Urinary 8-Isoprostane ELISA kit

Catalog Number: **8isoU1**

Store at -20°C.
FOR RESEARCH USE ONLY

Introduction

This competitive ELISA kit is for determination of free and glucronidated 8-isoprostane levels in biological samples. The isoprostanes are a family of eicosanoids of non-enzymatic origin produced by the random oxidation of tissues phospholipids by oxygen radicals. A recent NIH-sponsored study on Biomarkers of Oxidative Stress has indicated that 8-isoprostane is the best index of oxidative injury in a well-accepted oxidant stress rat model (1,2). In addition, plasma 8-isoprostane levels were found to be elevated in elderly subjects with severe hypertension (3) and in urine from subjects with high fat diet-induced liver steatosis (4).

High levels of the glucuronidated form of 8-isoprostane have been found in human urine.

This kit can be used for the determination of 8-isoprostane in diluted urine following proper isolation and purification of the eicosanoid from the isoprostane-containing sample. Instructions are provided as to the proper isolation and purification in the following pages.

Storage and Stability

This kit will obtain optimal results if all of the components are stored at the proper temperature prior to use. Items should be stored at the designated temperatures upon receipt of this kit.

All components are stored below -20°C and should not be re-frozen and thawed more than necessary.

Materials Provided

Part Number	Item	Description	Quantity
1	8-isoprostane ELISA Plate	Solid 96-well plate coated with anti 8-isoprostane antibody in each well	1
2	8-isoprotane Standard (10 ng in 2 µL)	Stock standard at a concentration of 5 µg/mL	1
3	8-isoprostane HRP Conjugates (12 µL)	1000 X concentrated solution	1
4	Sample Dilution Buffer (25 mL)	10 X solution of Tris-buffered saline with preservatives	1
5	HRP Buffer (15 mL)	1 X solution of Tris-buffered saline with preservatives	1
6	Wash Buffer Solution (25 mL)	10 X solution of Tris-buffered saline with detergents and preservatives	1
7	TMB Substrate	A solution of TMB (tetra methyl benzadine)	1

	(24 mL)		
8	β-glucuronidase enzyme (8 mg powder)	Solid enzyme for reconstitution	1
9	Citrate Buffer Solution (8 mL)	1.0 M solution of citric acid buffer, pH 5.5	1

Additional Required Materials (Not Provided)

- Plate reader with a 450 nm filter
- An 8-channel adjustable pipetter and an adjustable pipetter
- Storage bottles
- Costar® cluster tubes (1.2 mL) and microcentrifuge tubes

Precautions

- Please read all instructions carefully before beginning the assay.
- The reagents in this kit have been tested and formulated to perform optimally. This kit may not perform correctly if any of the reagents are replaced or any of the procedures are modified.
- This kit is intended for research use only and is not to be used as a diagnostic.

Procedural Notes

- Remove all of the reagents required, including the TMB, and allow them to equilibrate to room temperature before proceeding with the assay.
- It is necessary to thoroughly mix the concentrated buffer solutions. A stir bar is contained within each buffer solution.

Sample Preparations

A. Measurement of glucuronidated 8-isoprostane in Media and Urine (Note—Extraction is not necessary).

(i) Beta-Glucuronidase digestion

1. Pipet 1.0

mL of extracellular media or urine into two tubes.

2 .Add 125 uL of the beta-glucuronidase enzyme to each tube (see MATERIALS), final pH 5.5.

3. Immediately flash-freeze one of the tubes. This is the zero time point.

4. Incubate the other tube at 37ºC for 3 hours. This is the 3 hour time point.

(ii) ELISA

1. Follow instructions for ELISA kit. Samples may need to be diluted prior to adding to the ELISA plate. A 4X dilution is recommended.

There are different protocols for isolating and purifying 8-isoprostane depending on the medium it is in. Listed below are the different protocols. For optimal results follow the appropriate protocol based on the biological sample present.

Free 8-isoprostane measurement in urine

1. Dilute urine 4-fold with sample dilution buffer containing a final concentration of ~0.1 mM TPP (triphenylphosphine, 0.03-0.05 mg/mL). TPP is an antioxidant, which looks like a precipitate in samples because it does not easily dissolve. Before using the stored samples containing TPP, spin samples to separate the precipitated TPP from sample solution.
2. Perform the ELISA for 8-isoprostane (according to the instructions of the manufacturer).

Assay Preparations

The solid 96-well plate and TMB solution are provided ready to use. The preparations of other assay reagents are detailed below.

Wash Buffer: Mix the solution with a stir bar, applying low heat until a clear colorless solution is obtained. Dilute the entire contents of the Wash Buffer Concentrate (25 mL) with 225 mL of deionized water to yield a final volume of 250 mL of 1 X Wash Buffer. This can then be refrigerated for the entire life of the kit.

