Package Insert

15-Isoprostane F_{2t} ELISA

Catalog Number: 15I39-K01
96 Wells
For Research Use Only
v. 1.0

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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 a representative isoprostane, 15-

isoprostane F_{2t} (also known as 8-epi-$PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$ or 8-isoprostane). Levels of 15-isoprostane F_{2t} (15-IsoP F_{2t}) in biological fluids have been shown to be useful for assessment of oxidant stress *in vivo*. 15-IsoP 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.

The 15-Isoprostane F_{2t} ELISA kit can be used for the quantification of free 15-IsoP F_{2t} in urine, plasma, serum, and tissue samples following Solid Phase Extraction (SPE) of the isoprostane-containing fraction. Instructions are also provided for the quantification of total 15-IsoP F_{2t} following hydrolysis of phospholipids.

NOTE: Alternatively, urine samples may be analyzed without SPE using a separate available kit, ISO34-K01 – Urinary Isoprostane ELISA, also available from Eagle Biosciences, Inc.

PRINCIPLES OF PROCEDURE

15-Isoprostane F_{2t} ELISA kit is a competitive enzyme-linked immunoassay (ELISA) for determining levels of 15-IsoP F_{2t} in biological samples. Briefly, 15-IsoP F_{2t} in the samples or standards competes with 15-IsoP F_{2t} conjugated to horseradish peroxidase (HRP) for binding to a polyclonal antibody specific for 15-IsoP F_{2t} coated on the microplate. The HRP activity results in color development when the substrate is added, with the intensity of the color proportional to the amount of 15-IsoP F_{2t} bound and inversely proportional to the amount of unconjugated 15-IsoP F_{2t} in the samples or standards.

MATERIALS PROVIDED

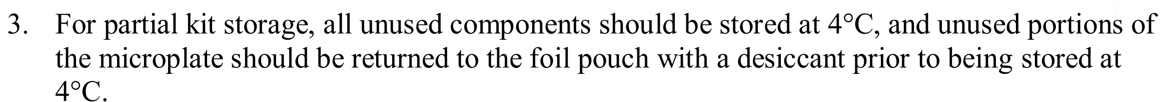
Component	Description	Volume	Storage	Cat. No.
Coated Plate	96-well microplate coated with15-Isoprostane F_{2t}.	1 Plate	4°C	EA84a
15-IsoP F_{2t} Standard	1 µg/mL 15-Isoprostane F_{2t} standard solution.	2 x 60 µL	4°C	EA84b
Wash Buffer	5x concentrated buffer used to wash the plate.	40 mL	4°C	EA84c
Dilution Buffer (5x)	Buffer used to dilute the standards and samples.	100 mL	4°C	EA84d
TMB Substrate	TMB Substrate used for color development.	25 mL	4°C	EA84e
15-IsoP F_{2t} Conjugate	15-Isoprostane F_{2t} HRP conjugate	100 µL	4°C	EA84f

MATERIALS NEEDED BUT NOT PROVIDED

1. Adjustable micropipettes (5 – 1000 µL) with tips
2. Multichannel pipette (50 – 200 µL) with tips
3. 96-well plate reader with a 450 nm filter
4. Deionized Water
5. 3 N Sulfuric Acid (H_2SO_4)
6. Materials and Reagents for Solid Phase Extraction (SPE). See Sample Preparation.

STORAGE

1. Store the components of this kit at the temperatures specified on the labels.
2. Unopened reagents are stable until the indicated kit expiration date.



3. For partial kit storage, all unused components should be stored at 4°C, and unused portions of the microplate should be returned to the foil pouch with a desiccant prior to being stored at 4°C.

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.

PROCEDURAL NOTES

1. The 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:
   -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.
3. Use aseptic technique when opening and dispensing reagents.
4. To minimize error due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel.

SAMPLE COLLECTION AND STORAGE

Biologically derived isoprostane samples should be stored at –80°C immediately upon collection. The use of preservatives such as BHT will aid in preventing the oxidation of isoprostanes whereas the use of enzyme blocking additives (indomethacin) will minimize the ex-vivo generation of isoprostanes. Prompt collection, processing and storage techniques should be used to ensure that isoprostane levels are more accurately reflective of those at the time of collection. There are no known incompatibilities with collection media such as EDTA or heparin when collecting blood.

