
# STAT-8-Isoprostane ELISA Kit

Item No. 500431

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

## Materials Supplied

| Item Number | Item | 96 wells Quantity/Size | 480 wells Quantity/Size |
|---|---|---|---|
| 400432 | STAT-8-Isoprostane ELISA Antiserum | 1 vial/100 dtn | 1 vial/500 dtn |
| 400430 | STAT-8-Isoprostane AP Tracer | 1 vial/100 dtn | 1 vial/500 dtn |
| 400434 | STAT-8-Isoprostane ELISA Standard | 1 vial | 1 vial |
| 400080 | Tris Buffer Concentrate (10X) | 2 vials/10 ml | 4 vials/10 ml |
| 411007 | AP Wash Buffer Concentrate (150X) | 1 vial/5 ml | 1 vial/12.5 ml |
| 400082 | DEA Buffer Concentrate (10X) | 1 vial/2.5 ml | 1 vial/12.5 ml |
| 400004/400006 | Mouse Anti-Rabbit IgG Coated Plate | 1 plate | 5 plates |
| 400012 | 96-Well Cover Sheet | 1 cover | 5 covers |
| 400090 | *p*NPP Tablets | 1 vial/5 ea | 1 vial/25 ea |
| 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 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** (see page 11).

# 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* $PGF_{2\alpha}$), 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 (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 STAT-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 23.4-3,000 pg/ml and a sensitivity (80% $B/B_0$) of approximately 45 pg/ml.

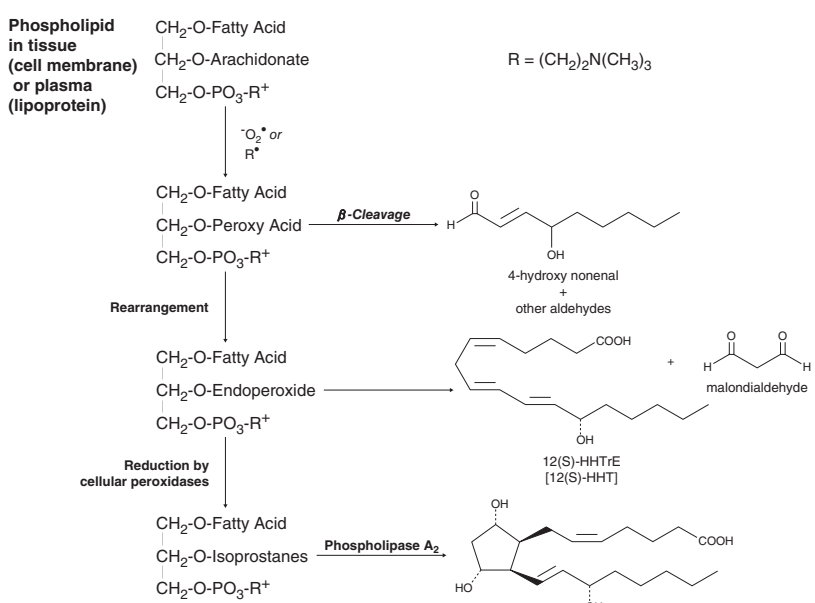



**Figure 1. The formation of 8-isoprostane**

## Description of AP Competitive ELISAs

This assay is based on the competition between 8-isoprostane and a 8-isoprostane alkaline phosphatase (AP) 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 8-isoprostane polyclonal antiserum will be inversely proportional to the concentration of 8-isoprostane in the well. This antiserum-8-isoprostane complex binds to mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then *para*-nitrophenyl phosphate (*p*NPP) 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 below in Figure 2.

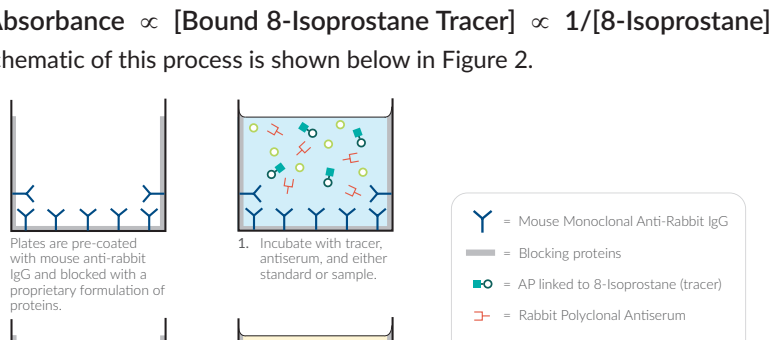



**Figure 2. Schematic of the ELISA**



## Definition of Key Terms

**Blank:** background absorbance caused by *p*NPP. 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 alkaline phosphatase-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. **Tris Buffer Preparation**

