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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-$PGF_{2\alpha}$ or 8-iso-$PGF_{2\alpha}$) 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 (lyophilized)	2 vials	4°C	GL85a
Acetate Buffer	Buffer used to reconstitute the β-Glucuronidase	500 μL	4°C	GL85b

MATERIALS NEEDED BUT NOT PROVIDED

1. Adjustable pipettes (10-1,000 µL) and disposable tips
2. Beakers, flasks, and cylinders as necessary for preparation of reagents
3. Microplate reader with 450 nm filter
4. Deionized Water
5. 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 1:30 with Enhanced Dilution Buffer. For performance of the entire assay at once, add 200 μL of conjugate to 5.8 mL of Enhanced Dilution Buffer.
3. **β-Glucuronidase:** Reconstitute each vial with 120 μL of Acetate Buffer, taking care to avoid bubbles. Prepare immediately prior to use and place on ice. Each vial is sufficient for 20 samples.

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.

β-Glucuronidase Treatment

1. For every 50 µL of stock 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.

Samples should be diluted with Enhanced Dilution Buffer prior to assay. Recommended starting dilutions are 1:4 or 1:8, regardless of pretreatment.

STANDARD PREPARATION

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

Table 1: Standard Stock Preparation

Standard	IsoP. Conc. (ng/mL)	Vol. of EIA Buffer (µL)	Transfer Vol. (µL)	Final Vol. (µL)
A	1000	-	Provided	60
B	20	450	50 µL of A	495
C	2	495	5 µL of B	495
D	0.2	495	5 µL of C	500

Table 2: Standard Curve Preparation

Standard	IsoP. Conc. (ng/mL)	Vol. of EIA Buffer (µL)	Vol. of Stock B (µL)	Vol. of Stock C (µL)	Vol. of Stock D (µL)
S_7	75	50	150	-	-
S_6	10	180	20	-	-
S_5	2.5	195	5	-	-
S_4	0.5	100	-	100	-
S_3	0.075	185	-	15	-
S_2	0.01	-	-	-	200
S_1	0.0025	150	-	-	50
B_0	0	200	-	-	-

ASSAY PROCEDURE

1. Add 50 µL of Standards or diluted unknowns to each well. Recommended sample dilutions are 1:4 or 1:8 with Enhanced Dilution Buffer. See Scheme I for a suggested plate layout.
2. Add 50 µL of diluted 15-isoprostane F_{2t} HRP Conjugate to each well omitting the Reagent Blank (RB); add 50 µL of Enhanced Dilution Buffer in lieu of Conjugate. 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 µL 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 150 µL of TMB Substrate to each well.
5. Incubate for 30 minutes until an appreciable blue hue is observed for the B_0.
6. Add 50 µL of 3 M Sulfuric Acid to each well to stop the reaction. The color with 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.

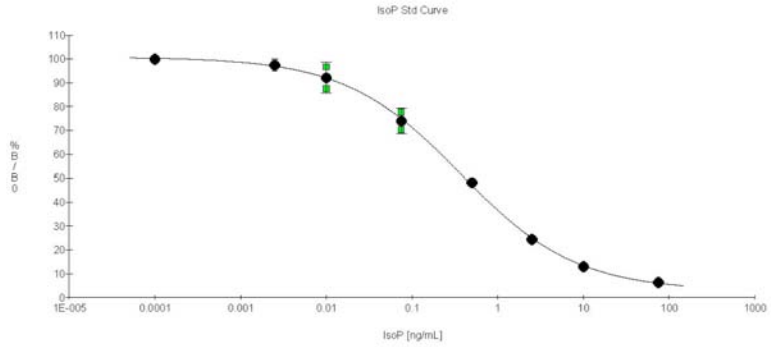
Scheme I:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S_7	S_7	U_1	U_1	U_9	U_9	U_{17}	U_{17}	U_{25}	U_{25}	U_{33}	U_{33}
B	S_6	S_6	U_2	U_2	U_{10}	U_{10}	U_{18}	U_{18}	U_{26}	U_{26}	U_{34}	U_{34}
C	S_5	S_5	U_3	U_3	U_{11}	U_{11}	U_{19}	U_{19}	U_{27}	U_{27}	U_{35}	U_{35}
D	S_4	S_4	U_4	U_4	U_{12}	U_{12}	U_{20}	U_{20}	U_{28}	U_{28}	U_{36}	U_{36}
E	S_3	S_3	U_5	U_5	U_{13}	U_{13}	U_{21}	U_{21}	U_{29}	U_{29}	U_{37}	U_{37}
F	S_2	S_2	U_6	U_6	U_{14}	U_{14}	U_{22}	U_{22}	U_{30}	U_{30}	U_{38}	U_{38}
G	S_1	S_1	U_7	U_7	U_{15}	U_{15}	U_{23}	U_{23}	U_{31}	U_{31}	U_{39}	U_{39}
H	B_0	B_0	U_8	U_8	U_{16}	U_{16}	U_{24}	U_{24}	U_{32}	U_{32}	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_0 value 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 1 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 1: Typical Standard Curve



Typical B/B_0: 20% - 4.2 ng/mL; 50% - 0.42 ng/mL; 80% - 0.05 ng/mL

PERFORMANCE CHARACTERISTICS

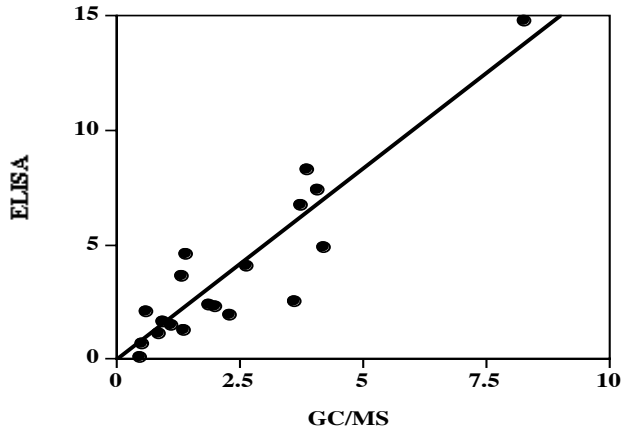
Cross reactivity at 50% B/B_0

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 (Figure 2).

Figure 2: ELISA Correlation with GC/MS



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