

8-Isoprostane EIA Kit

Item No. 516351



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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size	480 wells Quantity/Size
416352	8-Isoprostane EIA 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 EIA Standard	1 vial	1 vial
400060	EIA 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	EIA Tracer Dye	1 vial	1 vial
400042	EIA 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) 975-3999. We cannot accept any returns without prior authorization.



WARNING: This product is for laboratory research use only; not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

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

For research use only. Not for human or diagnostic use.

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 repeat pipettor.
3. A source of 'UltraPure' water. Water used to prepare all EIA 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 EIA. *NOTE: UltraPure water is available for purchase from Cayman (Item No. 400000).*
4. Materials used for **Sample Preparation** and **Purification Protocol** (see pages 13 and 34, 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_{2α}), has been shown to have biological activity. It is a potent pulmonary and renal vasoconstrictor¹ 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 (40-100 pg/ml) that increase with the age of the test subject.⁵ Normal human urinary levels range from 10-50 ng/mmol creatinine, which is 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 EIA Kit is a competitive assay that can be used for quantification of 8-isoprostane in plasma, urine, and other sample matrices. The EIA typically displays an IC₅₀ (50% B/B₀) of approximately 10 pg/ml and a detection limit (80% B/B₀) of approximately 2.7 pg/ml.

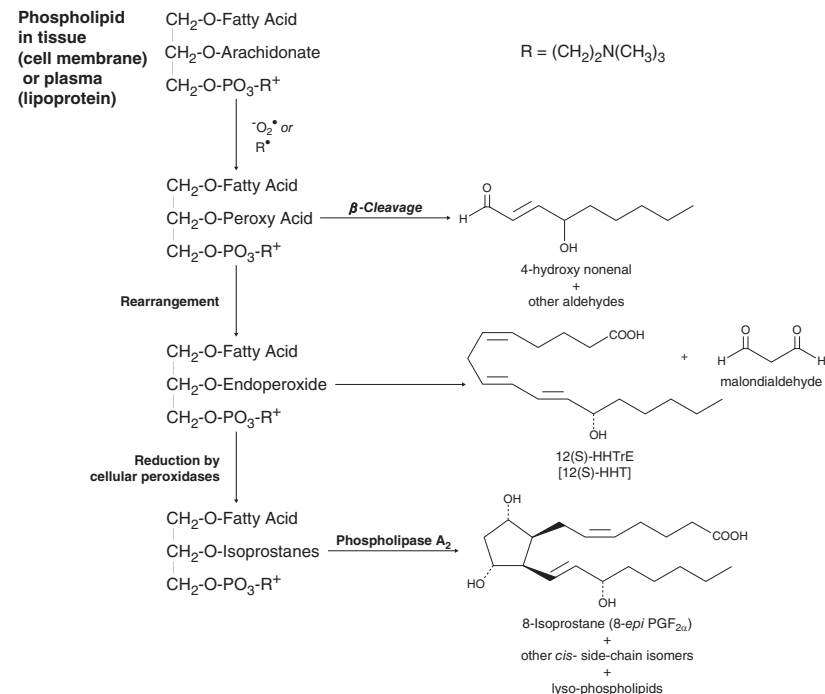


Figure 1. The formation of 8-Isoprostane

Description of ACE™ Competitive EIAs^{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.

