COX activity kit

Catalog No. ADI-907-003

96 Well Microtiter Plate Kit

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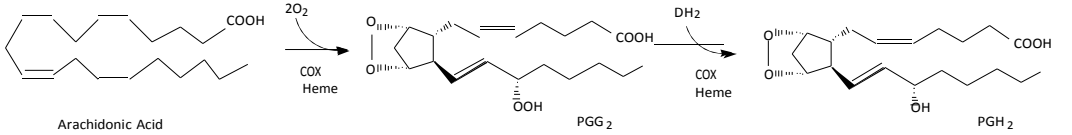
Description

The COX activity kit is for the quantitative determination of Cyclooxygenase activity in biological samples. Please read the complete kit insert before performing this assay.

Introduction

Cyclooxygenase (COX, also known as Prostaglandin G/H synthase) is a membrane bound enzyme responsible for the oxidation of arachidonic acid to Prostaglandin G $_2$ (PGG_2) and the subsequent reduction of PGG_2 to PGH_2[1,2]. The conversion is shown below. These reactions are the first steps in the formation of a variety of prostanoids. COX has been shown to be expressed in at least two different isoforms: a constitutively expressed form, COX-I, and an inducible form, COX-II. COX-I is thought to regulate a number of 'housekeeping' functions, such as vascular hemostasis, renal blood flow, and maintenance of glomerular function[3]. Inflammation mediators such as growth factors, cytokines and endotoxin induce COX-II expression in a number of cellular systems[4,5]. The effect of various non-steroidal anti-inflammatory drugs (NSAIDs) on the activity of COX-I and -II is an area of considerable interest. Some methods to determine COX activity involve procedures such as measuring uptake of oxygen using an oxygraph, measuring the conversion of radioactive arachidonic acid, or measuring the prostaglandins formed from PGH_2 (such as determining PGE_2 using immunoassays[6]). Most of these methods are complex, time consuming, and are prone to interferences.

The Cyclooxygenase Reaction



The COX activity kit uses a specific chemiluminescent substrate to detect the peroxidative activity of COX enzymes. After inhibition by NSAIDs, the direct residual activity of COX is measured by addition of a proprietary luminescent substrate and arachidonic acid. Light emission starts immediately and is directly proportional to the COX activity in the sample. The chemiluminescent signal is measured over 5 seconds.

Note: In this insert, one Unit of COX activity is defined as the amount of enzyme needed to consume 1 nmole of oxygen per minute at 37 °C.

Precautions

1. The luminescent substrate is an aromatic hydrocarbon molecule with unknown biological effects. Care should be used in handling this reagent.
2. The microtiter plate supplied in this kit has been selected for its low luminescent background and excellent reproducibility.
3. The NSAIDs supplied in the kit, Catalog Nos. 80-0276 and 80-1345, have known and unknown effects on biological tissue. Care should be taken in handling these materials.

Materials Supplied

1. **White 96 well microtiter plate, 12 strips of 8 wells, Catalog No. 80-0418**
   Microtiter plate selected for low background luminescence and reproducible light measurement.
2. **COX Chemiluminescent Substrate, 10 mL, Catalog No. 80-0275**
   A solution of chemiluminescent substrate in aqueous buffer. **Substrate is sensitive to light.** Protect solutions from ambient room light.
3. **Ibuprofen, 2mL, Catalog No. 80-0276**
   A 300 mM solution of ibuprofen (α-Methyl-4-(isobutyl)phenylacetic acid) in water.
4. **Meloxicam, 2 mL, Catalog No. 80-1345**
   A 1mM solution of Meloxicam (CAS No. 71125-38-7) in DMSO.
5. **Plate Sealers, 2 each, Catalog No. 30-0012**

Storage

All components of this kit, **except Ibuprofen and Meloxicam**, are stable at 4 °C until the kit's expiration date. The Ibuprofen and Meloxicam **must** be stored at -20 °C.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 25 µL and 1,000 µL.
3. Repeater pipets for dispensing 50 µL.
4. Disposable beakers and graduated cylinders.
5. Plate luminometer with dual injectors.
6. COX Inhibition Assay. This kit does not contain materials needed for an inhibition assay (cyclooxygenase, arachidonic acid, or hematin). These must be obtained from other sources. On page 5 we outline a typical inhibition assay protocol. These conditions have been used for the Quality Control testing of this detection kit. It is important that the end user optimize their inhibition assays in line with published studies[6-9].

