8-Isoprostane ELISA Kit

Item No. 516351

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

TABLE OF CONTENTS

GENERAL INFORMATION

3 Materials Supplied
4 Safey Data
4 Precautions
5 If You Have Problems
5 Storage and Stability
5 Materials Needed but Not Supplied

INTRODUCTION

6 Background
6 About This Assay
8 Description of AChE Competitive ELISAs
9 Biochemistry of Acetylcholinesterase
11 Definition of Key Terms

PRE-ASSAY PREPARATION

12 Buffer Preparation
13 Sample Preparation
17 Sample Purification

ASSAY PROTOCOL

20 Preparation of Assay-Specific Reagents
22 Plate Set Up
23 Performing the Assay

ANALYSIS

26 Calculations
28 Performance Characteristics

RESOURCES

32 Troubleshooting
33 References
34 Plate Template
35 Notes
35 Warranty and Limitation of Remedy

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	1 vial
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 cover	5 covers
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 AChE ELISA Kits. 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
Fax:	734-971-3641
Email:	techserv@caymanchem.com
Hours:	M-F 8:00 AM to 5:30 PM EST

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. Adjustable pipettes and a repeating 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. Materials used for **Sample Preparation** and **Purification Protocol** (see pages 13-19, respectively).

INTRODUCTION

Background

The isoprostanes are a family of eicosanoids of non-enzymatic origin produced by the random oxidation of tissue phospholipids by oxygen radicals. Isoprostanes appear as artifacts in tissue and plasma samples which have undergone oxidative degradation during prolonged or improper storage. They also appear in the plasma and urine under normal conditions and are elevated by oxidative stress.

At least one of the isoprostanes, 8-isoprostane (8-*iso* Prostaglandin $F_{2\alpha}$), has been shown to have biological activity. It is a potent pulmonary and renal vasoconstrictor[1] and has been implicated as a causative mediator of hepatorenal syndrome and pulmonary oxygen toxicity.[2] 8-Isoprostane has been proposed as a marker of antioxidant deficiency and oxidative stress and elevated levels have been found in heavy smokers.[3] 8-Isoprostane levels are also a relative indicator of sample integrity for lipid-containing samples such as serum, plasma, and whole cell preparations.[4] Plasma from healthy volunteers contains modest amounts of 8-isoprostane that increase with the age of the test subject.[5] Normal human urinary levels of 8-isoprostane can be at concentrations an order of magnitude higher than many enzymatically derived eicosanoids.[5,6] A scheme of 8-isoprostane generation is shown in Figure 1, on page 7.

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 from 0.8-500 pg/ml and a sensitivity (80% B/B_0) of approximately 3 pg/ml.

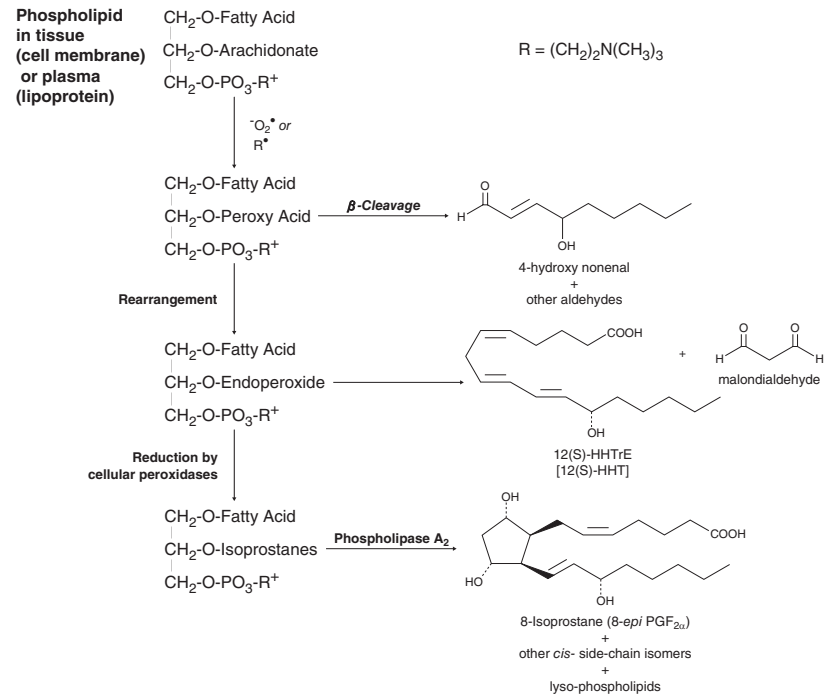


Figure 1. The formation of 8-Isoprostane

Description of AChE Competitive ELISAs[7,8]