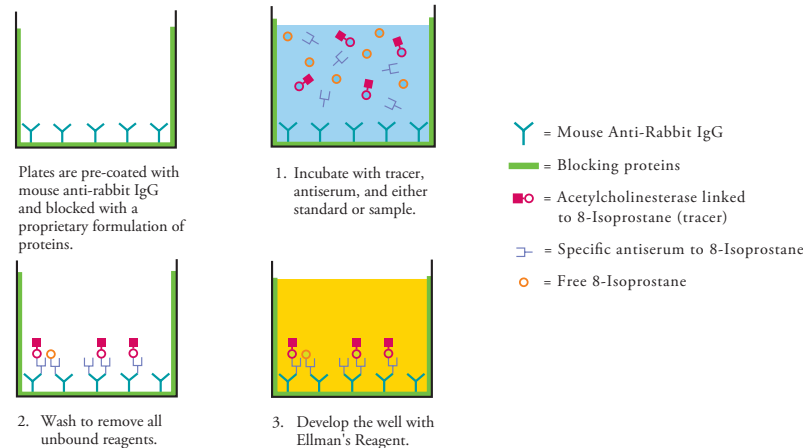


Figure 2. Schematic of the ACE™ EIA

Biochemistry of Acetylcholinesterase

The electric organ of the electric eel, *Electrophorus electricus*, contains an avid acetylcholinesterase (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 ($64,000\text{ s}^{-1}$) for the hydrolysis of acetylthiocholine.

A molecule of the analyte covalently attached to a molecule of AChE serves as the tracer in ACE™ 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 ($\epsilon = 13,600$).

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 re-development 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.

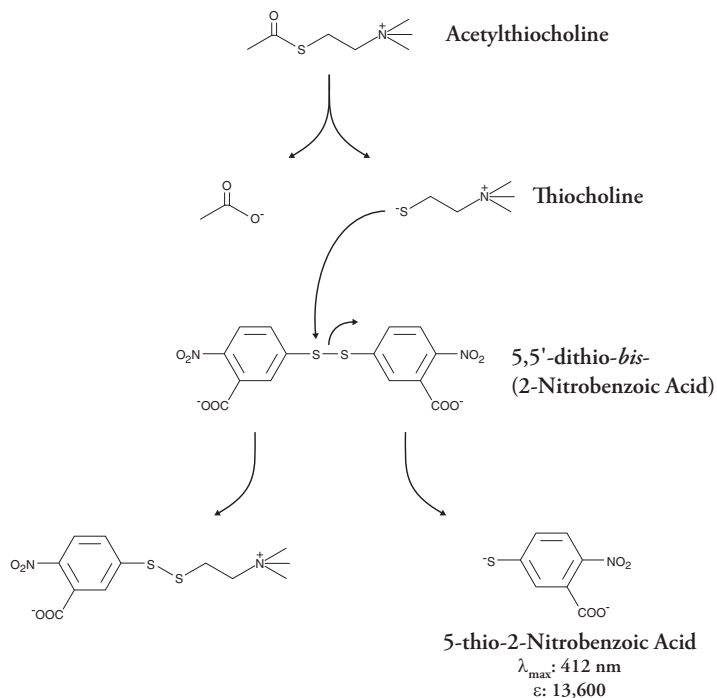


Figure 3. Reaction catalyzed by acetylcholinesterase

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.

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.

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.

PRE-ASSAY PREPARATION

NOTE: Water used to prepare all EIA 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 EIA. 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. EIA Buffer Preparation

Dilute the contents of one vial of EIA 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.

In general, tissue culture supernatant samples may be diluted with EIA Buffer and added directly to the assay well. Plasma, serum, urine, 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 ~5 and 100 pg/ml (*i.e.*, 20-80% B/B₀). 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.

Cayman offers an 8-Isoprostane Affinity Columns (Item Nos. 400058 and 400059) and Affinity Sorbent (Item No. 10010365) which are recommended as the easiest and most convenient purification format for 8-isoprostane. The affinity column purification procedures have been validated with plasma and urine samples. Recoveries average >90% with a variance of <20%. The SPE (solid phase extraction) purification methods described on pages 20-21 and continuing on pages 34-35 were validated by a comparison of the data from EIA and gas chromatography/negative ion chemical ionization-mass spectrometry (GC/NICI-MS) (see figures on pages 15 and 17). GC/NICI-MS analysis was performed on samples derivatized as pentafluorobenzyl esters and tert-butyldimethyl-silyl ethers.⁹

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 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.

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** above). 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

Urine samples measured by EIA give excellent correlation to GC/MS if purified by SPE and TLC (see Figure 4, on page 15) or immunoaffinity methods prior to analysis. Samples that cannot be assayed immediately should be stored at -80°C in the presence of 0.005% BHT (see **General Precautions** above).

