8-Isoprostane ELISA Kit

Item No. 516351

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TABLE OF CONTENTS

GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size	480 wells Quantity/Size
416352	8-Isoprostane ELISA Antiserum	1 vial/100 dtn	1 vial/500 dtn
416350	8-Isoprostane-AChE Tracer	1 vial/100 dtn	1 vial/500 dtn
416354	8-Isoprostane ELISA Standard	1 vial/0.5 ml	1 vial/0.5 ml
400060	ELISA Buffer Concentrate (10X)	2 vials/10 ml	4 vials/10 ml
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	1 vial/12.5 ml
400035	Polysorbate 20	1 vial/3 ml	1 vial/3 ml
400004/400006	Mouse Anti-Rabbit IgG-Coated Plate	1 plate	5 plates
400012	96-Well Cover Sheet	1 ea	5 ea
400050	Ellman's Reagent	3 vials/100 dtn	6 vials/250 dtn
400040	ELISA Tracer Dye	1 vial	1 vial
400042	ELISA Antiserum Dye	1 vial	1 vial

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 8-Isoprostane ELISA Kit. 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 at -20°C 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. An orbital microplate shaker
3. Adjustable pipettes; multichannel or repeating pipettor recommended
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** and the **Purification Protocol** (see pages 12 and 16, respectively)

INTRODUCTION

Background

8-Isoprostane, also known as 8-iso prostaglandin $F_{2\alpha}$ (8-iso $PGF_{2\alpha}$), is produced by the non-enzymatic peroxidation of arachidonic acid in membrane phospholipids.[1,2] 8-Isoprostane is present in human plasma and urine and levels increase with age.[1,3] Levels of 8-isoprostane in plasma and urine increase under conditions of oxidative stress, and urinary levels have been used as biomarkers for lipid peroxidation and oxidative stress.[3-5] 8-Isoprostane levels are increased in urinary, sputum, or exhaled breath condensate following exposure to bisphenol A (BPA), in smokers, and in patients with asthma, respectively.[4,6,7] 8-Isoprostane can be also formed in plasma samples that have undergone oxidative degradation during prolonged storage at -20°C.[1,8]

About This Assay

Cayman's 8-Isoprostane ELISA Kit is a competitive assay that can be used for quantification of 8-isoprostane in plasma, urine, and other sample matrices. The assay has a range of 0.8-500 pg/ml and a sensitivity (80% B/B_0) of approximately 3 pg/ml.

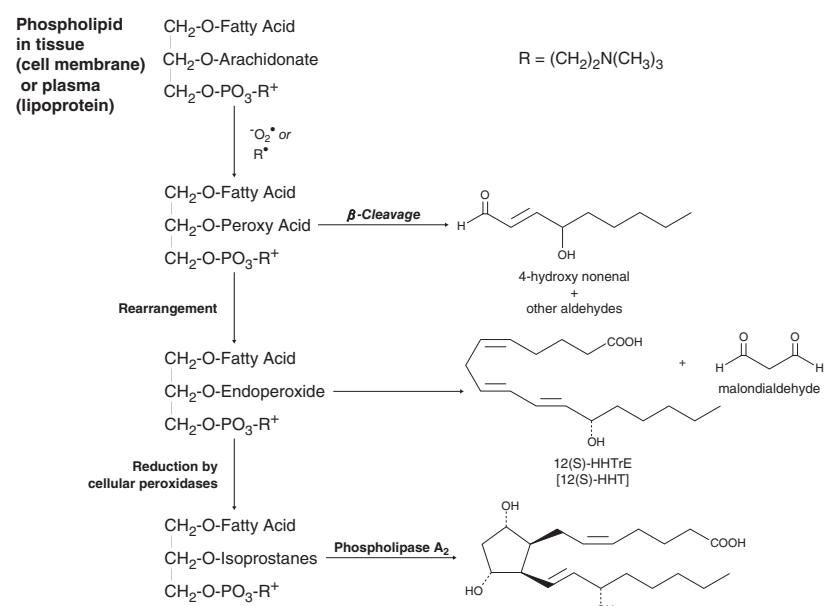


Figure 1. The formation of 8-isoprostane

Principle of This Assay

This assay is based on the competition between 8-isoprostane and an 8-isoprostane-acetylcholinesterase (AChE) conjugate (8-IsoprostaneAChE Tracer) for a limited number of 8-isoprostane-specific rabbit antibody binding sites. Because the concentration of the 8-Isoprostane-AChE Tracer is held constant while the concentration of 8-isoprostane varies, the amount of 8-Isoprostane-AChE Tracer that is able to bind to the rabbit antibody will be inversely proportional to the concentration of 8-isoprostane in the well. This rabbit antibody-8-isoprostane (either free or tracer) complex binds to the rabbit IgG mouse monoclonal 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 8-Isoprostane-AChE Tracer bound to the well, which is inversely proportional to the amount of free 8-isoprostane present in the well during the incubation; or

$$\text{Absorbance} \propto [\text{Bound 8-Isoprostane-AChE Tracer}] \propto 1/[\text{8-Isoprostane}]$$

A schematic of this process is shown in Figure 2, below.