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

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

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

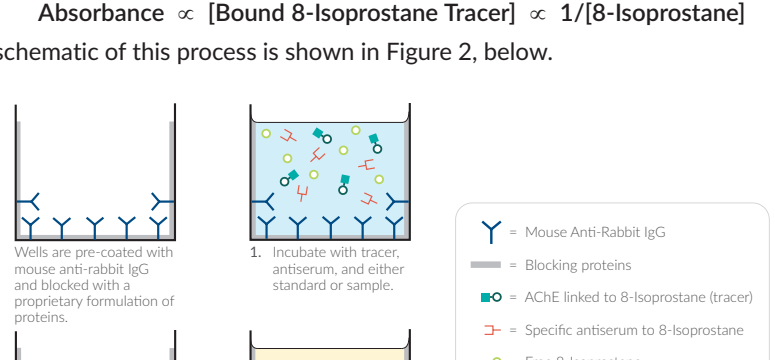


Figure 2. Schematic of the AChE ELISA

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 3, on page 10). 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.

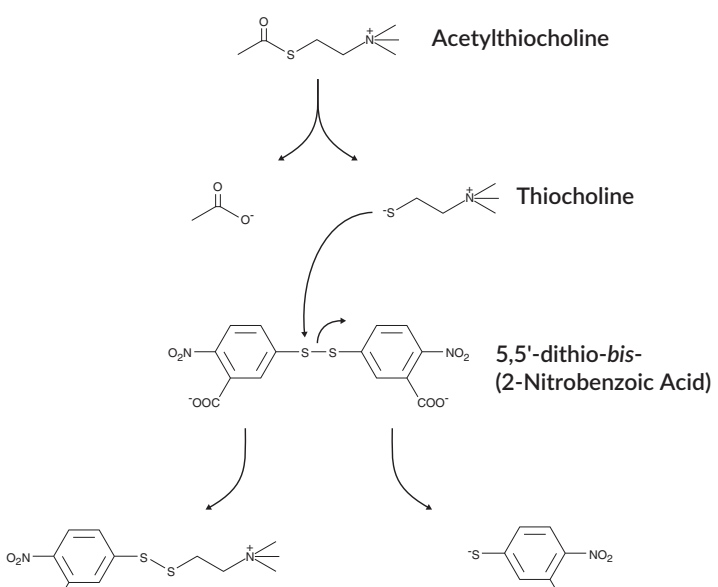


Figure 3. Reaction catalyzed by acetylcholinesterase

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:

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

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 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**

   5 ml vial Wash Buffer Concentrate (400X) (96-well kit; Item No. 400062): Dilute to a total volume of 2 liters 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 to a total volume of 5 liters with UltraPure water and add 2.5 ml of Polysorbate 20 (Item No. 400035).

Smaller volumes of Wash Buffer can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Polysorbate 20 (0.5 ml/liter of Wash Buffer).

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 for 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) and Affinity Sorbent (Item No. 401113) for sample purification. The affinity column purification procedures have been validated with plasma and urine samples and recoveries average >90%. You may also use SPE (solid phase extraction) purification methods described on page 19.

General Precautions

- All samples must be free of organic solvents prior to assay.
- AEBSF (Pefabloc SC®) and PMSF inhibit acetylcholinesterase. 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 which 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 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 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 ~5 and 100 pg/ml (*i.e.*, 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 you do not see good correlation of the different dilutions, 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 13). Be certain to dilute the standards in the same medium as your samples. *NOTE: If you obtain inconsistent results, SPE or immunoaffinity purification is warranted.*

Urine

In general, urine samples may be diluted with ELISA Buffer 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 13). *NOTE: If you obtain inconsistent results, 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 13). If the estimated concentration in your samples is too low to allow dilution with ELISA Buffer, be certain to dilute the 8-isoprostane standards in the same medium as your samples. *NOTE: If you obtain inconsistent results, 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 13).

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 page 17).

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

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. We recommend that you weigh each sample prior to homogenization. If you wish to determine protein concentration of the tissue lysate, we recommend the use of Cayman's Protein Determination Kit (Item No. 704002).

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 you wish to normalize your sample to protein concentration, 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 you wish to determine total 8-isoprostane in the tissue.

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 you wish to normalize your sample to protein concentration, 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 you wish to determine total 8-isoprostane in the tissue

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 your 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. If you wish to measure the total 8-isoprostane content of your samples, we recommend that you hydrolyze them prior to purification using the following procedure. If you wish to only measure free 8-isoprostane, proceed to either the 'Preparation for Affinity Purification' section or the 'SPE Purification Protocol' section, on page 19. To hydrolyze samples:

1. Add one volume of 15% (w/v) KOH to all sample tubes.
2. Incubate at 40°C for 60 minutes.
3. Neutralize samples by the addition of approximately 10 times the original sample volume of 1 M Potassium Phosphate Buffer, pH 7.0-7.4. (Standardize the pH adjustment using the sample matrix prior to proceeding with a large number of samples).
4. Proceed to either the 'Preparation for Affinity Purification' section or the 'SPE Purification Protocol' section on page 19.