   Dilute the contents of one vial of Tris Buffer Concentrate (10X) (Item No. 400080) 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. **AP Wash Buffer Preparation**

   **5 ml vial AP Wash Buffer Concentrate (150X) (96-well kit; Item No. 411007):** Dilute to a total volume of 750 ml with UltraPure water.

   **OR**

   **12.5 ml vial AP Wash Buffer Concentrate (150X) (480-well kit; Item No. 411007):** Dilute to a total volume of 1,875 ml with UltraPure water.

3. **DEA Buffer Preparation**

   **2.5 ml vial DEA Buffer Concentrate (10X) (96-well kit; Item No. 400082):** Dilute to a total volume of 25 ml with UltraPure water.

   **OR**

   **12.5 ml vial DEA Buffer Concentrate (10X) (480-well kit; Item No. 400082):** Dilute to a total volume of 125 ml with UltraPure water.



## 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 Tris 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 ~45 and 1,500 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.

Cayman offers an 8-Isoprostane Affinity Column and Affinity Sorbent (Item Nos. 401111 and 401113, respectively) 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 18-20 and continuing on pages 34-35 were validated by a comparison of the data from ELISA and gas chromatography/negative ion chemical ionization-mass spectrometry (GC/NICI-MS) (see figures on pages 13 and 15). GC/NICI-MS analysis was performed on samples derivatized as pentafluorobenzyl esters and *tert*-butyldimethyl-silyl ethers.[7]

## 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 ELISA give excellent correlation to GC/MS if purified by SPE and TLC (see Figure 3, on page 13) 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).

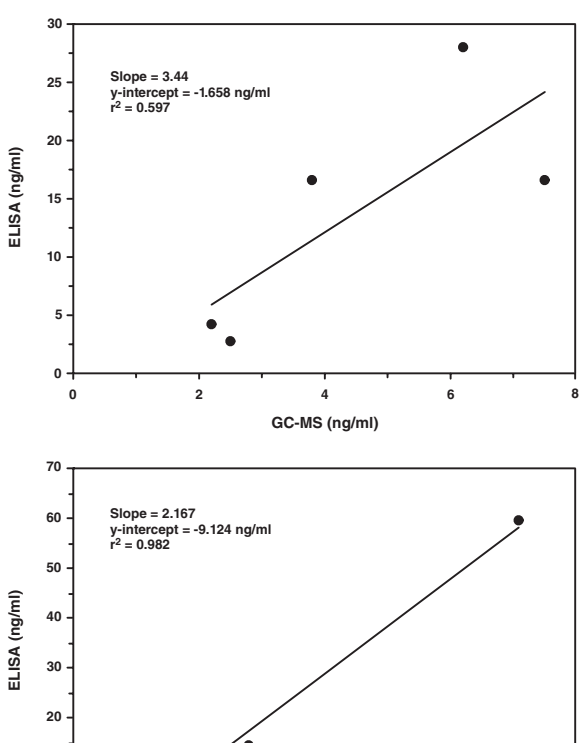



**Figure 3. Comparison of 8-isoprostane values in urine obtained by ELISA and GC-MS following purification by SPE (upper panel) and SPE/TLC (lower panel).** Urine samples were purified by SPE or SPE followed by TLC. Samples were then analyzed by GC-MS (x-axis) and ELISA (y-axis).

## 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 12). If the estimated concentration in your samples is too low to allow dilution with Tris 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 µM. 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 12).

Less than half 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 18).

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 5, page 16). Plasma samples measured by ELISA give excellent correlation to GC/MS if purified by SPE prior to analysis (see Figure 4, on page 15).

