15-Isoprostane F_{2t} Enzyme Immunoassay ELISA Kit Instructions

Please read all instructions carefully before beginning this assay

PRODUCT #430010

This product is sold for research use only.

Storage Conditions:
Do not freeze kit components
All kit components: 4°C
For information about partial kit storage, please see Procedural Notes #2.

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

This kit can be used for the quantification of free 15-isoprostane F_{2t} in urine, plasma, serum or tissue samples following solid phase extraction of the isoprostane-containing fraction. Instructions are also provided for the quantification of total 15-isoprostane F_{2t} following hydrolysis of phospholipids.

NOTE: Alternatively, urine samples may be analyzed without solid phase extraction using a separate kit (Product #430110) available from Neogen Corporation.

PRINCIPLES OF THE PROCEDURE

This kit is a competitive enzyme-linked immunosorbent assay (ELISA) for determining levels of 15-isoprostane F_{2t} in biological samples. Briefly, 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} bound and inversely proportional to the amount of unconjugated 15-isoprostane F_{2t} in the samples or standards.

MATERIALS PROVIDED

1. **15-ISOPROSTANE F_{2t} STANDARD:** Two 60 µL vials of 1µg/mL 15-isoprostane F_{2t} standard.
2. **15-ISOPROSTANE F_{2t} HRP CONJUGATE:** One 100 µL vials of 15-isoprostane F_{2t} conjugated to Horse Radish Peroxidase (HRP).
3. **15-ISOPROSTANE F_{2t} ANTIBODY-COATED MICROPLATE:** One 96 well microplate coated with anti-15-isoprostane F_{2t} capture antibody.
4. **5X DILUTION BUFFER:** One 100 mL bottle of enhanced dilution buffer allowing for extraction free analysis of 15-isoprostane F_{2t} in urine samples.
5. **5X WASH BUFFER:** One 40 mL bottle of 5x wash buffer.
6. ***K-BLUE* SUBSTRATE:** One 25 mL bottle of ready to use TMB substrate.
7. **MICROPLATE TEMPLATE:** To serve as a visual aid while pipetting.
8. **REAGENT TROUGH:** These troughs accommodate approximately 10 mL of solution and most 8-channel multi-pipettes.
9. **KIT INSTRUCTIONS**

MATERIALS NEEDED BUT NOT PROVIDED

1. Precision pipettes with a range of 5 µL to 1000 µL with disposable tips.
2. Multichannel pipette.
3. Beakers, flasks, cylinders necessary for preparation of reagents.
4. 96-well plate reader for measurement of absorbance at 450 nm.
5. Deionized water.
6. 3N Sulfuric Acid (H_2SO_4).
7. Materials and Reagents for Solid Phase Extraction (SPE). See Sample Preparation.

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 is not recommended as it 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:
   a. All unused components should be returned to storage at 4°C.
   b. Unused portions of the microplate should be returned to the zip lock pouch with desiccant prior to storage at 4°C.
   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.
   d. 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 fresh pipette tips when transferring or pipetting reagents from stock reagents.
4. Pipette tips should be pre-wetted or rinsed prior to dispensing reagent. To do this, adjust pipette to the volume desired and draw up reagent of choice into the tip 2-3 times before uptake for dispensing.
5. 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. The use of expeditious collection, processing and storage techniques should be employed ensure that isoprostane levels are more accurately reflective of those at the time of collection. There are no known incompatibilites 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 Neogen Corporation's extraction-free Urinary Isoprostane Immunoassay Kit, product number 430110.

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 sample 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-isoprostane 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; M-9272)
2. Butylated Hydroxytoluene (BHT) (Sigma; B1378)
3. Methanol (MeOH) (Alfa Aesar; 32435)
4. Chloroform ($CHCl_3$) (Sigma; C-2432)
5. Triphenylphosphine (TPP) (Sigma; T84409)
6. Potassium Hydroxide (KOH) (Sigma; P-1767)
7. Hydrochloric Acid (HCl) (Fisher; A481-212)
8. Ethyl Acetate (Aldrich; 27,098-9)
9. Heptane (Sigma; H9629)
10. Evaporation Apparatus Suitable for 50 mL conical tubes and 20 mL reagent volumes
11. Water Bath suitable for 37°C
12. Analytical grade Nitrogen gas for evaporation of samples (see your local gas supply company)
13. Silica Sep Pak (Waters; WAT043400)
14. C_{18} Sep Pak (Waters; WAT043395)
15. 20 Position Extraction Manifold (Waters; WAT200609)
16. 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; Chloroform: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:MeOH (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 hydrolize the ester bond and allow for the analysis of total isoprostane. Alternatively, if only the free isprostane at the time of collection is of interest, then 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 MeOH + 0.05% BHT solution directly to 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 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 manufacturer's instructions and affix the C_{18} Sep Pak column(s) to the manifold with an appropriate waste container below each column.
3. Prewash the C_{18} Sep Pak column with 5 mL of EtOH followed by 5 mL of pH 3 Deionized Water.

NOTE: When running solutions through 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 to the column and 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 manifold with an appropriate waste container below each column.
9. Prewash 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 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 as resulting from the drying procedure. Store at –80°C under inert gas until the time of assay.

15. Reconstitute the sample in an known amount of Dilution Buffer and proceed to the Assay Procedure.

REAGENT PREPARATION

The TMB Substrate and Coated 96-Well Microplate are supplied ready to use. The preparation of all other reagents is addressed below.

5x Wash Buffer: Dilute to 1x prior to use. Do this by combining 1 part of 5x Wash Buffer to 4 parts of Deionized Water relative to the amount required for the assay, either in whole or in part.

5x Dilution Buffer: Dilute to 1x prior to use. Do this by combining 1 part of 5x Dilution Buffer to 4 parts of Deionized Water relative to the amount required for the assay, either in whole or in part.

