

Enzyme Immunoassay for Urinary Isoprostane

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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-PGF_{2α} or 8-iso-PGF_{2α}) 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 immunoassay (ELISA) for determining levels of 15-isoprostane F_{2t} (the best characterized isoprostane) in urine samples. Briefly, urine samples are mixed with an enhanced dilution buffer that essentially eliminates interference 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

Component	Contents	Quantity	Storage	Cat. No.
Coated Plate	Anti-15-Isoprostane F _{2t} coated 96-well plate	1	4°C	EA85a
Standard	15-Isoprostane F _{2t} standard (1 µg/mL)	2 x 60 µL	4°C	EA85b
Enhanced Dilution Buffer	General buffer for diluting assay components	100 mL	4°C	EA85c
Wash Buffer	5x solution for washing plate	40 mL	4°C	EA85d
Substrate	TMB Substrate	25 mL	4°C	EA85e
HRP Conjugate	15-Isoprostane F _{2t} HRP conjugate	250 µL	4°C	EA85f
Glucuronidase	β-Glucuronidase for sample pretreatment	2 x 100 µL	4°C	GL85a

MATERIALS NEEDED BUT NOT PROVIDED

- Adjustable pipettes (10-1,000 µL) and disposable tips
- Microcentrifuge tubes
- Beakers, flasks, and cylinders as necessary for preparation of reagents
- Microplate reader with 450 nm filter
- Deionized water
- 3 M sulfuric acid

It is recommended that urine samples be normalized to creatinine or a comparable biomarker to allow reasonable sample-to-sample comparison of urinary 15-isoprostane F_{2t} values. Please inquire about our Creatinine Assay Kit (product number CR01) at 800-692-4633.

STORAGE CONDITIONS

1. Store this kit and its components at 4°C until use.
2. Do not freeze.

WARNINGS AND PRECAUTIONS

1. Use aseptic technique when opening and dispensing reagents.
2. This kit is designed to work properly as provided and instructed. Additions, deletions, or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.
3. Exercise universal precautions during the performance or handling of this kit or any component contained therein.

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 appropriate 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 and should be reconstituted as needed.
3. To minimize error due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel.

SAMPLE COLLECTION

This kit is designed for extraction-free analysis of urine or like media. Samples such as plasma, serum, tissues, and cultures should employ the use of Oxford Biomedical Research product number EA84. Please contact 800-692-4633 for further details.

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 assay at once, add 240 μ L of conjugate to 11.76 mL of Enhanced Dilution Buffer.

SAMPLE PREPARATION

Oxford Biomedical Research has found 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 μ L of urine to be assayed, add 5 μ L of Glucuronidase. Seal tube and vortex to mix.
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. A recommended starting dilution is 1:4, regardless of pretreatment.

STANDARD PREPARATION

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

Table 1: Preparation of Standard Curve

Standard	15-Isoprostane F _{2t} Concentration (ng/mL)	Enhanced Dilution Buffer (μ L)	Transfer Volume (μ L)	Transfer Source	Final Volume (μ L)
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 μ L of Standards or diluted unknowns to each well. A recommended sample dilution is 1:4 with Enhanced Dilution Buffer. See Scheme I for a suggested plate layout.
2. Add 100 μ L of diluted 15-isoprostane F_{2t} HRP Conjugate to each well omitting the Reagent Blank (RB). Add 100 μ L of Enhanced Dilution Buffer in lieu of Conjugate in the Reagent Blank. Incubate the plate for 2 hours at room temperature.
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 μ L of 1x Wash Buffer.
 - d. Let stand for 2-3 minutes.
 - e. Repeat procedure two more times, then proceed to step "f".
 - f. Remove the 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, then proceed to step 4.

4. Add 200 μ L of TMB Substrate to each well.
5. Incubate for 20 - 40 minutes until an appreciable blue hue is observed for the B₀.
6. Add 50 μ L of 3 M Sulfuric Acid to each well to stop the reaction. The color will change from blue to yellow.
7. Read the plate at 450 nm.

NOTE: The plate can be alternatively read at 650 nm in the absence of the addition of 3 M Sulfuric Acid in step 6 above.

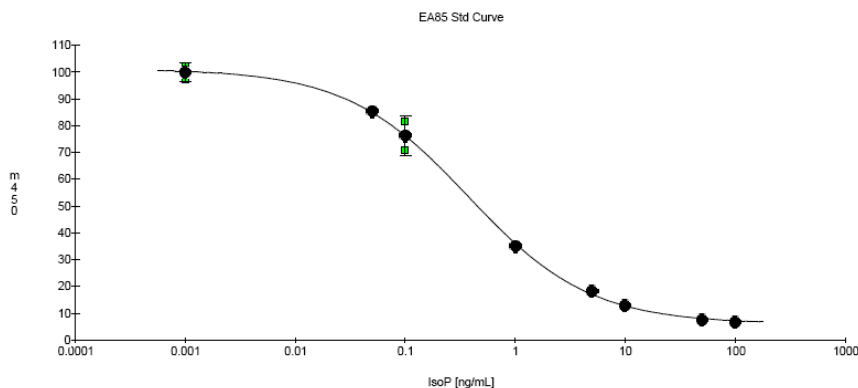
Scheme I:

	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	B ₀	B ₀	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 S₁ through S₇. Divide each average by the mean B₀ value and multiply the result by 100 to obtain %B₀ values.
3. Graph %B₀ values (y-axis-linear) vs. standard concentration (x-axis-logarithmic) to obtain a standard curve. Figure 1 is a Typical Standard Curve, which plots concentration vs. absorbance.
4. Average the replicates of each unknown and divide by the average B₀ value to obtain %B₀, then determine corresponding concentration using the standard curve and account for dilution factors.

Figure 1: Typical Standard Curve



Typical B/B₀: 20% - 3.5 ng/mL; 50% - 0.45 ng/mL; 80% - 0.08 ng/mL

PERFORMANCE CHARACTERISTICS

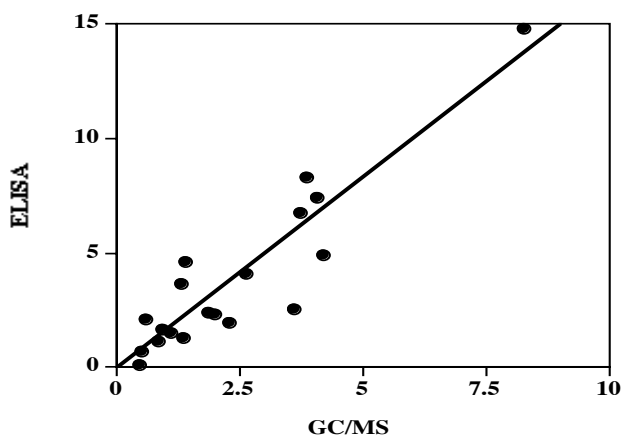
Cross reactivity at 50% B/B₀

15-isoprostane F_{2t}	100.0%
9 α ,11 β -PROSTAGLANDIN F _{2α}	4.1%
13,14-DIHYDRO-15-KETO-PGF _{2α}	3.0%
9 β ,11 α -PROSTAGLANDIN F _{2α}	<0.01%
PROSTAGLANDIN F _{2α}	<0.01%
6-KETO-PROSTAGLANDIN F _{1α}	<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 (r²) of > 0.8 was obtained (Figure 2).

Figure 2: ELISA Correlation with GC/MS



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