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8 isoprostane ELISA Kit

A competitive immunoenzymatic assay for the quantitative measurement of 8 isoprostane in urine, serum, plasma, cells and tissues.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Abcam's 8 isoprostane competitive in vitro ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the determination of 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. In addition, plasma 8 isoprostane levels were found to be elevated in elderly subjects with severe hypertension and in urine from subjects with high fat diet-induced liver steatosis.

This kit can be used for the determination of 8 isoprostane in diluted urine, serum, plasma, cells and tissues following proper isolation and purification of the eicosanoid from the isoprostane-containing sample.

2. Protocol Summary

Prepare all reagents, samples, and standards as instructed

Add standards and samples to each well used.

Add prepared HRP conjugate to each well and incubate at room temperature.

Add TMB substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at +4°C or -20°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage Condition
8-isoprostane ELISA Plate	96 wells	-20°C
8-isoprostane Standard (5 µg/mL)	2 µL (10ng)	-20°C
1,000X 8-isoprostane HRP Conjugates	12 µL	-20°C
10X Sample Dilution Buffer	25 mL	-20°C
HRP Buffer	15 mL	-20°C
10X Wash Buffer Solution	25 mL	-20°C
TMB Substrate	24 mL	-20°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Plate reader with a 450 nm filter.
- An 8-channel adjustable pipette and an adjustable pipette.
- Storage bottles.
- Costar cluster tubes (1.2 mL) and microcentrifuge tubes.
- Speed-vac (optional) or argon or nitrogen gas.
- 2N Sulfuric Acid Stop Solution.

8. Technical Hints

- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
- Use only clean pipette tips, dispensers, and lab ware.
- Do not interchange screw caps of reagent vials to avoid cross-contamination.
- Close reagent vials tightly immediately after use to avoid evaporation and microbial contamination.
- After first opening and subsequent storage check conjugate and control vials for microbial contamination prior to further use.
- To avoid cross-contamination and falsely elevated results pipette samples and dispense conjugate, without splashing, accurately to the bottom of wells.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary for accurate measurement readings.
- Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.
- It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than

one plate is used, it is recommended to repeat the dose response curve in each plate.

- The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells.
- Prepare only as much reagent as is needed on the day of the experiment.

9.1 1X Wash Buffer

Mix the 10X Wash Buffer Solution with a stir bar, applying low, gentle heat until a clear colorless solution is obtained. Dilute the entire contents of the 10X Wash Buffer Solution (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.

9.2 1X HRP Conjugate

Dilute 1 vial of the 8 isoprostane-HRP conjugate (12 µL) with 12 mL of 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.

9.3 1X Sample Dilution Buffer

Prepare 1X Sample Dilution Buffer by adding 25 mL of 10X Sample Dilution Buffer to 225 mL of dH20. Mix gently and thoroughly.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

10.1 Label 5 microtubes as Standard # 2 - 6.

10.2 Prepare a 5 ng/mL **Standard #1** by first spinning down the enclosed 8 isoprostane standard vial (2 µL, filled with inert gas) and then adding 1.998 mL of 1X Sample Dilution Buffer to obtain 2 mL of solution.

10.3 Prepare **Standard #2** by adding 400 µL of the Standard #1 and 1.6 mL of 1X Sample Dilution Buffer to the microtube labeled **Standard #2**. Mix thoroughly and gently.

10.4 Prepare **Standard #3** by adding 1 mL of the Standard #2 and 1 mL of 1X Sample Dilution Buffer to the microtube labeled **Standard #3**. Mix thoroughly and gently.

10.5 Using the table below as a guide, repeat for tubes #4 through #6.

10.6 Standard Bo contains no protein and is blank control.

Standard #	Volume to dilute (µL)	Volume Diluent (µL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	Step 10.2			5,000
2	400 µL Standard #1	1,600	5,000	1,000
3	1,000 µL Standard #2	1,600	1,000	500
4	400 µL Standard #3	1,600	500	100
5	1,000 µL Standard #4	1,600	100	50
6	400 µL Standard #5	1,600	50	10

7 (Blank)	N/A	1,600	-	-

11. Sample Preparation

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

Δ Note 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-formamide (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.