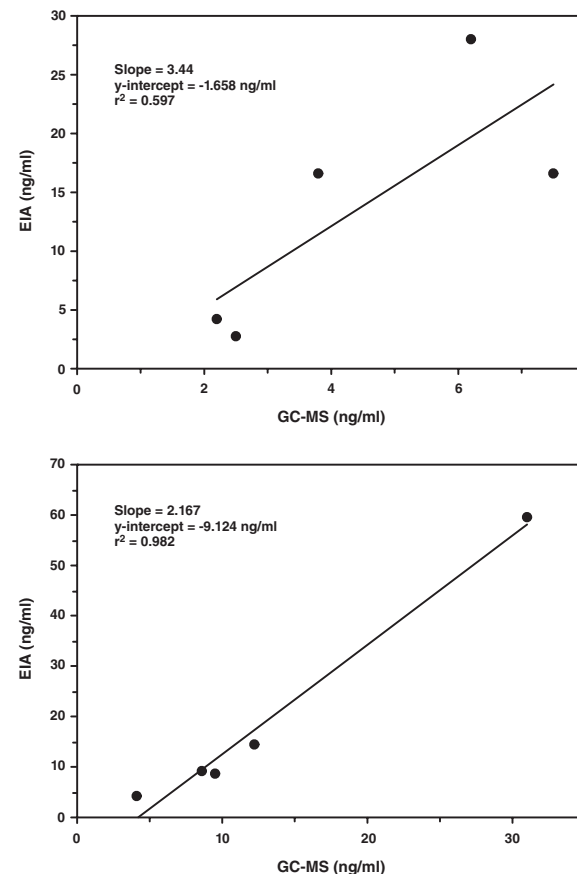


Figure 4. Comparison of 8-isoprostane values in urine obtained by EIA and GC-MS following purification by SPE (upper panel) and SPE/TLC (lower panel). Urine samples were purified by SPE or SPE followed by TLC. Samples were then analyzed by GC-MS (x-axis) and EIA (y-axis).

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 14). If the estimated concentration in your samples is too low to allow dilution with EIA 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. Vacutainers can also be supplemented with indomethacin to give a final concentration of at least $10\text{ }\mu\text{M}$. Indomethacin will prevent *ex vivo* formation of prostaglandins, which have the potential to interfere with this assay (although most prostaglandins do not appear to exhibit any cross-reactivity (see page 33)). Samples that cannot be assayed immediately should be stored at -80°C in the presence of 0.005% BHT (see **General Precautions** on page 14).

Less than half of total plasma 8-isoprostane is present as the free acid, while the remainder is esterified in phospholipids.³ Direct EIA of plasma samples without hydrolysis will measure only the free 8-isoprostane fraction. Total plasma 8-isoprostane determination requires an alkaline hydrolysis prior to EIA (see page 20).

Analysis of plasma samples without purification may lead to inconsistent results. If inconsistent results are obtained, we recommend the immunoaffinity purification as the easiest and most convenient purification format (see Figure 6, page 18). Plasma samples measured by EIA give excellent correlation to GC/MS if purified by SPE prior to analysis (see Figure 5, on page 17).

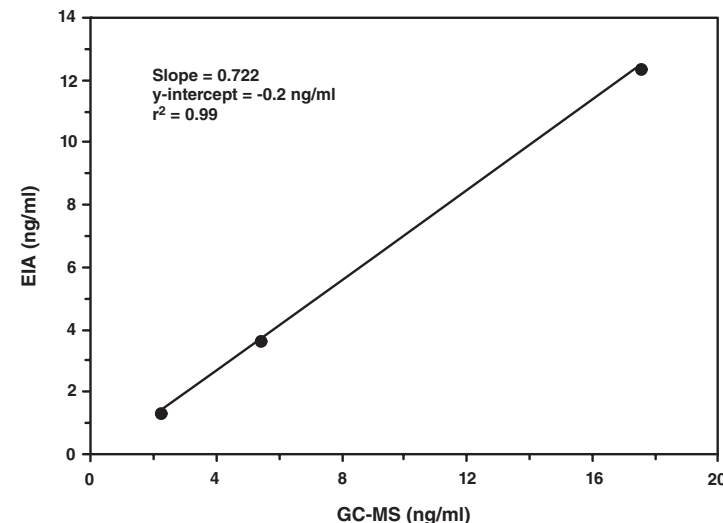


Figure 5. Comparison of 8-isoprostane values in plasma obtained by EIA and GC-MS following purification by SPE. Plasma samples were purified by SPE. Samples were then analyzed by GC-MS (x-axis) and EIA (y-axis).