Sample Handling

The COX activity kit is compatible with samples in a wide range of matrices. The procedure described in the Typical Inhibition Assay section is for reference only and the end user must independently determine the proper format and matrix for their inhibition studies. We have tested this assay in a number of buffer systems, but the end user will have to obtain information about compatability of the assay to measure cyclooxygenase activity in some samples, especially those from tissue sources.

We recommend that for NSAID inhibitor studies at least 10 Units/mL (i.e., approximately 0.5 Units per well) of either COX-I or COX-II are used. Even though this assay will allow accurate measurements of COX activity down to less than 5 Units/mL, for accurate inhibitor calculations we recommend using higher levels of enzyme.

Procedural Notes

1. Do not mix reagents from different lot numbers or use reagents beyond expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
4. Add the reagents to the side of the well to avoid contamination.
5. Mix the substrate well prior to use.
6. **This assay uses a luminescent measurement of COX activity. The luminescent signal is typically represented as Relative Light Units (RLU). Different luminometers will display different RLU readings. Please see the luminometer Instruction Manual for details.**
7. **In this insert one Unit of COX activity is defined as the amount of enzyme needed to consume 1 nmole of oxygen per minute at 37 °C.**

Typical Inhibition Assay

Preparation Protocol

(1) Store COX-I and/or COX-II at -70 °C or lower. Enzyme dilutions in 100 mM phosphate, pH 7.5, used for inhibition reactions, must be kept at 0-4 °C in an ice bath. These enzyme dilutions are stable for 2-8 hours.

(2) Prepare hematin (Sigma H-3281, porcine) by dissolving in DMSO at 0.380 mg/mL. The concentrated stock can be stored at -80 °C in single use volumes. Dilute in 100 mM phosphate, pH 7.5 to a final concentration of 0.12 µM. Diluted hematin is stable for up to 8 hours at room temperature.

Avoid exposure to light.

Higher concentrations of hematin give rise to increasing background signals and do not result in increased cyclooxygenase actvity or increased inhibition by NSAIDs.

(3) Prepare a 100 mM Tris, 0.5mM phenol buffer, pH 7.3. Buffer pH **must** be measured at 37 °C for correct pH measurement.

(4) Arachidonic acid stock solution in ethanol is prepared by taking a freshly opened vial of arachidonic acid and adding ethanol under argon or nitrogen gas to give a 50 mg/mL solution. Store in the dark at -70 °C or lower.

(5) Activation of arachadonic acid is carried out by the following procedure. Add 94 µL of 0.1N sodium hydroxide to a glass vial. Add 6 µL of the ethanolic arachadonic acid solution to the vial. Vortex. Dilute the mixture with 9.9 mL of deionized water. Store the prepared solution of arachidonic acid at 0-4 °C in an ice bath; it is stable for up to 3 hours.

For best reproducibility we recommend storing the prepared arachadonic acid solution at 0-4 °C in the luminometer. Injection of the cold arachadonic acid solution is recommended.

Luminescent Assay Procedure

All samples should be run in duplicate.

All samples should be allowed to warm to room temperature and all activity measurements should be run at room temperture.

Assay Protocol

1. Pipet 50 µL of the Tris-phenol buffer into all wells.
2. Add 50 µL of the hematin solution into all wells.
3. Add 50 µL COX-I or COX-II preparations to all wells, except for the Blank and Zero Activity wells.
4. Pre-incubate at room temperature for 5 minutes.
5. Add 25 µL of NSAID inhibitor solution to appropriate wells.
6. Incubate at room temperature for 5-120 minutes (dependent on inhibitor).
7. Place microtiter plate in luminometer for the chemiluminescent measurement.
8. Inject 50 µL of **cold** COX Chemiluminescent Substrate.
9. Immediately inject 50 µL of diluted **cold** arachidonic acid solution.
10. Immediately read in a luminometer for 5 seconds. Determine integrated light output for the 5 second read time in Relative Light Units (RLU).

If your luminometer has automated injection capabilities, program the instrument to carry out steps 8-10 with minimum delay between steps 8 and 9, and with zero delay between step 9 and light detection.

