COX Activity Assay Kit

Item No. 760151

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

TABLE OF CONTENTS

GENERAL INFORMATION

Materials Supplied

Kit components may be stored at -80°C prior to use. After opening the kit, store individual components as stated below.

Item Number	Item	Quantity/Size	Storage Temperature
760114	Assay Buffer (10X)	1 vial/5 ml	-20°C
760116	Hemin	1 vial/300 µl	-20°C
760152	COX-1 (ovine)	1 vial/100 µl	-80°C
760113	Arachidonic Acid (substrate)	1 vial/400 µl	-80°C
760115	Potassium Hydroxide	1 vial/500 µl	-20°C
760117	Colorimetric Substrate	1 vial/3 ml	-20°C
760158	DuP-697 Assay Reagent	1 vial/1 ml	-20°C
760159	SC-560 Assay Reagent	1 vial/1 ml	-20°C
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate	RT
400012	96-Well Cover Sheet	1 ea	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.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's COX Activity 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

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 (see 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 absorbance between 590-611 nm
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. A source of pure water; glass-distilled or HPLC-grade water is acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. Boiling water bath or other equipment to boil samples

INTRODUCTION

Background

Cyclooxygenase 1 (COX-1) and COX-2, also known as prostaglandin H synthase-1 (PGHS-1) and PGHS-2, respectively, are bifunctional enzymes that exhibit both COX and peroxidase activities and catalyze the first step in the biosynthesis of prostaglandins, thromboxanes, and prostacyclins.[1,2] The COX component converts arachidonic acid to the hydroperoxy endoperoxide, prostaglandin G_2 (PGG_2), and the peroxidase component reduces the endoperoxide to the corresponding alcohol, PGH_2, which is the precursor of PGs, thromboxanes, and prostacyclins. COX-1 is the target of many non-steroidal anti-inflammatory drugs (NSAIDs) and is responsible for undesirable gastrointestinal and renal side effects, such as ulcer formation and reductions in the glomerular filtration rate, respectively.[3,4] *COX2* expression is induced by a variety of stimuli, including phorbol esters, LPS, and cytokines, and COX-2 is responsible for the biosynthesis of PGs under acute inflammatory conditions.[5,6] Thus, COX-2 has been the focus of attention for NSAID development.

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.[7] It can be used with 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)**

   This vial contains 5 ml of Assay Buffer (10X). Dilute the contents of the vial with 45 ml of pure water. Be certain to rinse the vial to remove any salts that may have precipitated. The Assay Buffer (1X) will be stable for two months when stored at 4°C.

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

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

3. **COX-1 (ovine) - (Item No. 760152)**

   This vial contains 100 µl of ovine COX-1. It is included as a positive control and should be thawed on ice. Briefly centrifuge before opening. For use in the assay, add 300 µl of Assay Buffer (1X) and store on ice.

   For positive control background wells, transfer 125 µl of diluted enzyme to a microcentrifuge tube. Boil for 5 minutes before centrifuging at 8,000 x g for 5 minutes. Transfer the supernatant to a clean tube.

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

   The vial contains 500 µl of 0.1 M potassium hydroxide and is ready to use to prepare the arachidonic acid solution.

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

   The vial contains 400 µl of arachidonic acid in ethanol.

   To make a 2.2 mM arachidonic acid solution, dilute 300 µl of Arachidonic Acid with 300 µl of Potassium Hydroxide and 2.4 ml of pure water. Vortex. This is a sufficient volume for a full plate; scale as needed. Use within one hour. When used as directed, the final concentration is 210 µM in the wells.

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

   This vial contains a solution of TMPD. Immediately prior to use in the assay, dilute the Colorimetric Substrate by adding 950 µl to 1,250 µl of pure water. This is a sufficient volume for a full plate; scale as needed. The diluted Colorimetric Substrate will be stable for 2 hours at room temperature.

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

   The vial contains 60 µM DuP-697 in DMSO and is ready to use as supplied. DuP-697 is a potent inhibitor of COX-2.[8]

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

   The vial contains 66 µM SC-560 in DMSO and is ready to use as supplied. SC-560 is a potent and selective COX-1 inhibitor.[9]

Sample Preparation

If a buffer not specified in the protocols below must be used, please see page 18 for a list of compatible reagents.

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.

