*Oxis*Research™

A Division of OXIS Health Products, Inc.

BIOXYTECH® 8-Isoprostane Assay Kit

Enzyme Immunoassay for 8-epi-Prostaglandin $F_{2\alpha}$

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number 21019. This product is patent protected.

INTRODUCTION

The Analyte

Isoprostanes are prostanglandin-like compounds that are produced by peroxidation of lipoproteins (1,2). 8-epi-prostaglandin-$F_{2\alpha}$ has been shown to be a potent vasoconstrictor in rat kidneys (3) and rabbit lungs (4). Isoprostanes may also play a role in atherosclerosis (5,6). Measurement of isoprostane concentration may be helpful in assessment of oxidative stress, hepatorenal syndrome, rheumatoid arthritis, atherosclerosis and carcinogenesis (7).

This kit can be used to determine free 8-epi-prostaglandin-$F_{2\alpha}$ in urine, serum or tissue samples following solid phase extraction of the 8-Isoprostane-containing fraction. Total 8-epi-prostaglandin-$F_{2\alpha}$ may also be determined following hydrolysis of phospholipids.

Principles of the Procedure

The BIOXYTECH® 8-Isoprostane Assay is a competitive enzyme-linked immunoassay (ELISA) for determining levels of 8-epi-prostaglandin-$F_{2\alpha}$ in biological samples. Briefly, 8-EPI in the samples or standards competes for binding (to the antibody coated on the plate) with 8-EPI conjugated to horseradish peroxidase (HRP). The peroxidase activity results in color development in the substrate when added. The intensity of the color is proportional to the amount of 8-EPI-HRP bound and inversely proportional to the amount of 8-EPI in the samples or standards.

REAGENTS

Materials Provided

Item	Quantity
• 96-well micortiter plate, pre-coated with 8-epi-prostaglandin-$F_{2\alpha}$ antibody	1
• 8-epi-prostaglandin-$F_{2\alpha}$ Standard	2 x 5 µL
• Wash Buffer (5X)	40 mL
• Dilution Buffer (5X)	100 mL
• Substrate	25 mL
• 8-epi-HRP-Conjugate	200 µL
• Disposable reagent troughs for a multichannel pipettor	2
• ELISA Template	1

Materials Required But Not Provided

- Precision pipettes with a range of 5 µL to 1,000 µL with disposable tips.
- Beakers, flasks, cylinders necessary for preparation of reagents.
- 96-well plate reader for measurement of absorbance at 450 or 650 nm.
- Reagents and chromatographic medium for pretreatment/extraction of biological samples.

Optional
1. 3M sulfuric acid.
2. A multichannel pipette and/or repeat pipettor is helpful, but not required.

Warnings and Precautions
- Do not smoke, eat or drink in areas where samples and reagents are handled.
- Wear disposable gloves when handling samples and reagents.
- Do not pipette reagents or samples by mouth.
- In case of accidental exposure of skin, mucous membranes or eyes to the components of this kit, thoroughly wash the exposed area with water.
- Reagents may contain sodium azide. Sodium azide may react with lead and copper plumbing to form explosive metal azides. On disposal of reagents, flush with large volumes of water to prevent azide accumulation.
- For *in vitro* use only. For research purposes only. Not for use in diagnostic procedures.

Reagent Storage and Handling
Store all components at 4°C until immediately before use. Do not freeze.

PROCEDURE

The following instructions are based on using the entire kit (all of the wells at one time). If portions of the kit are to be used at a later time, one may desire to prepare smaller quantities and save the remaining stock for later use.

Sample Preparation
Solid phase extraction is required to remove interferences from serum, plasma and urine* before determining free 8-epi-PGF2a. Since tissue culture media are generally supplemented with serum, solid phase extraction is also required for these samples.

*Urine can contain significant quantities of immunoreactive 8-epi- PGF2α metabolites. Unextracted urine samples can be assayed, but higher analyte concentrations than GC/MS will be obtained. Nevertheless, such unextracted urine determinations may be suitable for evaluation of oxidative stress. Following solid phase extraction using the protocol provided, 8-epi - PGF2a values obtained by ELISA and GC/MS are comparable.

Urine may be collected any time of day. Urine samples should be frozen at -20°C or below if extraction/assay will not be performed immediately.

Reagent Preparation
1. Substrate**:** Ready to use.
2. Add 40 mL Wash Buffer (5X) to 160 ml of deionized water, mix well.
3. Add 100 mL Dilution Buffer (5X) to 400 ml of deionized water, mix well
4. HRP Conjugate:
   a. Centrifuge vial before removing the cap.
   b. Add 90 µL HRP Conjugate to 11.91 mL Dilution Buffer (1X).
5. Dilute extracted samples or unknowns in Dilution Buffer (1X).

