Urinary Isoprostane (Enzyme Immunoassay) ELISA Kit Instructions

Please read all instructions carefully before beginning this assay

PRODUCT #430110
For Research Use Only

This product is protected by US Patents 5,700,654; 5,858,696; 5,891,622

Storage Conditions:
All kit components: 4°C until immediately before use.
Do not freeze
Only use the 96-well Precoated plate supplied with the kit.

INTRODUCTION

Isoprostanes are prostaglandin-like compounds that are produced by free radical mediated peroxidation of lipoproteins. This kit is for the quantification of 15-isoprostane F_{2t} (also known as 8-epi-PGF2α or 8-iso-PGF2α) in urine samples. Levels of 15-isoprostane F_{2t} in urine are useful for the non-invasive assessment of oxidant stress *in vivo*. 15-isoprostane F_{2t} has also been shown to be a potent vasoconstrictor in rat kidneys and rabbit lungs, and plays a causative role in atherogenesis. Elevated isoprostane levels are associated with hepatorenal syndrome, rheumatoid arthritis, atherosclerosis, and carcinogenesis.

PRINCIPLES OF PROCEDURE

This kit is a competitive enzyme-linked immunosorbent assay (ELISA) for determining levels of 15-isoprostane F_{2t} (the best characterized isoprostane) in urine samples. Briefly, urine samples are mixed with an enhancing reagent that essentially eliminates interferences due to non-specific binding. The 15-isoprostane F_{2t} in the samples or standards competes with 15-isoprostane F_{2t} conjugated to horseradish peroxidase (HRP) for binding to a polyclonal antibody specific for 15-isoprostane F_{2t} coated on the microplate. The HRP activity results in color development when substrate is added, with the intensity of the color proportional to the amount of 15-isoprostane F_{2t}- HRP bound and inversely proportional to the amount of unconjugated 15-isoprostane F_{2t} in the samples or standards.

MATERIALS PROVIDED

1. ***96-WELL MICROTITER PLATE:*** 1 plate precoated with Anti-15-Isoprostane F_{2t}
2. ***15-ISOPROSTANE F_{2t} STANDARD (1µg/mL) :*** 2 x 60 µL.
3. ***ENHANCED DILUTION BUFFER:*** 100 mL. General buffer for diluting assay components.
4. ***WASH BUFFER (5X):*** 40 mL. Solution for washing plates.
5. ***K-BLUE SUBSTRATE:*** 25 mL. TMB Substrate.
6. ***15-ISOPROSTANE F_{2t} HRP CONJUGATE:*** 250 µL.
7. ***GLUCURONIDASE:*** 2 x 100 µL. β-Glucuronidase for sample pretreatment.

MATERIALS NEEDED BUT NOT PROVIDED

1. Precision pipetttes with a range of 10 µL tp 1,000 µL with disposable tips. A multichannel pipette is helpful, but not required.
2. Beakers, flasks, and cylinders necessary for preparation of reagents.
3. 96-well microplate reader for measurement of absorbance at 450 nm.
4. Dionized water.
5. 3 M sulfuric acid.

OPTIONAL MATERIALS:

1. 96-well plate washing/aspiration device

PROCEDURAL NOTES

1. Reagents can be used immediately upon removal from refrigeration.
2. Performance of the entire kit at once is not required. When performing this kit in part, please adhere to the following:
   - All unused components should be returned to storage at 4°C.
   - Unused portions of the microplate should be returned to the zip lock pouch with desiccant prior to storage at 4°C.
   - The Isoprostane HRP Conjugate is most stable at the stock concentration as provided; use only the appropri ate amount of this stock and store remaining for subsequent uses.
   - Create a standard curve for each performance of the assay. Two vials of Standard are provided for added ease and convenience of use.
   - Each vial of β-Glucuronidase is sufficient for treating 20 samples.
3. To minimize error due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel.

NOTES: This assay is non-species specific. This product is intended for use with urine samples and has not been validated for use with serum, tissue culture supernatants or tissue extracts. We recommend that such samples be subjected to solid phase extraction and analyzed using Neogen's Isoprostane Test Kit (Product #430010).