SAMPLE PREPARATION

Biological samples often contain high concentrations of proteins and other substances that interfere with immunoassays. For this reason, purification by way of Solid Phase Extraction (SPE) is required prior to use in this assay. The following is an SPE protocol that accommodates plasma, serum, and tissue homogenates. Alternatively, urine samples can be analyzed using Eagle Biosciences’s extraction-free Urinary Isoprostane Immunoassay Kit, product number ISO34-K01.

ABOUT SPE: The SPE process can vary considerably with different needs, objectives and available equipment. Equipment such as columns, reagents, vacuum, extraction manifold and alternatives for each contribute considerably to this variation. The SPE protocol as written below is intended for use with the SPE Reagents and Materials list located in this section. This protocol may be amended to accommodate the specific needs of the testing lab, but should be done so at their own risk and discretion.

This procedure is intended for use with 1 mL of plasma, serum or tissue homogenate sample. This sample volume represents the minimum recommended volume for preparation in this assay. If attempting to prepare more or less than the 1 mL of sample mentioned, the proportion of materials and reagents should be adjusted accordingly. Things to consider when establishing the sample volume to be extracted and the final reconstitution volume are the anticipated concentration of isoprostane, the number of replicates to be performed in the assay, the

anticipated IC_{50} value and the stringency of the experiment. Customarily, the stringency for this assay is at the 20% and 80% B/B_0. For higher stringency, adjust the $\%B/B_0$ acceptance thresholds to favor the 50% B/B_0 value.

NOTE: It is necessary to determine and adjust for incomplete recovery of isoprostane from the extraction columns. This can be achieved by the addition of a known quantity of 15-IsoP F_{2t} standard (e.g. 5 ng) to an aliquot of one unknown prior to extraction, then analyzing both the spiked and unspiked samples. Upon analysis, calculate the difference to determine the percent recovery.

SPE MATERIALS AND REAGENTS

1. Magnesium Chloride ($MgCl_2$) (Sigma; M9272)
2. Butylated Hydroxytoluene (BHT) (Sigma; B1378)
3. Methanol (Alfa Aesar; 32435)
4. Ethanol (EMD; 4450)
5. Chloroform ($CHCl_3$) (Sigma; C2432)
6. Triphenylphosphine (TPP) (Sigma; T84409)
7. Potassium Hydroxide (KOH) (Sigma; P1767)
8. Hydrochloric Acid (HCl) (Fisher; A481-212)
9. Ethyl Acetate (Aldrich; 27,098-9)
10. Heptane (Sigma; H9629)
11. Evaporation Apparatus Suitable for 50 mL conical tubes and 20 mL reagent volumes
12. Water Bath suitable for 37°C
13. Analytical grade Nitrogen gas for evaporation of samples (see your local gas supply company)
14. Silica Sep Pak (Waters; WAT043400)
15. C_{18} Sep Pak (Waters; WAT043395)
16. 20 Position Extraction Manifold (Waters; WAT200609)
17. Vacuum source (Waters 110V, 60 Hz Vacuum pump; WAT085114 or equivalent)

SPE REAGENT PREPARATION

1. 0.043% $MgCl_2$ (store on ice prior to use)
2. MeOH + 0.05% BHT (w/v)
3. Folch Solution; Chlorform:Methanol (2:1) + 0.05% BHT (w/v) + 0.05% TPP (w/v) (store on ice prior to use)
4. Ethyl Acetate:Heptane (1:1)
5. Ethyl Acetate:Methanol (1:1)
6. pH 3 Deionized Water (pH with HCl and NaOH)
7. 15% KOH (w/v)

FREEING ESTERIFIED ISOPROSTANES

Only free isoprostanes are detected with this assay. Isoprostanes may be found adjoined to other molecules by an ester bond. For these esterified isoprostanes to be reflected in the assayed values they must have the ester bond removed. The following procedure will hydrolyze the ester bond and allow for the analysis of total isoprostane. Alternatively, if only the free isoprostane is of interest at the time of collection, skip this step and go directly to the Solid Phase Extraction Procedure later in this section.

1. Add 20 mL of ice-cold Folch Solution to a 50 mL conical tube followed by 1 mL of sample or tissue homogenate and vortex on high for 1 minute. Please note that the Folch Solution has a very low surface tension and may leak out of a poorly sealed tube during mixing.

