Prostaglandin D_2-MOX ELISA Kit

Item No. 512011

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GENERAL INFORMATION

Materials Supplied

Item Number	Item Name	96 wells Quantity/Size	480 wells Quantity/Size	Storage Temperature
412012	Prostaglandin D_2-MOX ELISA Antiserum	1 vial/100 dtn	1 vial/500 dtn	-20°C
412010	Prostaglandin D_2-MOX-AChE Tracer	1 vial/100 dtn	1 vial/500 dtn	-20°C
412014	Prostaglandin D_2 ELISA Standard	1 vial	1 vial	-80°C
400060	ELISA Buffer Concentrate (10X)	2 vials/10 ml	4 vials/10 ml	RT
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	1 vial/12.5 ml	RT
400035	Polysorbate 20	1 vial/3 ml	1 vial/3 ml	RT
400004/ 400006	Mouse Anti-Rabbit IgG-Coated Plate	1 plate	5 plates	4°C
400012	96-Well Cover Sheet	1 cover	5 covers	RT
400050	Ellman's Reagent	3 vials/100 dtn	6 vials/250 dtn	-20°C
412015	Prostaglandin D_2-MOX Control	1 vial	1 vial	-20°C
400036	Methoxylamine HCl	1 vial/100 dtn	1 vial/500 dtn	RT
400037	Sodium Acetate	1 vial/100 dtn	1 vial/500 dtn	RT
400040	ELISA Tracer Dye	1 vial	1 vial	RT
400042	ELISA Antiserum Dye	1 vial	1 vial	RT

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.

This kit may not perform as described if any reagent or procedure is replaced or modified.

When compared to quantification by LC/MS or GC/MS, it is not uncommon for immunoassays to report higher analyte concentrations. While LC/MS or GC/MS analyses typically measure only a single compound, antibodies used in immunoassays sometimes recognize not only the target molecule, but also structurally related molecules, including biologically relevant metabolites. In many cases, measurement of both the parent molecule and metabolites is more representative of the overall biological response than is the measurement of a short-lived parent molecule. It is the responsibility of the researcher to understand the limits of both assay systems and to interpret their data accordingly.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

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 directed in the Materials Supplied section (see 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; multichannel or repeating pipettor recommended
3. An orbital microplate shaker
4. A source of ultrapure water, with a resistivity of 18.2 MΩ·cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
5. Materials used for **Sample Preparation** (see page 14)

INTRODUCTION

Background

Prostaglandin D_2 (PGD_2) is biosynthesized in the brain by a soluble, 26 kDa glutathione-independent lipocalin-type PGD_2 synthase.[1] This PGD_2 accumulates in the cerebrospinal fluid (CSF), where it induces physiologic sleep in rats and humans.[2] PGD_2 is also synthesized in mast cells and leukocytes by a cellular, myeloid-type, glutathione-dependent PGD synthase. The PGD_2 formed in the intracellular and vascular compartments is rapidly metabolized to 11β-$PGF_{2\alpha}$.[3] Thus, urinary measurements of PGD synthesis are most appropriately focused on the measurement of 11β-$PGF_{2\alpha}$ or tetranor-PGDM , the major urinary metabolite of PGD_2. Measurement of the parent eicosanoid, PGD_2, is appropriate in the supernatants of cell cultures, where PGD_2 levels may reach several ng/ml, and in CSF, where concentrations of several hundred pg/ml have been measured.[4]

All studies of PGD_2 biosynthesis should take into consideration the chemical instability of PGD_2 and its rapid degradation in the presence of serum proteins such as albumin.[5] PGD_2 also readily degrades in both acidic and basic media to give a variety of decomposition products. These include PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (see Figure 1 on page 8). Similarly, antigenic protein conjugates of PGD_2, synthesized for the production of antisera, also show considerable amounts of decomposition. Thus, the resulting antibody response is heterogeneous with poor specificity. This makes PGD_2 assay systems based on the parent compound unreliable and difficult to interpret.

About This Assay

This PGD_2-Methoxime (PGD_2-MOX) ELISA is based on the conversion of PGD_2 to a stable MOX derivative. Treatment of the sample with methoxylamine HCl converts PGD_2 into PGD_2-MOX, preventing its further chemical degradation. The antiserum used in the assay was developed using conjugates of this derivative and is very specific for PGD_2-MOX.[6] The assay has been validated against stable isotope dilution GC-MS. Measurements using both techniques on identical samples showed a correlation coefficient of 0.97.