SAMPLE COLLECTION

- This kit is designed for extraction-free analysis of urine or like media.
- Sample collection and preparation is subject to the discretion and approval of the principal investigator.
- Spot or 24-hour urine should be collected then aliquoted and stored immediately at –80°C. Additives such as 0.02% thimerosal and 0.005% BHT may be used as preservatives where applicable but is typically not required.

REAGENT PREPARATION

1. **5X Wash Buffer:** Dilute to 1X with deionized water and mix prior to use.
2. **15-isoprostane F_{2t} HRP Conjugate:** Dilute 1:50 with Enhanced Dilution Buffer. For performance of the entire as say at once, add 240 µL of conjugate to 11.76 mL of Enhanced Dilution Buffer.

SAMPLE PREPARATION

Research has shown that an average of 50% of the isoprostane excreted in human urine is conjugated to glucuronic acid. The extent of glucuronidation among individuals ranges significantly from 28% to 80%. In light of this information it is strongly recommended that specimens be pretreated with β-glucuronidase prior to analysis to provide a more accurate assessment of oxidative stress. This kit provides sufficient materials and methods for the treatment of 40 samples allowing the user to differentiate the inter-individual differences in glucuronidation and measure the total systemic isoprostane output. If interested only in free isoprostane, do not treat samples with glucuronidase.

β-Glucuronidase Treatment

1. For every 100 µL of urine to be assayed, add 5 µL of Glucuronidase. Seal container and mix solution by inversion.
2. Incubate the mixture at 37°C for 2 hours.
3. Your sample is now ready for dilution and assay. Alternatively, the samples may be frozen at -70°C and assayed at a later date.

Samples should be diluted with Enhanced Dilution Buffer prior to assay. Recommended starting dilutions are 1:4, regardless of pretreatment.

PREPARATION OF STANDARDS

The 15-Isoprostane F_{2t} Standard is provided as a 1 µg/mL stock solution. Use the following table to construct an eight-point standard curve.

TABLE 1: STANDARD CURVE PREPARATION

Standard	15-Isoprostane F_{2t} Conc. (ng/mL)	Enhanced Dilution Buffer (µL)	Transfer Volume (µL)	Transfer Source	Final Volume (µL)
S_7	100	450	50	Standard Stock	300
S_6	50	200	200	S_7	300
S_5	10	400	100	S_6	300
S_4	5	200	200	S_5	300
S_3	1	400	100	S_4	400
S_2	0.1	900	100	S_3	500
S_1	0.05	500	500	S_2	1,000
B_0	0	300	---	---	300

ASSAY PROCEDURE

1. Add 100 µL of standards or diluted unknowns to each well. Recommended sample dilutions are 1:4 with Enhanced Dilution Buffer. See Figure 1 for a suggested plate layout.
2. Add 100 µL of diluted 15-isoprostane F_{2t} HRP conjugate to each well omitting the Reagent Blank (RB). Add 100 µL Enhanced Dilution Buffer in lieu of conjugate. Allow the plate to incubate for 2 hours at RT.
3. Wash wells according to the following wash procedure:
   a. Remove the contents of each well by inversion of the plate.
   b. Tap out the remaining contents of the plate onto a lint free paper towel.
   c. Add 300 µL of 1X Wash Buffer.
   d. Let stand for 2-3 minutes.
   e. Repeat steps a - d two additional times.
   f. Remove contents of each well by inversion of plate into an appropriate disposal device.
   g. Tap out the remaining contents of the plate onto a lint free paper towel.
4. Add 200 µL of K-Blue Substrate to each well.

5. Incubate for 20-40 minutes until an appreciable blue hue is observed for the color B_0.
6. Add 50 µL of 3 M sulfuric acid to each well to stop the reaction. The color will change from blue to yellow.
7. Read plate at 450 nm.

NOTE: Plate can be alternatively read at 650 nm in the absence of the addition of 3 M sulfuric acid in step 6 above.