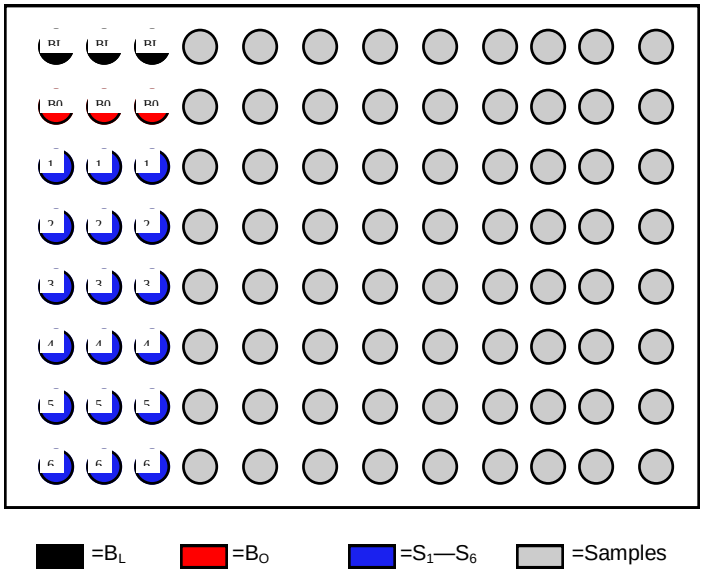
HRP Conjugate: Dilute 1 vial of the 8-isoprostane-HRP conjugate (0.012 mL) with 12.00 mL of 1 X HRP buffer. One vial makes enough conjugate for one plate. The conjugate must be used the same day and should not be stored for later use.

Standards: Label 5 microtubes as Standard 1 through Standard 5. Dilute the entire contents of Sample Dilution Stock buffer (25 mL) with 225 mL deionized water to yield a final volume of 250 mL of 1 X Sample Dilution Buffer. Spin down the enclosed 8-isoprostane standard vial (2 µL, filled with inert gas) and add 1.998 mL of Sample Dilution Buffer to obtain 2 mL of solution. Label this Standard 6. Add 1.6 mL of 1 X Sample Dilution Buffer to Standard 5; 1 mL of Sample Dilution Buffer to Standard 4; 1.6 mL of 1 X Sample Dilution Buffer into Standard 3; 1 mL of 1 X Sample Dilution Buffer into Standard 2; and 1 mL of Sample Dilution Buffer into Standard 1. Take 400 µL of Standard 6 (5 ng/mL) into Standard 5 (1 ng/mL). Take 1 mL of the Standard 5 solution into Standard 4 (500 pg/mL). From Standard 4, take 400 µL into Standard 3 (100 pg/mL). Take 1 mL of Standard 3 into Standard 2 (50 pg/mL). The last step is to add 400 µL of Standard 2 to Standard 1 (10 pg/mL). The serially diluted standards for 1 to 6 are ready for analysis from concentration 10 pg/mL to 5000 pg/mL.

Samples: Samples can be directly diluted into the 1 X Sample Dilution Buffer if it is in solution. For extracted and dried samples, it is recommended to dissolve the dried-up samples with a minimal amount of ethanol of N, N-dimethyl-formanmide (DMF, 10 µL to 20 µL) and vortex well. Before ELISA assay, add 100 µL of 1 X Sample Dilution Buffer to make the stock sample solution ready for quantification with ELISA. The stock sample solution can be further diluted to a proper range of concentration for ELISA test

Performing the Assay

Plate Setup: Each plate must contain a minimum of three blank wells (B_L), three maximum binding wells (B_O), and a six point standard curve (S_1-S_6). Each sample should be assayed in triplicate. A suggested plate format is shown below:



Standard Dilutions Table

Standards	Final Concentration (pg/mL)	Add Sample Dilution Buffer (mL)	Serial Dilutions Procedure
No. 6	5,000	1.998	2 µL of stock solution.
No. 5	1,000	1.600	Add 0.4 mL of No. 6
No. 4	500	1.000	Add 1.0 mL of No. 5
No. 3	100	1.600	Add 0.4 mL of No. 4
No. 2	50	1.000	Add 1.0 mL of No. 3
No. 1	10	1.600	Add 0.4 mL of No. 2

Assay Procedure

Step 1: Load 200 microliters of Sample Dilution Buffer into the blank (B_L) wells and 100 microliters of Sample Dilution Buffer into the maximum binding (B_O) wells.

Step 2: Load 100 microliters of each of the standards into the appropriate wells.

Step 3: Load 100 microliters of each of the samples into the appropriate wells.

Step 4: Load 100 microliters of the diluted 8-isoprostane-HRP conjugate in the B_O wells, the standard wells, and the sample wells. Do NOT add HRP conjugate into the B_L wells.

Step 5: Incubate the plate at room temperature for two hours.

