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Northwest
Life Science Specialties, LLC

Premier Products for Superior Life Science Research

NWLSS™ Isoprostane ELISA

Specific for 15-isoprostane F_{2t}

Product NWK-ISO01

For Research Use Only

Assay system for measurement of 15-isoprostane F_{2t} in urine, 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.

nwkiso01rev050406

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User Notes:

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REFERENCES

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Statement of Limited Warranty:

Northwest Life Science Specialties, LLC (NWLSS) makes no guarantee of any kind, expressed or implied, that extends beyond the description of the material in this kit, except that they will meet our specifications at the time of delivery. Customer's remedy and NWLSS' sole liability is limited to, at NWLSS' option, refund of the purchase price, or the replacement of material not meeting our specification. By acceptance of our product, customer assumes all liability and will indemnify and hold NWLSS harmless for the consequence of this product's use or misuse by the customer, its employees, or others. Refund or replacement is conditioned of customer notifying NWLSS within twenty-one (21) days of the receipt of product. Failure to give notice within 21 days shall constitute a waiver by the customer of all claims hereunder with respect to said product.

Introduction:

Isoprostanes are prostaglandin-like compounds that are produced by free radical mediated peroxidation of lipoproteins. Levels of 15-isoprostane F_{2t}, a representative isoprostane 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, athero-sclerosis, and carcinogenesis.

Intended Use:

This kit is intended for the quantification of 15-isoprostane F_{2t} (also known as 8-epi-$PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$ or more commonly 8-Isoprostane) and can be used with urine, 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 the NWK-ISO2 kit.

Test Principle:

This assay utilizes a competitive enzyme-linked immunoassay (ELISA) format. Briefly, 15-isoprostane F_{2t} in the samples or standards is allowed to compete with 15-isoprostane F_{2t} conjugated to horseradish peroxidase (HRP) for binding to a polyclonal antibody specific for 15-isoprostane F_{2t} coated on a microplate. Subsequent TMB substrate addition results in a blue color development that is inversely proportional to the quantity of 15-isoprostane F_{2t} in the original samples or standards. Addition of an acid stop solution causes a color change to yellow where absorbance is read at 450 nM.

General Specifications:

Format:	96 well competitive ELISA
Number of tests:	Triplicate = 24 Duplicate= 40
Specificity:	15-isoprostane F_{2t} (8-Isoprostane)
Sensitivity:	0.01 ng (10 pg)
Effective Range:	0.05ng/mL - 10 ng/mL

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Kit Contents

Microwells precoated with Anti-15-isoprostane F_{2t}:	1 X 96 wells
15-isoprostane F_{2t} Standard:	2 X 60 µL
5X Wash Buffer:	1 X 40 mL
5X Dilution Buffer:	1 X 100 mL
TMB Substrate:	1 X 25 mL
15-isoprostane F_{2t} HRP Conjugate:	1 X 100 µL
Reagent Trough for multi-channel pipettor:	2 Each

Required Materials Not Provided:

Adjustable pipettes with a range of 5 µL to 1,000 µL with disposable tips.

Glassware for preparation of reagents.

Reagents for hydrolysis of phospholipids and extraction of isoprostanes from biological samples.

Deionized water.

3M sulfuric acid.

Required Instrumentation:

Microtiter plate reader with 450 nm capability.

Warnings, Limitations, Precautions:

Individual components may be harmful if swallowed, inhaled or absorbed through the skin. Contact should be minimized through the use of gloves and standard good laboratory practices. If contact with skin or eyes occurs, rinse the site immediately with water and consult a physician.

Storage Instructions:

Store all components at 4°C until immediately before use. Do not freeze.

Assay Preparation

1. Determine the number of wells required to assay standards, samples and controls for the appropriate replicate.

2. Create an assay template showing positioning of standards, controls and samples. Include a blank as well.

3. Bring all samples and reagents to room temperature before use. To avoid condensation, do not open foil pouches containing the microtiter strips until after they have reached room temperature.

4. Next remove the required number of strips and place in the frame supplied. Return unused wells to the storage bag, seal and store at 2-8ºC.