2. Add 10 mL of ice-cold 0.043% $MgCl_2$ directly to the 50 mL conical tube and vortex on high for 1 minute.
3. Centrifuge for 3 minutes at 2500 x g to separate the phases of this mixture.
4. There will now be three phases. Remove the upper layer by aspiration and discard. Poke through the remaining middle layer with a pipette and carefully transfer the lower organic layer to a separate 50 mL conical test tube.
5. Evaporate the lower organic layer in the 50 mL conical tube under a stream of N_2. The dried sample will appear as an oily residue at the bottom of the vial.
6. Once dried, add 1 mL of Methanol + 0.05% BHT solution directly to the sample and swirl by hand for 30 seconds to ensure the sample is adequately dissolved.
7. Add 2 mL of 15% KOH and swirl mixture for 30 seconds.
8. Incubate this mixture at 37°C for 30 minutes.
9. After incubation, add 17 mL of pH 3 Water directly to the 50 mL conical tube. Your sample is now ready for SPE.

SOLID PHASE EXTRACTION

The following procedure is performed under a constant vacuum. A negative pressure of ~5 psi is an appropriate benchmark but may require deviation for optimal flow rates and results.

1. The sample should be at a pH of 3 prior to SPE. Check and adjust the pH with 1 N HCl and 1 N NaOH accordingly.
2. Setup the extraction manifold and vacuum apparatus according to the manufacturer's instructions and affix the C_{18} Sep Pak column(s) to the manifold with an appropriate waste container below each column.
3. Pre-wash the C_{18} Sep Pak column with 5 mL of Ethanol followed by 5 mL of pH 3 Deionized Water.
   NOTE: When running solutions through the columns during washes, stop the solution when the solution meets the bed volume – do not allow the bed volume to run dry except when specified.
4. Load the sample onto the column and allow it to flow through the column at a flow rate of 1 mL per minute.
5. Wash the column with 10 mL of pH 3 Deionized Water followed by 10 mL of Heptane.
6. Remove waste material collection device and insert sample collection tubes appropriate for the volume and solvent being used.
7. Elute the sample from the column with 10 mL Ethyl Acetate:Heptane until the column is dry.
8. Remove the eluted sample and set aside for the next phase in extraction. Affix the Silica Sep Pak column(s) to the manifold with an appropriate waste container below each column.
9. Pre-wash the Silica Sep Pak column with 5 mL of Methanol followed by 5 mL of Ethyl Acetate.
10. Load the sample collected from the C_{18} Sep Pak to the column and allow it to flow through the column at a flow rate of 1 mL per minute.
11. Wash the column with 5 mL of Methanol followed by 5 mL of Ethyl Acetate.
12. Remove waste material collection device and insert sample collection tubes appropriate for the volume and solvent being used.
13. Elute the sample from the column with 5 mL Ethyl Acetate:Methanol until the column is dry
14. Evaporate the eluted sample under a stream of N_2. The dried sample will appear as an oily residue at the bottom of the vial.
    NOTE: Storage of isoprostane samples is ideal in the concentrated lipid form resulting from the drying procedure. Store at –80°C under inert gas until the time of assay.
15. Reconstitute the sample in a known amount of Dilution Buffer and proceed to the Assay Procedure.

REAGENT PREPARATION

1. **Wash Buffer:** Dilute from 5x to 1x prior to use with deionized water. (40 mL Wash Buffer + 160 mL dH_2O)
2. **5x Dilution Buffer:** Dilute from 5x to 1x prior to use with deionized water. (100 mL Dilution Buffer + 400 mL dH_2O)
3. **15-IsoP F_{2t} Conjugate:** When performing the entire assay at once, combine 90 µL of Conjugate with 11.910 mL of 1x Dilution Buffer.

STANDARD CURVE PREPARATION

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

Table 1: Standard Curve Preparation

Standard	15-IsoP F_{2t} Concentration (ng/mL)	Vol. of 1x Dilution Buffer (µL)	Transfer Vol. (µL)	Transfer Source	Final Vol. (µL)
S_7	100	450	50	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 Sample or Standard to each well. See **Scheme I** for a suggested plate layout.
2. Add 100 µL of diluted 15-IsoP F_{2t} HRP Conjugate to each well omitting the Reagent Blank (RB) (add 100 µL of 1x Dilution Buffer in lieu of conjugate). Incubate at room temperature for 2 hours.
3. Wash wells according to the following 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. Remove the contents of each well by inversion of plate into an appropriate disposal device.
   f. Repeat procedure two more times and proceed to step "g".
   g. Tap out the remaining contents of the plate onto a lint free paper towel then proceed to step 4.
4. Add 200 µL of TMB Substrate to each well. Incubate for 30 minutes at room temperature until an appreciable blue hue is observed for the B_0.
5. Add 50 µL of 3 N H_2SO_4 to each well to stop the reaction. The color will change from blue to yellow.
6. Read plate at 450 nm. Please note that the plate can be alternatively read at 650 nm in the absence of the addition of 3 N H_2SO_4 in step 6 above.