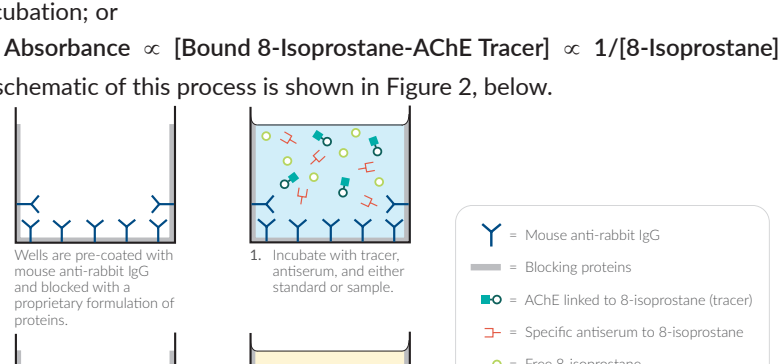


Figure 2. Schematic of the ELISA

Definition of Key Terms

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

TA (Total Activity): total enzymatic activity of the 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 that 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): 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:

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

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 ultrapure water.*

2. **Wash Buffer (1X) Preparation**

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

   OR

   12.5 ml vial Wash Buffer Concentrate (400X) (480-well kit; Item No. 400062): Dilute the contents of one vial of Wash Buffer Concentrate (400X) 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 0.5 ml of Polysorbate 20 per 1 L of Wash Buffer (1X).

NOTE: 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 a wide range of samples, including urine and plasma. Proper sample storage and preparation are essential for consistent and accurate results. Please read this section thoroughly before beginning the assay.

Cayman offers an 8-Isoprostane Affinity Column (Item No. 401111), Affinity Sorbent (Item No. 401113), and Eicosanoid Affinity Column Buffer (Item 400220), available separately or in the 8-Isoprostane Affinity Purification Kit (Item No. 501110), for sample purification. The affinity column purification procedures have been validated with plasma and urine samples and recoveries average >90%. Alternatively, a solid phase extraction (SPE) purification method (described on page 17) may be used, with the average recovery of 90%.

General Precautions

- All samples must be free of organic solvents prior to assay.
- AEBSF (Pefabloc SC®) and PMSF inhibit AChE. Samples containing these protease inhibitors should not be used in this assay.
- Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C in the presence of 0.005% BHT (10 µl of 5 mg/ml solution in ethanol per 1 ml sample). Storage at -20°C is not sufficient to prevent oxidative formation of 8-isoprostane.[9] BHT has limited solubility in water. Precipitate may form when BHT is added to aqueous solution.
- Samples of rabbit origin may contain antibodies that interfere with the assay by binding to the mouse anti-rabbit IgG-coated plate. We recommend that all rabbit samples be purified prior to use in this assay.

Testing for Interference

In general, tissue culture supernatant samples may be diluted with ELISA Buffer (1X) and added directly to the assay well. Plasma, serum, urine, as well as other heterogeneous mixtures such as lavage fluids and aspirates, often contain contaminants that 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 20-80% B/B_0. If the two different dilutions of the sample show good correlation (differ by 20% or less) in the final calculated 8-isoprostane concentration, purification is not required. If good correlation of the different dilutions is not seen, purification is advised.