Careful sample collection and preparation is critical for the successful measurement of PGD_2. Samples should be collected into media containing a cyclooxygenase inhibitor, methoximated, and assayed at once. For additional details on sample handling, refer to the procedure beginning on page 15.

Cayman's PGD_2-MOX ELISA Kit is a competitive assay that can be used for quantification of PGD_2-MOX in plasma, serum, and cell culture supernatants. The assay has a range of 2-250 pg/ml with a midpoint of approximately 10-35 pg/ml (50% B/B_0) and a sensitivity (80% B/B_0) of approximately 3 pg/ml.

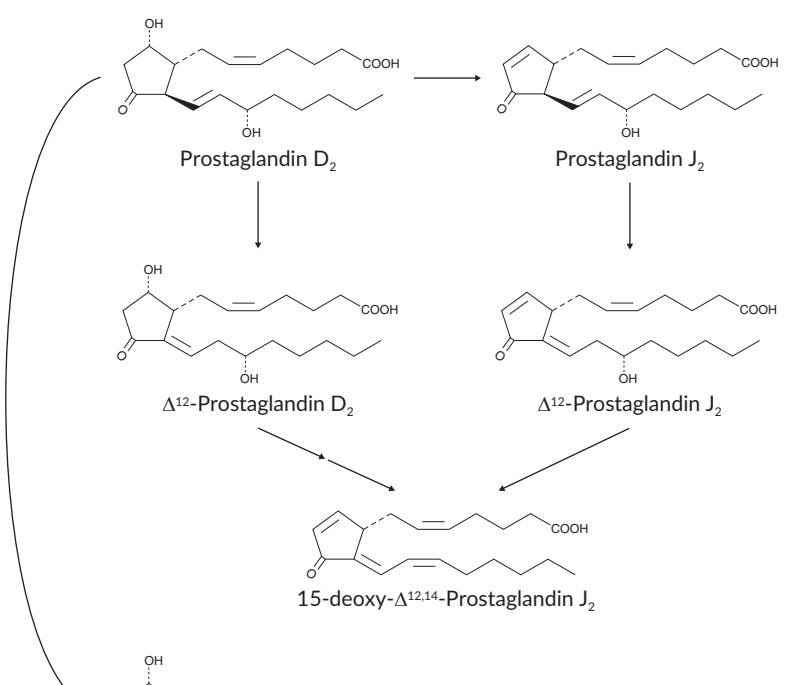


Figure 1. Metabolism of PGD_2

Principle Of This Assay

This assay is based on the competition between PGD_2-MOX and a PGD_2-MOX-acetylcholinesterase (AChE) conjugate PGD2-MOX-AChe Tracer for a limited amount of PGD_2-MOX-specific rabbit antiserum. Because the concentration of the PGD_2-MOX-AChE Tracer is held constant while the concentration of PGD_2-MOX varies, the amount of PGD_2-MOX-AChE Tracer that is able to bind to the PGD_2-MOX-specific rabbit antiserum will be inversely proportional to the concentration of PGD_2-MOX in the well. This antibody-PGD_2-MOX (either free or tracer) complex binds to the mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and 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 that can be measured at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of PGD_2-MOX-AChE Tracer bound to the well, which is inversely proportional to the amount of free PGD_2-MOX present in the well during the incubation, as described in the equation:

$$\text{Absorbance} \propto [\text{Bound } PGD_2\text{-MOX-AChE Tracer}] \propto 1/[PGD_2\text{-MOX}]$$

A schematic of this process is shown in Figure 2, on page 10.