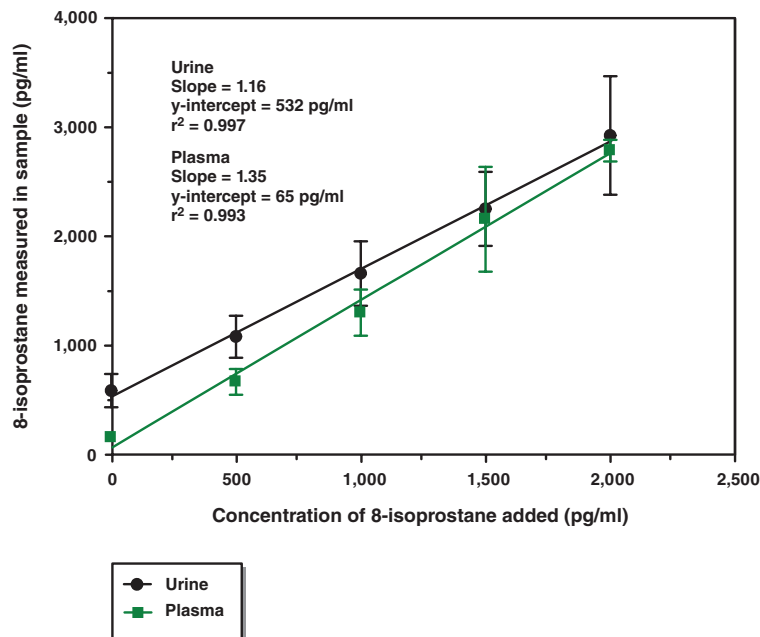


Figure 6. Recovery of 8-isoprostane from urine and plasma following immunoaffinity purification. Urine and plasma samples were spiked with 8-isoprostane, purified using immunoaffinity chromatography and analyzed using the 8-Isoprostane EIA Kit. The y-intercepts correspond to the amount of 8-isoprostane measured in the unspiked samples.

Tissue

Tissue samples should be homogenized in 0.1 M phosphate, pH 7.4, containing 1 mM EDTA and 0.005% BHT using a Polytron type homogenizer and then processed as described for plasma samples. As described in the 'Plasma' section on page 16, most of the 8-isoprostane will be esterified in lipids, so hydrolysis must be performed in order to determine total amounts of 8-isoprostane.

Tissue Homogenization using the Precellys 24 Homogenizer

Snap-freeze tissues in liquid nitrogen immediately upon collection and store at -80°C . Add 1 ml 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). Spin the tissue homogenates at $8,000 \times g$ for 10 minutes. Collect supernatant and assay as described below. Samples will need to be diluted appropriately for the assay. Tissue samples should be normalized using a protein assay. Cayman's Protein Determination Kit (Item No. 704002) may be used to normalize protein samples.

NOTE: For total 8-isoprostane content, samples must be hydrolyzed. See Free versus Total 8-Isoprostane Measurement, on page 20, for the hydrolyzing procedure.

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 Sorbent/Column Purification' section or the 'Preparation for SPE Purification' section, on page 21. 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 three volumes (six 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 Sorbent/Column Purification' section or the 'Preparation for SPE Purification' section on page 21.

Preparation for Affinity Sorbent/Column Purification

All samples must be free of particulates and precipitates to avoid plugging the column. This can be achieved either by filtration or by centrifugation. All samples must be at approximately neutral pH (6.5-7.5).

1. Urine samples should be centrifuged briefly to remove sediment and 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 which 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.
2. Proceed with purification following the protocol described in the product insert for the 8-isoprostane Affinity Sorbent (Item No. 10010365), Column (Item Nos. 400058 or 400059), or Purification Kit (Item Nos. 10367 or 10368).