Lavage Fluids and Aspirates

Some lavage fluids may be assayed without purification. Samples that cannot be assayed immediately should be stored at -80°C in the presence of 0.005% BHT (see **General Precautions** on page 12). Be certain to dilute the standards in the same medium as the samples. *NOTE: If inconsistent results are obtained, SPE or immunoaffinity purification is warranted.*

Urine

In general, urine samples may be diluted with ELISA Buffer (1X) and added directly to the well. Samples that cannot be assayed immediately should be stored at -80°C in the presence of 0.005% BHT (see **General Precautions** on page 12). *NOTE: If inconsistent results are obtained, SPE or immunoaffinity purification is warranted.*

Culture Medium Samples

Most culture medium samples can be assayed without purification. Samples that cannot be assayed immediately should be stored at -80°C in the presence of 0.005% BHT (see **General Precautions** on page 12). If the estimated concentration in the samples is too low to allow dilution with ELISA Buffer (1X), be certain to dilute the 8-isoprostane standards in the same medium as the samples. *NOTE: If inconsistent results are obtained, SPE or immunoaffinity purification is warranted.*

Plasma

Plasma samples should be collected in vacutainers containing sodium citrate, heparin, or EDTA. Samples that cannot be assayed immediately should be stored at -80°C in the presence of 0.005% BHT (see **General Precautions** on page 12).

A portion of total plasma 8-isoprostane is present as the free acid, while the remainder is esterified in phospholipids.[3] Direct ELISA of plasma samples without hydrolysis will measure only the free 8-isoprostane fraction. Total plasma 8-isoprostane determination requires an alkaline hydrolysis prior to ELISA (see **Sample Purification** on page 16).

Analysis of plasma samples without purification may lead to inconsistent results. If inconsistent results are obtained, immunoaffinity or SPE purification is recommended.

Tissue

Tissue can be homogenized either manually or using a Precellys™ 24 homogenizer. When assaying tissue, 8-isoprostane concentrations are usually normalized using either the wet weight of the tissue or the protein concentration of the lysate. It is recommended to weigh each sample prior to homogenization. If determining the protein concentration of the tissue lysate is desired, the use of Cayman's Protein Determination Kit (Item No. 704002) is recommended.

Tissue - Manual Homogenization

Add 1 ml of Homogenization Buffer (0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and 0.005% BHT) per 100 mg of tissue. Homogenize the sample using either a Polytron™-type homogenizer or a sonicator. After homogenization, centrifuge the sample at 8,000 x g for ten minutes to pellet particulate matter. Transfer the supernatant to a clean tube. If normalization of the sample to protein concentration is desired, reserve an aliquot of this supernatant for use in a protein assay. Process samples as described above for plasma samples. Most of the 8-isoprostane in tissues will be esterified with lipids, so hydrolysis must be performed if determination of the total 8-isoprostane in the tissue is desired (see **Sample Purification** on page 16).

Tissue Homogenization using the Precellys™ 24 Homogenizer

Add 1 ml of Homogenization Buffer (0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and 0.005% BHT) per 100 mg of tissue. Homogenize the sample with the Precellys™ 24 using the appropriate settings (see Table 1). After homogenization, centrifuge the sample at 8,000 x g for ten minutes to pellet particulate matter. Transfer the supernatant to a clean tube. If normalization of the sample to protein concentration is desired, reserve an aliquot of this supernatant for use in a protein assay. Process samples as described above for plasma samples. Most of the 8-isoprostane in tissues will be esterified with lipids, so hydrolysis must be performed if determination of the total 8-isoprostane in the same is desired (see **Sample Purification** on page 16).

Organ	Speed (rpm)	Cycle Length (seconds)	Beads
Lung	5,200	20	CK28 Large Ceramic (Item No. 10011151)
Brain	5,500	20	CK28 Large Ceramic (Item No. 10011151)
Liver	5,200	15	CK28 Large Ceramic (Item No. 10011151)
Kidney	5,200	20	CK14 Small Ceramic (Item No. 10011152)
Heart	5,200	30	CK14 Small Ceramic (Item No. 10011152)

Table 1. Precellys™ settings

Sample Purification

Free *versus* Total 8-Isoprostane Measurement

Depending on the sample type, a large percentage of 8-isoprostane may be esterified in lipids within the sample and will not be detected by measurement of free 8-isoprostane. For the total 8-isoprostane measurement, it is necessary to perform a sample hydrolysis (procedure below), followed by sample purification. For the free 8-isoprostane measurement, proceed to either the **Preparation for Affinity Purification** section below or the **SPE Purification Protocol** section on page 17. To hydrolyze samples:

1. Aliquot samples into clean glass tubes (300 µl recommended)
2. Add equal volume of 15% (w/v) KOH to all sample tubes.
3. Incubate at 37°C for 60 minutes.
4. Neutralize samples by the addition of approximately 600 µl of 1 N HCl to each sample.
5. Proceed to either the **Preparation for Affinity Purification** section below or the **SPE Purification Protocol** section on page 17.

