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# Produktinformation

- Forschungsprodukte & Biochemikalien
- Zellkultur & Verbrauchsmaterial
- Diagnostik & molekulare Diagnostik
- Laborgeräte & Service

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See the following pages for more information!

## Lieferung & Zahlungsart

siehe unsere Liefer- und Versandbedingungen

## Zuschläge

- Mindermengenzuschlag
- Trockeneiszuschlag
- Gefahrgutzuschlag
- Expressversand


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# COX (ovine/human) Inhibitor Screening Assay Kit

Item No. 560131

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

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# GENERAL INFORMATION

## Materials Supplied

Kit will arrive packaged as a -80°C kit. After opening the kit, store individual components as started below.

| Item Number | Item | Quantity | Storage |
|---|---|---|---|
| 414016 | PG Screening ELISA Antiserum | 1 vial | -20°C |
| 414006 | PG Screening AChE Tracer | 1 vial | -20°C |
| 414026 | PG Screening ELISA Standard | 1 vial | -20°C |
| 400060 | ELISA Buffer Concentrate (10X) | 2 vials | RT |
| 400062 | Wash Buffer Concentrate (400X) | 1 vial | RT |
| 400035 | Polysorbate 20 | 1 vial | RT |
| 400004 | Mouse Anti-Rabbit IgG Coated Plate | 1 plate | 4°C |
| 400012 | 96-Well Cover Sheet | 1 cover | RT |
| 400050 | Ellman's Reagent | 3 vials | -20°C |
| 460104 | Reaction Buffer (10X) | 1 vial | -20°C |
| 460100 | COX-1 (ovine) | 1 vial | -80°C |
| 460121 | COX-2 (human recombinant) | 1 vial | -80°C |
| 460102 | Heme | 1 vial | -20°C |
| 460103 | Arachidonic Acid (substrate) | 1 vial | -20°C |
| 460105 | Potassium Hydroxide | 1 vial | -20°C |
| 460106 | Hydrochloric Acid | 1 vial | -20°C |
| 460107 | Stannous Chloride | 1 vial | -20°C |

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 AChE ELISA Kits. 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

**Fax:** 734-971-3641

**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 specified in the **Materials Supplied** section, on 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 405-420 nm.
2. Adjustable pipettes and a repeating or multichannel pipettor.
3. A source of 'UltraPure' water. Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA. *NOTE: UltraPure water is available for purchase from Cayman (Item No. 400000).*
4. A 37°C water bath or heat block.
5. Reaction tubes.
6. Materials used for purification procedure (optional, see page 30).

# INTRODUCTION

## Background

Cyclooxygenase (COX, also called Prostaglandin H Synthase or PGHS) is a bifunctional enzyme exhibiting both COX and peroxidase activities. The COX component converts arachidonic acid to a hydroperoxy endoperoxide ($PGG_2$), and the peroxidase component reduces the endoperoxide to the corresponding alcohol ($PGH_2$), the precursor of prostaglandins (PGs), thromboxanes, and prostacyclins.[1,2]

It is now well established that there are two distinct isoforms of COX. COX-1 is constitutively expressed in a variety of cell types and is involved in normal cellular homeostasis. A variety of stimuli, such as phorbol esters, lipopolysaccharides, and cytokines, lead to the induced expression of a second isoform of COX, COX-2. COX-2 is responsible for the biosynthesis of PGs under acute inflammatory conditions.[3,4] This inducible COX-2 is believed to be the target enzyme for the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs.[4]

## About This Assay

The COX (ovine/human) Inhibitor Screening Assay directly measures $PGF_{2\alpha}$ by $SnCl_2$ reduction of COX-derived $PGH_2$ produced in the COX reaction. The prostanoid product is quantified *via* enzyme immunosorbent assay (ELISA) using a broadly specific antiserum that binds to all the major PG compounds.

This assay includes both ovine COX-1 and human recombinant COX-2 enzymes allowing the user to screen isozyme-specific inhibitors. This assay is an excellent tool which can be used for general inhibitor screening, or to eliminate false positive leads generated by less specific methods.



## Description of AChE Competitive ELISAs[5,6]

This assay is based on the competition between PGs and a PG-acetylcholinesterase (AChE) conjugate (PG tracer) for a limited amount of PG antiserum. Because the concentration of the PG tracer is held constant while the concentration of PG varies, the amount of PG tracer that is able to bind to the PG antiserum will be inversely proportional to the concentration of PG in the well. This rabbit antiserum-PG (either free or tracer) complex binds to a mouse monoclonal anti-rabbit antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of PG tracer bound to the well, which is inversely proportional to the amount of free PG present in the well during the incubation; or

$$\text{Absorbance} \propto [\text{Bound PG Tracer}] \propto 1/[\text{PG}]$$

A schematic of this process is shown in Figure 1, below.