Preparation for SPE Purification - ethanol precipitation and acidification

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. Acidify samples to pH 4.0 by the addition of 30% acetic acid. (Standardize the pH adjustment using the sample matrix prior to proceeding with a large number of samples.) *NOTE: Acidification of samples using acetate buffer or citrate is not advised.*
5. Proceed with the SPE/TLC purification protocol described in the **Appendix** (see pages 34-35).

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

8-Isoprostane EIA 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 μ l of the 8-Isoprostane EIA Standard (Item No. 416354) into a clean test tube, then dilute with 900 μ l 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 EIA Buffer, culture medium should be used in place of EIA Buffer for dilution of the standard curve.

To prepare the standard for use in EIA: obtain eight clean test tubes and number them #1 through #8. Aliquot 900 μ l EIA Buffer to tube #1 and 750 μ l EIA Buffer to tubes #2-8. Transfer 100 μ l 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 μ l from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 μ l 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.

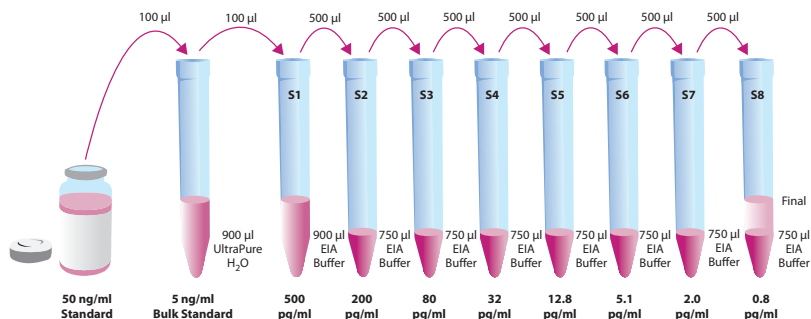


Figure 7. Preparation of the 8-isoprostane standards

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 EIA Buffer.

OR

500 dtn 8-Isoprostane AChE Tracer (480-well kit; Item No. 416350): Reconstitute with 30 ml EIA 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 μ l of dye to 6 ml tracer or add 300 μ l of dye to 30 ml of tracer).

8-Isoprostane EIA Antiserum

Reconstitute the 8-Isoprostane EIA Antiserum as follows:

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

OR

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

Store the reconstituted 8-Isoprostane EIA 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 μ l of dye to 6 ml antiserum or add 300 μ l 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 8, 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 28, for more details). We suggest you record the contents of each well on the template sheet provided (see page 39).

	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 8. 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. EIA Buffer

Add 100 μ l EIA Buffer to Non-Specific Binding (NSB) wells. Add 50 μ l EIA Buffer to Maximum Binding (B_0) wells. If culture medium was used to dilute the standard curve, substitute 50 μ l of culture medium for EIA Buffer in the NSB and B_0 wells (*i.e.*, add 50 μ l culture medium to NSB and B_0 wells and 50 μ l EIA Buffer to NSB wells).

2. 8-Isoprostane EIA Standard

Add 50 μ l from tube #8 to both of the lowest standard wells (S8). Add 50 μ l 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 μ l 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 μ l to each well *except* the Total Activity (TA) and the Blank (Blk) wells.

5. 8-Isoprostane EIA Antiserum

Add 50 μ l to each well *except* the Total Activity (TA), the Non-Specific Binding (NSB), and the Blank (Blk) wells.

Well	EIA Buffer	Standard/ Sample	Tracer	Antibody
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 Total Activity 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.*, B₀ 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 B₀ wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the B₀ 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/B₀ versus log concentration using a four-parameter logistic fit or as logit B/B₀ 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/eia) 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 B₀ wells.
3. Subtract the NSB average from the B₀ average. This is the corrected B₀ or corrected maximum binding.
4. Calculate the B/B₀ (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 B₀ (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain %B/B₀ for a logistic four-parameter fit, multiply these values by 100.)