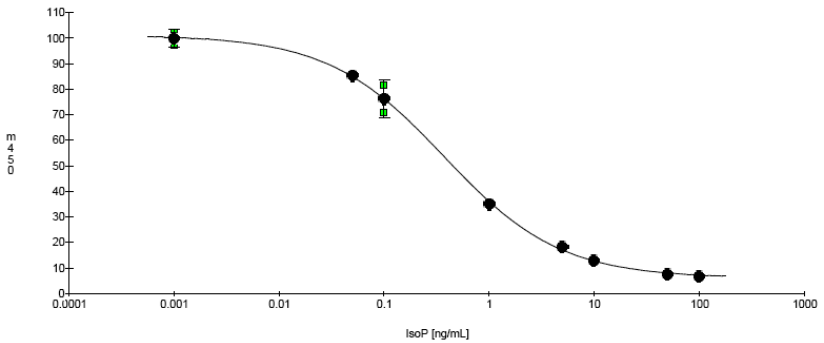
FIGURE 1: SAMPLE PLATE TEMPLATE

	1	2	3	4	5	6	7	8	9	10	11	12
A	S7	S7	U1	U1	U9	U9	U17	U17	U25	U25	U33	U33
B	S6	S6	U2	U2	U10	U10	U18	U18	U26	U26	U34	U34
C	S5	S5	U3	U3	U11	U11	U19	U19	U27	U27	U35	U35
D	S4	S4	U4	U4	U12	U12	U20	U20	U28	U28	U36	U36
E	S3	S3	U5	U5	U13	U13	U21	U21	U29	U29	U37	U37
F	S2	S2	U6	U6	U14	U14	U22	U22	U30	U30	U38	U38
G	S1	S1	U7	U7	U15	U15	U23	U23	U31	U31	U39	U39
H	B0	B0	U8	U8	U16	U16	U24	U24	U32	U32	RB	RB

CALCULATIONS

1. Average the REAGENT BLANK (RB) absorbance values and subtract this average from the value obtained for all other wells. Most modern microplate readers are capable of doing this automatically.
2. Average replicates of each standard S_1 through S_7. Divide each average by the mean B_0value and multiply the result by 100 to obtain $\%B_0$ values.
3. Graph $\%B_0$ values (y-axis-linear) vs. standard concentration (x-axis-logarithmic) to obtain a standard curve. Figure 2 is a Typical Standard Curve which plots concentration vs. absorbance.
4. Average the replicates of each unknown and divide by the average B_0 value to obtain $\%B_0$, then determine corresponding concentration using the standard curve and account for dilution factors.

FIGURE 2: TYPICAL STANDARD CURVE



Typical B/B_0: 20% - 3.5 ng/mL; 50% - 0.45 ng/mL; 80% - 0.08 ng/mL

PERFORMANCE CHARACTERISTICS

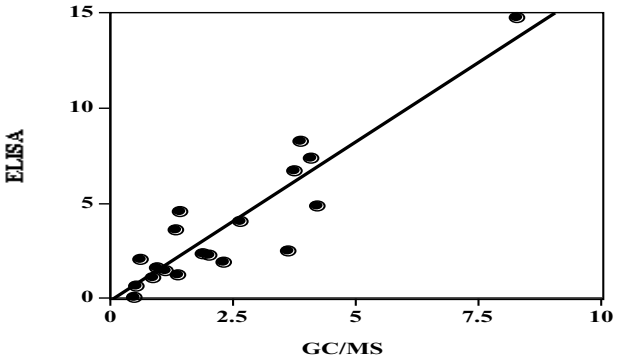
Cross reactivity at 50% B/Bo

15-isoprostane F_{2t}	100.0%
9α,11β-PROSTAGLANDIN $F_{2\alpha}$	4.1%
13,14-DIHYDRO-15-KETO-$PGF_{2\alpha}$	3.0%
9β,11α-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%

VALIDATION

The concentrations of 15-isoprostane F_{2t} in several human urine samples were determined by immunoassay and by GC/MS following solid phase extraction of separate aliquots, and a correlation (r^2) of > 0.8 was obtained. See Figure 3.

FIGURE 3: ELISA CORRELATION WITH GC/MS



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