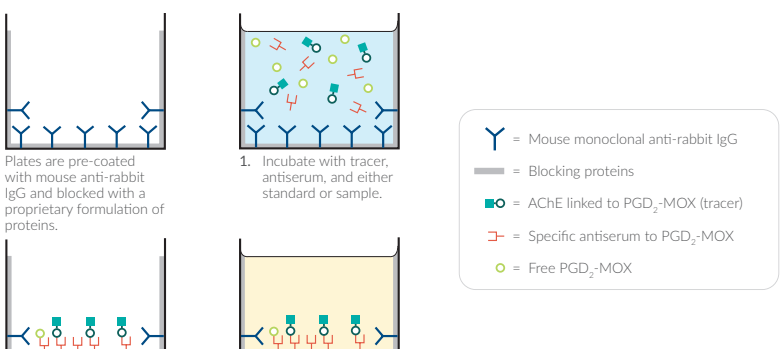


Figure 2. Schematic of the AChE ELISA

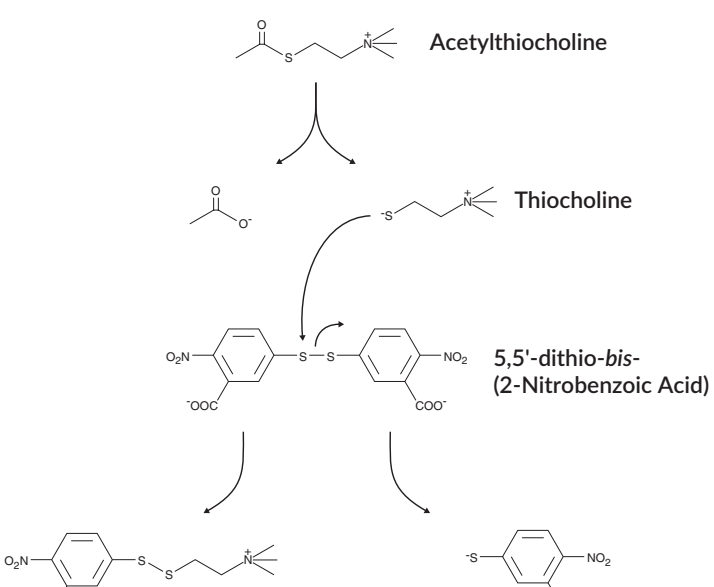


Figure 3. Reaction catalyzed by PDG_2-MOX

Definition of Key Terms

Blank (Blk): background absorbance caused by Ellman's Reagent. The blank absorbance should be subtracted from the absorbance readings of all the other wells, including the non-specific binding (NSB) wells.

Total Activity (TA): total enzymatic activity of the PGD_2-MOX AChE-linked 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.

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 sample or standard well to the average absorbance of the maximum binding (B_0) wells.

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:

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

PRE-ASSAY PREPARATION

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).

Buffer Preparation

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

1. **ELISA Buffer (1X) 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 (1X) Preparation**

   5 ml vial Wash Buffer Concentrate (400X) (96-well kit; Item No. 400062): Dilute to a total volume of 2 L with ultrapure water and add 1 ml of Polysorbate 20 (Item No. 400035) to an end concentration of 0.5 ml/L.

   OR

   12.5 ml vial Wash Buffer Concentrate (400X) (480-well kit; Item No. 400062): Dilute to a total volume of 5 L with ultrapure water and add 2.5 ml of Polysorbate 20 (Item No. 400035). Smaller volumes of Wash Buffer (1X) can be prepared by diluting the Wash Buffer Concentrate (400X) 1:400 and adding Polysorbate 20 (Item No. 400035) to an end concentration of 0.5 ml/L.

   NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with ultrapure water. Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.

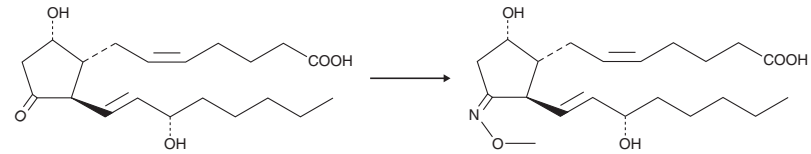
Sample Preparation

This assay has been validated in plasma, serum, and cell culture supernatants. Proper sample storage and preparation are essential for consistent and accurate results. PGD_2 is chemically unstable in biological samples, especially those containing albumin.[5] For this reason, it is necessary to convert PGD_2 to a stable MOX derivative prior to assay to obtain accurate quantification of this prostanoid. Please read this section thoroughly before beginning the assay.

General Precautions

- All samples must be free of organic solvents prior to assay.
- Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C, where they will be stable for approximately six months.
- Samples of rabbit origin may contain antibodies that interfere with the assay by binding to the mouse anti-rabbit plate. We recommend that all rabbit samples be purified prior to use in this assay.
- AEBSF (Pefabloc SC®) and PMSF inhibit AChE. Samples containing these protease inhibitors should not be used in this assay.

