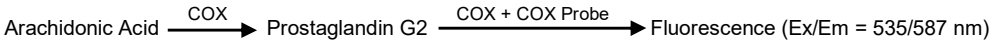
Cyclooxygenase (COX) Activity Assay Kit (Fluorometric) (BN00779)

(Catalog # BN00779; 100 assays; Store at -20°C)

I. Introduction:

Cyclooxygenase (COX), also known as prostaglandin-endoperoxide synthase (PTGS, EC 1.14.99.1), 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. Assay Genie's COX Activity Assay Kit provides a simple, sensitive, and high-throughput adaptable 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. Detection limit: 6 µU/mg.



II. Application:

- Measurement of COX activity in various biological samples and purified/crude enzyme preparations

III. Sample Type:

- Adherent and suspension cells
- Animal tissues such as rat liver
- Purified enzyme

IV. Kit Contents:

Components	BN00779	Cap Code	Part Number
COX Assay Buffer	25 ml	WM	BN00779-1
COX Probe (in DMSO)	200 µl	Red	BN00779-2
COX Cofactor (in DMSO)	20 µl	Violet	BN00779-3
Arachidonic Acid	50 µl	Blue	BN00779-4
NaOH	500 µl	Clear	BN00779-5
COX-1 Positive Control	1 vial	Green	BN00779-6
Resorufin Standard (5 mM, in DMSO)	50 µl	Yellow	BN00779-7
SC560 (COX-1 inhibitor in DMSO)	100 µl	Orange	BN00779-8
Celecoxib (COX-2 inhibitor in DMSO)	100 µl	Brown	BN00779-9

V. User Supplied Reagents and Equipment:

- 96-well white opaque plate with flat bottom
- Multi-well spectrophotometer (fluorescence plate reader)
- Multi-channel pipette (adjustable to 10 µl)
- DMSO, 1X PBS, lysis buffer (*e.g.*1 X PBS with 1% NP40), Protease inhibitor Cocktail (Cat. # K272 or equivalent)

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Briefly spin small vials prior to opening. Read entire protocol before performing the assay. Unless specified, bring components to room temperature (RT) before use.

- **COX-1 Positive Control:** Reconstitute with 20 µl of sterile ddH_2O. Aliquot and store at -80°C. Avoid repeated freeze/thaw. Use within two months. For short-term storage (~ 2 weeks), Positive Control can be stored at -20°C. Keep on ice while in use. It's stable for at least ~30 min. on ice. **Note:** we recommend not keeping the enzyme on ice for long.

VII. Cox Activity Assay Protocol:

1. **Sample Preparation:** To prepare cell lysate, wash cells (~2-6 X 10^6) once with 10 ml PBS (1X). Resuspend in 1 ml PBS (1X) and transfer cells to a 1.5 ml tube. Centrifuge at 500 x g for 3 min. Discard supernatant and resuspend cell pellet in 0.2-0.5 ml of lysis buffer with protease inhibitor cocktail (not provided). Vortex and incubate on ice for 5 min. To prepare tissue homogenate, wash tissue (~50-100 mg wet weight) three times with PBS (1X). Add 0.2-0.5 ml of lysis buffer with protease inhibitor cocktail (not provided) and quickly homogenize tissue on ice. Centrifuge the cell lysate & tissue homogenate at 12,000 X g, 4°C for 3 min. Collect supernatant and keep on ice.

   Notes:

   a. We recommend using perfused tissue samples for preparing tissue homogenates.

   b. For unknown samples, we recommend doing a pilot experiment & testing several doses to ensure the readings are within the Standard Curve range.

   c. Adherent cells can be scraped off from the culture plate.

2. **Standard Curve Preparation:** Dilute Resorufin Standard to 10 µM (10 pmol/µl) by adding 2 µl of 5 mM Resorufin Standard to 998 µl COX Assay Buffer. Dilute further to 1 µM (1 pmol/µl) by adding 50 µl of 10 µM Resorufin Standard into 450 µl COX Assay Buffer. Add

0, 4, 8, 12, 16, and 20 µl of 1 pmol/µl Resorufin Standard into a series of wells in a 96-well plate to generate 0, 4, 8, 12, 16, and 20 pmol/well of Resorufin Standard. Adjust the volume to 100 µl/well with COX Assay Buffer.

Note: We don't recommend storing the diluted Standard.