We have supplied solutions of the COX inhibitors Ibuprofen and Meloxicam to allow users of this kit to distinguish between COX-I and COX-II activity in some samples. Meloxicam is a specific inhibitor of COX-II. Ibuprofen is a non-selective inhibitor of both COX-I and -II.

Calculation of Results

Several options are available for the calculation of the inhibition of cyclooxygenase in the samples. We recommend that the data be handled by an software package utilizing a curve fitting program. If data reduction software is not readily available the data can be transformed as follows:

1. Calculate the average net Relative Light Units (RLU) for each standard and sample by subtracting the average blank RLU from the average RLU for the standards and samples:

   Average Net RLU = Average RLU - Average Blank RLU

2. Percent inhibition should be calculated using the following formula for each inhibitor:

   $$\text{Percent Inhibition} = \left(1 - \frac{\text{Average Net Inhibitor RLU}}{\text{Average Net RLU for non-inhibited Enzyme}}\right) \times 100$$

Typical Results

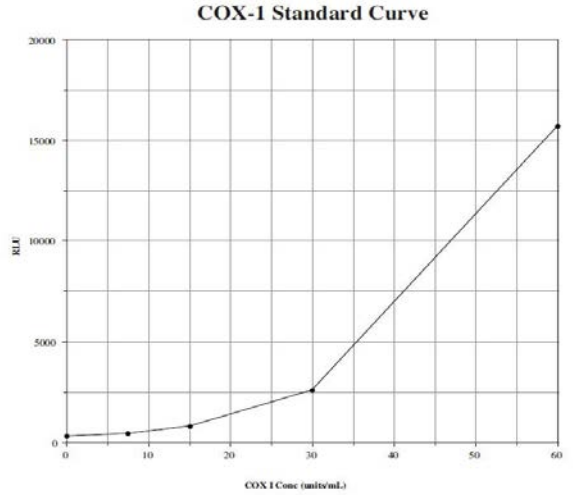
The results shown below are for illustration only and **should not** be used to calculate results from another assay.

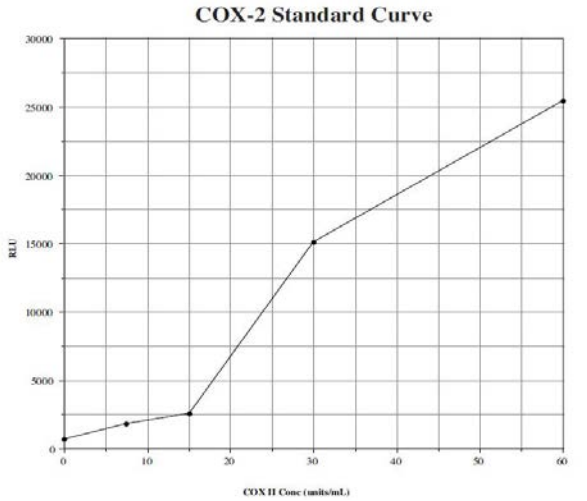
	Cyclooxygenase-I			**Cyclooxygenase-II**		
Sample	COX-I (Units/mL)	Net RLU	**Percent Inhibition**	COX-II (Units/mL)	Net RLU	**Percent Inhibition**
S0	0	311		0	655	
S1	60	15,691		60	25,442	
S2	30	2,603		30	15,075	
S3	15	812		15	2,531	
S4	7.5	429		7.5	1,835	
Ibuprofen	60	1,748	**88.9%***	60	2,451	**90.4%***
Meloxicam	60	7,246	**53.8%***	60	10,070	**60.4%***

***Actual level of inhibition seen will be dependent on assay conditions used.**

Typical Standard Curves

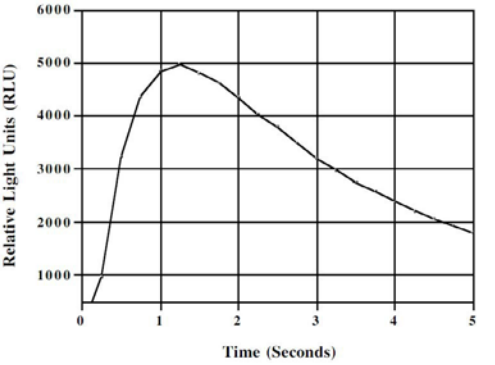
Typical standard curves are shown below. The concentrations of COX-I and COX-II were varied from 0 to 60 Units/mL and run as described in Step 1 of the Typical Inhibition Assay on page 5. These curves **must not** be used to calculate COX concentrations; each user must run a standard curve for each assay.