Derivatization of PGD_2 to PGD_2-MOX



Prostaglandin D_2 Prostaglandin D_2-Methoxime

1. **Preparation of the Methyloximating Reagent**

   NOTE: The methyloximating reagent is not stable. Prepare fresh reagent each time you wish to use it. The instructions below are written for preparation of the entire contents of the vials supplied in the kit. Smaller amounts of methyloximating reagent can be prepared by proportionally decreasing the amount of each reagent used.

   To prepare 100 dtn of methyloximating reagent (sufficient for 1 x 96-well plate): Prepare 10 ml of a 10:90 solution of ethanol:water. Add a small amount of the ethanol:water solution to the vial labeled 100 dtn (0.1 g) of Methoxylamine HCl (Item No. 400036). Vortex to mix. Transfer to the vial labeled 100 dtn (0.82 g) of Sodium Acetate (Item No. 400037). Vortex. Transfer this mixture to a tube capable of holding at least 10 ml. Rinse each of the vials with a portion of the remaining ethanol:water solution and add to the same tube. Transfer the rest of the ethanol:water solution to this same tube. Mix thoroughly before use.

   OR

   To prepare 500 dtn of methyloximating reagent (sufficient for 5 x 96-well plates): Prepare 50 ml of a 10:90 solution of ethanol:water. Add a small amount of the ethanol:water solution to the vial labeled 500 dtn (0.5 g) of Methoxylamine HCl (Item No. 400036). Vortex to mix. Transfer to the vial labeled 500 dtn (4.1 g) of Sodium Acetate (Item No. 400037). Vortex. Transfer this mixture to a tube capable of holding at least 50 ml. Rinse each of the vials with a portion of the remaining ethanol:water solution and add to the same tube. Transfer the rest of the ethanol:water solution to this same tube. Mix thoroughly before use.

2. **Derivatization of the PGD_2 ELISA Standard**

   Equilibrate a pipette tip in ethanol by repeatedly filling and expelling the tip with ethanol several times. Using the equilibrated pipette tip, transfer 100 µl of the PGD_2 ELISA Standard (Item No. 412014) into a clean test tube, then dilute with 900 µl ultrapure water. Heat a 1:1 solution of the diluted standard and methyloximating reagent at 60°C for 30 minutes. The concentration of this methoximated solution (the bulk standard) will be 20 ng/ml. The methoximated standard will be used to prepare the standard curve. Store this solution at 4°C, where it will be stable for approximately six weeks.

3. **Derivatization of the PGD_2 Samples**

   Add 200 µl of the prepared methyloximating reagent to 200 µl of sample. Heat this solution at 60°C for 30 minutes and then cool to room temperature. Samples can now be purified if necessary and tested in the assay. If immediate purification and testing are not possible, samples should be stored at -80°C.

Testing for Interference

In general, methoximated tissue culture supernatant samples may be diluted with ELISA Buffer (1X) and added directly to the assay well. Plasma, serum, and whole blood, as well as other heterogeneous mixtures such as lavage fluids and aspirates, often contain contaminants, which can interfere in the assay. It is best to check for interference before embarking on a large number of sample measurements. To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between 100 and ~5 pg/ml (*i.e.*, between 20-80% B/B_0). If two different dilutions of the sample show good correlation (differ by 20% or less) in the final calculated PGD_2-MOX concentration, purification is not required. If you do not see good correlation of the different dilutions, purification is advised. Purification methods need to be determined by end user and tested for compatibility in the assay.

Plasma

Collect blood in vacutainers containing heparin, EDTA, or sodium citrate for plasma and in serum vacutainers for serum. Indomethacin should be added immediately after whole blood collection (sufficient to give a 10 µM final concentration). Indomethacin will prevent *ex vivo* formation of eicosanoids, which have the potential to interfere with this assay (although most eicosanoids do not appear to exhibit any cross reactivity (see page 33)). Samples should be derivatized immediately to stabilize PGD_2.

1. Add 1,200 µl of ethyl acetate to the derivatized sample (400 µl). Vortex and then incubate for 5 min at room temperature.
2. Centrifuge at 5,000 x g for 5 minutes.
3. Transfer the ethyl acetate (upper) fraction into a clean, glass test tube.
4. Repeat extraction 2 times and combine all ethyl acetate fractions in one tube.
5. Evaporate solvent under a gentle stream of nitrogen while heating samples up to 30°C (heating is optional).
6. Re-suspend sample in 400 µl of ELISA Buffer (1X) (dilution factor 2) and use in the assay.