Step 6: Wash the plate three times with 400 microliters of the diluted Wash Buffer per well.

Step 7: After the last of the three wash cycles pat the plate dry onto some paper toweling.

Step 8: Add 200 microliters of the TMB substrate to all of the wells (including B_L wells).

Step 9: Incubate the plate at room temperature for 15-30 minutes.

Step 10: Add 50 micoliters of 2 N sulfuric acid to all of the wells.

Step 11: Read the plate at 450 nm.

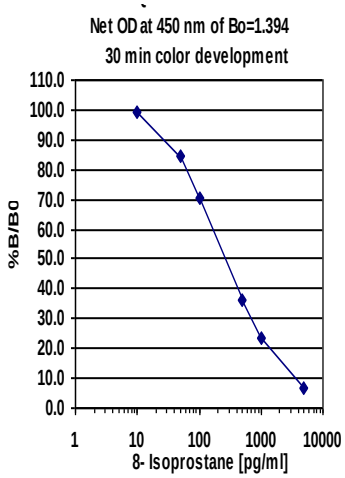
Calculating the Results

Most plate readers provide data reduction software that can be used to plot the standard curve and determine the sample concentrations. If your plate reader does not have this option, then a data reduction program can be used (4 parameter of log-log curve fit).

If you do not have these options, the results can be obtained manually as follows:

1. Average the absorbance readings from the blanks and subtract that value from each well of the plate to obtain the corrected readings. (Note: Some plate readers do this automatically. Consult the user manual of your plate reader.)
2. Average the corrected absorbance readings from the B_O wells. This is your maximum binding.
3. Calculate the $\%B/B_O$ for Standard 1 by averaging the corrected absorbance of the two S_1 wells, divide the average by the maximum binding, then multiply by 100. Repeat this formula for the remaining standards.
4. Plot the $\%B/B_O$ versus the concentration of 8-isoprostane from the standards using semi-log paper.
5. Calculate the $\%B/B_O$ for the samples and determine the concentrations, utilizing the standard curve.
6. Multiply the concentrations obtained for each of the samples by their corresponding dilution factor.

Typical Results



The data shown here is an example of typical results obtained using the Detroit R & D 8-isoprostane ELISA kit. These results are only a guideline, and should not be used to determine values from your samples. The user must run their own standard curve every time.

B_L wells = 0.14

B_O wells = 1.387

Standard	Concentration	O.D.	%B/B_O
No. 1	10 pg/mL	1.394	100.0
No. 2	50 pg/mL	1.174	73.4
No. 3	100 pg/mL	0.985	70.7
No. 4	500 pg/mL	0.504	36.2
No. 5	1000 pg/mL	0.328	23.5
No. 6	5000 pg/mL	0.090	6.4

Specificity of anti-8-isoprostane IgG

The specificity of the 8-isoprostane ELISA was investigated using authentic 8-isoprostane and a panel of eicosanoids structurally similar to the 8-isoprostane.

8-isoprostane	100.00 %
2,3-dinor-8-isoPGF2α	< 0.01 %
2,3-dinor-11β-PGF2α	< 0.01 %
PGF 1α	< 0.01 %
8-iso PGE2	< 0.01 %
8-iso PGE1	< 0.01 %

Troubleshooting

No color present in standard wells.

- The HRP conjugate was not added. Redo the assay and add the conjugate at the proper step.
- The HRP conjugate was not incubated for the proper time. Redo the assay and incubate for the proper time.

No color in any wells, including the TA wells.

- The TMB substrate was not added. Add substrate.
- The TMB substrate was not incubated for the proper time. Continue incubation until desired color is reached.

The color is faint.

- One or all of the incubation times were cut short. Redo the assay with the proper incubation times.
- The TMB substrate was not warmed up to room temperature. Redo the assay making sure all reagents are at room temperature.
- The lab is too cold. Be sure the lab temperature is between 21-27°C and redo the assay.

The background color is very high.

- The TMB substrate has been contaminated. Redo the assay with a fresh bottle of substrate.

Scattered O.D. obtained from the sample.

- Redo assay using an 8-channel pipetman making sure that 8 channels are equal volume while loading.

References

1. Morrow JD. Quantification of isoprostanes as indices of oxidant stress and the risk of atherosclerosis in humans. Arterioscler Thromb Vasc Biol. 2005; 25:279-86.
2. Gross M, et al. Plasma F2-isoprostanes and coronary artery calcification: the CARDIA Study. Clin Chem. 2005; 51:125-31.
3. Hozawa A, et al. Increased plasma 8-isoprostane levels in hypertensive subjects: the Tsurugaya Project. Hypertens Res. 2004; 27:557-61.
4. Carabelli, J, et al. High fat diet-induced liver steatosis promotes an increase in liver mitochondrial biogenesis in response to hypoxia. J. Cell. Mol. Med. 2010.

Warranty

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