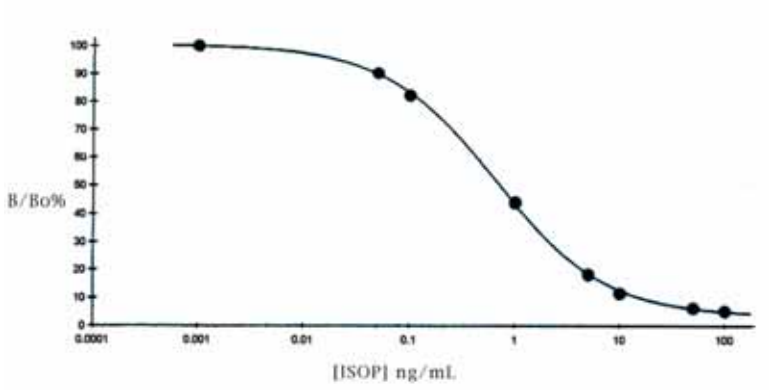
Data Analysis

1. Average the reagent blank absorbance values and subtract this 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 S_0 (Zero Bound or B_0) to express data as a percent of B_0.

3. Graph $\%B_0$ values (y-axis-linear) vs. standard concentration (x-axis-logarithmic) to obtain a standard curve. Figure 1 shows a typical curve obtained when plotting concentration vs. percent bound this fashion.

Figure 1.



4. Average the replicates of each unknown and divide by the average S_0 (B_0) value to express data in terms of $\%B_0$, then determine corresponding concentration using the standard curve.

PERFORMANCE Details

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-$PGF_{2\alpha}$	3.0%
9β,11α-Prostaglandin $F_{2\alpha}$	< 0.01%
Prostaglandin F2α	< 0.01%
6-KETO-Prostaglandin $F_{1\alpha}$	< 0.01%
Prostaglandin E $_{\alpha}$	< 0.01%
ProstaglandinDIN D $_{\alpha}$	< 0.01%
Arachidonic Acid	< 0.01%

6. Add 4 mL 0.9% NaCl.

7. Vortex vigorously and centrifuge for 2-3 min.

8. Discard the upper layer (MeOH/saline).

9. Remove the lower phase to 100 mL Rota-VAP flask or 50 mL conical tube, avoiding the protein layer.

10. Evaporate and add 2-4 mL MeOH with 5 mg BHT/100 mL and an equal volume of 15% KOH.

11. Place in a water bath at 37-40° for 30 minutes.

12. Dilute to 40 – 80 mL with pH 3 water so the MeOH is ≤ 5% of the total volume. The sample is now ready for solid phase extraction as described above.

From Urine samples
Following the solid phase extraction procedure described for serum samples, urine samples may be analyzed using this kit. Alternatively, unextracted urine samples can be analyzed directly using NWLSS product number NWK-ISO02.

Assay Protocol:
Make dilutions of extracted samples in Working Dilution Buffer. The extent of dilution will vary depending on sample 15-isoprostane F_{2t} levels and must be determined by individual users.

1. Add 100 µL of Standards and diluted samples to wells according to assay template.

2. Add 100 µL of Diluted HRP Conjugate to each well except Blanks. Add 100 µL Working Dilution Buffer to Blank wells and allow plate to stand at room temperature 2 hours.

3. Invert plate and empty contents. Pat dry upside-down on a lint free towel.

4. Wash each well 3 times with 400 µL Working Wash Buffer.(allow to stand 2 minutes, invert, empty contents, and pat dry between washes).

5. Add 200 µL Substrate to each well, allow to stand for 20-30 minutes.

6. Add 50 µL of 3M sulfuric acid to each well to stop the reaction.

7. Read the microplate at 450 nm on a plate reader.

Reagent Preparation:
The following instructions are based on the user using the entire kit at one time. If portions of the kit are to be used at a later time, smaller quantities may be prepared saving the remaining stock for later use.

5X Wash Buffer
Add the contents of the 5X Wash Buffer to 160 mL deionized H_2O, mix well and label as **Working Wash Buffer.**

5X Dilution Buffer
Add 100 mL of 5X Dilution Buffer to 400 mL deionized H_2O, mix well and label as **Working Dilution Buffer.**

15-isoprostane F_{2t} HRP-Enzyme Conjugate
Briefly centrifuge the vial to remove liquid from cap and vial walls. Pre-wet the pipet tip in the conjugate then add 90 µL conjugate to 11.91 mL Working Dilution Buffer. Label as **Diluted HRP-Conjugate**

Substrate is supplied ready to use.

Standard Preparation:
Stock Standard Supplied: 15-isoprostane F_{2t}: 2 X 60 µL at 1 µg/mL.

