ab139432

Cyclooxygenase (COX) Activity Assay Kit (Luminometric)

Instructions for Use

The COX activity kit is for the quantitative determination of Cyclooxygenase activity in biological samples.

View kit datasheet: www.abcam.com/ab139432
(use www.abcam.cn/ab139432 for China, or www.abcam.co.jp/ab139432 for Japan)

This product is for research use only and is not intended for diagnostic use.

Version 1 Last Updated 6 February 2019

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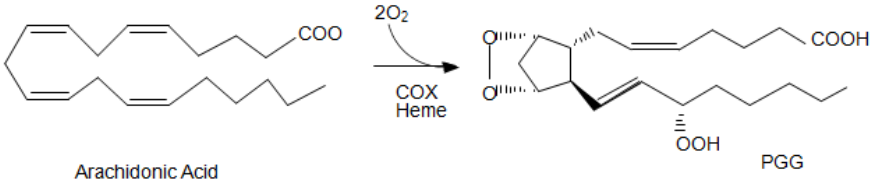
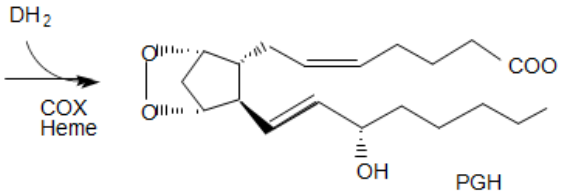
1. Introduction

Cyclooxygenase (COX, also known as Prostaglandin G/H synthase) is a membrane bound enzyme responsible for the oxidation of arachidonic acid to Prostaglandin G (PGG) and the subsequent reduction of PGG to PGH 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. Inflammation mediators such as growth factors, cytokines and endotoxin induce COX-II expression in a number of cellular systems. 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 (such as determining PGE using immunoassays Most of these methods are complex, time consuming, and are prone to interferences.



Arachidonic Acid

PGG



PGH

2. Principle of Assay

ab139432 COX activity kit is for the quantitative determination of Cyclooxygenase activity in biological samples. Please read the complete kit insert before performing this assay.

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 signals 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.

3. Assay Summary

Dilute inhibitor, substrate and enzyme and warm to reaction temperature

Pipette assay buffer into the appropriate wells and allow to equilibrate to reaction temperature

Add Hematin solution into all wells

Add enzyme to appropriate wells.

Pre-Incubate 5 mins at 37°C

Add inhibitor incubate at room temperature 5-120 minutes

Place microtiter plate in luminometer for the chemiluminescent measurement

Inject cold COX Chemiluminescent Substrate

Immediately inject diluted cold arachidonic acid solution.

Analyze Data

4. Components and Storage

A. Kit Contents

Item	Quantity	Storage Temperature
White 96 well microtiter plate	12 strips of 8 wells	+4°C
COX Chemiluminescent Substrate	10 mL	+4°C
Ibuprofen	2mL	≤ -20°C
Meloxicam	2 mL	≤ -20°C
Plate Sealers	2 each	+4°C

B. Storage and Handling

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

The COX activity kit is compatible with samples in a wide range of matrices. The procedure described in the Assay Protocol 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.

C. Additional Materials Required

- Deionized or distilled water.
- Precision pipettes for volumes between 25 µL and 1,000 µl.
- Repeater pipettes for dispensing 50 µL.
- Disposable beakers and graduated cylinders.
- Plate luminometer with dual injectors.
- 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. 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.

5. Pre-Assay Preparation

- Do not mix reagents from different lot numbers.
- Allow all reagents to warm to room temperature for at least 30 minutes before opening.
- Pre-rinse the pipette tip with reagent, use fresh pipet tips for each sample, standard and reagent.
- Add the reagents to the side of the well to avoid contamination.
- Mix the substrate well prior to use.
- 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.
- 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.

A. Reagent Preparation

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 (porcine) by dissolving in DMSO at 0.38 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, 0.5 mM 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.

6. Assay Protocol

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 temperature.

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 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.