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 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 (Item No. 401113), Column (Item No. 401111), or Purification Kit (Item No. 501110).

SPE Purification Protocol

NOTE: Precipitation of proteins using ethanol is optional and may not be needed if samples are clean enough to flow through the SPE. If ethanol precipitation is not required, proceed to step 4 of this section.

1. Add 2-4 volumes of ethanol to all tubes prepared above. Vortex to mix thoroughly. Allow the samples to stand at 4°C for five minutes, then centrifuge at 1,500 x g for ten minutes to remove precipitated proteins.
2. Decant supernatants to clean tubes.
3. Evaporate the ethanol by either vacuum centrifugation or under a gentle stream of nitrogen.
4. Resuspend samples in 1M acetate buffer, pH 4.0.
5. Activate a SPE Cartridges (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 become dry.
6. Pass the sample through the SPE cartridge. Rinse the cartridge with 5 ml UltraPure water, followed by 5 ml HPLC grade hexane. Allow the column to become dry after this step. Discard both the washes.
7. Elute the 8-isoprostane with 5 ml ethyl acetate containing 1% methanol.*
8. Evaporate the ethyl acetate solution to dryness by evaporation under a stream of dry nitrogen.

   *If it is necessary to stop during this purification, samples may be stored in the ethyl acetate/methanol solution at -80°C.
9. Add 500 µl of ELISA Buffer and vortex. It is common for an insoluble precipitate to remain after the addition of ELISA Buffer; this will not affect the assay. The sample is now ready for use in the immunoassay.

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 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, culture medium should be used in place of ELISA Buffer 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 to tube #1 and 750 µl ELISA Buffer 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.

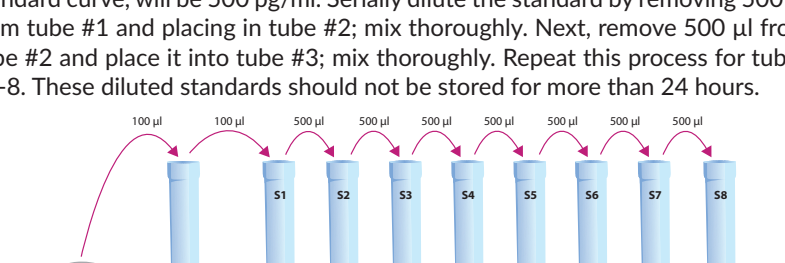


Figure 4. Preparation of the 8-Isoprostane standards

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.

OR

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

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.

OR

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

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 blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (B_0), 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, we recommend assaying samples in triplicate.

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 26, for more details). We suggest you record the contents of each well on the template sheet provided (see page 34).

	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**

   Add 100 µl ELISA Buffer to NSB wells. Add 50 µl ELISA Buffer to B_0 wells. If culture medium was used to dilute the standard curve, substitute 50 µl of culture medium for ELISA Buffer 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 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 the Blk wells.

5. **8-Isoprostane ELISA Antiserum**

   Add 50 µl 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 2. Pipetting summary

Incubation of the Plate

Cover each plate with plastic film (Item No. 400012) and incubate 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.
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(s) to develop in the dark at room temperature. 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. 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 405-420 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; 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 29). 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****).*

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 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

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

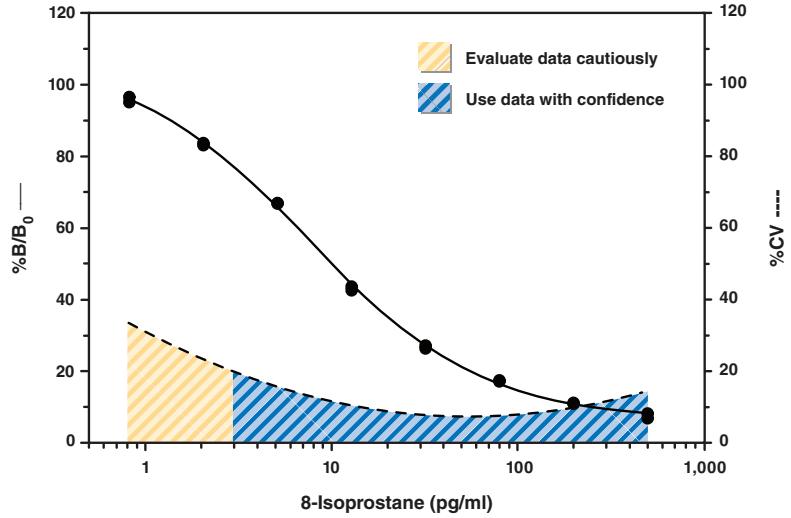


Figure 6. Typical standard curve

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 29 and in the table below.