Sample Background

Performing the assay in the absence of COX will result in background (non-specific) absorbance. To determine this non-specific absorbance, it is necessary to inactivate the COX enzyme. The simplest way is by boiling a portion of each sample.

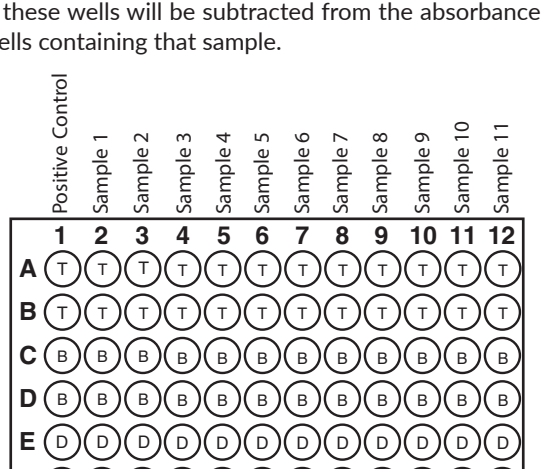
1. Transfer 125 µl of each sample to a microcentrifuge tube.
2. Boil for 5 minutes.
3. Centrifuge at 8,000 x g for 5 minutes.
4. Transfer the supernatant to a clean tube.

ASSAY PROTOCOL

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 21).

It is necessary to have two background wells for each sample tested. The absorbance of these wells will be subtracted from the absorbance measured for all the other wells containing that sample.



T = Total Activity Wells
B = Background Wells
D = COX-1 Specific Wells (Optional)
S = COX-2 Specific Wells (Optional)

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- 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.
- All reagents should be prepared as described in the Reagent Preparation Section. The COX-1 (ovine) should be kept on ice and all other reagents should be kept at room temperature before beginning the assay.
- The assay is performed at room temperature.
- It is recommended that the samples be assayed in duplicate.
- The included inhibitors, DuP-697 and SC-560, are optional and can be used to distinguish COX-1 from COX-2 activity.
- Monitor the absorbance at 590-611 nm.

Performing the Assay

For the positive control and for each sample tested, it is necessary to run both Total Activity and Background wells. The addition of inhibitor wells will allow the user to determine which COX isoform(s) is/are present in samples. Inhibitor wells may be run with the positive control as well, in order to confirm specificity of the inhibitor. SC-560 should inhibit COX activity in the positive control sample; DuP-697 should be without effect.

1. Add the appropriate amount of each reagent to the designated wells as follows:

Reagent	Total Activity (T)	Background (B)	*(Optional)* COX-1-specific (D)	*(Optional)* COX-2-specific (S)
Assay Buffer (1X)	120 µl	120 µl	110 µl	110 µl
Positive Control/Sample	40 µl	40 µl (boiled)	40 µl	40 µl
DuP-697	--	--	10 µl	--
SC-560	--	--	--	10 µl
Hemin	10 µl	10 µl	10 µl	10 µl

Table 1. Pipetting summary

2. Carefully shake the plate for a few seconds to mix and incubate at room temperature for 5 minutes.
3. Add 20 µl of diluted Colorimetric Substrate to each well.
4. Initiate reactions by adding 20 µl of the arachidonic acid solution to each well.
5. Carefully shake the plate for a few seconds to mix and incubate at room temperature for 5 minutes.
6. Read the absorbance at 590-611 nm.

ANALYSIS

Calculations

1. Calculate the average absorbance for each sample.
2. Subtract the background values from the absorbance for the corresponding samples.
3. Use the following formula to calculate COX activity. One unit is defined as the amount of enzyme that will catalyze the oxidation of 1 nmol of TMPD per minute at 25°C.

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

*Two molecules of TMPD are required to reduce PGG_2 to PGH_2

4. Use the following formula to determine the percentage of isoform-specific COX activity:

$$\%\text{COX-1 Activity} = \frac{\text{Activity in the presence of DuP-697}}{\text{Total Activity}} \text{ X } 100$$

$$\%\text{COX-2 Activity} = \frac{\text{Activity in the presence of SC-560}}{\text{Total Activity}} \text{ X } 100$$

See Table 2, on page 17, for examples.