Standard 7 (S_7): Add 50 µL of Stock Standard to 450 µL Working Dilution Buffer. *Label as* S_7 *with a concentration of* ***100 ng/mL.***

Standard 6 (S_6): Add 200 µL of S_7 to 200 µL Working Dilution Buffer and vortex. *The* S_6 *concentration is now* ***50 ng/mL.***

Standard 5 (S_5): Add 100 µL of S_6 to 400 µL Working Dilution Buffer and vortex. *The* S_5 *concentration is now* ***10 ng/mL.***

Standard 4 (S_4): Add 200 µL of S_5 to 200 µL Working Dilution Buffer and *vortex. The* S_4 *concentration is now* ***5 ng/mL.***

Standard 3 (S_3): Add 100 µL of S_4 to 400 µL Working Dilution Buffer and vortex. *The* S_3 *concentration is now* ***1 ng/mL.***

Standard 2 (S_2): Add 100 µL of S_3 to 900 µL Working Dilution Buffer and vortex. *The* S_2 *concentration is now* ***0.1 ng/mL****.*

Standard 1 (S_1): Add 500 µL of S_2 to 500 µL Working Dilution Buffer and vortex. *The* S_1 *concentration is now* ***0.05 ng/mL.***

Standard 0 (S_0): Aliquot 500 µL Working Dilution Buffer only to this tube. It will be used to determine maximal color development.

Sample Handling/Preparation

Extraction Of 15-IsoprostaneF$_{2t}$ From Biological Samples

Serum & Plasma Samples

Serum and plasma contain high concentrations of proteins and other substances that interfere with immunoassays. Partial purification of isoprostanes is therefore required prior to assay. The following solid phase extraction protocol suitable for extraction of isoprostanes prior to assay. NOTE: It is necessary to determine and adjust for incomplete recovery of isoprostanes from the extraction columns. Addition of a known quantity of 15-isoprostane F_{2t} standard (e.g. 5 ng) to one aliquot of one unknown, followed by immunoassay of both samples, and computation of the percent recovery is sufficient.

Solid Phase Extraction

1. Add 3 mL of pH 3 water to 10 mL of the sample

2. Adjust pH to 3.0 with 1 N HCl

3. C18 Sep-Pak

Prewash with 5 mL of ethanol
Follow with 5 mL of pH 3 water
Load the sample
Wash with 10 mL of pH 3 water
Wash with 10 mL of Heptane

Elute with 10 mL Ethyl Acetate:Heptane (1:1) into a plastic tube.
Add one scoop (pea sized amount) sodium sulfate to eluant; add more sodium sulfate if needed.
The Ethyl Acetate:Heptane sample solution should be aspirated with a pipet or decanted using caution not to transfer sodium sulfate to the silica sep pak in step 4.

4. *Silica Sep-Pak*

Prewash with 5 mL of Methanol
Follow with 5 mL of Ethyl Acetate
Load Ethyl Acetate:Heptane eluant (sample) collected from the C18 Sep-Pak.
Wash with 5 mL Ethyl Acetate

Elute with 5 mL Ethyl Acetate:Methanol (1:1) into a plastic tube.

5. Evaporate under N_2
Dissolve residue in a known volume of dilution buffer for immunoassay.

Tissue culture media

Since tissue culture medium is generally supplemented with serum, we recommend that isoprostanes be extracted using the same protocol used for plasma samples.

Determination of total (free + esterified) isoprostanes

From plasma or other fluids

1. Prepare Folch solution (2:1 $CHCl_3$/MeOH) with butylated hydroxytoluene (BHT)(5 mg/100 mL) and triphenylphosphine (TPP) (50 mg/100 mL).

2. Add 20 mL Folch solution to a 50 mL conical tube and place on ice.

3. Add 1 mL plasma or other fluid and shake or vortex well for 1 min.

5. Add ice cold 0.043% $MgCl_2$ (10 mL) and shake or vortex well for 1 min.

6. Centrifuge for 2-3 min.

7. Aspirate off the top layer ($MgCl_2$/MeOH) and transfer the organic layer to another 50 mL tube, being careful not to transfer any protein layer that may be present.

8. Dry the organic layer under N_2.

9. Add 0.5 to 2 mL MeOH (depending on the amount of lipid present) containing BHT (5 mg/100 mL) and an equal volume of 15% KOH. Swirl after each addition.

10. Allow sample to stand at 37 °C for 30 minutes.

11. Dilute with pH 3 water so that the volume of MeOH added is ≤ 5% of the total volume. The sample is now ready for solid phase extraction as described above.

From tissue samples

1. Prepare Folch solution (2:1 $CHCl_3$/MeOH) with BHT (5 mg/100 mL).

2. Add 20 mL Folch solution to a 40 mL flat bottom tube and place on ice.

3. Weigh 0.5 to 1 gram tissue and add to tube on ice then shake or vortex well for 1 min.

4. Homogenize with blade homogenizer or sonicator for 30 seconds

5. Allow to sit under N_2 in a sealed tube for one hour at room temp, vortexing occasionally.