11.1 8 isoprostane measurement in urine:

11.1.1 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.

11.1.2 Perform the ELISA for 8 isoprostane (according to the instructions of the manufacturer).

11.2 8-isoprostane measurement in cells:

11.2.1 Collect and homogenize and/or sonicate the cells using a solution 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.

11.2.2 Acidify the whole homogenized cells with acetic acid to a pH of approximately 3-4. Measure using standard pH paper.

11.2.3 Extraction with ethyl acetate. Add an equal volume of ethyl acetate to the homogenized cells and vortex very well. Place the upper organic phase into a fresh clean tube after centrifugation. Then add another equal volume of ethyl

acetate to the homogenized cells to start the second-time extraction. It is strongly recommended that extraction is performed three times.

11.2.4 Evaporate the pooled ethyl acetate from the extractions until all has dried up under argon or nitrogen gas.

11.2.5 Saponification if needed (see below)

11.2.6 Add 10 µL to 20 µL ethanol, or N, N-dimethyl-formamide (DMF), to dissolve the dried-up residue from above step #4. Add 0.5 mL of 1x Sample Dilution Buffer (provided in kit). Load 100 µL in each well, in triplicates, on the ELISA plate. (Note: We recommend measuring a different dilution of sample in attempt to fit the results to the standard curve. e.g., add 3 wells with 50 µL of the rest of sample plus 50 µL 1x Sample Dilution Buffer and 3 wells of 10 µL of the rest of sample and 90 µL of 1x Sample Dilution Buffer.)

11.2.7 Perform the ELISA for 8 isoprostane (according to the instructions of the manufacturer).

11.3 Saponification (to cleave fatty acid from glycerol backbone):

11.3.1 Dissolve dried fatty acids (obtained from 3X ethyl acetate extractions) in 2 mL of 20% KOH solution (make working solution: 1 mL of 2 M KOH + 4 mL methanol so that the final conc. of KOH = 0.4 N).

11.3.2 Vortex and incubate for 1 h at 50°C.

11.3.3 Add 1.5 X H_2O to the solution and adjust pH with 20% formic acid to pH~5.

11.3.4 Re-extract the solution with ethyl acetate (1 part aqueous solution + 1 part ethyl acetate) and dry.

11.4 8-isoprostane measurement in tissues:

11.4.1 Homogenize 1 g of tissue, 4 mL of H_2O, and 0.01mg TPP.

11.4.2 Acidify the homogenate by adding 8 µL of acetic acid to each homogenate.

11.4.3 Extract with an equal amount of ethyl acetate, vortex thoroughly, spin down, and collect the organic phase. Repeat this extraction twice more and combine all of the organic phases.

11.4.4 Dry the organic phase with argon or nitrogen gas.

11.4.5 Saponification if needed (see Section 11.3)

11.4.6 Dissolve the dried residue from above step with ethanol or DMF. (Add approximately 20 µL of ethanol or DMF to reconstitute the dried-up residue.)

11.4.7 Dilute further with 1X Sample Dilution Buffer: Add approximately 500 µL of 1x Sample Dilution Buffer and centrifuge at 10,000 rpm for five minutes at room temperature. The supernatant will be used for ELISA.