Sample Matrix Properties

Spike and Recovery

Plasma and serum were spiked with different amounts of PGD_2, immediately derivatized and purified as described in the Sample Preparation section (see page 14), and analyzed using the PGD_2-MOX ELISA Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

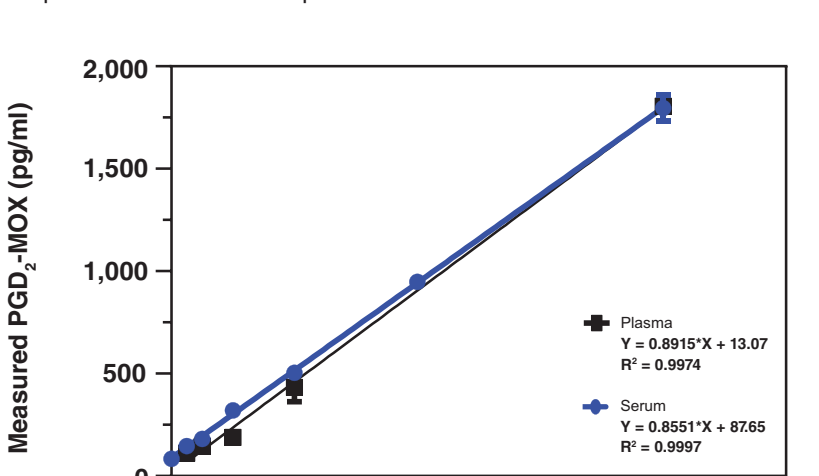


Figure 4. Spike and recovery in plasma and serum

Parallelism

To assess parallelism, samples were derivatized as described on page 16. Plasma and serum were then purified as described in the Sample Preparation section, serially diluted, and evaluated with the PGD_2-MOX ELISA Kit. Concentrations were plotted as a function of the sample dilution. The results are shown below.

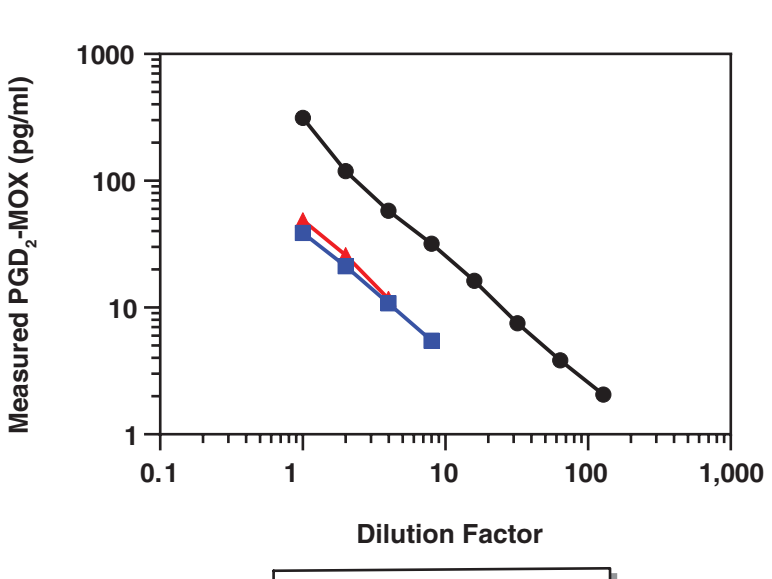


Figure 5. Parallelism of plasma and serum in the PGD_2-MOX ELISA Kit

Preparation of Assay-Specific Reagents

Methoximated PGD_2 ELISA Standard

Transfer 200 µl of the PGD_2 ELISA methoximated bulk standard (20 ng/ml, prepared as described in Step 2 on page 16), into a clean test tube and dilute with 600 µl of ultrapure water. The concentration of this solution will be 5,000 pg/ml.

NOTE: If assaying culture medium samples that have not been diluted with ELISA Buffer (1X), culture medium should be used in place of ELISA Buffer (1X) for dilution of the standard curve.

To prepare the standard for use in ELISA: Obtain eight clean test tubes and label them #1-8. Aliquot 950 µl ELISA Buffer (1X) to tube #1 and 500 µl ELISA Buffer (1X) to tubes #2-8. Transfer 50 µl of the diluted standard (5,000 pg/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 it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than 24 hours.