Dose (pg/ml)	%CV* Intra-assay variation	%CV* Inter-assay variation
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 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
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

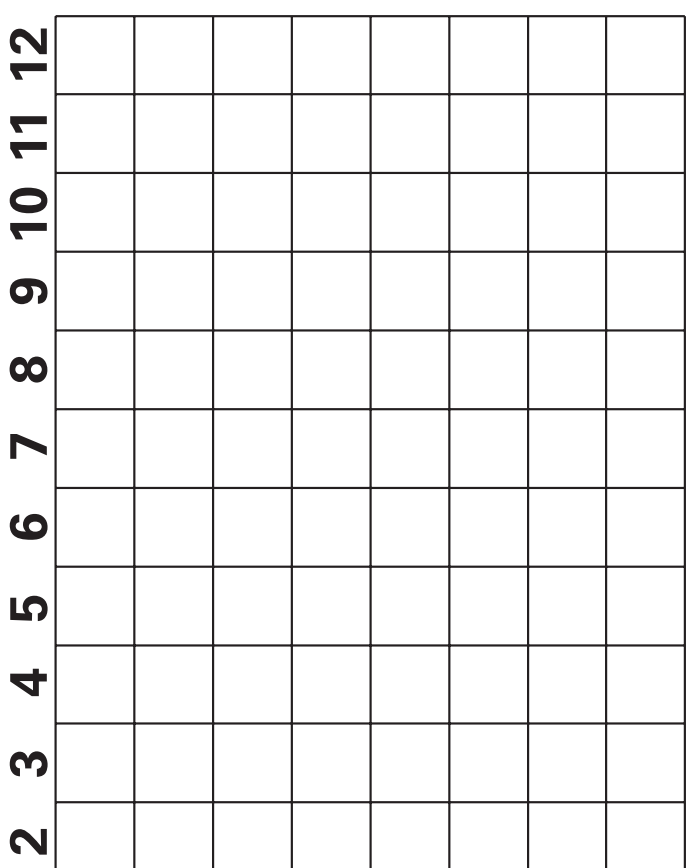
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique	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. 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[10]
Only Total Activity (TA) wells develop	Trace organic contaminants in the water source	Replace activated carbon filter or change source of UltraPure water

References

1. Banerjee, M., Kang, K.H., Morrow, J.D., *et al.* Effects of a novel prostaglandin, 8-*epi*-$PGF_{2\alpha}$, in rabbit lung *in situ*. *Am. J. Physiol.* **263**, H660-H663 (1992).
2. Vacchiano, C.A. and Tempel, G.E. Role of nonenzymatically generated prostanoid, 8-*iso*-PGF_2, in pulmonary oxygen toxicity. *J. Appl. Physiol.* **77**, 2912-2917 (1994).
3. Morrow, J.D., Frei, B., Longmire, A.W., *et al.* Increase in circulating products of lipid peroxidation (F_2-isoprostanes) in smokers. *N. Engl. J. Med.* **332**, 1198-1203 (1995).
4. Morrow, J.D., Hill, K.E., Burk, R.F., *et al.* A series of prostaglandin F_2-like compounds are produced *in vivo* in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc. Natl. Acad. Sci. USA* **87**, 9383-9387 (1990).
5. Wang, Z., Ciabattoni, G., Créminon, C., *et al.* Immunological characterization of urinary 8-*epi*-prostaglandin $F_{2\alpha}$ excretion in man. *J. Pharmacol. Exp. Ther.* **275**, 94-100 (1995).
6. Reilly, M.P., Barry, P., Lawson, J.A., *et al.* Urinary 8-*epi* $PGF_{2\alpha}$: An index of oxidant stress *in vivo*. *Fibrinolysis & Proteolysis* **11**, 81-84 (1997).
7. 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).
8. Pradelles, P., Grassi, J. and Maclouf, J. Enzyme immunoassays of eicosanoids using AChE as label: An alternative to radioimmunoassay. *Anal. Chem.* **57**, 1170-1173 (1985).
9. Morrow, J.D., Harris, T.M., and Roberts, L.J., II. Noncyclooxygenase oxidative formation of a series of novel prostaglandins: Analytical ramifications for measurement of eicosanoids. *Anal. Biochem.* **184**, 1-10 (1990).
10. Maxey, K.M., Maddipati, K.R. and Birkmeier, J. Interference in enzyme immunoassays. *J. Clin. Immunoassay* **15**,116-120 (1992).



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.