11.4.8 Perform the ELISA for 8 isoprostane (according to the instructions of the manufacturer).

11.5 8 isoprostane measurement in plasma or serum:

11.5.1 Combine 1.8 mL of plasma (adjusted with approximately 20 µL of acetic acid to pH 4) and 1.8 mL of ethyl acetate. Vortex thoroughly. Centrifuge at 2000 rpm for ten minutes at 22°C. Three phases should result:

11.5.1.1 Upper organic phase – ethyl acetate phase (lipoproteins)

11.5.1.2 Interphase – proteins

11.5.1.3 Lower phase – aqueous phase

11.5.2 Collect the upper organic phase (a) and set aside.

11.5.3 Discard the interphase. Transfer the lower phase with a glass pipette to a new tube, and repeat the ethyl acetate extraction step 2 more times.

11.5.4 Evaporation of pooled organic phase: There should be approximately 5-6 mL of the ethyl acetate phase (a). Dry the pooled organic phase in a Speedvac to get the extracted sediment (b).

11.5.5 Saponification (to cleave fatty acid from glycerol backbone): Dissolve the dried residues (b) in 2 mL of 20% KOH solution (for preparation see 8 isoprostane measurement in cells). Vortex thoroughly and incubate for 1 h at 50°C. This will yield an aqueous solution (c).

11.5.6 Dilute 2 mL of the aqueous solution (c) with 3 mL of H_2O. Adjust the pH using 20% formic acid (132 µL) to pH~5.5. Add 2 mL ethyl acetate (1 part aqueous solution (c) + 1 part ethyl acetate), vortex thoroughly, and centrifuge at 2,000 rpm for ten minutes at 22°C. Repeat the procedure twice more using an equal volume of ethyl acetate per sample. Collect the upper phase containing saponified lipids.

11.5.7 Dry the pooled ethyl acetate upper phase (d) and dry in a Speedvac, yielding the dried sample-sediment (e). Store the sediment (e) at -20°C. For ELISA assay, dissolve the dried sample-residue (e) in 20 µL of ethanol, and then add 130 µL of 1x Sample Dilution Buffer.

11.5.8 For the competitive 8 isoprostane ELISA, the above 150 µL sample needs to be further diluted: Dilute 1:4 (e.g., 80 µL sample + 320 µL 1x Sample Dilution Buffer). Check the final pH (should be pH 7.4). When calculating the concentration, consider the dilution factor. In this case, 150 µL total sample volume from 1.8 mL plasma (12-fold concentration) and then, 80 sample in 400 µL SDB (5-fold dilution). Since, the samples are concentrated 2.4-fold; to get the actual concentration, you must divide by 2.4.

11.5.9 Perform the ELISA for 8 isoprostane (according to the instructions of the manufacturer).

12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents
- Unused well strips should be returned to the plate packet and stored at 4°C
- For each assay performed, a minimum of 2 wells must be used as a blank, omitting sample and conjugate from well addition. Another 2 wells must be used for a maximum binding control
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)

13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- Please read the test protocol carefully before performing the assay. Result reliability depends on strict adherence to the test protocol as described.
- If performing the test on an automatic ELISA system we recommend increasing the washing steps from three to five and the volume of 1X Wash Buffer from 300 µL to 350 µL to avoid washing effects.
- We recommend that you assay all standards, controls and samples in duplicate.

13.1 Add 200 µL of 1X Sample Dilution Buffer into the blank wells and 100 µL of 1X Sample Dilution Buffer into maximum binding control wells.
13.2 Add 100 µL of each of the standards or samples into the appropriate wells.
13.3 Add 100 µL of the 1X-HRP conjugate in the all wells except the blank control wells.
13.4 Incubate the plate at room temperature for two hours.
13.5 Wash the plate three times with 400 µL of 1X Wash Buffer per well.
13.6 After the last of the three wash cycles pat the inverted plate dry onto some paper towels.
13.7 Add 200 µL of the TMB substrate to all of the wells.
13.8 Incubate the plate at room temperature for 15-30 minutes.
13.9 Add 50 µL of 2 N sulfuric acid to all of the wells.
13.10 Read the plate at 450 nm.

