COX Activity Assay Kit

Item No. 760151

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GENERAL INFORMATION

Materials Supplied

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

Item Number	Item	Quantity	Storage
760114	Assay Buffer (10X)	1 vial	-20°C
760116	Hemin	1 vial	-20°C
760152	COX Standard	1 vial	-80°C
760113	Arachidonic Acid (substrate)	1 vial	-80°C
760115	Potassium Hydroxide	1 vial	-20°C
760117	Colorimetric Substrate	1 vial	-20°C
760158	DuP-697 Assay Reagent	1 vial	-20°C
760159	SC-560 Assay Reagent	1 vial	-20°C
400014	96-Well Plate	1 plate	RT
400012	96-Well Cover Sheet	1 cover	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 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 absorbance between 590-611 nm
2. Adjustable pipettes and a repeating 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 cyclooxygenase and peroxidase activities. The cyclooxygenase component converts arachidonic acid to a hydroperoxy endoperoxide (Prostaglandin G_2; PGG_2), and the peroxidase component reduces the endoperoxide to the corresponding alcohol (prostaglandin H_2; PGH_2), the precursor of prostaglandins, thromboxanes, and prostacyclins.[1,2]

It is now well established that there are two distinct isoforms of cyclooxygenase. 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 prostaglandins 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 Activity Assay Kit measures the peroxidase activity of COX. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm.[4] It can be used with both crude (cell lysates/tissue homogenates) and purified enzyme preparations. The kit includes isozyme-specific inhibitors for distinguishing COX-2 activity from COX-1 activity.

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 two months.

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

   The vial contains 300 µl of Hemin in DMSO. Dilute 88 µl of Hemin with 1,912 µl of diluted Assay Buffer. The diluted Hemin is stable for 12 hours at room temperature.

3. **COX Standard - (Item No. 760152)**

   The vial contains 50 µl of ovine COX-1 and should be kept on ice when thawed. The Standard is ready to use as supplied. A 10 µl aliquot of the enzyme per well causes an absorbance of approximately 0.28 under the standard assay conditions described below.

4. **Arachidonic Acid - (Item No. 760113)**

   The vial contains 400 µl of Arachidonic Acid in ethanol. Transfer 100 µl of the supplied solution to another vial, add 100 µl of KOH (Item No. 760115), vortex, and dilute with 800 µl of HPLC-grade water to achieve a final concentration of 2.2 mM. This quantity is sufficient for 50 reactions. Use the prepared Arachidonic Acid solution within 30 minutes. A 20 µl aliquot will yield a final concentration of 210 µM in the wells. If a lower concentration is desired, dilute further with HPLC-grade water and use within 30 minutes.

5. **Potassium Hydroxide - (Item No. 760115)**

   The vial contains 500 µl of Potassium Hydroxide (KOH). The reagent is ready to use as supplied.

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

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

7. **DuP-697 Assay Reagent - (Item No. 760158)**

   The vial contains 60 µM of DuP-697 in dimethylsulfoxide. DuP-697 is a potent and time-dependent inhibitor of COX-2.[5] The reagent is ready to use as supplied.

8. **SC-560 Assay Reagent - (Item No. 760159)**

   The vial contains 66 µM of SC-560 in dimethylsulfoxide. SC-560 is a potent and selective COX-1 inhibitor.[6] The reagent is ready to use as supplied.

Sample Preparation

Listed below are some suggested procedures for preparing tissue homogenates and cell lysates for activity determination. Table 2 (see page 15) lists some common reagents that can be used in the sample preparation or should not be used because they will interfere with the assay.

Tissue Homogenate

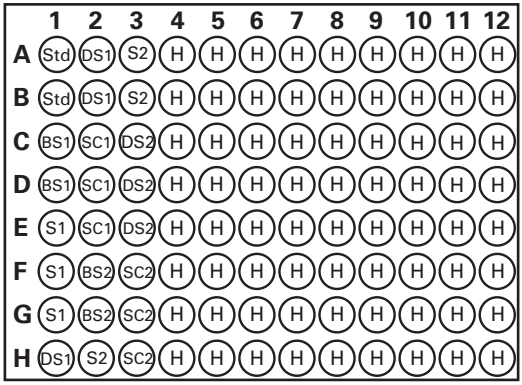
1. Prior to dissection, perfuse or rinse tissue with a Tris Buffer, pH 7.4, to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold buffer (*i.e.*, 0.1 M Tris-HCl, pH 7.8, containing 1 mM EDTA) per gram tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Cell Lysate

1. Collect cells (>1 x 10^8) by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. Homogenize or sonicate cell pellet in cold buffer (*i.e.*, 0.1 M Tris-HCl, pH 7.8, containing 1 mM EDTA). Keep sample as concentrated as possible (*i.e.*, if the cell pellet is ~100 µl add no more than 400 µl of buffer).
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout is shown in Figure 1. We suggest you record the contents of each well on the template sheet provided (see page 18). It is also necessary to have two wells designated as background wells. The absorbance of these wells must be subtracted from the absorbance measured in the sample wells. We suggest that each sample be assayed in triplicate and that the COX standard be run in duplicate.



