof, E. *Biochim. Biophys. Acta* **326**, 448-461 (1973).
2. Hamberg, M. and Samuelsson, B. *Proc. Natl. Acad. Sci. USA* **70**, 899-903 (1973).
3. Xie, W., Chipman, J.G., Robertson, D.L., *et al. Proc. Natl. Acad. Sci. USA* **88**, 2692-2696 (1991).
4. Kulmacz, R.J. and Lands, W.E.M. *Prostaglandins* **25**, 531-540 (1983).
5. Jang, M., Cai, L., Udeani, G.O., *et al. Science* **275**, 218-220 (1997).



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.