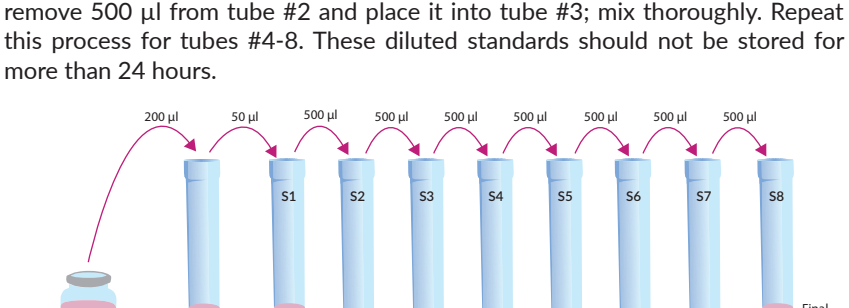


Figure 6. Preparation of the methoximated PGD_2 standards

PGD_2-MOX-AChE Tracer

Reconstitute the PGD_2-MOX-AChE Tracer as follows:

100 dtn PGD_2-MOX-AChE Tracer (96-well kit; Item No. 412010): Reconstitute with 6 ml ELISA Buffer (1X).

OR

500 dtn PGD_2-MOX-AChE Tracer (480-well kit; Item No. 412010): Reconstitute with 30 ml ELISA Buffer (1X).

Store the reconstituted PGD_2-MOX-AChE Tracer at 4°C *(do not freeze!)* and use within four weeks. A 20% surplus of tracer has been included to account for any incidental losses.

Tracer Dye Instructions (optional)

This dye may be added to the tracer, if desired, to aid in visualization of tracer-containing wells. Add the dye to the reconstituted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer or add 300 µl of dye to 30 ml of tracer). *NOTE: Do not store tracer with dye for more than 24 hours.*

PGD_2-MOX ELISA Antiserum

Reconstitute the PGD_2-MOX ELISA Antiserum as follows:

100 dtn PGD_2-MOX ELISA Antiserum (96-well kit; Item No. 412012): Reconstitute with 6 ml ELISA Buffer (1X).

OR

500 dtn PGD_2-MOX ELISA Antiserum (480-well kit; Item No. 412012): Reconstitute with 30 ml ELISA Buffer (1X).

Store the reconstituted PGD_2-MOX ELISA Antiserum at 4°C and use within four weeks. A 20% surplus of antiserum has been included to account for any incidental losses.

Antiserum Dye Instructions (optional)

This dye may be added to the antiserum, if desired, to aid in visualization of antiserum-containing wells. Add the dye to the reconstituted antiserum at a final dilution of 1:100 (add 60 µl of dye to 6 ml antiserum or add 300 µl of dye to 30 ml of antiserum). *NOTE: Do not store antiserum with dye for more than 24 hours.*

PGD_2-MOX Control

This control is supplied in methoximated form. Do not perform the methoximation procedure. Use of this control is optional, but it is provided as a tool to monitor the degree of methoximation of the PGD_2 ELISA Standard and samples (usually 60-70%). To use this control for ELISA, equilibrate a pipette tip in ethanol by repeatedly filling and expelling the tip with ethanol several times. Using the equilibrated pipette tip, transfer 100 µl of the PGD_2-MOX Control (Item No. 412015) to a clean test tube and dilute with 900 µl ultrapure water. The concentration of this solution is 5 ng/ml. Dilute this solution 1:50 (100 pg/ml) and 1:500 (10 pg/ml) in ELISA Buffer (1X) and use in the assay.

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 Blk, two NSB, and two B_0 wells, 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 sample should be assayed at a minimum of two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown in Figure 7, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 28, for more details). We suggest you record the contents of each well on the template sheet provided (see page 37).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	S1	S1	1	1	1	9	9	9	17	17	17
B	Blk	S2	S2	2	2	2	10	10	10	18	18	18
C	NSB	S3	S3	3	3	3	11	11	11	19	19	19
D	NSB	S4	S4	4	4	4	12	12	12	20	20	20
E	B_0	S5	S5	5	5	5	13	13	13	21	21	21
F	B_0	S6	S6	6	6	6	14	14	14	22	22	22
G	B_0	S7	S7	7	7	7	15	15	15	23	23	23
H	TA	S8	S8	8	8	8	16	16	16	24	24	24

Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S8 - Standards 1-8
1-24 - Samples

Figure 7. Sample plate format

Performing the Assay

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 (1X)**

   Add 100 µl ELISA Buffer (1X) to NSB wells. Add 50 µl ELISA Buffer (1X) to B_0 wells. If culture medium was used to dilute the standard curve, substitute 50 µl of culture medium for ELISA Buffer (1X) in the NSB and B_0 wells (*i.e.*, add 50 µl culture medium to NSB and B_0 wells and 50 µl ELISA Buffer (1X) to NSB wells).