Std - COX Standard
BS1 & BS2 - Background Samples 1 & 2
S1 & S2 - Samples 1 & 2
DS1 & DS2 - DuP-697-treated Samples 1 & 2
SC1 & SC2 - SC-560-treated Samples 1 & 2
H - Other Samples

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Use different tips to pipette each reagent.
- 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 210 µl in all the wells.
- It is not necessary to use all the wells on the plate at one time.
- Use the Assay Buffer (dilute) in the assay and pre-equilibrate to 25°C.
- You do not have to use both inhibitors (DuP-697 and SC-560). It is the user's discretion.
- We recommend assaying samples in triplicate, but it is the user's discretion.

Performing the Assay

1. **Preparation of Background values -** Transfer 150 µl of each sample to a 500 µl microfuge tube then place in boiling water for five minutes. Centrifuge the microfuge tube at 8,000 x g for one minute in a microcentrifuge. Use the supernatant to generate the background value. *NOTE: Each sample should have a background value.*
2. **COX Standard Wells -** Add 150 µl of Assay Buffer, 10 µl of Hemin, and 10 µl of Standard per well in the designated wells on the plate (see **Plate Set Up**, Figure 1, page 9).
3. **Background Wells -** Add 120 µl of Assay Buffer, 10 µl of Hemin, and 40 µl of inactive sample to two wells per sample.
4. **Sample Wells -** Add 120 µl of Assay Buffer, 10 µl of Hemin, and 40 µl of sample to three wells. To obtain reproducible results, the amount of cyclooxygenase added to the well should fall within the range of the assay. When necessary, samples should be diluted with Assay Buffer (dilute) or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 30,000 to bring the enzymatic activity to this level.
5. **Inhibitor Wells -** Add 110 µl of Assay Buffer, 10 µl of Hemin, and 40 µl of sample to the wells in triplicate. Add either 10 µl of DuP-697 or SC-560 to the three wells (see **Plate Set Up**, Figure 1, page 9). *NOTE: DuP-697 will eliminate all COX-2 activity and SC-560 will eliminate all COX-1 activity. If you already know which COX isoform (COX-1 or COX-2) is present in your sample, you can skip this step. If it is unknown which isoform is present, then you can pick either inhibitor or use an additional three wells and do both inhibitors.*
6. Carefully shake the plate for a few seconds to mix and incubate for five minutes at 25°C.
7. Add 20 µl Colorimetric Substrate to every well that you are using.
8. Initiate the reactions by adding 20 µl of arachidonic acid solution to all the wells you are using. Carefully shake the plate for a few seconds to mix and incubate for five minutes at 25°C.
9. Read the absorbance at 590 nm using a plate reader.

Calculations

1. Calculate the average absorbance for each background, sample, and inhibitor treated sample.
2. Subtract the background value from its corresponding sample and inhibitor treated sample. These are the corrected absorbances (ΔA_{590}).
3. Use the following formula to calculate the Total COX activity for each sample. The reaction rate at 590 nm can be determined using the TMPD extinction coefficient of 0.00826 μM^{-1}.[4] One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of TMPD per minute at 25°C. *NOTE: The actual coefficient for TMPD has been adjusted for the pathlength of the solution in the well.*

Total COX Activity =

$$\frac{\Delta A_{590}/5 \text{ min.}}{0.00826\ \mu M^{-1}} \times \frac{0.21 \text{ ml}}{0.04 \text{ ml}} \div 2^{*} = \text{nmol/min/ml (U/ml)}$$

*It takes two molecules of TMPD to reduce PGG_2 to PGH_2.

4. Subtract the Total COX Activity of each Inhibitor treated Sample from the Total COX Activity of its corresponding Sample, then divide by the Total COX Activity of the Sample, and multiply by 100 to give the percent inhibition. The amount of inhibition corresponds to the amount of either COX-1 or COX-2 in the sample (see Table 1, for some examples).

Sample	Total COX Activity (U/ml)	COX-1 (%)	COX-2 (%)
Sample 1	10	unknown	unknown
DuP-697 treated	0	0	100
SC-560 treated	10	0	100
Sample 2	20	unknown	unknown
DuP-697 treated	20	100	0
SC-560 treated	0	100	0
Sample 3	30	unknown	unknown
DuP-697 treated	15	50	50
SC-560 treated	15	50	50
Sample 4	20	unknown	unknown
DuP-697 treated	5	25	75
SC-560 treated	15	25	75

Table 1. Interpreting sample data

Performance Characteristics

Precision:

When a series of nine COX measurements were performed on the same day, the intra-assay coefficient of variation was 2.6%. When a series of nine COX measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 5.4%.