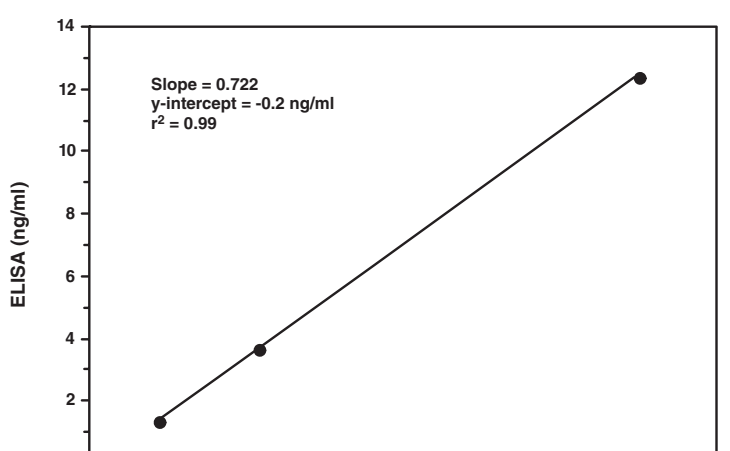



**Figure 4. Comparison of 8-isoprostane values in plasma obtained by ELISA and GC-MS following purification by SPE.** Plasma samples were purified by SPE. Samples were then analyzed by GC-MS (x-axis) and ELISA (y-axis).

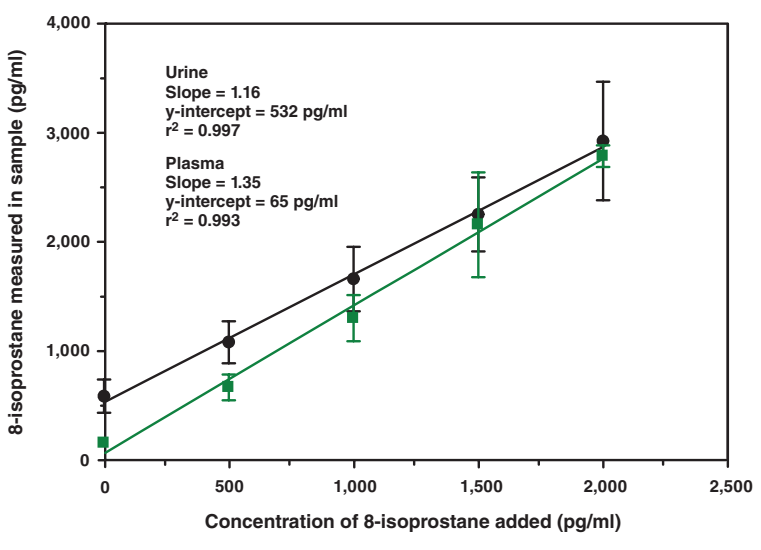



**Figure 5. Recovery of 8-isoprostane from urine and plasma following immunoaffinity purification.** Urine and plasma samples were spiked with 8-isoprostane, purified using immunoaffinity chromatography and analyzed using the 8-Isoprostane ELISA Kit. The y-intercepts correspond to the amount of 8-isoprostane measured in the unspiked samples.

## Tissue

Tissue samples should be homogenized in 0.1 M Tris-HCl, 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 14, 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 Tris-HCl, 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 x 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 19, 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

### Determination of Recovery

Determination of percent recovery is recommended when any sample purification is performed. Each sample should be split prior to purification and an appropriate known amount of 8-isoprostane added to one aliquot of each sample. The spiked samples are then purified and assayed *via* ELISA alongside the unspiked samples. Calculations for determining recovery are found in the **Analysis** section beginning on page 27. If you wish to purify your samples, we recommend that you determine recovery using the following procedure:

1. Aliquot a known amount of sample into each of two tubes (500 µl is recommended). Label the first tube 'sample #' and the second tube 'sample # + spike'. If your samples need to be concentrated, a larger volume should be used (*e.g.*, a 5 ml sample will be concentrated by a factor of 10, a 10 ml sample will be concentrated by a factor of 20, etc.).
2. Add a cold spike of 8-isoprostane to the 'sample + spike' tubes. The samples can now be used for purification of either free or total 8-isoprostane, as described on page 19.



### 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 20. To hydrolyze samples:

1. Add an equal volume of 15% (w/v) KOH to both your 'sample' and 'sample + spike' tubes.
2. Incubate at 40°C for 60 minutes.
3. Neutralize samples by the addition of approximately three volumes of 1 M Tris-HCl, 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 20.