Standard Preparation

Prepare a series of standards by diluting the 10µg/ml standard provided to the following concentrations: 100, 50, 10, 5, 1, 0.1 and 0.05 ng/ml. Vortex to mix each standard level.

- S7: Add 495 µL of Dilution buffer (1X) to one supplied standard vial. = 100 ng/mL
- S6: Add 200 µL of S7 to 200 µL of Dilution Buffer (1X) = 50 ng/mL
- S5: Add 100 µL of S6 to 400 µL of Dilution Buffer (1X) = 10 ng/mL
- S4: Add 200 µL of S5 to 200 µL of Dilution Buffer (1X) = 5 ng/mL
- S3: Add 100 µL of S4 to 400 µL of Dilution Buffer (1X) = 1 ng/mL
- S2: Add 100 µL of S3 to 900 µL of Dilution Buffer (1X) = 0.1 ng/mL
- S1: Add 500 µL of S2 to 500 µL of Dilution Buffer (1X) = 0.05 ng/mL
- S0: Dilution Buffer (1x) only. = 0 ng/mL

Assay Procedure

1. Remove plate from foil pouch. Apply 100 µL of standards and diluted samples to wells (for suggested plate arrangement, see Microtiter plate template).
2. Add 100 µL of diluted HRP Conjugate to each well and allow plate to stand at room temperature for 2 hours.
3. Carefully invert plate to empty contents. Pat dry upside-down on a lint free towel.
4. Wash each well with 400 µL Wash Buffer (1X) (add Wash Buffer to each well, allow to stand 2 minutes, empty contents, and pat dry).
5. Repeat step 4 two more times.
6. Add 200 µL Substrate to each well, allow to stand 30 minutes.
7. Measure absorbance at 650 nm. (*Optional*: Add 50 µL of 3 M sulfuric acid to each well and read plate at 450 nm.)

SOLID PHASE EXTRACTION PROCEDURE

Notes:

1. Some isoprostanes will be retained on the extraction column, so a recovery correction for the column should be determined: Add a known quantity of isoprostane standard (e.g., 5 ng) to an aliquot of a single unknown, perform the extraction, assay both samples and compute the percent recovery.
2. This procedure requires 500 mg sorbent for C18 and silica. A 6 mL column is recommended.
3. To determine free isoprostanes, follow the Solid Phase Extraction Procedure below.
4. To determine total (free + esterified) isoprostanes, additional treatment of the sample is required prior to performing the solid phase extraction. See Pretreatment for Total Isoprostanes below.
5. This is a suggested protocol. Varying compositions of biological fluids may alter extraction efficiency.

Procedure

1. Add 3 mL of 1 mM HCl to 10 mL of the sample.
2. Adjust pH to 3.0 with 1 N HCl.
3. *C18 Sep Pak™*
   - Prewash with 5 mL of Ethanol.
   - Follow with 5 mL of 1 mM HCl.
   - Load sample.
   - Wash with 10 mL of 1 mM HCl.
   - Wash with 10 mL of Heptane.
   - Elute sample with 10 mL Ethyl Acetate:Heptane (1:1) into plastic tube.
   - Add Sodium Sulfate (200 mg) to eluant. Take care not to transfer Sodium Sulfate to the Silica Sep Pak of step 4.
4. *Silica Sep Pak™*
   - Prewash with 5 mL of Methanol.
   - Follow with 5 mL of Ethyl Acetate.
   - Load Ethyl Acetate:Heptane eluate from C18 Sep Pak™.
   - Wash with 5 mL Ethyl Acetate.
   - Elute sample with 5 mL of fresh Ethyl Acetate:Methanol (1:1) , into plastic tube.

5. Evaporate under N_2.
6. Dissolve the sample residue in 1 mL of Dilution Buffer (1X) and assay 0.1 mL as described under Assay Procedure.

PRETREATMENT FOR TOTAL ISOPROSTANES (FREE + ESTERIFIED)

Procedure for Plasma, Serum, Urine and other fluids

1. Prepare Folch solution (2:1 CHC_{13}:Methanol) with Butylated Hydroxytoluene (BHT) (5 mg/100 mL) and Triphenylphosphine (TPP) (50 mg/100 mL).
2. Add 20 mL Folch solution to a 50 mL conical tube and place on ice.
3. Add 1 mL plasma or other fluid.
4. Shake or vortex well for 1 minute.
5. Add10 mL ice cold 0.043% $MgCl_2$ and shake or vortex well for 1 minute.
6. Centrifuge for 2 – 3 minutes.
7. Aspirate off the top layer ($MgCl_2$/Methanol) and transfer the organic layer to another 50 mL tube, being careful not to transfer any protein layer that may be present.
8. Dry the organic layer under N_2.
9. Add 0.5 to 2 mL Methanol (depending on the amount of lipid present) containing BHT (5 mg/100 mL) and an equal volume of 15% KOH. Swirl after each addition.
10. Incubate sample at 37 C for 30 minutes.
11. Dilute with 1 mM HCl so that the volume of Methanol added is ≤ 5% of the total volume. Proceed with solid phase extraction described above.

