Technical Bulletin

COX Activity Assay Kit (Fluorometric)

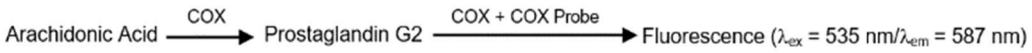
Catalog Number MAK414

Product Description

Cyclooxygenase (COX), also known as prostaglandin-endoperoxide synthase (PTGS), is an enzyme that is responsible for the formation of important biological mediators called prostanoids, including prostaglandins, prostacyclin and thromboxane. COX is the central enzyme in the biosynthetic pathway to prostanoids from arachidonic acid. There are two known isoenzymes: COX-1 and COX-2. COX-1 is constitutively expressed in many tissues and is the predominant form in gastric mucosa and in kidney. COX-2 is not expressed under normal conditions in most cells, but elevated levels are observed during inflammation. Pharmacological inhibition of COX by non-steroidal anti-inflammatory drugs (NSAID) can provide relief from the symptoms of inflammation and pain.

The COX Activity Assay Kit provides a simple and sensitive method to detect the peroxidase activity of COX in biological samples or purified/crude enzyme preparations. The kit includes COX-1 and COX-2 specific inhibitors to differentiate the activity of COX-1 and COX-2, as well as other peroxidases, which may be present in the sample. The method is suitable for high-throughput applications and has a detection limit of 6 μU/mg.

The kit is suitable for the measurement of COX activity in various biological samples such as adherent and suspension cells and tissues, and purified/crude enzyme preparations.



Components

The kit is sufficient for 100 fluorometric assays in 96-well plates.

- COX Assay Buffer — 25 mL
  Catalog Number MAK414A
- COX Probe (in DMSO) — 200 μL
  Catalog Number MAK414B
- COX Cofactor (in DMSO) — 20 μL
  Catalog Number MAK414C
- Arachidonic Acid — 1 vial
  Catalog Number MAK414D
- NaOH — 500 μL
  Catalog Number MAK414E
- COX-1 Positive Control — 1 vial
  Catalog Number MAK414F
- Resorufin Standard (5 mM, in DMSO) — 50 μL
  Catalog Number MAK414G
- SC560 (COX-1 inhibitor in DMSO) — 100 μL
  Catalog Number MAK414H
- Celecoxib (COX-2 inhibitor in DMSO) — 100 μL
  Catalog Number MAK414I

Reagents and Equipment Required but Not Provided

- Pipetting devices and accessories (e.g., multichannel pipettor)
- Fluorescence multiwell plate reader
- Opaque flat-bottom 96-well plates. Cell culture or tissue culture treated plates are not recommended.
- Dounce tissue grinder set (Catalog Number D9063 or equivalent)
- Microcentrifuge capable of RCF ≥12,000 × *g*
- Dimethyl Sulfoxide (DMSO), anhydrous (Catalog Number 276855 or equivalent)
- Phosphate Buffered Saline (Catalog Number P3813)
- IGEPAL® CA-630 (Catalog Number I8896 or equivalent)
- Protease Inhibitor Cocktail (Catalog Number P8340 or equivalent)
- Ethanol, 200 Proof (Catalog Number E7023 or equivalent)
- Bicinchoninic Acid Kit for Protein Determination (Catalog Number BCA1 or equivalent)

Precautions and Disclaimer

For Research Use Only. Not for use in diagnostic procedures. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on wet ice. Store components at -20 °C, protected from light.

Preparation Instructions

Briefly centrifuge small vials prior to opening. Unless specified, bring components to room temperature prior to use.

Lysis Buffer: Prepare buffer by adding 1% IGEPAL® CA-630 (not included) to 1× PBS (not included). Prepare enough for the assay.

COX Assay Buffer, COX Probe (in DMSO), COX Cofactor (in DMSO), NaOH, Resorufin Standard (5 mM, in DMSO), SC560 (COX-1 inhibitor in DMSO) and Celecoxib (COX-2 inhibitor in DMSO): Ready to use as supplied.