7. Data Analysis

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

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:

$$Average\ Net\ RLU \quad = \quad Average\ RLU - Average\ Blank\ RLU$$

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

Percent Inhibition =

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

Typical Results

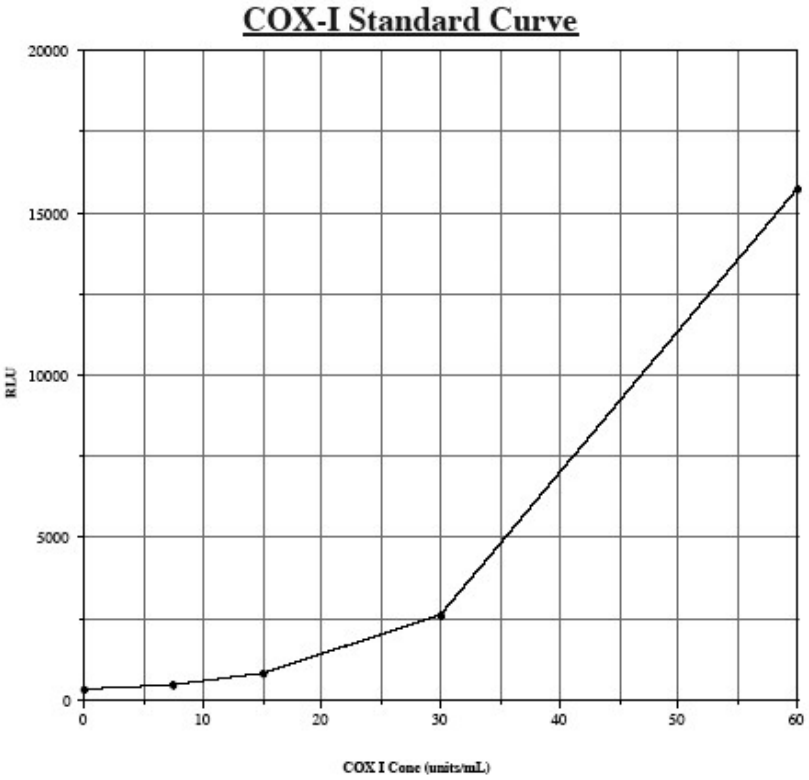
The results shown below are for illustration only and **should not** be used to calculate results from another assay.

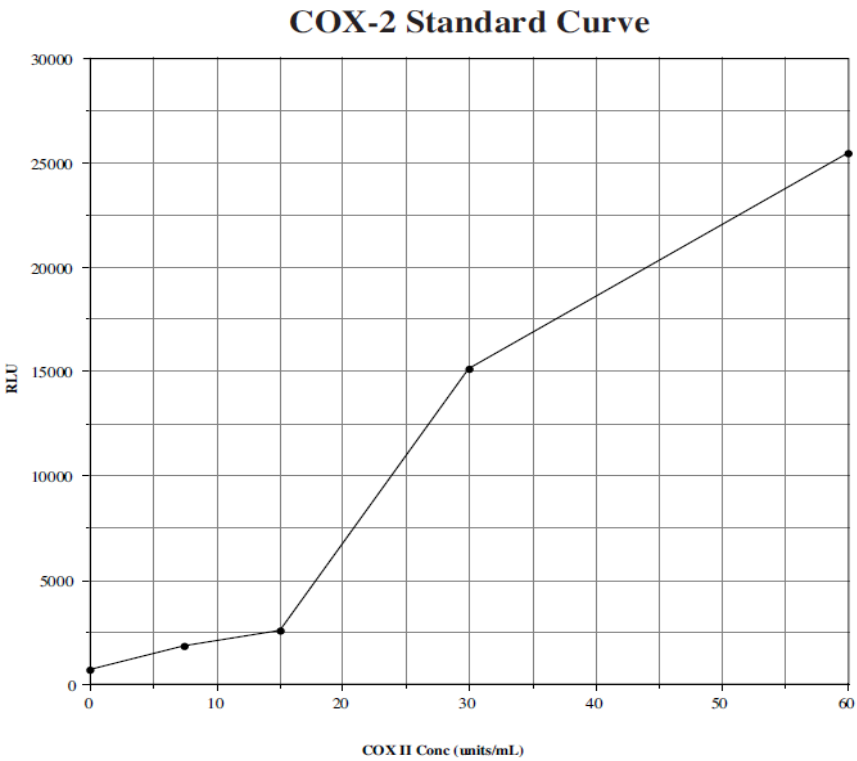
	Cyclooxygenase-I			**Cyclooxygenase-II**		
Sample	COX-1 (Units/ mL)	Net RLU	% Inhibition	COX-1 (Units/ mL)	Net RLU	% Inhibition
S0	0	311		0	655	
S1	60	15691		60	25442	
S2	30	2,603		30	15075	
S3	15	812		15	2531	
S4	7.5	429		7.5	1835	
Ibuprofen	60	1748	88.9%*	60	2451	90.4%*
Meloxicam	60	7246	53.8%*	60	10070	60.4%*

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

Typical Standard Curves

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




COX-2 Standard Curve
RLU
30000
25000
20000
15000
10000
5000
0
0
10
20
30
40
50
60
COX II Conc (units/mL)

8. 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·

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	=	10667	±	544 (5.1%)
Delta RLU's (7.5-0 Units/mL	=	9725		
2 SD's of 0 Units/mL Standard	=	190		

Sensitivity = 190/9725 x 7.5 Units/mL = 0.147 Units/mL

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).