Procedure for Tissue Samples

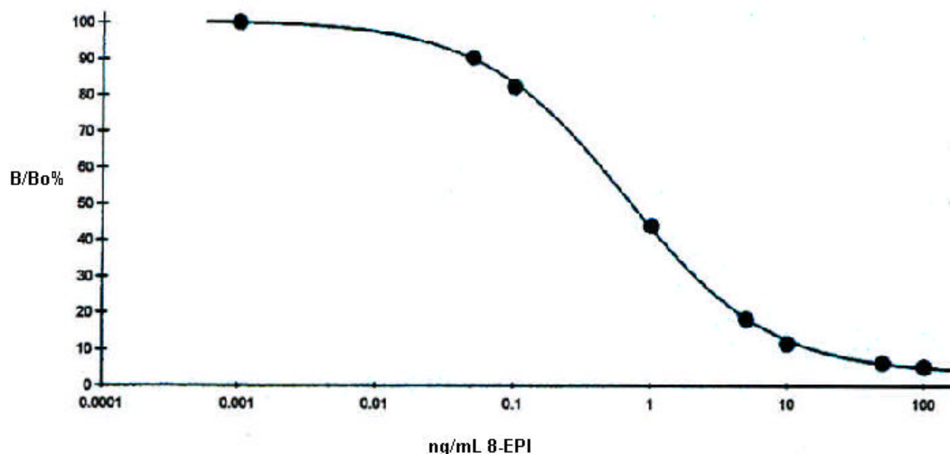
1. Prepare Folch solution (2:1 CHC_{13}:Methanol) with BHT (5 mg/100 mL).
2. Add 20 mL Folch solution to a 40 mL flat bottom tube and place on ice.
3. Weigh 0.5 – 1 gram of tissue and add to tube on ice.
4. Shake or vortex well for 1 minute.
5. Homogenize with blade homogenizer or sonicator for 30 seconds.
6. Incubate under N_2 in a sealed tube at room temperature for 1 hour, vortexing occasionally.
7. Add 4 mL 0.9% NaCl.
8. Vortex vigorously and centrifuge for 2 – 3 minutes.
9. Discard the upper layer (Methanol/Saline).
10. Remove the lower phase to a 100 mL rotary evaporator flask or 50 mL conical tube, avoiding the protein layer.
11. Evaporate and add 2 – 4 mL Methanol with 5 mg BHT/100 mL and an equal volume of 15% KOH.
12. Place in 37 ^{o}C water bath for 30 minutes.
13. Dilute to 40 – 80 mL with 1 mM HCl so that the volume of Methanol added is ≤ 5% of the total volume. Proceed with solid phase extraction described above.

CALCULATIONS

1. Average standard replicates (S_1 through S_7) and divide by the average of the S_0 (B_0) values (this will give the decimal equivalent of the %B_0 value).
2. Graph %B_0 (y-axis-linear) vs. standard concentration (x-axis-logarithmic) to obtain a standard curve.
3. Average the replicates of each unknown and divide by the average S_0 (B_0) values to obtain the %B_0, then determine the corresponding concentration using the standard curve.
4. Note that while the detection limit is approximately 0.05 ng/mL, data obtained in the steeper and more linear region of the curve is the most precise.

Typical Standard Curve

Figure 1: 8-epi-Prostaglandin-F_{2a} Standard Curve



Typical B/Bo: 20% 3.5 ng/mL; 50% 0.7 ng.mL; 80% 0.2 ng/mL

PERFORMANCE CHARACTERISTICS

Specificity*

8-epi-Prostaglandin $F_{2\alpha}$	**100.0%**
9_{α},11â-Prostaglandin $F_{2\alpha}$	4.1%
13,14-Dihydro-15-Keto-$PGF_{2\alpha}$	3.0%
9_{α},11â-Prostaglandin $F_{2\alpha}$	<0.01%
Prostaglandin $F_{2\alpha}$	<0.01%
6-Keto-Prostaglandin $F_{1\alpha}$	<0.01%
Prostaglandin E_2	<0.01%
Prostaglandin D_2	<0.01%
Arachidonic Acid	<0.01%

* Cross reactivity at 50% B/B_0

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