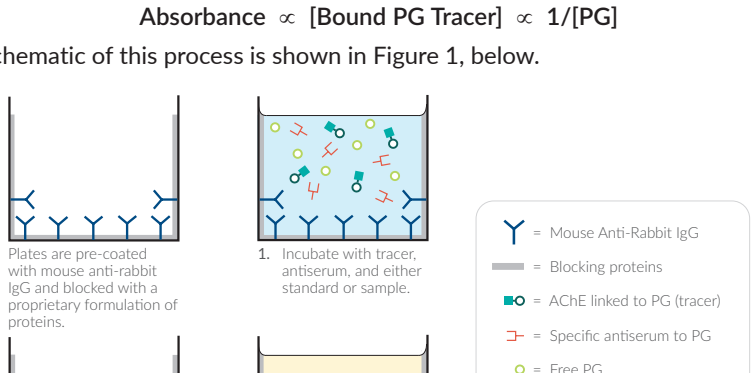



**Figure 1. Schematic of the AChE ELISA**

## Biochemistry of Acetylcholinesterase

The electric organ of the electric eel, *E. electricus*, contains an avid AChE capable of massive catalytic turnover during the generation of its electrochemical discharges. The electric eel AChE has a clover leaf-shaped tertiary structure consisting of a triad of tetramers attached to a collagen-like structural fibril. This stable enzyme is capable of high turnover (64,000 $s^{-1}$) for the hydrolysis of acetylthiocholine.

A molecule of the analyte covalently attached to a molecule of AChE serves as the tracer in AChE enzyme immunoassays. Quantification of the tracer is achieved by measuring its AChE activity with Ellman's Reagent. This reagent consists of acetylthiocholine and 5,5'-dithio-*bis*-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine (see Figure 2, on page 9). The non-enzymatic reaction of thiocholine with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm (ε = 13,600).

AChE has several advantages over other enzymes commonly used for enzyme immunoassays. Unlike horseradish peroxidase, AChE does not self-inactivate during turnover. This property of AChE also allows redevelopment of the assay if it is accidentally splashed or spilled. In addition, the enzyme is highly stable under the assay conditions, has a wide pH range (pH 5-10), and is not inhibited by common buffer salts or preservatives. Since AChE is stable during the development step, it is unnecessary to use a 'stop' reagent, and the plate may be read whenever it is convenient.

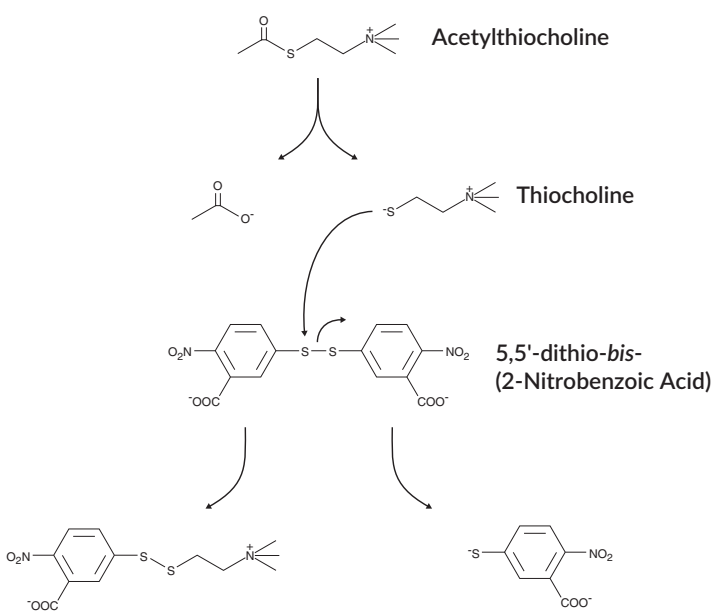



**Figure 2. Reaction catalyzed by acetylcholinesterase**

# COX REACTION PROCEDURE

*The use of both enzymes is not a requirement of the assay; one or both enzymes may be used depending on the nature of the study. The EIA plate will allow for 36 COX reactions (32 inhibitors, in duplicate) at one dilution or 18 COX reactions (15 inhibitors, in duplicate) at two dilutions.*

**IMPORTANT: Please read both COX Reaction Procedure and ELISA Procedure sections carefully before initiating your experiments!**

## COX Reagent Preparation

1. **Reaction Buffer (10X) - (Item No. 460104)**

   Dilute 5 ml of Reaction Buffer concentrate with 45 ml of UltraPure water. This final Reaction Buffer (0.1 M Tris-HCl, pH 8.0, containing 5 mM EDTA and 2 mM phenol) is used in the COX reactions and for dilution of Heme. When stored at room temperature, this diluted Reaction Buffer is stable for at least one month. Equilibrate the diluted Reaction Buffer to 37°C before using in the COX reactions.

2. **COX-1 (ovine) - (Item No. 460100)**

   This vial contains a solution of ovine COX-1 and should be kept on ice when thawed. Dilute 80 µl of enzyme with 320 µl of diluted Reaction Buffer and store on ice. This quantity is sufficient for 40 reactions. The diluted enzyme is stable for one hour on ice. If less than 40 reactions will be performed, it is recommended that the undiluted enzyme be aliquoted into several small vials and stored at -80°C to avoid repeated freeze thaw cycles.

3. **COX-2 (human recombinant) - (Item No. 460121)**

   This vial contains a solution of human recombinant COX-2 and should be kept on ice when thawed. Dilute 80 µl of enzyme with 320 µl of diluted Reaction Buffer and store on ice. This quantity is sufficient for 40 reactions. The diluted enzyme is stable for one hour on ice. If less than 40 reactions will be performed, it is recommended that the undiluted enzyme be aliquoted into several small vials and stored at -80°C to avoid repeated freeze thaw cycles.



4. **Heme - (Item No. 460102)**

   This vial contains a solution of Heme in dimethylsulfoxide (DMSO). Dilute 40 µl of Heme with 960 µl of 1X Reaction Buffer prior to use. The diluted Heme is stable for 12 hours at room temperature.