15-isoprostane F_{2t} Standard: Please refer to the to Standard Curve Preparation.

15-isoprostane F_{2t} HRP Conjugate: When performing the entire assay at once, combine 90 µL of conjugate with 11.910 mL of 1x Dilution Buffer.

STANDARD CURVE PREPARATION

Begin set up for the standard curve preparation by labeling dilution tubes and dispensing the indicated volume of Dilution Buffer according to Table 1 below. Follow by transferring the indicated amount of standard also as indicated in Table 1.

TABLE 1: PREPARATION OF THE STANDARD CURVE

Standards	*15-isoprostane F_{2t} Concentration (ng/mL)*	*Enhanced Dilution Buffer (µL)*	*Transfer Volume (µL)*	*Transfer Source*	*Final Volume (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

The following instructions are based on the user 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.

1. Add 100 µL of Sample or Standard to each well. See **Scheme I** for a suggested plate layout.
2. Add 100 mL of diluted 15-isoprostane F_{2t} HRP CONJUGATE to each well omitting the reagent blank (add 100 µL of ENHANCED DILUTION BUFFER in lieu of conjugate). Allow the plate to incubate for 2 hours at RT.
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. Repeate 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 µL 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 N sulfuric acid to each well to stop the reaction. The color will change from blue to yellow.
7. 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 sulfuric acid 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

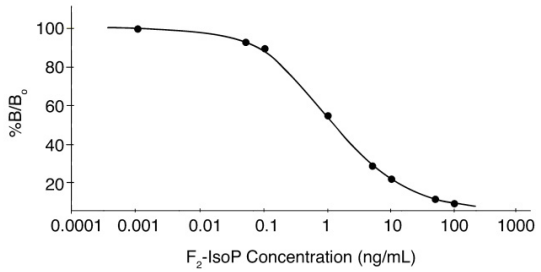
	1	2	3	4	5	6	7	8	9	10	11	12
A	B_0	B_0	U_1	U_1	U_9	U_9	U_{17}	U_{17}	U_{25}	U_{25}	U_{33}	U_{33}
B	S_1	S_1	U_2	U_2	U_{10}	U_{10}	U_{18}	U_{18}	U_{26}	U_{26}	U_{34}	U_{34}
C	S_2	S_2	U_3	U_3	U_{11}	U_{11}	U_{19}	U_{19}	U_{27}	U_{27}	U_{35}	U_{35}
D	S_3	S_3	U_4	U_4	U_{12}	U_{12}	U_{20}	U_{20}	U_{28}	U_{28}	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 the average from each well. Most modern microplate readers are capable of doing this automatically.
2. Average standard replicates (S_1 through S_7) and divide by the average obtained for B_0 and multiply 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

Figure 2: Typical Standard Curve



PERFORMANCE CHARACTERISTICS

Cross reactivity at 50% B/B_0:

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

REFERENCES

1. Morrow, J., Harris, T., & Roberts, L., Anal. Biochem. 14:1-10 (1990).
2. Morrow, J. D., Hill, K. , Burke, R. F., Nammour, T. M., Badr, K. F., and Roberts, L. J., Proc. Natl. Acad. Sci. U.S.A. 87:9383-9387 (1990).
3. Morrow, et. al., The Isoprostanes: Unique Bioactive Products of Lipid Peroxidation. Prog. Lipid Res. 36:1-21, 1997.
4. Morrow, J., Awad, J. A., Boss, H. J., Blair, I. A., and Roberts, L. J., Proc. Natl Acad. Sci USA 89:10721-10725 (1992).
5. Wang, et al., Immunological characterization of urinary 8-epi-prostaglandin $F_{2\alpha}$ excretion in man. J. Pharm. Exp. Ther. 275:94-100 (1995).
6. Morrow, J., Zackert, W., Yang, J., Kuhrts, E., Callewaert, D., Taber, D., Oates, J., Roberts, J., Quantitation of the major urinary metabolite of the isoprostane 15-F_{2t}-Isoprostane (8-iso-$PGF_{2\alpha}$) by a stable isotope dilution mass spectrometric assay, Analytical Biochem. 269: 326-331 (1999).
7. Morrow, J.D., Roberts II, L.J., Mass Spectrometric Quantification of F2-Isoprostanes in Biological Fluids and Tissues as Measure of Oxidant Stress. Meth. Enz.. 300:3-12 (1999).
8. Roberts II, L.J., Morrow, J.D., Measurement of F2-isoprostanes as an index of oxidative stress in vivo, Free Radical. Biol. Med. 28:505-513 (2000).

DISCLAIMER

This information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. Neogen corporation shall not be held liable for any damage resulting from handling or form contact with the above product. See catalog for additional terms and conditions of sale.

TECHNICAL SUPPORT

If you need technical information or assistance with assay procedures, call our Technical Support Department at 1-800-477-8201 or 1-859-254-1221. Our staff will be happy to answer your questions about this or any other product our line. Technical assistance is available Monday-Friday, between 8:00 a.m. and 6:00 p.m. EST.

WARRANTY

Neogen Corporation makes no warranty of any kind, either expressed or implied, except that the material from which its products are made are of standard quality. If any materials are defective, Neogen Corporation will provide a replacement product. Buyer assumes all risk and liability resulting from the use of this product and any of the predictive models. There is no warranty of merchantability of this product, or of the fitness of the product for any purpose. Neogen Corporation shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product.

COPYRIGHT

NEOGEN® CORPORATION

944 Nandino Blvd • Lexington KY 40511-1205 USA
859/254-1221 or 800/477-8201 USA/CANADA
Fax: 859/255-5532 • email: inform@neogen.com

D430010-3/23/10