2. **PGD_2 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. **Samples and PGD_2-MOX Control**

   Add 50 µl sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. **PGD_2-MOX-AChE Tracer**

   Add 50 µl to each well *except* the TA and the Blk wells.

5. **PGD_2-MOX ELISA Antiserum**

   Add 50 µl to each well *except* the TA, NSB, and Blk wells.

Well	ELISA Buffer (1X)	Standard/ Control/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/ Control	-	50 µl	50 µl	50 µl

Table 1. Pipetting summary

Incubation of the Plate

Cover each plate with the 96-well Cover Sheet (Item No. 400012) and incubate overnight at 4°C.

Development of the Plate

1. Reconstitute Ellman's Reagent immediately before use (20 ml of reagent is sufficient to develop 100 wells):

   100 dtn vial Ellman's Reagent (96-well kit; Item No. 400050): Reconstitute with 20 ml of ultrapure water.

   OR

   250 dtn vial Ellman's Reagent (480-well kit; Item No. 400050): Reconstitute with 50 ml of ultrapure water.

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 be run on different days.

2. Empty the wells and rinse five times with Wash Buffer (1X).
3. Add 200 µl of Ellman's Reagent to each well.
4. Add 5 µl of the reconstituted tracer to the TA wells.
5. Cover the plate with the 96-well Cover Sheet. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark. This assay typically develops (*i.e.*, B_0 wells ≥0.3 A.U. (blank subtracted)) in 90-120 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.*
3. Read the plate at a wavelength of 414 nm.

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 either %B/B_0 *versus* log concentration using a four-parameter logistic fit or as logit B/B_0 *versus* log concentration using a linear 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). Repeat for S2-S8 and all sample wells. (To obtain %B/B_0 for a logistic four-parameter fit, multiply these values by 100.)

NOTE: The TA values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool. Erratic absorbance values could indicate the presence of organic solvents in the buffer or other technical problems (see page 34 for ***Troubleshooting****). Low or no absorbance from a TA well could indicate a dysfunction in the enzyme-substrate system.*

Plot the Standard Curve

Plot %B/B_0 for standards S1-S8 *versus* PGD_2-MOX concentration using linear (y) and log (x) axes and perform a four-parameter logistic fit.

Alternative Plot - The data can also be linearized 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

Calculate the B/B_0 (or $\%B/B_0$) value for each sample. 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. *NOTE: Remember to account for any dilution of the original sample concentration prior to its addition to the well.* Samples with $\%B/B_0$ values greater than 80% or 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 indicates interference, which could be eliminated by purification.

NOTE: If there is an error in the B_0 wells it is possible to calculate sample concentrations by plotting the absorbance values and back calculating sample absorbance off the standard curve.

Performance Characteristics

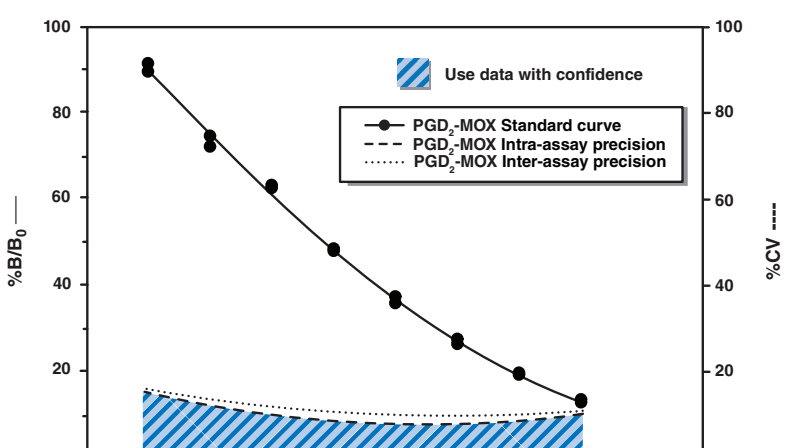
Representative Data

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 values of your samples.