5. **Arachidonic Acid (Substrate) - (Item No. 460103)**

   This vial contains a solution of Arachidonic Acid in ethanol. Transfer 50 µl of the supplied Substrate to another vial, add 50 µl of Potassium Hydroxide (Item No. 460105), vortex, and dilute with 4.9 ml UltraPure water to achieve a final concentration of 200 µM. Use the prepared Arachidonic Acid Solution within one hour. A 10 µl aliquot of the prepared substrate will yield a final concentration of 10 µM in the reaction.

6. **Potassium Hydroxide - (Item No. 460105)**

   This vial contains 0.1 M Potassium Hydroxide (KOH). The reagent is ready to use as supplied.

7. **Hydrochloric Acid - (Item No. 460106)**

   This vial contains 1 M Hydrochloric Acid (HCl). The HCl is used to prepare the saturated Stannous Chloride Solution.

8. **Stannous Chloride - (Item No. 460107)**

   This vial contains crystalline Stannous Chloride. Add 5 ml of Hydrochloric Acid (Item No. 460106) and vortex to produce a saturated solution of Stannous Chloride. (This saturated Stannous Chloride Solution may be cloudy.) This solution is stable for eight hours at room temperature. If not performing all of the COX reactions in one day, weigh 125 mg of Stannous Chloride into another vial and add 2.5 ml of Hydrochloric Acid (Item No. 460106). 30 µl of saturated Stannous Chloride is required for each reaction. *NOTE: Stannous Chloride is used to reduce $PGH_2$, produced in the COX reaction, to a more stable PG, $PGF_{2\alpha}$*

## Performing COX Reactions

**Pipetting Hints**

- It is recommended that a repeating or multichannel pipettor be used to deliver Arachidonic Acid to the reaction tubes. This saves time and helps to maintain more precise incubation times.
- Use different tips to pipette the buffer, enzyme, Heme, inhibitor, and Arachidonic Acid.
- 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 reaction tube.

- *The final volume of the reaction is 230 µl in all the reaction tubes.*
- *You do not have to use both enzymes. You can use either COX-1 or COX-2 depending on your experimental design. The ELISA plate will allow for 36 COX reactions (32 inhibitors) in duplicate at one dilution or 18 COX reactions (15 inhibitors) in duplicate at two dilutions.*
- *Use the 1X Reaction Buffer in the reactions and pre-equilibrate to 37°C.*
- *Set the water bath or heat block temperature to 37°C before initiating the reactions.*
- *It is recommended that no more than 18 reactions be performed at one time.*
- *Aliquot reagents into reaction tubes that are at 37°C.*

1. **Background tubes** - Inactivate COX-1 and COX-2 by transferring 20 µl of each enzyme diluted to a 500 µl microfuge tube and placing the tube in boiling water for three minutes. The inactivated enzymes will be used to generate the background values. Add the following reagents to two reaction tubes: 160 µl of Reaction Buffer, 10 µl of Heme, and 10 µl of inactive COX-1 or inactive COX-2.
2. **COX-1 100% Initial Activity tubes** - Add 160 µl of Reaction Buffer, 10 µl of Heme, and 10 µl of COX-1 to two reaction tubes.
3. **COX-1 Inhibitor tubes** - Add 160 µl of Reaction Buffer, 10 µl of Heme, and 10 µl of COX-1 to six reaction tubes.
4. **COX-2 100% Initial Activity tubes** - add 160 µl of Reaction Buffer, 10 µl of Heme, and 10 µl of COX-2 to two reaction tubes.
5. **COX-2 Inhibitor tubes** - add 160 µl of Reaction Buffer, 10 µl of Heme, and 10 µl of COX-2 to six reaction tubes.
6. Add 10 µl of inhibitor* to the COX-1 and -2 inhibitor tubes and 10 µl of inhibitor vehicle to the 100% Initial Activity and Background tubes.
7. Incubate for 10 minutes at 37°C. *NOTE: Most inhibitors exhibit time-dependent inhibition of COX activity. Altering incubation times with the inhibitor can significantly change the apparent $IC_{50}$ value of the compound. Determining the optimal pre-incubation times for inhibitors is suggested.*
8. Initiate the reaction by adding 10 µl of Arachidonic Acid to all the reaction tubes. Quickly mix and incubate for EXACTLY two minutes at 37°C.
9. Add 30 µl of the saturated Stannous Chloride solution to each reaction tube to stop enzyme catalysis. Remove tubes from the water bath and vortex. Incubate for five minutes at room temperature. The reaction mixture will be cloudy.
10. Repeat steps 2-8 if performing more reactions.
11. The prostaglandins are quantified by ELISA. Proceed to the **ELISA Procedure**. The reactions are stable for one week at 0-4°C if tightly capped.

*Inhibitors can be dissolved in methanol, dimethylsulfoxide, or ethanol and should be added to the assay in a final volume of 10 µl. In the event that the appropriate concentration of inhibitor is completely unknown, we recommend that several dilutions of the inhibitor be made.

# ELISA PROCEDURE

*NOTE: Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA. UltraPure water may be purchased from Cayman (Item No. 400000).*

## ELISA Buffer Preparation

*Store all diluted buffers at 4°C; they will be stable for about two months.*

1. **ELISA Buffer Preparation**

   Dilute the contents of one vial of ELISA Buffer Concentrate (10X) (Item No. 400060) with 90 ml of UltraPure water. Be certain to rinse the vial to remove any salts that may have precipitated. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.*

2. **Wash Buffer Preparation**

   Dilute the contents of the vial of Wash Buffer Concentrate (400X) (Item No. 400062) to a total volume of 2 liters with UltraPure water and add 1 ml of Polysorbate 20 (Item No. 400035). A smaller volume of Wash Buffer can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Polysorbate 20 (0.5 ml/L of Wash Buffer). The diluted buffer will be stable for two months at 4°C.