### 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, Column or Purification Kit (Item Nos. 401111, 401113, or 501110).

### 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 'sample' and 'sample + spike' 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

### STAT-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 STAT-8-Isoprostane ELISA Standard (Item No. 400434) into a clean test tube, then dilute with 900 µl UltraPure water. The concentration of this solution (the bulk standard) will be 30 ng/ml.

*NOTE: If assaying culture media samples that have not been diluted with Tris Buffer, culture medium should be used in place of Tris 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 Tris Buffer to tube #1 and 500 µl Tris Buffer to tubes #2-8. Transfer 100 µl of the bulk standard (30 ng/ml) to tube #1 and mix thoroughly. The concentration of this standard, the first point on the standard curve, will be 3 ng/ml (3,000 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.

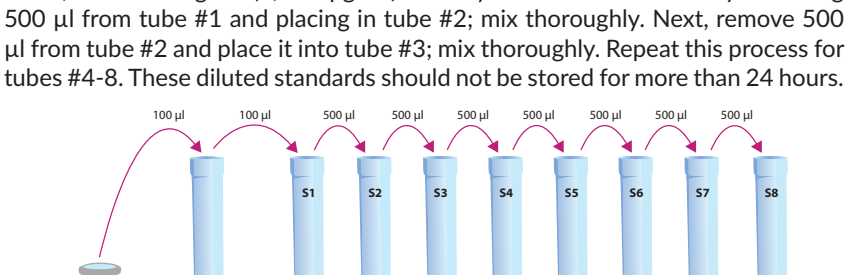



**Figure 6. Preparation of the STAT-8-Isoprostane standards**

### STAT-8-Isoprostane AP Tracer

Reconstitute the STAT-8-Isoprostane AP Tracer as follows:

**100 dtn STAT-8-Isoprostane AP Tracer (96-well kit; Item No. 400430):** Reconstitute with 6 ml Tris Buffer.

**OR**

**500 dtn STAT-8-Isoprostane AP Tracer (480-well kit; Item No. 400430):** Reconstitute with 30 ml Tris Buffer.

Store the reconstituted STAT-8-Isoprostane AP Tracer at 4°C *(do not freeze!)* and use within four weeks. A 20% surplus of tracer has been included to account for any incidental losses

**Tracer Dye Instructions (optional)**

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

### STAT-8-Isoprostane ELISA Antiserum

Reconstitute the STAT-8-Isoprostane ELISA Antiserum as follows:

**100 dtn STAT-8-Isoprostane ELISA Antiserum (96-well kit; Item No. 400432):** Reconstitute with 6 ml Tris Buffer.

**OR**

**500 dtn STAT-8-Isoprostane ELISA Antiserum (480-well kit; Item No. 400432):** Reconstitute with 30 ml Tris Buffer.

Store the reconstituted STAT-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). *NOTE: Do not store antiserum with dye for more than 24 hours.*



## 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 are using a strip plate and do not 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 7, (see 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 27, **Analysis** for more details). We suggest you record the contents of each well on the template sheet provided (see page 38).

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

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

**Figure 7. Sample plate format**

## Performing the Assay

**Pipetting Hints**

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

### Addition of the Reagents

1. **Tris Buffer**

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

2. **STAT-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. **STAT-8-Isoprostane AP Tracer**

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

5. **STAT-8-Isoprostane ELISA Antiserum**

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



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

**Table 1. Pipetting summary**

### Incubation of the Plate

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

### Development of the Plate

1. When ready to develop the plate(s), reconstitute 5 *p*NPP tablets in 25 ml DEA Buffer (25 ml is sufficient to develop 100 wells). Reconstituted *p*NPP is not stable, so we recommend that you store the reconstituted *p*NPP for no more than 24 hours. If the entire plate is not used at once, dissolve just enough *p*NPP for the number of wells used.
2. Empty the wells and rinse five times with Wash Buffer.
3. Add 200 µl of *p*NPP solution 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. This assay typically develops (*i.e.*, $B_0$ wells ≥0.3 A.U. (blank subtracted)) in 60-90 minutes.

### Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Remove the plate cover being careful to keep *p*NPP solution has not splashed up on the plate cover. *NOTE: Any loss of p*NPP *will affect the absorbance readings. If p*NPP *is present on the cover, use a pipette to transfer the p*NPP *into the well.*
3. Read the plate at a wavelength between 405 and 420 nm. The absorbance may be checked periodically until the $B_0$ wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the $B_0$ wells are in the range of 0.3-1.0 A.U. (blank subtracted).



# ANALYSIS

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

## Calculations

### Preparation of the Data

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

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

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

*NOTE: The TA values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool; 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 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 36 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 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.

The original concentration of the sample and recovery factor can be determined by the following method:

V = ELISA determined concentration of the unspiked sample (pg/ml)

S = concentration of the spike (pg/ml)

Y = ELISA determined concentration of the spiked sample (pg/ml)

$$\text{Purification Recovery Factor} = \left[\frac{Y-V}{S}\right]$$

$$\text{8-Isoprostane (pg) in purified sample} = \left[\frac{V}{\text{Recovery Factor}}\right] \times 0.5 \text{ ml}^*$$

$$\text{8-Isoprostane in original sample} = \frac{\text{8-Isoprostane (pg) in purified sample}}{\text{Volume of sample used for purification (ml)}}$$

*Volume of reconstituted sample after purification; adjust this number accordingly if a different volume of Tris Buffer was used to reconstitute the sample after purification.

## Performance Characteristics

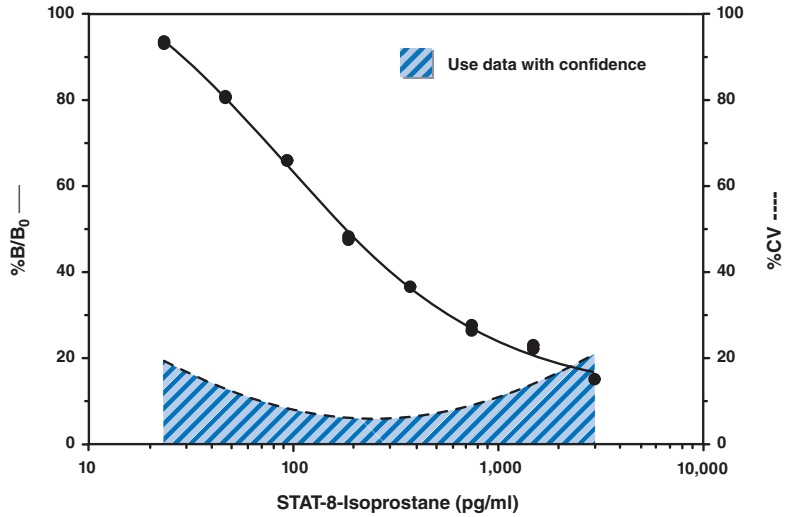
### Sample Data

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 value of your samples. Your results could differ substantially.

| | Raw Data | | Average | Corrected |
|---|---|---|---|---|
| **Total Activity** | 2.185 | 2.213 | 2.200 | |
| **NSB** | 0.000 | 0.000 | 0.000 | |
| **$B_0$** | 0.827 | 0.841 | | |
| | 0.849 | 0.859 | 0.844 | 0.844 |

| Dose (pg/ml) | Raw Data | | Corrected | | %B/$B_0$ | |
|---|---|---|---|---|---|---|
| 3,000 | 0.128 | 0.127 | 0.128 | 0.127 | 15.2 | 15.1 |
| 1,500 | 0.186 | 0.194 | 0.186 | 0.194 | 22.0 | 22.9 |
| 750 | 0.223 | 0.233 | 0.223 | 0.233 | 26.4 | 27.6 |
| 375 | 0.299 | 0.309 | 0.299 | 0.309 | 35.5 | 36.6 |
| 187.5 | 0.408 | 0.401 | 0.408 | 0.401 | 48.3 | 47.5 |
| 93.8 | 0.557 | 0.557 | 0.557 | 0.557 | 66.0 | 66.0 |
| 46.9 | 0.683 | 0.380 | 0.683 | 0.380 | 80.9 | 80.6 |
| 23.4 | 0.789 | 0.791 | 0.789 | 0.791 | 93.5 | 93.7 |

Table 2. Typical results




**Assay Range** = 23.4-3,000 pg/ml
**Sensitivity** (defined as 80% $B/B_0$) = 45 pg/ml
**Mid-point** (defined as 50% $B/B_0$) = 100-300 pg/ml

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

Figure 8. 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 |
|---|---|---|
| 3,000 | 24.1 | 15.4 |
| 1,500 | 10.1 | 10.3 |
| 750 | 7.6 | 10.2 |
| 375 | 6.3 | 8.2 |
| 187.5 | 7.4 | 7.3 |
| 93.8 | 9.1 | 10.0 |
| 46.9 | 14.6 | 10.5 |
| 23.4 | 17.3 | 14.7 |

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

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

†Outside of the recommended usable range of the assay.