*NOTE: The total activity (TA) values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool; the corrected B₀ 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 30). 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 35 for **Troubleshooting**).*

Plot the Standard Curve

Plot %B/B₀ 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 linearized using a logit transformation. The equation for this conversion is shown below. *NOTE: Do not use %B/B₀ in this calculation.*

$$\text{logit (B/B}_0\text{)} = \ln [\text{B/B}_0\text{}/(1 - \text{B/B}_0\text{)}]$$

Plot the data as logit (B/B₀) versus log concentrations and perform a linear regression fit.

Determine the Sample Concentration

Calculate the B/B₀ (or %B/B₀) 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/B₀ 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

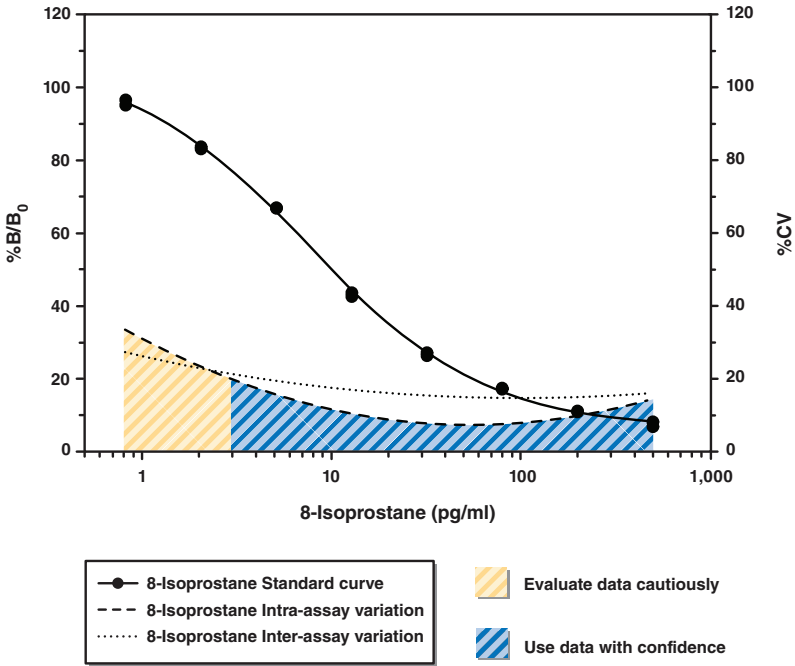
Sample Data

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



50% B/B₀ - 10 pg/ml
Detection Limit (80% B/B₀) - 2.7 pg/ml

Figure 9. 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 31 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.

Specificity:

Compound	Cross Reactivity	Compound	Cross Reactivity
8-Isoprostane	100%	2,3-dinor-6-keto Prostaglandin F _{1α}	0.09%
8-iso Prostaglandin F _{2α} ethanolamide	100%	8-iso Prostaglandin F _{1β}	0.08%
8-iso Prostaglandin F _{3α}	20.6%	Thromboxane B ₂	0.08%
2,3-dinor-8-iso Prostaglandin F _{2α}	4.00%	11-dehydro Thromboxane B ₂	0.07%
8-iso Prostaglandin E ₂	1.84%	11β-Prostaglandin F _{2α}	0.03%
2,3-dinor-8-iso Prostaglandin F _{1α}	1.70%	Prostaglandin E ₂	0.02%
8-iso Prostaglandin E ₁	1.56%	8-iso-15(R)-Prostaglandin F _{2α}	0.02%
Prostaglandin F _{1α}	0.71%	8,12-epi iPF _{2α} -III	0.01%
Prostaglandin F _{3α}	0.66%	iPF _{2α} -VI	<0.01%
Prostaglandin E ₁	0.39%	8,12-epi iPF _{2α} -VI	<0.01%
Prostaglandin D ₂	0.16%	tetranor-PGEM	<0.01%
6-keto Prostaglandin F _{1α}	0.14%	tetranor-PGFM	<0.01%
Prostaglandin F _{2α}	0.14%	13,14-dihydro-15-keto Prostaglandin F _{2α}	<0.01%

Table 5. Specificity of the 8-Isoprostane EIA Antiserum

Appendix

Purification Protocol (SPE/TLC method)