Precision

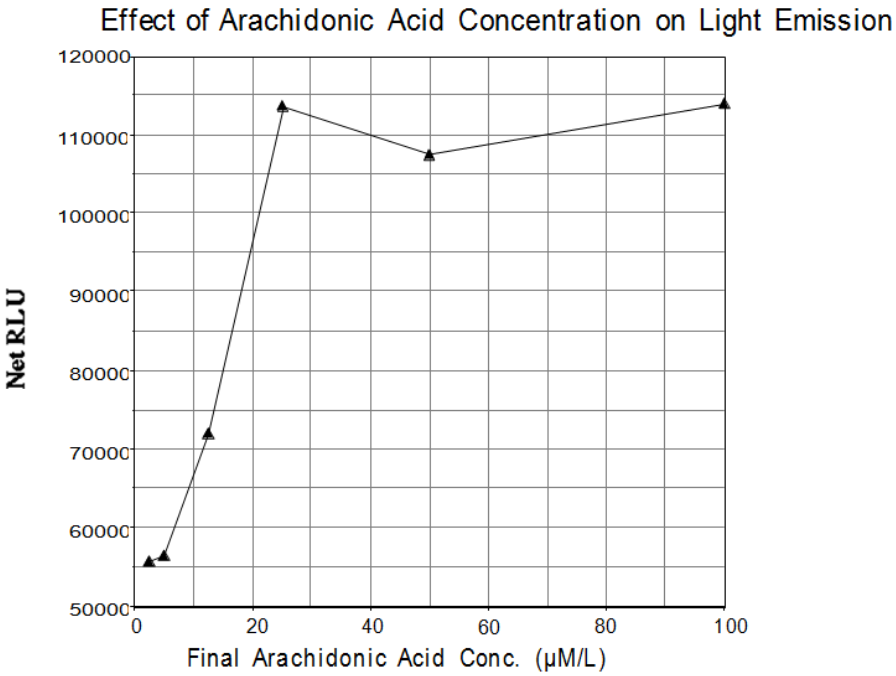
Intra-assay precision was determined by taking samples containing low and high concentrations of COX-I and running these samples multiple times (n=16) in the same assay. The precision numbers listed below represent the percent coefficient of variation for the concentrations of COX-I determined in these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

	COX-I Concentration (Units/mL)	**RLU's**	**RLU Intra-assay (%CV)**
High	30	78972	7.0
Low	7.5	19193	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)	2.5 mM	12.7
Tween 20	0.1%	5
EDTA	0.1%	7.3
Protein	100%	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

9. Troubleshooting

Problem	Reason	Solution
Assay not working	Assay buffer at wrong temperature	Assay buffer must not be chilled – needs to be at RT
	Protocol step missed	Re-read and follow the protocol exactly
	Plate read at incorrect wavelength	Ensure you are using appropriate reader and filter settings

	Unsuitable microtiter plate for assay	Fluorescence: Black plates (clear bottoms); Luminescence: White plates; Colorimetry: Clear plates. If critical, protocol will indicate whether to use flat- or U-shaped wells
Unexpected results	Measured at wrong wavelength	Ensure you are using appropriate reader and filter settings
	Unsuitable sample type	Refer to datasheet for details about incompatible samples
	Sample readings are outside linear range	Concentrate/ dilute samples to be in linear range

Samples with inconsistent readings	Unsuitable sample type	Refer to datasheet for details about incompatible samples
	Too many freeze/thaw cycles	Aliquot samples to reduce the number of freeze/thaw cycles
	Samples are too old or incorrectly stored	Use fresh made samples and store at recommended temperature until use
Lower/higher readings in samples and standards	Not fully thawed kit components	Wait for components to thaw completely and gently m ix prior use
	Reagents sitting for extended periods on ice	Try to prepare a fresh reaction mix prior to each use

	Incorrect amounts used	Check pipette is calibrated correctly (always use smallest volume pipette that can pipette entire volume)
Standard curve is not linear	Not fully thawed kit components	Wait for components to thaw completely and gently m ix prior use
	Pipetting errors when setting up the standard curve	Try not to pipette too small volumes
	Incorrect pipetting when setting up the reaction mix	Always prepare a master mix

	Air bubbles in wells	Air bubbles will interfere with readings; try to avoid producing air bubbles and always remove bubbles prior to reading plates

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