Preparation for Affinity Purification

1. All samples must be free of particulates and precipitates to avoid plugging the column. This may be achieved either by filtration or centrifugation. All samples must be approximately neutral pH (6.5-7.5).
2. Urine samples, after removal of any sediment, may be applied directly to the column or sorbent. Plasma samples should be diluted 1:5 with Eicosanoid Affinity Column Buffer and applied to the column or sorbent. Samples that have been hydrolyzed and then neutralized for measurement of total 8-isoprostane should be further diluted with 1/3 volume of Eicosanoid Affinity Column Buffer (1X) before being applied to the column or sorbent.
3. Proceed with purification following the protocol described in the product insert for the 8-Isoprostane Affinity Sorbent, Column, or Purification Kit.

SPE Purification Protocol

1. Centrifuge sample at 1,000 x g for 10 minutes and transfer the supernatant into a clean test tube. It will be used for SPE purification.
2. Activate an 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 cartridge to dry.
3. Pass the sample through the SPE cartridge. Rinse the cartridge with 5 ml ultrapure water and allow it to become dry after this step. Discard both washes.
4. Elute the 8-isoprostane with 2 ml of methanol.*
5. Evaporate methanol solution under a stream of dry nitrogen.

   *If it is necessary to stop during this purification, samples may be stored in the methanol solution at -80°C.
6. Add 300 µl of ELISA Buffer (1X) and vortex. It is common for an insoluble precipitate to remain after the addition of ELISA Buffer (1X); this will not affect the assay. The sample is now ready for use in the immunoassay.

Spike and Recovery:
Human plasma treated with 0.005% BHT was spiked with different amounts of 8-isoprostane, purified using the SPE purification protocol described on page 17, then serially diluted with ELISA Buffer (1X), and evaluated using the 8-Isoprostane ELISA Kit. The error bars represent standard deviations obtained from multiple dilutions of each sample.

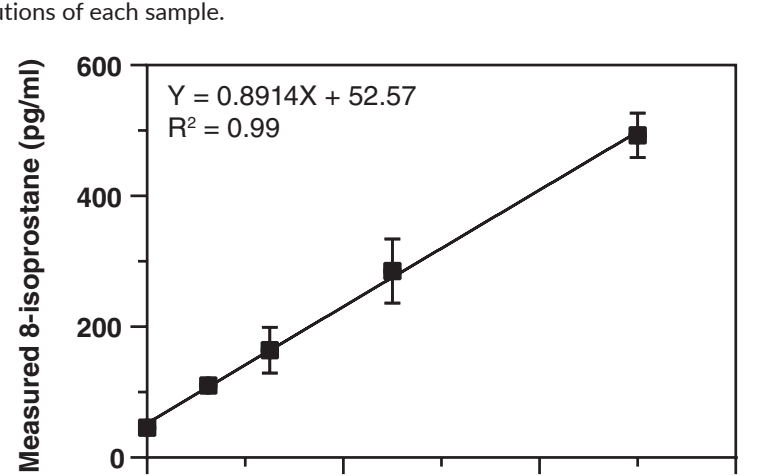


Figure 3. Spike and recovery of free 8-isoprostane in human plasma

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

8-Isoprostane 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 8-Isoprostane ELISA Standard (Item No. 416354) into a clean test tube, then dilute with 900 µl of ultrapure water. The concentration of this solution (the bulk standard) will be 5 ng/ml. Stored at 4°C, the standard will be stable for up to six weeks.

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 number them #1 through #8. Aliquot 900 µl ELISA Buffer (1X) to tube #1 and 750 µl ELISA Buffer (1X) to tubes #2-8. Transfer 100 µl of the bulk standard (5 ng/ml) to tube #1 and mix thoroughly. The concentration of this standard, the first point on the standard curve, will be 500 pg/ml. 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.

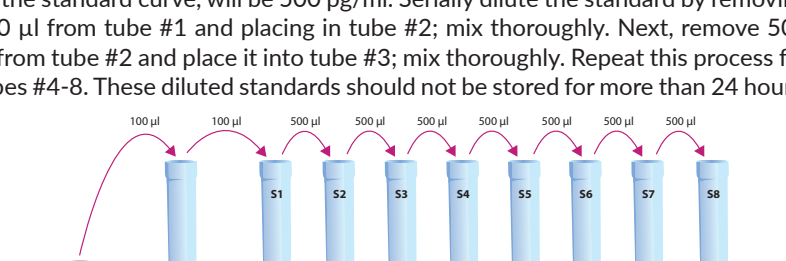


Figure 4. Preparation of the 8-isoprostane standards

8-Isoprostane-AChE Tracer

Reconstitute the 8-Isoprostane-AChE Tracer as follows:

100 dtn 8-Isoprostane-AChE Tracer (96-well kit; Item No. 416350): Reconstitute with 6 ml ELISA Buffer (1X).