1. Activate a SPE Cartridge (C-18) (Item No. 400020) by rinsing with 5 ml methanol and then with 5 ml UltraPure water. Do not allow the cartridge to become dry.
2. 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.
3. Elute the 8-isoprostane with 5 ml ethyl acetate containing 1% methanol.*
4. Evaporate the ethyl acetate solution to dryness by evaporation under a stream of dry nitrogen. For plasma, serum, and most lavage fluids, skip to step 8. We recommend further purification of urine samples by TLC as described in steps 5-8.
5. Dissolve the sample in a small amount of acetone and spot in the preadsorbent zone of a channeled 20 x 20 cm TLC plate without any fluorescent indicator (*e.g.*, Analtech 31911, Whatman 4865-821). The preadsorbent zone of the plates will concentrate the sample into a thin line at the solvent front so there is no need for special precautions when spotting the sample. Spot at least 1 µg of authentic 8-isoprostane (Item No. 16350) on one of the edge lanes of each plate to help locate the appropriate bands in your sample. Develop the plate using chloroform/methanol/acetic acid/water (80:18:1:0.8, v/v).
6. After the solvent has traveled to the top of the plate, remove the plate from the solvent chamber and allow to dry. The lanes containing 8-isoprostane may be detected in one of two ways: masking all of the lanes except the one containing the authentic standard and spraying this lane with 3.5% phosphomolybdic acid, or by carefully spreading a mixture of iodine and silica gel on the 8-isoprostane lane and removing after five minutes.

*If it is necessary to stop during this purification, samples may be stored in the ethyl acetate/methanol solution at -80°C.

7. Carefully scrape the band corresponding to 8-isoprostane from each sample lane onto a piece of weighing paper and transfer into a clean test tube. Elute the sample by adding 4 ml ethanol, vortexing, and then centrifuging at 1,500 x g for 10 minutes. Decant the supernatant into a clean test tube and evaporate the solvent using a stream of nitrogen.
8. Add 500 µl of EIA Buffer and vortex. It is common for an insoluble precipitate to remain after the addition of EIA Buffer; this will not affect the assay. The sample is now ready for use in the immunoassay.

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 (>0.035)	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>i.e.</i> , more than 20% difference)	Interfering substances are present	Purify sample prior to analysis by EIA ¹⁰
Only Total Activity (TA) wells develop	Trace organic contaminants in the water source	Replace activated carbon filter or change source of UltraPure water

Additional Reading

Go to www.caymanchem.com/516351/references for a list of publications citing the use of Cayman's 8-Isoprostane EIA Kit.

References

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Related Products

Aconitase Fluorometric Assay Kit - Item No. 700600
Ascorbate Assay Kit - Item No. 700420
Catalase Assay Kit - Item No. 707002
Creatinine (serum) Assay Kit - Item No. 700460
DNA/RNA Oxidative Damage EIA Kit - Item No. 589320
Lipid Hydroperoxide (LPO) Assay Kit - Item No. 705002
8-*iso* Prostaglandin F_{2α} - Item No. 16350
8-Isoprostane Affinity Column (4 ml) - Item No. 400058
8-Isoprostane Affinity Column (20 ml) - Item No. 400059
8-Isoprostane Affinity Purification Kit (4 ml) - Item No. 10367
8-Isoprostane Affinity Purification Kit (20 ml) - Item No. 10368
8-Isoprostane Affinity Sorbent - Item No. 10010365
8-Isoprostane Express EIA Kit - Item No. 516360
Prostaglandin F_{2α} EIA Kit - Item No. 516011
ent-Prostaglandin F_{2α} EIA Kit - Item No. 10010382
Protein Carbonyl Colorimetric Assay Kit - Item No. 10005020
Protein Carbonyl Fluorometric Assay Kit - Item No. 700490
SPE Cartridges (C-18) - Item No. 400020
L-Theanine Fluorometric Assay Kit - Item No. 700570
UltraPure Water - Item No. 400000
Xanthine Oxidase Assay Kit - Item No. 10010895

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NOTES

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