	Raw Data		Average	Corrected
Total Activity	0.232	0.388	0.310	
NSB	0.000	0.000	0.000	
B_0	1.046	1.073		
	1.025	1.107	1.063	1.063

Dose (pg/ml)	Raw Data		Corrected		$\%B/B_0$	
250	0.135	0.142	0.135	0.142	13	13
125	0.208	0.203	0.208	0.203	20	19
62.5	0.279	0.291	0.279	0.291	26	27
31.3	0.380	0.396	0.380	0.396	36	37
15.6	0.514	0.509	0.514	0.509	48	48
7.8	0.671	0.664	0.671	0.664	63	63
3.9	0.766	0.793	0.766	0.793	72	75
2.0	0.952	0.972	0.952	0.972	90	91

Table 2. Typical results



Assay Range = 2-250 pg/ml
Sensitivity (defined as 80% B/B_0) = 3 pg/ml
Mid-point (defined as 50% B/B_0) = 10-35 pg/ml

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted with ELISA Buffer (1X).

Figure 8. Typical standard curve

Compound	Cross Reactivity
PGD_2-MOX	100%
PGD_2	0.2%
tetranor-PGEM	<0.01%
tetranor-PGFM	<0.01%
PGE_2-MOX	<0.01%
6-keto $PGF_{1\alpha}$-MOX	<0.01%
$PGF_{2\alpha}$	<0.01%
Thromboxane B_2-MOX	<0.01%

Table 3. Cross reactivity of the PGD_2-MOX ELISA

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique	A. Replace activated carbon filter or change source of ultrapure water
High NSB (>0.100)	A. Poor washing B. Exposure of NSB wells to specific antibody	A. Rewash plate and redevelop
Very low B_0	A. Trace organic contaminants in the water source B. Plate requires additional development time C. Dilution error in preparing reagents	A. Replace activated carbon filter or change source of ultrapure water B. Return plate to shaker and re-read later
Low sensitivity (shift in dose-response curve)	Standard is degraded or low efficiency of the methyloximating agent	Replace standard
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present	Purify sample prior to analysis by ELISA[7]
Only TA wells develop	Trace organic contaminants in the water source	Replace activated carbon filter or change source of ultrapure water

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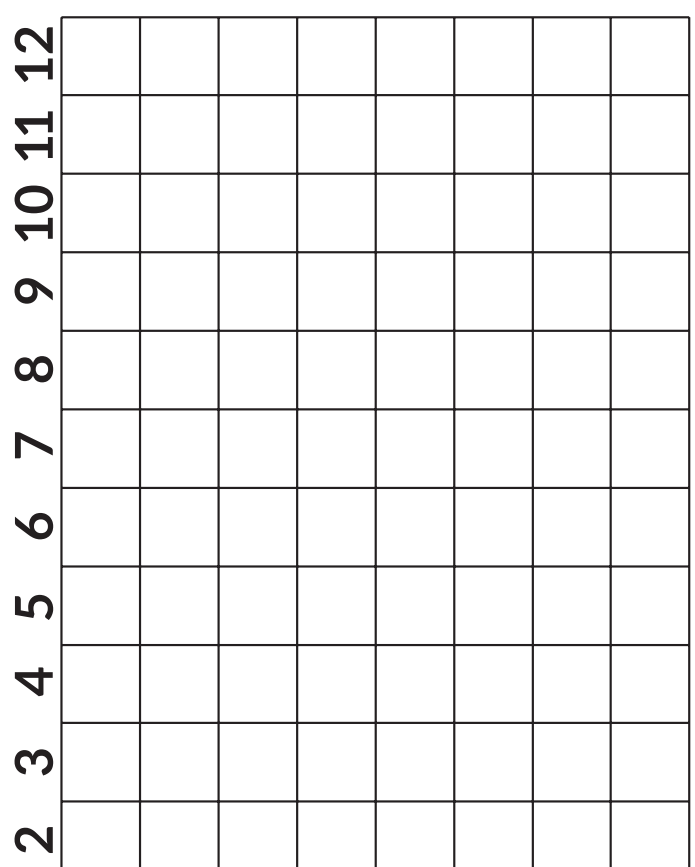

NOTES

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