OR

500 dtn 8-Isoprostane-AChE Tracer (480-well kit; Item No. 416350): Reconstitute with 30 ml ELISA Buffer (1X).

Store the reconstituted 8-Isoprostane-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).

8-Isoprostane ELISA Antiserum

Reconstitute the 8-Isoprostane ELISA Antiserum as follows:

100 dtn 8-Isoprostane ELISA Antiserum (96-well kit; Item No. 416352): Reconstitute with 6 ml ELISA Buffer (1X).

OR

500 dtn 8-Isoprostane ELISA Antiserum (480-well kit; Item No. 416352): Reconstitute with 30 ml ELISA Buffer (1X).

Store the reconstituted 8-Isoprostane 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.

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).

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 two dilutions and each dilution should be assayed in duplicate. For statistical purposes, assaying samples in triplicate is reommended.

A suggested plate format is shown in Figure 5, 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 25, for more details). It is recommended to record the contents of each well on the template sheet provided (see page 33).

	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 5. 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. **8-Isoprostane 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**

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

4. **8-Isoprostane-AChE Tracer**

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

5. **8-Isoprostane ELISA Antiserum**

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

Well	ELISA Buffer (1X)	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 2. Pipetting summary

Incubation of the Plate

Cover each plate with the 96-Well Cover Sheet (Item No. 400012) and incubate for 18 hours 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 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 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 at room temperature. This assay typically develops (*i.e.*, B_0 wells ≥0.6 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. 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 (1X) and repeat the development with fresh Ellman's Reagent.*
3. Read the plate at a wavelength between 405-420 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 0.3-1.0 A.U. (blank subtracted). If the absorbance of the wells exceeds 2.0, 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 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 Chemical 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. 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* 8-isoprostane 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 using the equation obtained from the standard curve plot. *NOTE: Remember to account for any concentration or dilution of the sample prior to the 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.

Performance Characteristics

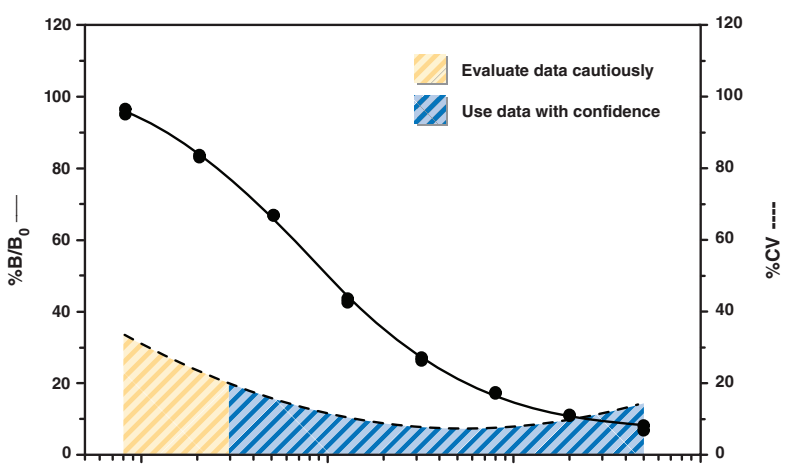
Sample Data

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 values of your samples. Your results could differ substantially.

	Raw Data		Average	Corrected
Total Activity	0.639	0.592	0.616	
NSB	0.000	0.000	0.000	
B_0	0.730	0.662		
	0.625	0.780	0.699	0.699

Dose (pg/ml)	Raw Data		Corrected		%B/B_0	
500	0.048	0.057	0.048	0.057	6.9	8.2
200	0.078	0.076	0.078	0.076	11.2	10.9
80	0.120	0.122	0.120	0.122	17.2	17.4
32	0.184	0.190	0.184	0.190	26.3	27.2
12.8	0.298	0.305	0.298	0.305	42.6	43.6
5.1	0.467	0.467	0.467	0.467	66.8	66.8
2.0	0.581	0.585	0.581	0.585	83.1	83.7
0.8	0.675	0.665	0.675	0.665	96.5	95.1

Table 3. Typical results



Assay Range = 0.8-500 pg/ml
Sensitivity (defined as 80% B/B_0) = 3 pg/ml
Mid-point (defined as 50% B/B_0) = 7-20 pg/ml

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

Figure 6. 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 28 and in the table below.