## Cross Reactivity:

| Compound | Cross Reactivity | Compound | Cross Reactivity |
|---|---|---|---|
| 8-Isoprostane | 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 $F_{2\alpha}$ | 4.0% | 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% | $PGE_2$ | 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% |
| 2,3-dinor-6-keto Prostaglandin $F_{1\alpha}$ | 0.09% | | |

**Table 4. Cross Reactivity of the STAT-8-Isoprostane ELISA Antiserum**

## Appendix

### Purification Protocol (SPE/TLC method)

1. Activate a SPE Cartridge (C-18) (6 ml) (Item No. 400020) by rinsing with 5 ml methanol and then with 5 ml UltraPure water. Do not allow the 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).

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

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.
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 Tris Buffer and vortex. It is common for an insoluble precipitate to remain after the addition of Tris Buffer; this will not affect the assay. The sample is now ready for use in the immunoassay.

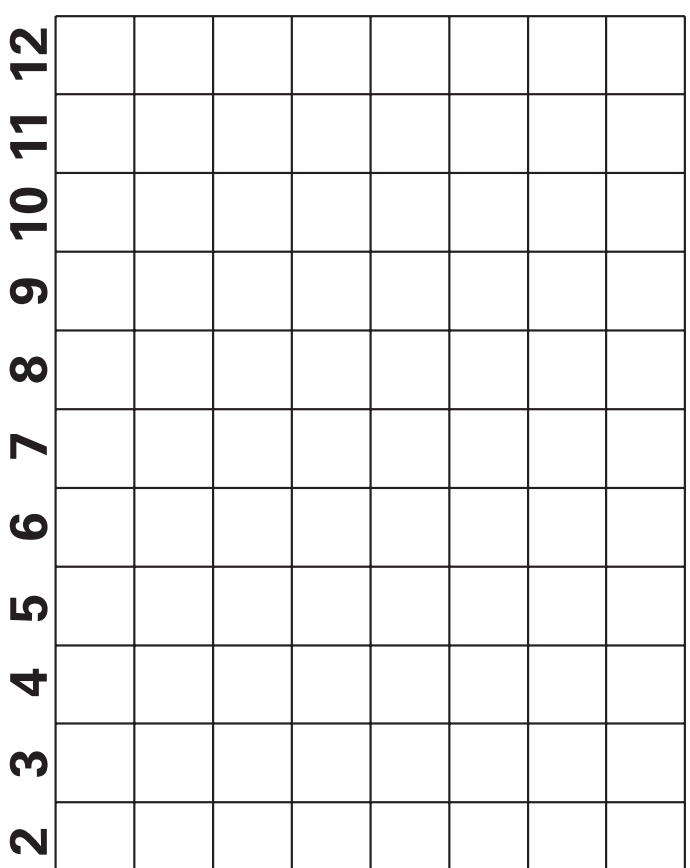
## Troubleshooting

| Problem | Possible Causes | Recommended Solutions |
|---|---|---|
| Erratic values; dispersion of duplicates | A. Trace organic contaminants in the water source<br>B. Poor pipetting/technique | A. Replace activated carbon filter or change source of UltraPure water |
| High NSB (>10% of $B_0$) | A. Poor washing<br>B. Exposure of NSB wells to specific antibody | A. Rewash plate and redevelop |
| Very low $B_0$ | A. Trace organic contaminants in the water source<br>B. Plate requires additional development time<br>C. Dilution error in preparing reagents | A. Replace activated carbon filter or change source of UltraPure water<br>B. Return plate to shaker and re-read later |
| Low sensitivity (shift in dose response curve) | Standard is degraded | Replace standard |
| Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference) | Interfering substances are present | Purify sample prior to analysis by ELISA[8] |
| 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. 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).
8. 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.