   *NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a pipette. A positive displacement device such as a syringe should be used to deliver small quantities accurately.*



## Preparation of Assay-Specific Reagents

### Prostaglandin Screening Standard

Dissolve the lyophilized PG Screening ELISA Standard (Item No. 414026) in 1 ml of ELISA Buffer. The concentration of this solution (the bulk standard) will be 10 ng/ml. Store this solution at 4°C; it will be stable for approximately six weeks.

To prepare the standard for use in ELISA: Obtain eight clean test tubes and number them #1 through #8. Aliquot 800 µl ELISA Buffer to tube #1 and 500 µl ELISA Buffer to tubes #2-8. Transfer 200 µl of the bulk standard (10 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than 24 hours.

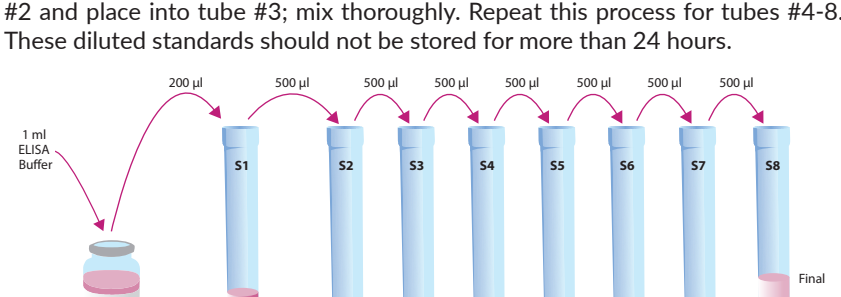



**Figure 3. Preparation of the PG standards**

### Prostaglandin Screening AChE Tracer

Reconstitute the 100 dtn PG Screening AChE Tracer (Item No. 414006) with 6 ml ELISA Buffer. Store the reconstituted PG Screening AChE Tracer at 4°C *(do not freeze!)* and use within two weeks. A 20% surplus of tracer has been included to account for any incidental losses.

### Prostaglandin Screening ELISA Antiserum

Reconstitute the 100 dtn PG Screening ELISA Antiserum (Item No. 414016) with 6 ml ELISA Buffer. Store the reconstituted PG Screening ELISA Antiserum at 4°C. It will be stable for at least four weeks. A 20% surplus of antiserum has been included to account for any incidental losses.



## COX Reaction Dilutions

### Background Samples

Obtain two clean test tubes and label them BC1 and BC2. Aliquot 990 µl of ELISA Buffer to each test tube. Add 10 µl of background COX-1 to the tube labeled BC1, 10 µl of background COX-2 to the tube labeled BC2, and mix thoroughly. Each test tube contains a 1:100 dilution of the original sample.

### COX 100% Initial Activity Samples

Obtain three clean test tubes per sample and number them IA1 through IA3. Aliquot 990 µl of ELISA Buffer to tube IA1, add 10 µl of COX-1 or COX-2 100% Initial Activity sample, and mix thoroughly. Aliquot 950 µl of ELISA Buffer to tube IA2, add 50 µl of tube IA1 to tube IA2, and mix thoroughly. Tube IA2 contains a 1:2,000 dilution of the original sample. Aliquot 500 µl of ELISA Buffer to tube IA3, add 500 µl of tube IA2 to tube IA3, and mix thoroughly. Tube IA3 contains a 1:4,000 dilution of the original sample. Tubes IA2 and IA3 will be run in the ELISA. Do not use test tube IA1 in the ELISA, this tube is too concentrated (this dilution is outside the usable range of the assay).

### COX Inhibitor Samples

Obtain three clean test tubes per sample and number them C1 through C3. Aliquot 990 µl of ELISA Buffer to tube C1, add 10 µl of sample, and mix thoroughly. Aliquot 950 µl of ELISA Buffer to tube C2, add 50 µl of tube C1 to tube C2, and mix thoroughly. Tube C2 contains a 1:2,000 dilution of the original sample. Aliquot 500 µl of ELISA Buffer to tube C3, add 500 µl of tube C2 to tube C3, and mix thoroughly. Tube C3 contains a 1:4,000 dilution of the original sample. Tubes C2 and C3 will be run in the ELISA. Do not use test tube C1 in the ELISA, this tube is too concentrated (this dilution is outside the usable range of the assay).

## Definition of Key Terms

**Blank:** background absorbance caused by Ellman's Reagent. The blank absorbance should be subtracted from the absorbance readings of all the other wells, including NSB wells.

**Total Activity:** total enzymatic activity of the AChE-linked tracer. This is analogous to the specific activity of a radioactive tracer.

**NSB (Non-Specific Binding):** non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well; the NSB is a measure of this low binding. Do not forget to subtract the Blank absorbance values.

**$B_0$ (Maximum Binding):** maximum amount of the tracer that the antibody can bind in the absence of free analyte.

**%B/$B_0$ (%Bound/Maximum Bound):** ratio of the absorbance of a particular sample or standard well to that of the maximum binding ($B_0$) well.

**Standard Curve:** a plot of the %B/$B_0$ values *versus* concentration of a series of wells containing various known amounts of analyte.