Dose (pg/ml)	%CV* Intra-Assay Precision	%CV* Inter-Assay Precision
500	12.6	10.5
200	11.7	16.4
80	9.5	20.2
32	6.4	24.3
12.8	7.2	15.5
5.1	20.0	12.5
2.0	19.9	9.6
0.8	†	†

Table 4. Intra- and inter-assay precision
*%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
8-Isoprostane	100%	2,3-dinor-6-keto Prostaglandin $F_{1\alpha}$	0.09%
8-*iso* Prostaglandin $F_{2\alpha}$ ethanolamide	100%	8-*iso* Prostaglandin $F_{1\beta}$	0.08%
8-*iso* Prostaglandin $F_{3\alpha}$	20.6%	Thromboxane B_2	0.08%
2,3-dinor-8-*iso* Prostaglandin $F_{2\alpha}$	4.00%	11-dehydro Thromboxane B_2	0.07%
8-*iso* Prostaglandin E_2	1.84%	11β-Prostaglandin $F_{2\alpha}$	0.03%
2,3-dinor-8-*iso* Prostaglandin $F_{1\alpha}$	1.70%	Prostaglandin E_2	0.02%
8-*iso* Prostaglandin E_1	1.56%	8-*iso*-15(R)-Prostaglandin $F_{2\alpha}$	0.02%
Prostaglandin $F_{1\alpha}$	0.71%	8,12-*epi* $iPF_{2\alpha}$-III	0.01%
Prostaglandin $F_{3\alpha}$	0.66%	$iPF_{2\alpha}$-VI	<0.01%
Prostaglandin E_1	0.39%	8,12-*epi* $iPF_{2\alpha}$-VI	<0.01%
Prostaglandin D_2	0.16%	tetranor-PGEM	<0.01%
6-keto Prostaglandin $F_{1\alpha}$	0.14%	tetranor-PGFM	<0.01%
Prostaglandin $F_{2\alpha}$	0.14%	13,14-dihydro-15-keto Prostaglandin $F_{2\alpha}$	<0.01%

Table 5. Cross reactivity of the 8-Isoprostane 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	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	Re-wash plate and re-develop
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	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[9]
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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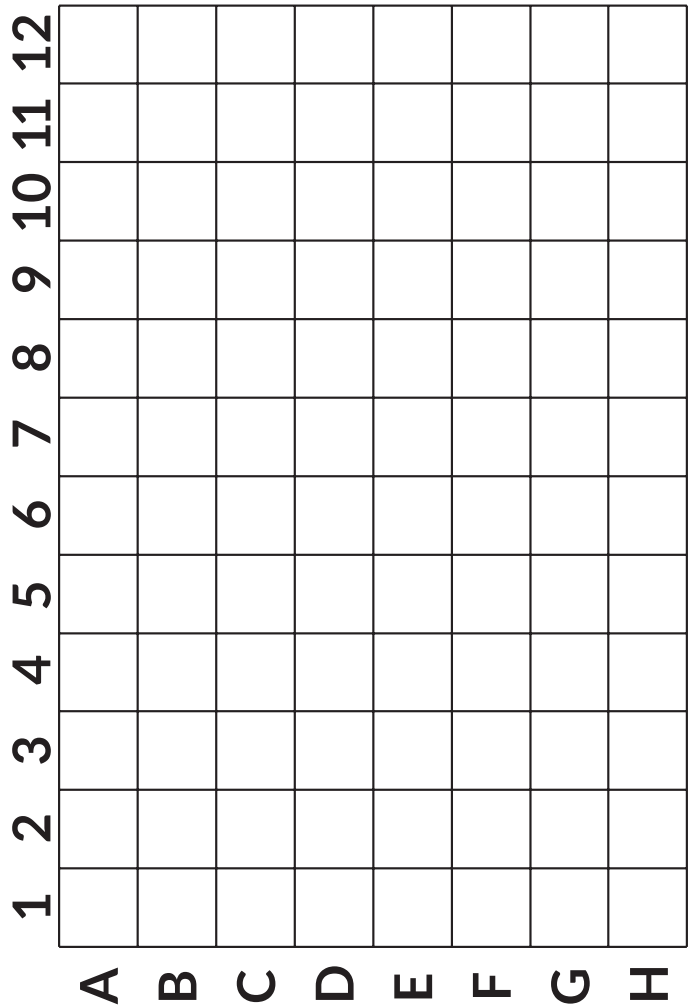

NOTES

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