COX-1 Positive Control: Reconstitute vial with 20 µL of purified water. Aliquot and store at -80 °C. Avoid repeated freeze/thaw cycles. Use within two months of reconstitution. For short-term storage (~ two weeks), Positive Control can be stored at -20 °C. Keep on ice while in use. **Aliquot is only stable for ~30 minutes on ice.**

Arachidonic Acid: Reconstitute vial with 55 µL of 100% ethanol (not included) and vortex for 15-30 seconds.

Procedure

All samples and standards should be run in duplicate.

Sample Preparation

Cells

Note: Adherent cells can be scraped off from the culture plate.

1. To prepare cell lysate, wash cells (~2-6 × 10^6 cells) once with 10 mL of 1× PBS.
2. Centrifuge at 12,000 × *g* at 4 °C for 15 minutes.
3. Resuspend in 1 mL of 1× PBS and transfer cells to a 1.5 mL tube.
4. Centrifuge at 500 × *g* for 3 minutes.
5. Discard the supernatant and resuspend cell pellet in 0.2-0.5 mL of Lysis Buffer supplemented with Protease Inhibitor Cocktail (not included).
6. Vortex and incubate on ice for 5 minutes.
7. Measure the protein concentration of the supernatant from Step 6 using BCA or preferred method.

Tissue

Note: Perfused tissue samples are recommended for preparing tissue homogenates.

1. Wash tissue (~50-100 mg wet weight) three times with 1× PBS.
2. Add 0.2-0.5 mL of Lysis Buffer supplemented with Protease Inhibitor Cocktail (not included) and quickly homogenize tissue on ice.
3. Centrifuge the cell lysate and tissue homogenate at 12,000 × *g* at 4 °C for 3 minutes.
4. Collect the supernatant and keep on ice.
5. Measure the protein concentration of the supernatant using BCA or preferred method.

Standard Curve Preparation

1. Prepare a 10 µM (10 pmol/ µL) Resorufin Standard by diluting 2 µL of the 5 mM Resorufin Substrate Solution with 998 µL of COX Assay Buffer.
2. Prepare a 1 µM (1 pmol/ µL) Resorufin Standard by diluting 50 µL of the 10 µM Resorufin Standard from Step 1 with 450 µL of COX Assay Buffer. Prepare Resorufin Standards according to Table 1. Mix well. Discard standard dilutions after use, do not store.

Table 1.
Preparation of Resorufin Standards

Well	1 µM (1 pmol/µL) Resorufin Standard	COX Assay Buffer	Resorufin (pmol/ well)
1	0 µL	100 µL	0
2	4 µL	96 µL	4
3	8 µL	92 µL	8
4	12 µL	88 µL	12
5	16 µL	84 µL	16
6	20 µL	80 µL	20

COX Activity

1. Dilute COX Cofactor 1:200 by adding 2 µL of COX Cofactor to 398 µL of COX Assay Buffer just before use. Mix well. Diluted COX Cofactor is stable for 1 hour at room temperature. Do not store the Diluted COX Cofactor solution.
2. Prepare Arachidonic Acid solution by adding 5 µL of supplied Arachidonic Acid to 5 µL of NaOH just before use. Vortex briefly to mix. Dilute Arachidonic Acid/NaOH solution 1:10 by adding 90 µL of purified water to the vial and vortex briefly to mix. Make as much as needed. Diluted Arachidonic Acid/NaOH solution is stable for at least 1 hour on ice. Do not store the Diluted Arachidonic Acid/NaOH solution.
3. For each Sample and the Positive Control, prepare 2 parallel wells.
   a. To one of the wells, add 2 µL of DMSO for total activity assay of Sample (S).
   b. To the second well, add 2 µL of either COX-1 or COX-2 inhibitor for partial activity of Sample + Inhibitor (SI). To measure COX-1 activity, add COX-1 Inhibitor (SC560). To measure COX-2 activity, add COX-2 Inhibitor (Celecoxib).

4. Prepare the appropriate Reaction Mix for the 2 parallel wells of Sample or Positive Control according to Table 2. Mix well. Note: The Reaction Mix instructions prepare sufficient reagent for **both** the Sample and Sample + Inhibitor (S and SI) wells or for **both** Positive Control and Positive Control + Inhibitor wells.