**Dtn:** determination, where one dtn is the amount of reagent used per well.

**Cross Reactivity:** numerical representation of the relative reactivity of this assay towards structurally related molecules as compared to the primary analyte of interest. Biomolecules that possess similar epitopes to the analyte can compete with the assay tracer for binding to the primary antibody. Substances that are superior to the analyte in displacing the tracer result in a cross reactivity that is greater than 100%. Substances that are inferior to the primary analyte in displacing the tracer result in a cross reactivity that is less than 100%. Cross reactivity is calculated by comparing the mid-point (50% B/$B_0$) value of the tested molecule to the mid-point (50% B/$B_0$) value of the primary analyte when each is measured in assay buffer using the following formula:

$$\%\ \text{Cross Reactivity} = \left[ \frac{50\%\ B/B_0 \text{ value for the primary analyte}}{50\%\ B/B_0 \text{ value for the potential cross reactant}} \right] \times 100\%$$



## Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. *NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

Each plate or set of strips must contain a minimum of two Blanks (Blk), two Non-Specific Binding wells (NSB), two Maximum Binding wells ($B_0$), one Total Activity well (TA), and an eight point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.* Each COX reaction sample should be assayed at two dilutions and each dilution should be assayed in duplicate. A minimum of one 100% Initial Activity Sample should be assayed for both COX-1 and -2.

A suggested plate format is shown in Figure 4, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 23, for more details). We suggest you record the contents of each well on the template sheet provided (see page 34).

Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
$B_0$ - Maximum Binding
S1-S8 - Standards 1-8
BC1 - Background COX-1
BC2 - Background COX-2
‡ - 100% Initial Activity Samples (IA2 & IA3)
H - COX Inhibitor Samples (C2 & C3)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Blk | S1 | S1 | BC1 | BC1 | H | H | H | H | H | H | H |
| B | Blk | S2 | S2 | BC2 | BC2 | H | H | H | H | H | H | H |
| C | NSB | S3 | S3 | ‡ | ‡ | H | H | H | H | H | H | H |
| D | NSB | S4 | S4 | ‡ | ‡ | H | H | H | H | H | H | H |
| E | $B_0$ | S5 | S5 | ‡ | ‡ | H | H | H | H | H | H | H |
| F | $B_0$ | S6 | S6 | ‡ | ‡ | H | H | H | H | H | H | H |
| G | $B_0$ | S7 | S7 | H | H | H | H | H | H | H | H | H |
| H | TA | S8 | S8 | H | H | H | H | H | H | H | H | H |

**Figure 4. Sample plate format**

## Performing the ELISA

**Pipetting Hints**

- Use different tips to pipette each reagent.
- 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.

### Addition of the Reagents

1. **ELISA Buffer**

   Add 100 µl of ELISA Buffer to NSB wells. Add 50 µl of ELISA Buffer to $B_0$ wells.

2. **Prostaglandin Screening ELISA Standard**

   Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. **Background Samples (test tubes BC1 and BC2)**

   Add 50 µl of sample per well. Each sample should be assayed in duplicate.

4. **COX 100% Initial Activity Samples (test tubes IA2 and IA3)**

   Add 50 µl of sample per well. We recommend that you assay each sample at the two dilutions with each dilution assayed in duplicate.

5. **COX Inhibitor Samples (test tubes C2 and C3)**

   Add 50 µl of sample per well. We recommend that you assay each sample at the two dilutions with each dilution assayed in duplicate.

6. **Prostaglandin Screening AChE Tracer**

   Add 50 µl of PG Screening AChE Tracer to each well *except* the TA and the Blk wells.

7. **Prostaglandin Screening ELISA Antiserum**

   Add 50 µl of PG Screening ELISA Antiserum to each well *except* the TA, the NSB, and the Blk wells.

| Well | ELISA Buffer | Standard/Sample | Tracer | Antiserum |
|---|---|---|---|---|
| Blk | - | - | - | - |
| TA | - | - | 5 µl (at devl. step) | - |
| NSB | 100 µl | - | 50 µl | - |
| $B_0$ | 50 µl | - | 50 µl | 50 µl |
| Std/Sample | - | 50 µl | 50 µl | 50 µl |

**Table 1. Pipetting summary**

### Incubation of the Plate

Cover each plate with plastic film (Item No. 400012) and incubate for 18 hours at room temperature on an orbital shaker.

### Development of the Plate

1. When ready to develop the plate, reconstitute one 100 dtn vial of Ellman's Reagent (Item No. 400050) with 20 ml of UltraPure water. This reagent is sufficient to develop 100 wells.

*NOTE: Reconstituted Ellman's Reagent is unstable and should be used the same day it is prepared; protect the Ellman's Reagent from light when not in use. Extra vials of the reagent have been provided should a plate need to be re-developed or multiple assays run on different days.*

2. Empty the wells and rinse five times with Wash Buffer.
3. Add 200 µl of Ellman's Reagent to each well.
4. Add 5 µl of tracer to the TA wells.
5. Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate to develop in the dark. This assay typically develops (*i.e.*, $B_0$ wells ≥0.3 A.U. (blank subtracted)) in 60-90 minutes.

### Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Remove the plate cover being careful to keep Ellman's Reagent from splashing on the cover. *NOTE: Any loss of Ellman's Reagent will affect the absorbance readings. If Ellman's Reagent is present on the cover, use a pipette to transfer the Ellman's Reagent into the well. If too much Ellman's Reagent has splashed on the cover to easily redistribute back into the wells, wash the plate three times with wash buffer and repeat the development with fresh Ellman's Reagent.*
3. Read the plate at a wavelength between 405 and 420 nm. The absorbance may be checked periodically until the $B_0$ wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the $B_0$ wells are in the range of 0.3-0.8 A.U. (blank subtracted). If the absorbance of the wells exceeds 1.5, wash the plate, add fresh Ellman's Reagent and let it develop again.



# ANALYSIS

Many plate readers come with data reduction software that plot data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as $\%B/B_0$ *versus* log concentration using either a 4-parameter logistic or log-logit curve fit. *NOTE: Cayman has a computer spreadsheet available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/analysis/elisa) to obtain a free copy of this convenient data analysis tool.*

## Calculations

### Preparation of the Data

The following procedure is recommended for preparation of the data prior to graphical analysis.

*NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.*

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the $B_0$ wells.
3. Subtract the NSB average from the $B_0$ average. This is the corrected $B_0$ or corrected maximum binding.
4. Calculate the $\%B/B_0$ (% Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected $B_0$ (from Step 3). Multiply by 100 to obtain $\%B/B_0$. Repeat for S2-S8 and all sample wells.

*NOTE: The TA values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool; the corrected $B_0$ divided by the actual TA (10X measured absorbance) will give the %Bound. This value should closely approximate the %Bound that can be calculated from the* ***Sample Data*** *(see page 26). Erratic absorbance values and a low (or no) %Bound could indicate the presence of organic solvents in the buffer or other technical problems (see page 32 for* ***Troubleshooting****).*

If you have purified your samples (see **Interference**, page 30), the final sample concentrations can be determined as follows:

## Calculations

$$\text{Recovery Factor} = \frac{10 \times \text{cpm of sample}}{[^3\text{H}]\text{-PGE}_2 \text{ added to sample (cpm)}}$$

$$\text{PG (pg) in purified sample} = \left[\frac{\text{Value from ELISA (pg/ml)}}{\text{Recovery Factor}}\right] \times 0.5 \text{ ml} - \text{added } [^3\text{H}]\text{-PGE}_2 \text{ (pg)}$$

$$\text{Total PG in sample (pg/ml)} = \frac{\text{PG (pg) in purified sample}}{\text{Volume of sample used for purification (ml)}}$$

## Plot the Standard Curve

Plot $\%B/B_0$ for standards S1-S8 *versus* Prostaglandin concentration using linear (y) and log (x) axes and perform a 4-parameter logistic fit.

Alternative Plot - The data can also be lineraized using a logit transformation. The equation for this conversion is shown below. *NOTE: Do not use $\%B/B_0$ in this calculation.*

$$\text{logit } (B/B_0) = \ln [B/B_0/(1 - B/B_0)]$$

Plot the data as logit $(B/B_0)$ *versus* log concentrations and perform a linear regression fit.

## Determine the Sample Concentration

1. Calculate the $\%B/B_0$ value for each sample.
2. Determine the concentration of each sample by identifying the $\%B/B_0$ on the standard curve and reading the corresponding values on the x-axis. $\%B/B_0$ values greater than 80% and less than 20% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample could indicate interference which may be eliminated by purification. Remember to multiply the COX samples by the appropriate dilution factor (BC1 and BC2 = 100; IA2 and C2 = 2,000; IA3 and C3 = 4,000).
3. Subtract the background values (BC1 and BC2) from the 100% Initial Activity and COX Inhibitor samples.
4. Subtract each Inhibitor Sample from the 100% Initial Activity Sample, then divide by the 100% Initial Activity Sample, and multiply by 100 to give the percent inhibition.
5. Graph the percent inhibition by the inhibitor concentration to determine the $IC_{50}$ value (concentration at which there was 50% inhibition).

## ELISA Performance Characteristics

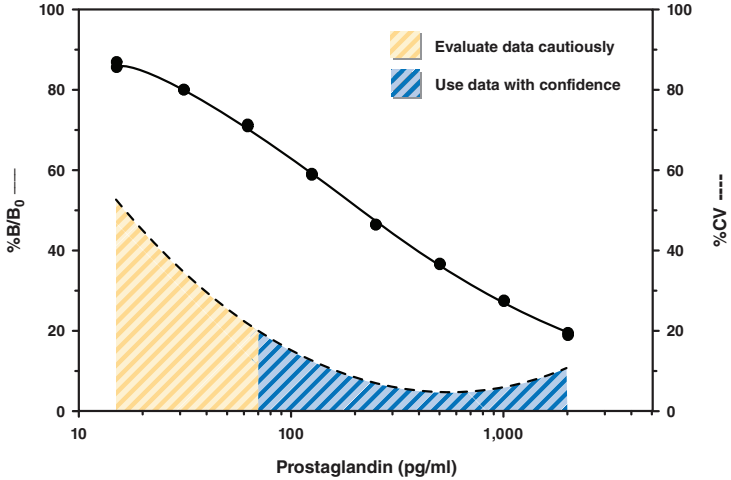
### Sample Data

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the value of your samples. Your results could differ substantially.