Table 2: Quick Reference Assay Guide

	2 hour Incubation			Let Stand 2-3 minutes	30 minute Development
Reagent:	**Analyte**	**Conjugate**	**Buffer**	**Wash x3**	**Substrate**
Standard	100 µL	100 µL	---	300 µL	200 µL
Sample	100 µL	100 µL	---	300 µL	200 µL
Reagent Blank	---	---	200 µL	300 µL	200 µL

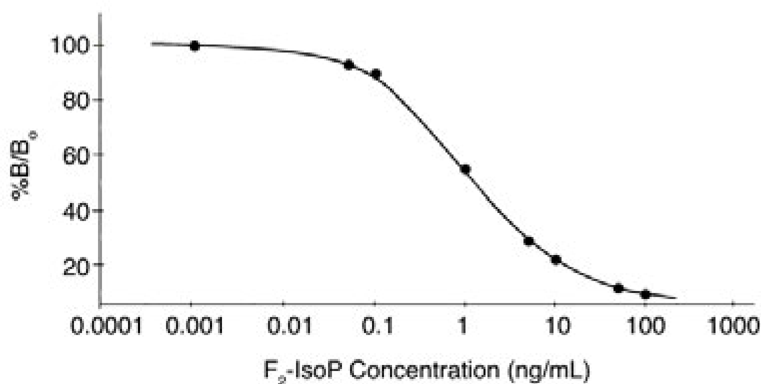
Scheme I:

	1	2	3	4	5	6	7	8	9	10	11	12
A	B_0	B_0	U_1	U_1	U_9	U_9	U_{17}	U_{17}	U_{25}	U_{25}	U_{33}	U_{33}
B	S_1	S_1	U_2	U_2	U_{10}	U_{10}	U_{18}	U_{18}	U_{26}	U_{26}	U_{34}	U_{34}
C	S_2	S_2	U_3	U_3	U_{11}	U_{11}	U_{19}	U_{19}	U_{27}	U_{27}	U_{35}	U_{35}
D	S_3	S_3	U_4	U_4	U_{12}	U_{12}	U_{20}	U_{20}	U_{28}	U_{28}	U_{36}	U_{36}
E	S_4	S_4	U_5	U_5	U_{13}	U_{13}	U_{21}	U_{21}	U_{29}	U_{29}	U_{37}	U_{37}
F	S_5	S_5	U_6	U_6	U_{14}	U_{14}	U_{22}	U_{22}	U_{30}	U_{30}	U_{38}	U_{38}
G	S_6	S_6	U_7	U_7	U_{15}	U_{15}	U_{23}	U_{23}	U_{31}	U_{31}	U_{39}	U_{39}
H	S_7	S_7	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 the 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 2** is a Typical Standard Curve, which plots concentration vs. $\%B/B_0$.
4. Average the replicates of each unknown and divide by the average B_0 value to obtain $\%B_0$, then determine the corresponding concentration using the standard curve and account for dilution factors.

Figure 2: Typical Standard Curve



PERFORMANCE CHARACTERISTICS

Cross reactivity at 50% B/B0	
15-Isoprostane F_{2t}	**100.0%**
9α,11β-PROSTAGLANDIN $F_{2\alpha}$	4.1%
13,14-DIHYDRO-15-KETO-$F_{2\alpha}$	3.0%
9β,11α-PROSTAGLANDIN $F_{2\alpha}$	< 0.01%
PROSTAGLANDIN $F_{2\alpha}$	< 0.01%
6-KETO-PROSTAGLANDIN $F_{2\alpha}$	< 0.01%
PROSTAGLANDIN E_2	< 0.01%
PROSTAGLANDIN D_2	< 0.01%
ARACHIDONIC ACID	< 0.01%

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For further information about this kit, its application or the procedures in this insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.

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