3. **COX Activity:** Dilute COX Cofactor 200 times by adding 2 µl of COX Cofactor to 398 µl of COX Assay Buffer just before use. Mix well. 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 10 times by adding 90 µl ddH2O, vortex briefly to mix. Make as much as needed.
For Positive Control and each sample, prepare 2 parallel wells. To one of the wells, add 2 µl of DMSO (for total activity) and to other well add 2 µl of either COX-1 or COX-2 inhibitor. To measure COX-1 activity, add COX-1 Inhibitor (SC560) and to measure COX-2 activity, add COX-2 Inhibitor (Celecoxib). Prepare reaction mix for the 2 parallel wells according to the layout below:

	Reaction Mix
COX Assay Buffer	Adjust total volume to 176 µl
COX Probe	2 µl
Diluted COX Cofactor	4 µl
Sample	2-20 µl

Add 88 µl of Reaction Mix into each parallel well in 96-well plate. For Positive Control, replace sample with 2 µl of Positive Control in the reaction mix. Use a multi-channel pipette to add 10 µl diluted Arachidonic Acid/NaOH Solution into each well to initiate the reaction at the same time.

Notes:

a. Diluted COX Cofactor is stable for 1 hr at RT. We don't recommend storing the diluted COX Cofactor.

b. Diluted Arachidonic Acid/NaOH solution is stable for at least 1 hr on ice. We don't recommend storing diluted Arachidonic Acid/NaOH solution.

c. Preset the plate reader to avoid delay in measurement after addition of Arachidonic Acid/NaOH solution.

4. **Measurement** After addition of the Arachidonic Acid**,** measure fluorescence (Ex/Em = 535/587 nm) immediately in a kinetic mode once every 15 sec. for 30 min.

Note: Incubation time depends on sample's COX activity. We recommend measuring fluorescence in a kinetic mode and choosing two time points (T_1 and T_2) in the linear range to calculate the COX activity of the sample (RFU_S) and sample with inhibitor (RFU_I). The Standard Curve can be read in the end point mode (i.e. at the end of incubation time).

5. **Calculation:** Subtract 0 Standard reading from all Standard readings. Plot the Resorufin Standard Curve. Calculate COX activity of the sample: $\Delta RFU = (RFU_{S2} - RFU_{S1}) - (RFU_{I2} - RFU_{I1})$. Apply the ΔRFU to the Resorufin Standard Curve to get B pmol of Resorufin generated by the respective COX isoenzyme during the reaction time ($\Delta T = T2 - T1$).

$$\textbf{Sample Cox Activity} = \frac{B}{\Delta T \times M}\left(\frac{pmol}{min.mg}\right) \text{ or } \left(\frac{\mu U}{mg}\right) f$$

Where: **B** is amount of resorufin from Standard Curve (pmol)
ΔT is incubation time (min.)
M is protein amount added into the reaction well (mg)

Unit Definition: One unit of COX activity is the amount of enzyme that generates 1.0 µmol of resorufin per min. at pH 8.0, 25°C.

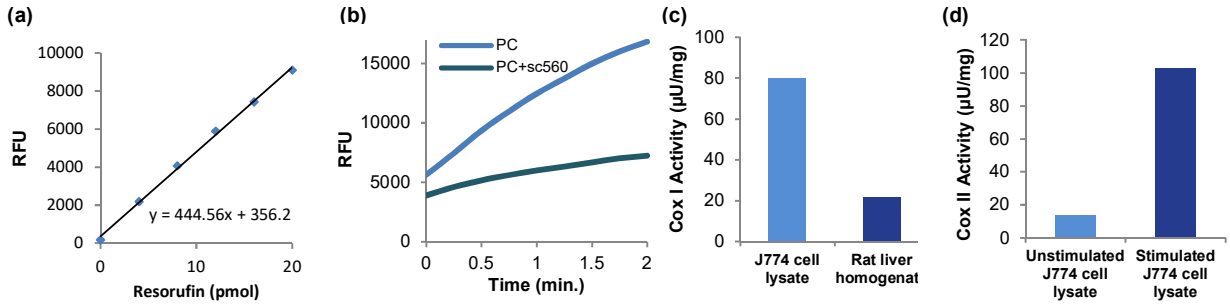


Figure: (a) Resorufin Standard Curve. **(b)** Measurement of Cox-1 Positive Control (PC) activity. **(c)** Detection of endogenous Cox I activity in J774 cell lysate (6 µg) and rat liver homogenate (210 µg). **(d)** Detection of endogenous Cox II activity in J774 cell lysate (7 µg) stimulated with or without 100 ng/ml LPS and 100 ng/ml murine IFN-gamma. Assays were performed following the kit protocol.

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