| | Raw Data | | Average | Corrected |
|---|---|---|---|---|
| **Total Activity** | 1.850 | 2.019 | 1.935 | |
| **NSB** | 0.003 | 0.002 | 0.003 | |
| **$B_0$** | 0.779 | 0.766 | | |
| | 0.771 | 0.778 | 0.774 | 0.771 |

| Dose (pg/ml) | Raw Data | | Corrected | | %B/$B_0$ | |
|---|---|---|---|---|---|---|
| 2,000 | 0.148 | 0.153 | 0.145 | 0.150 | 18.9 | 19.5 |
| 1,000 | 0.214 | 0.215 | 0.211 | 0.212 | 27.4 | 27.6 |
| 500 | 0.286 | 0.285 | 0.283 | 0.282 | 36.8 | 36.6 |
| 250 | 0.362 | 0.361 | 0.359 | 0.358 | 46.6 | 46.5 |
| 125 | 0.457 | 0.459 | 0.454 | 0.456 | 58.9 | 59.2 |
| 62.5 | 0.554 | 0.550 | 0.551 | 0.547 | 71.5 | 71.0 |
| 31.3 | 0.620 | 0.621 | 0.617 | 0.618 | 80.1 | 80.2 |
| 15.6 | 0.664 | 0.674 | 0.661 | 0.671 | 85.8 | 87.1 |

Table 2. Typical results




**Assay Range** = 15.6-2,000 pg/ml
**Sensitivity** (defined as 80% B/$B_0$) = 29 pg/ml
**Mid-point** (defined as 50% B/$B_0$) = 125-250 pg/ml

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted with ELISA Buffer.

Figure 5. Typical standard curve

## Precision:

The intra- and inter-assay CVs have been determined at multiple points on the standard curve. These data are summarized in the graph on page 27 and in the table below.

| Dose (pg/ml) | %CV* Intra-assay variation | %CV* Inter-assay variation |
|---|---|---|
| 2,000 | 7.9 | 13.1 |
| 1,000 | 8.1 | 9.8 |
| 500 | 8.2 | 6.8 |
| 250 | 8.6 | 9.7 |
| 125 | 10.6 | 10.2 |
| 62.5 | 11.0 | 8.8 |
| 31.3 | † | 19.4 |
| 15.6 | † | † |

**Table 3. Intra- and inter-assay variation**

*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve.

†Outside of the recommended usable range of the assay.

## Cross Reactivity:

| Compound | Cross Reactivity | Compound | Cross Reactivity |
|---|---|---|---|
| Prostaglandin $E_1$ | 100% | Leukotriene $D_4$ | 0.2% |
| Prostaglandin $E_2$ | 100% | Arachidonic Acid | <0.01% |
| Prostaglandin $F_{1\alpha}$ | 100% | Leukotriene $B_4$ | <0.01% |
| Prostaglandin $F_{2\alpha}$ | 100% | Leukotriene $C_4$ | <0.01% |
| Prostaglandin $F_{3\alpha}$ | 51.3% | Leukotriene $E_4$ | <0.01% |
| Prostaglandin $E_2$ Ethanolamide | 44.0% | Misoprostol | <0.01% |
| 6-keto Prostaglandin $F_{1\alpha}$ | 43.6% | Misoprostol (free acid) | <0.01% |
| 8-*iso* Prostaglandin $F_{2\alpha}$ | 38.4% | Prostaglandin $A_1$ | <0.01% |
| 8-*iso* Prostaglandin $E_2$ | 28.5% | Prostaglandin $A_2$ | <0.01% |
| Prostaglandin $D_2$ | 26.6% | Prostaglandin $A_3$ | <0.01% |
| 8-*iso*-2,3-dinor Prostaglandin $F_{1\alpha}$ | 20.0% | Prostaglandin $B_1$ | <0.01% |
| Prostaglandin $E_3$ | 9.5% | 15-keto Prostaglandin $E_2$ | <0.01% |
| Thromboxane $B_2$ | 5.0% | 13,14-dihydro-15-keto Prostaglandin $F_{2\alpha}$ | <0.01% |
| 12(S)-HHTrE | 0.25% | | |

**Table 4. Cross Reactivity of the PG Screening Assay**

## Interference

It is possible that a COX inhibitor will interfere with the ELISA and thus appear to exhibit no enzyme inhibition or exhibit a higher prostaglandin value than the 100% initial activity well. If the inhibitor exhibits no inhibition, you can either repeat the COX reaction using a higher concentration of inhibitor or purify the sample and repeat the ELISA. If the sample exhibits a higher PG value than the 100% initial activity well, purify the sample and repeat the ELISA. You can also test for inhibitor interference by adding the inhibitor to a boiled enzyme sample as a control. Treat the control as a normal COX Inhibitor Sample. The sample should not yield any PGs. If the inhibitor is detected by the antiserum, the inhibitor is interfering with the ELISA.