Sample	Total COX Activity (U/ml)	COX Activity in Presence of DuP-697 (U/ml)	COX Activity in Presence of SC-560 (U/ml)	% COX-1 Activity	% COX-2 Activity
1	10	0	10	0	100
2	20	20	0	100	0
3	20	5	15	25	75

Table 2. 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 U/ml can be assayed without further dilution or concentration.

Interferences

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

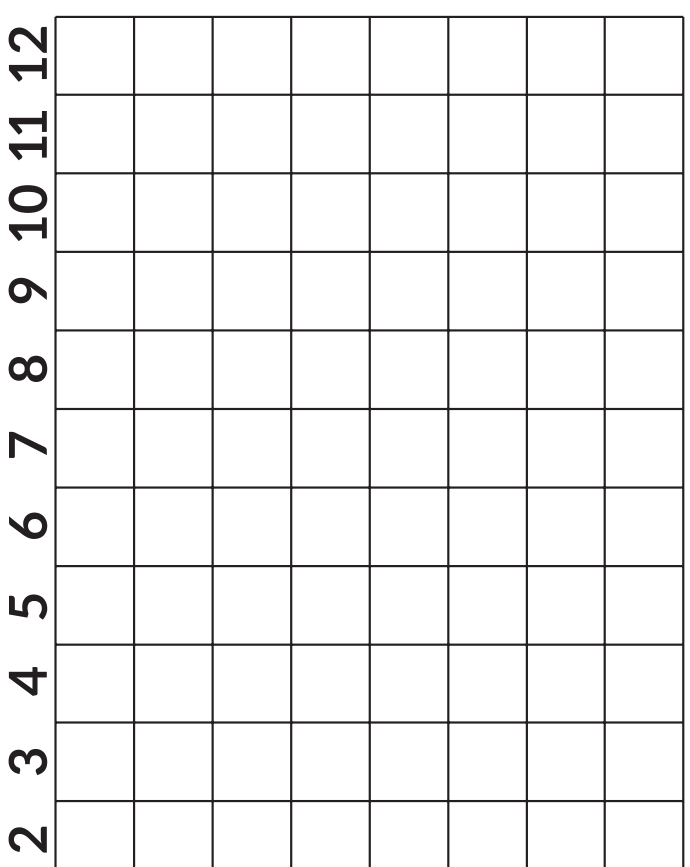
Reagent		Will Interfere (Yes or No)
Buffers	Tris	No
	HEPES	No
	Phosphate	Yes
Detergents	SDS	Yes
	Polysorbate 20 (>0.1%)	Yes
	Triton X-100 (>0.1%)	Yes
	CHAPS (>0.1%)	Yes
Protease Inhibitors/ Chelators/ Enzymes	EDTA (≤5 mM)	No
	EGTA	Yes
	Trypsin (≤0.1 mg/ml)	No
	PMSF (≤1 mM)	No
	Leupeptin (≤1 mg/ml)	No
	Antipain	Yes
	Chymotrypsin (≤0.1 mg/ml)	No
	Chymostatin (≤1 mg/ml)	No
Solvents	Ethanol (10 µl)	No
	Methanol (10 µl)	No
	DMSO (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 replicates	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 positive control 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 (1X) and re-assay B. Check for possible Interferences (see page 18)

References

1. Nugteren, D.H. and Hazelhof, E. Isolation and properties of intermediates in prostaglandin biosynthesis. *Biochim. Biophys. Acta* **326(3)**, 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(3)**, 899-903 (1973).
3. Gierse, J.K., Hauser, S.D., Creely, D.P., *et al.* Expression and selective inhibition of the constitutive and inducible forms of human cyclo-oxygenase. *Biochem. J.* **305(Pt. 2)**, 379-484 (1995).
4. Frölich, J.C. A classification of NSAIDs according to the relative inhibition of cyclooxygenase isoenzymes. *Trends Pharmacol. Sci.* **18(1)**, 3-34 (1997).
5. Kang, Y.-J., Mbonye, U.R., DeLong, C.J., *et al.* Regulation of intracellular cyclooxygenase levels by gene transcription and protein degradation. *Prog. Lipid Res.* **46(2)**, 108-125 (2007).
6. Blobaum, A.L. and Marnett, L.J. Structural and functional basis of cyclooxygenase inhibition. *J. Med. Chem.* **50(7)**, 1425-1441 (2007).
7. 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).
8. 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).
9. 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).



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.