14. Calculations

If data redaction software is not available on your plate reader then the results can be obtained manually as follows:

14.1 Average the absorbance (Abs) readings from the blank wells 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.)

14.2 Average the corrected absorbance readings from the maximum binding control wells. This is your maximum binding.

14.3 Calculate the % Abs for Standard 1 by averaging the corrected absorbance of the two wells; divide the average by the Maximum Binding Control well average absorbance, then multiply by 100. Repeat this formula for the remaining standards.

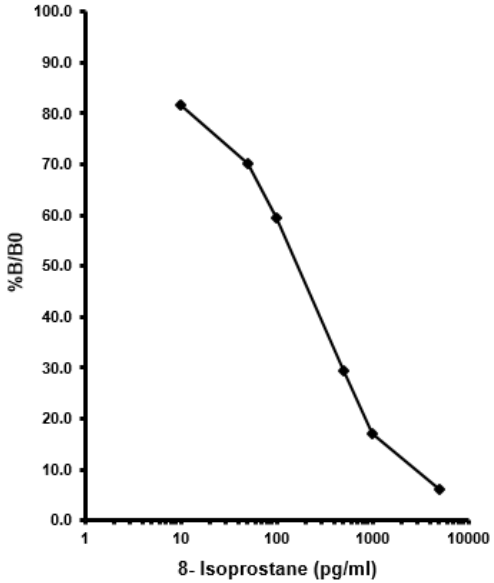
14.4 Plot the % Abs versus the concentration of 8 isoprostane from the standards using semi-log paper.

14.5 Calculate the % Abs for the samples and determine the concentrations, utilizing the standard curve.

14.6 Multiply the concentrations obtained for each of the samples by their corresponding dilution factor.

15. Typical Data

Typical standard curve – data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.



Conc. (pg/mL)	% (B/B_0)
0	81.6
50	70.2
100	59.5
500	29.4
1,000	17.1
5,000	6.2

Figure 1. Example of typical results obtained using the Abcam's 8 isoprostane ELISA kit. These results are only a guideline, and should not be used to determine values from your samples.

16. Assay Specificity

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

Eiconsanoid	Reactivity
8 isoprostane	100.00%
2,3-dinor-8-isoPGF2a	<0.01%
2,3-dinor-11β-PGF2a	<0.01%
PGF 1a	<0.01%
8-iso PGE2	<0.01%
8-iso PGE1	<0.01%

SENSITIVITY-

The calculated minimal detectable (MDD) dose is 1 pg/mL. The MDD was determined by calculating the mean of zero standard replicates.

Please contact our Technical Support team for more information.

17. Troubleshooting

Problem	Reason	Solution
Low signal	Incubation time too short	Try overnight incubation at 4 °C
	Precipitate can form in wells upon substrate addition when concentration of target is too high	Increase dilution factor of sample
	Using incompatible sample type (e.g. serum vs. cell extract)	Detection may be reduced or absent in untested sample types
	Sample prepared incorrectly	Ensure proper sample preparation/dilution
Large CV	Bubbles in wells	Ensure no bubbles present prior to reading plate
	All wells not washed equally/thoroughly	Check that all ports of plate washer are unobstructed/wash wells as recommended
	Incomplete reagent mixing	Ensure all reagents/master mixes are mixed thoroughly
	Inconsistent pipetting	Use calibrated pipettes & ensure accurate pipetting
	Inconsistent sample preparation or storage	Ensure consistent sample preparation and optimal sample storage conditions (e.g. minimize freeze/thaws cycles)

High background	Wells are insufficiently washed	Wash wells as per protocol recommendations
	Contaminated wash buffer	Make fresh wash buffer
	Waiting too long to read plate after adding stop solution	Read plate immediately after adding stop solution
Low sensitivity	Improper storage of ELISA kit	Store all reagents as recommended. Please note all reagents may not have identical storage requirements.
	Using incompatible sample type (e.g. Serum vs. cell extract)	Detection may be reduced or absent in untested sample types

18. Notes

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