**Sample purification procedure to be followed only if an inhibitor is interfering with the assay:**

1. Add 10,000 cpm of radiolabeled $PGE_2$ ([$^3$H]-$PGE_2$) to the sample. We recommend that a high specific activity tracer be used in order to minimize the amount of radioactive $PGE_2$ added. The ELISA will be able to detect the added $PGE_2$ and therefore the amount added should be insignificant in comparison to the endogenous analyte, yet should be sufficient for accurate scintillation counting.
2. Adjust the pH of the sample to ~4.0 using 1.0 M acetate buffer or citrate buffer (pH 4.0). (Standardize the pH adjustment using the sample matrix prior to proceeding with a large number of samples; approximately 1-2 equivalents of buffer is required for most biological samples.) If the samples are cloudy or contain precipitate, either filter or centrifuge to remove the precipitate. Particulate matter in the sample may clog the SPE (solid phase extraction) cartridge, resulting in loss of the sample.
3. Activate a SPE Cartridge (C-18) (6 ml) (Item No. 400020) by rinsing with 5 ml methanol and then with 5 ml UltraPure water. Do not allow the SPE cartridge to dry. *NOTE: Use one cartridge per sample.*
4. Pass the sample through the SPE cartridge. Rinse the cartridge with 5 ml UltraPure water. Discard the washes. Elute the $PGE_2$ with 5 ml ethyl acetate containing 1% methanol. Higher recovery and better reproducibility may be obtained if the sample is applied and eluted by gravity. The wash steps may be performed under vacuum or pressure.
5. Remove 500 µl for scintillation counting.
6. Evaporate the ethyl acetate/methanol to dryness either by vacuum centrifugation or by evaporation under a stream of dry nitrogen. It is imperative that all of the organic solvent is removed, as even trace quantities will adversely affect the ELISA.
7. Add 1.035 ml of ELISA Buffer and vortex. Proceed to **COX Reaction Dilutions** (see page 17) for the sample dilutions.

## Troubleshooting

| Problem | Possible Causes | Recommended Solutions |
|---|---|---|
| Erratic values; dispersion of duplicates | A. Trace organic contaminants in the water source<br>B. Poor pipetting/technique | Replace activated carbon filter or change source of UltraPure water |
| High NSB (>10% of $B_0$) | A. Poor washing<br>B. Exposure of NSB wells to specific antibody | Rewash plate and redevelop |
| Very low $B_0$ | A. Trace organic contaminants in the water source<br>B. Plate requires additional development time<br>C. Dilution error in preparing reagents | A. Replace activated carbon filter or change source of UltraPure water<br>B. Return plate to shaker and re-read later |
| Low sensitivity (shift in dose response curve) | Standard is degraded | Replace standard |
| Analyses of two dilutions do not agree (*i.e.*, more than 20% difference) | Interfering substances are present | See **Interference** section on page 30 |
| Only Total Activity (TA) wells develop | Trace organic contaminants in the water source | Replace activated carbon filter or change source of UltraPure water |
| No inhibition seen with compound | A. The concentration of the compound is not high enough<br>B. The compound is not an inhibitor of the enzyme | Increase the compound concentration and re-assay |

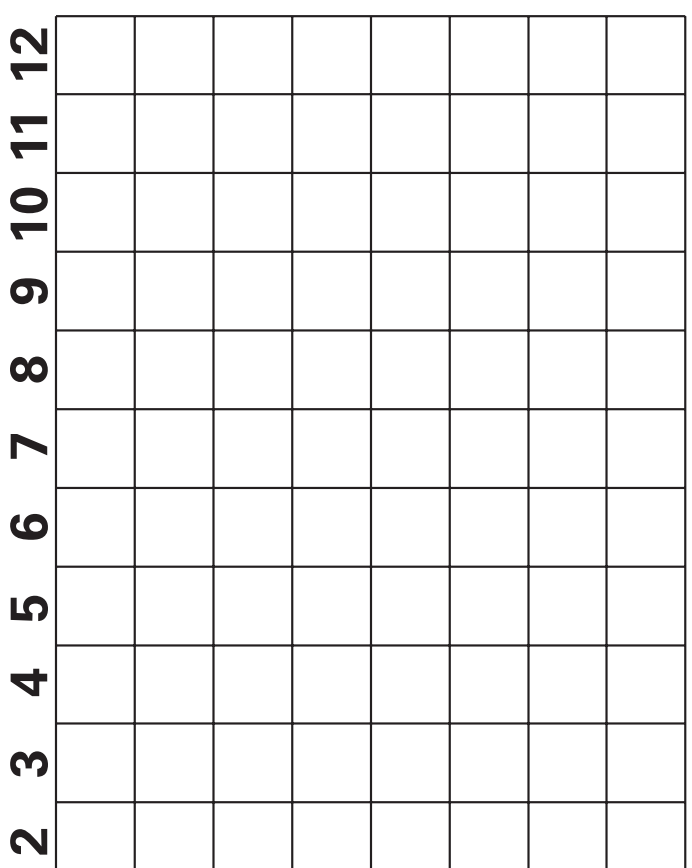


## Additional Reading

Go to **www.caymanchem.com/560131/references** for a list of publications citing the use of Cayman's COX (ovine/human) Inhibitor Screening Assay Kit.

## References


1. Nugteren, D.H. and Hazelhof, E. Isolation and properties of intermediates in prostaglandin biosynthesis. *Biochim. Biophys. Acta* **326**, 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**, 899-903 (1973).
3. Xie, W., Chipman, J.G., Robertson, D.L., *et al.* Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. USA* **88**, 2692-2696 (1991).
4. Blobaum, A.L. and Marnett, L.J. Structural and functional basis of cyclooxygenase inhibition. *J. Med. Chem.* **50(7)**, 1425-1441 (2007).
5. Maclouf, J., Grassi, J., and Pradelles, P. Development of enzyme-immunoassay techniques for the measurement of eicosanoids, Chapter 5, *in* Prostaglandin and Lipid Metabolism in Radiation Injury. Walden, T.L., Jr. and Hughes, H.N., editors, Plenum Press, Rockville, 355-364 (1987).
6. Pradelles, P., Grassi, J., and Maclouf, J.A. Enzyme immunoassays of eicosanoids using acetylcholinesterase as label: An alternative to radioimmunoassay. *Anal. Chem.* **57**, 1170-1173 (1985).






## 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.