Typical Kinetic Output

A typical kinetic profile of the light emission is also shown to the right.



Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[10].

Sensitivity

Sensitivity was calculated by determining the average RLUs for sixteen (16) wells run with 0 Units/mL, and comparing to the average RLUs for sixteen (16) wells run with Standard #4. The detection limit was determined as the concentration of COX-I measured at two (2) standard deviations from the 0 Units/mL Standard along the standard curve.

Average RLU for S0	=	942	±	95 (10.1%)
Average RLU for Standard #4	=	10,667	±	544 (5.1%)

Delta RLU's (7.5-0 Units/mL) = 9,725

2 SD's of 0 Units/mL Standard = 190

$$\text{Sensitivity} = \frac{190}{9{,}725} \times 7.5 \text{ Units/mL} = 0.147 \text{ Units/mL COX-I}$$

Using a 50 µL sample, 0.00732 Units of COX-I activity can be detected (corresponds to 7.32 mUnits of COX activity using 1 Unit = 1 nmole of oxygen consumed at 37 °C. See # 7 on page 4.)

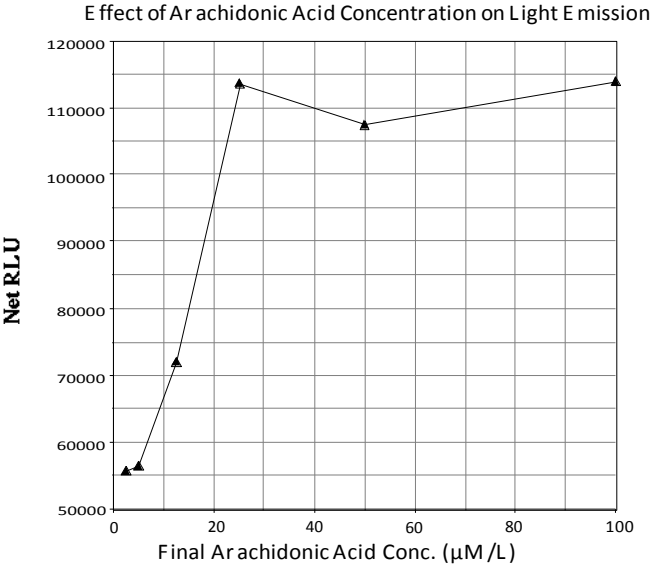
Precision

Intra-assay precision was determined by taking samples containing low and high concentrations of COX-I and running these samples multiple times (n=16) in the same assay. The precision numbers listed below represent the percent coefficient of variation for the concentrations of COX-I determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	COX-I Concentration (Units/mL)	RLU's	RLU Intra-assay (%CV)
High	30	78,972	7.0
Low	7.5	19,193	3.1

Effect of Arachidonic Acid

The following graph shows the effect of varying arachidonic acid concentration on light emission. In this example 2 Units of COX-I were used per well. Note that any change in arachidonic acid concentration above 25 µM has very little effect on light output.



Interfering Substances

The following commonly used substances were tested for interference with the luminescent signal generated in the Cyclooxygenase Activity Kit. At the concentrations listed, the following change in luminescent signal generation was observed.

Substance	Tested Conc.	Signal Change (%)
Ethanol	5%	6
Methanol	5%	1
Dimethyl sulfoxide (DMSO)	10%	1
N,N-Dimethyl formamide (DMF)	1%	12.7
Tween 20	0.1%	5
EDTA	2.5mM	7.3
Protein	0.1%	0
Protease Inhibitors*	0.1%	3.3
Tissue Culture Media	100%	14.8
PBS	100%	15.9
Sodium Azide	0.1%	5.5
Gentamicin	0.01%	0.1

*****Contains Peafabloc, pepstatin, leupeptin, E-64, bestatin & aprotinin

References

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Notes

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Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending.

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