Table 2.
Preparation of Reaction Mix

Reagent	Sample Reaction Mix	Positive Control Reaction Mix
COX Probe	2 µL	2 µL
Diluted COX Cofactor	4 µL	4 µL
Sample (see Note*)	2-20 µL	-
COX-1 Positive Control	-	2 µL
COX Assay Buffer	Adjust total volume to 176 µL	168 µL

* Note: For unknown samples, test different amounts of Sample in the reaction (parallel wells for S and SI) to ensure the readings are within the Standard Curve range.

5. Preset the plate reader to λ_{Ex} = 535 nm/λ_{Em} = 587 nm in kinetic mode to read the plate every 15 seconds for a total of 30 minutes at room temperature. Presetting the instrument will avoid a delay in measurement after addition of Arachidonic Acid/NaOH solution.
6. Add 88 µL of the appropriate Reaction Mix into each parallel well.
7. Using a multi-channel pipette, add 10 µL of Diluted Arachidonic Acid/NaOH Solution into each Sample and Positive Control well (with and without inhibitor) to initiate the reaction at the same time. Do **not** add Diluted Arachidonic Acid/NaOH Solution to the standard wells.

Measurement

Immediately after addition of the Arachidonic Acid**,** measure the fluorescence (RFU) using the preset plate reader settings (λ_{Ex} = 535 nm/λ_{Em} = 587 nm) in kinetic mode reading every 15 seconds for a total of 30 minutes at room temperature. The Standard Curve can be read in end point mode at the end of the incubation time.

Results

1. Subtract the 0 Standard RFU reading from all Standard RFU readings.
2. Plot the Resorufin Standard Curve.
3. Choose two time points (T_1 and T_2) in the linear range of the Sample (S and SI) RFU reading curves to calculate the COX activity of the Sample (RFU_S) and Sample + Inhibitor (RFU_{SI}).
4. Calculate ΔT ($\Delta T = T_2 - T_1$).
5. Calculate COX isozyme (COX-1 or COX-2) activity of the Sample (S):

   ΔRFU_S =
   $(RFU_{S2} - RFU_{S1}) - (RFU_{SI2} - RFU_{SI1})$.

6. Apply the ΔRFU_S to the Resorufin Standard Curve to determine B pmol of Resorufin generated by the respective COX isoenzyme during the reaction time (ΔT).

   COX isozyme activity (pmol/minute/mg or µU/mg) =

   $$B/(\Delta T \times M)$$

where:

B = Resorufin amount from Standard Curve (pmol)

ΔT = Reaction time ($T_2 - T_1$) (minutes)

M = Protein amount added into the reaction well (mg)

Unit Definition: One unit of Cyclooxygenase (COX) activity is the amount of enzyme that will generate 1.0 μmol of resorufin per minute at pH 8.0 and 25 °C.

Figure 1.
Typical Resorufin Standard Curve.

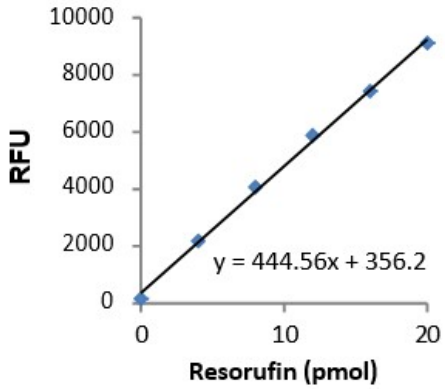


Figure 2.
Measurement of COX-1 Positive Control (PC) activity with and without addition of SC560 COX-1 inhibitor.

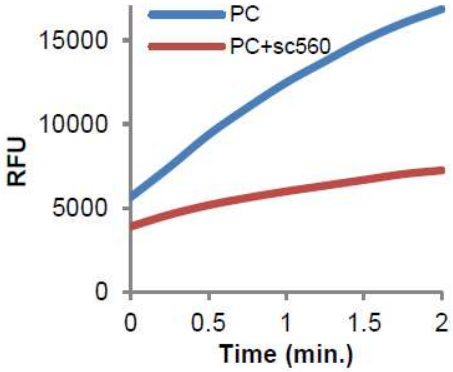


Figure 3.
Detection of endogenous COX-1 activity in J774 cell lysate (6 μg) and rat liver homogenate (210 μg).