Sensitivity:

Samples containing COX activity between 13-63 nmol/min/ml can be assayed without further dilution or concentration.

Interferences

The following reagents were tested in the assay for interference in the assay:

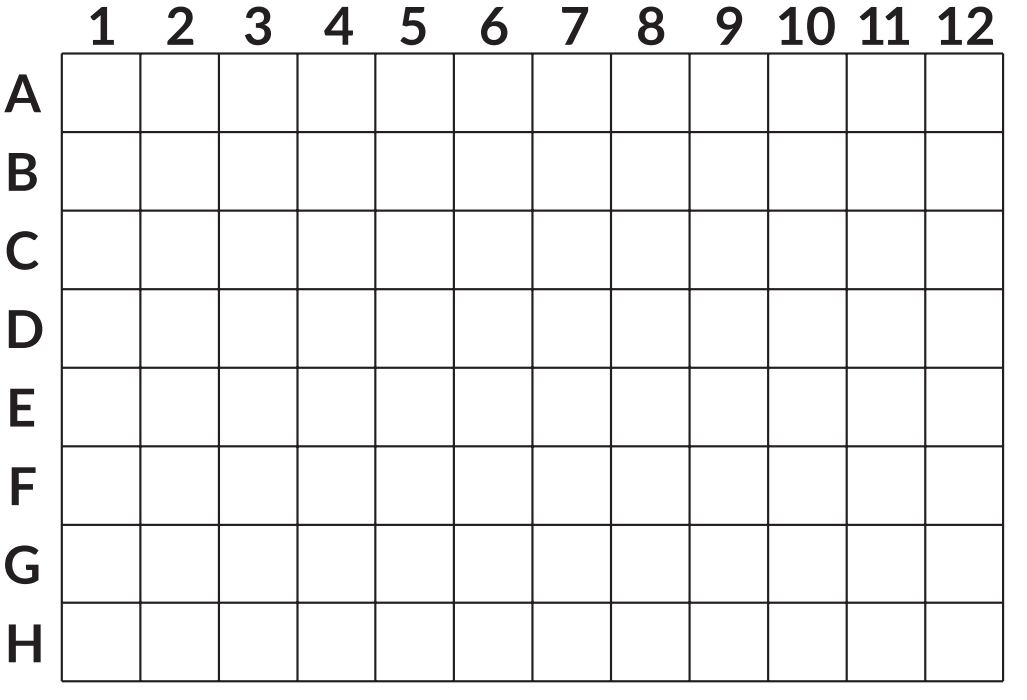
	Reagent	Will Interfere (Yes or No)
Buffers	Tris	No
	HEPES	No
	Phosphate	Yes
Detergents	SDS	Yes
	Polysorbate 20 (>0.1%)	Yes
	Triton X-100 (>0.1%)	Yes
	CHAPS (>0.1%)	Yes
Protease Inhibitors/ Chelators/ Enzymes	EDTA (≤5 mM)	No
	EGTA	Yes
	Trypsin (≤0.1 mg/ml)	No
	PMSF (≤1 mM)	No
	Leupeptin (≤1 mg/ml)	No
	Antipain	Yes
	Chymotrypsin (≤0.1 mg/ml)	No
	Chymostatin (≤1 mg/ml)	No
Solvents	Ethanol (10 µl)	No
	Methanol (10 µl)	No
	Dimethylsulfoxide (10 µl)	No
Others	BSA (≤0.1%)	No
	Antioxidants (*i.e.*, Glutathione)	Yes
	Glycerol (≤5%)	No
	Thiol compounds (*i.e.*, Dithiothreitol)	Yes

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 activity was detected in the sample	Sample was too dilute	Concentrate the sample with an Amicon centrifuge concentrator with a molecular weight cut-off of 30,000 to bring the enzymatic activity to fall within the sensitivity of the assay and re-assay
The absorbance in the wells is less than 0.1, including the COX standard wells	Arachidonic acid or colorimetric substrate was not added to the wells	Make sure to add all components to the wells and re-assay
The initial absorbance in the sample wells is above 1.2	A. The sample contains a significant amount of COX activity B. Something is interfering with the assay	A. Dilute your sample with Assay Buffer (dilute) and re-assay B. Check the interference section for possible Interferences (see page 15)

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. Kulmacz, R.J. and Lands, W.E.M. Requirements for hydroperoxide by the cyclooxygenase and peroxidase activities of prostaglandin H synthase. *Prostaglandins* **25**, 531-540 (1983).
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).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NOTES

Warranty and Limitation of Remedy

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