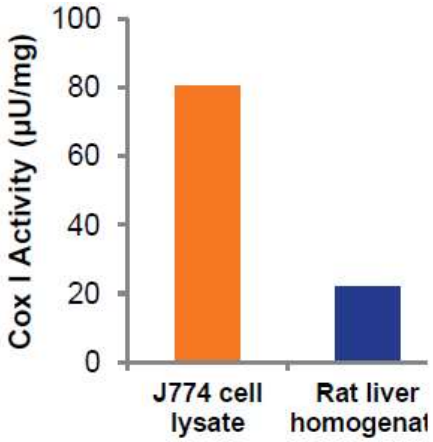
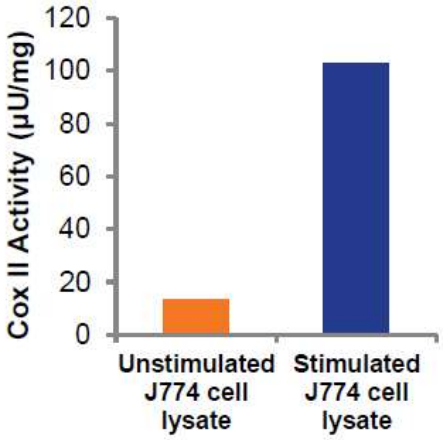


Figure 4.
Detection of endogenous COX-2 activity in J774 cell lysate (7 μg) stimulated with or without 100 ng/mL LPS and 100 ng/mL murine IFN-γ. Assays were performed following the kit protocol.



Frequently Asked Questions

Can this kit be used with samples like bacteria, plants, drosophila, yeast, etc.?

The kit has been standardized for mammalian cells only.

Can frozen samples be used with this assay?

Fresh samples are recommended. However, frozen samples can be used provided:

- Samples are frozen immediately after isolation, and
- Have not undergone multiple freeze thaw cycles.

It is recommended to aliquot samples prior to freezing.

What is the exact volume of sample required for this assay?

There is no specific recommended volume for each sample to be used since activity is based on sample concentration and quality based. Perform pilot experiments with multiple sample volumes to determine the optimal volume which gives a reading within the linear range of the standard curve.

Why are my standard curve values lower than those shown on the datasheet?

Multiple factors influence the signals such as incubation times, room temperature, and sample handling. In general, to increase the RFU reading value of the standards, increase the incubation time. As long as the standard curve is linear, it should be fine to use, since the samples will be measured under the same conditions on this curve.

How do I normalize my samples against protein concentration?

Use a protein quantitation assay on the cell/tissue lysates supernatants or with any other liquid sample in the assay buffer.

Can an alternate buffer be used for sample preparation (cell lysis, sample dilutions etc.)?

The COX Activity Assay Kit buffer and the Lysis Buffer (not included) have been optimized for the reaction. The COX Assay Buffer contains proprietary components required for the assay reactions. Therefore, it is highly recommended to use the buffer provided in the kit for the best results.

Should I make a standard curve for every experiment I do, or is one curve/kit enough?

A standard curve should be prepared for each experimental run. Varying conditions from run to run may cause a difference in assay results.

What can cause a high background in the 0 Standard?

The high background indicates decomposition of the standard. The resorufin standard is extremely susceptible to handling and should be kept cold at all times. Aliquot and store at -20 °C, avoid repeated freeze/thaw cycles. When in use, the standard should be kept on ice.

Notice

We provide information and advice to our customers on application technologies and regulatory matters to the best of our knowledge and ability, but without obligation or liability. Existing laws and regulations are to be observed in all cases by our customers. This also applies in respect to any rights of third parties. Our information and advice do not relieve our customers of their own responsibility for checking the suitability of our products for the envisaged purpose.

The information in this document is subject to change without notice and should not be construed as a commitment by the manufacturing or selling entity, or an affiliate. We assume no responsibility for any errors that may appear in this document.

Contact Information

For the location of the office nearest you, go to SigmaAldrich.com/offices.

Technical Service

Visit the tech service page on our web site at SigmaAldrich.com/techservice.

Standard Warranty

The applicable warranty for the products listed in this publication may be found at SigmaAldrich.com/terms.