



Product Manual

8-iso-PGF_{2α} ELISA kit

Catalog #: ADI-900-010

96 Well Kit

For use with culture supernatants, urine, and tissue.



Product Manual

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Please read
entire booklet
before
proceeding with
the assay.



Carefully note
the handling
and storage
conditions of
each kit
component.



Please contact
Enzo Life
Sciences
Technical
Support if
necessary.

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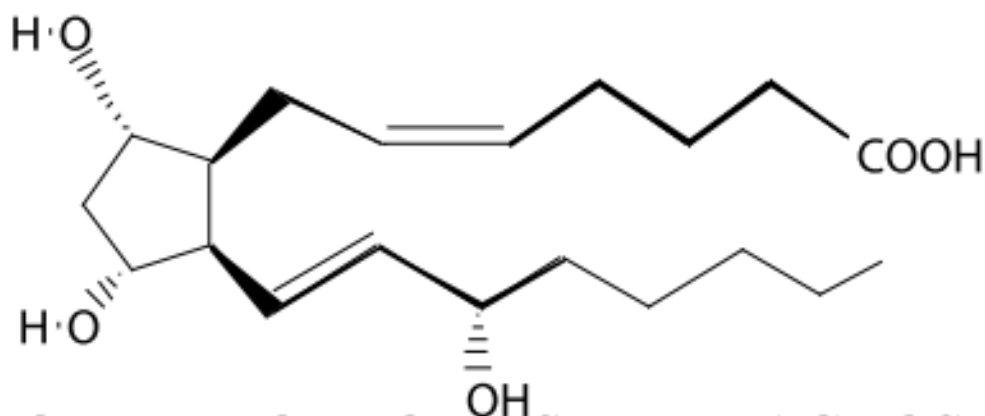
DESCRIPTION

The 8-iso-PGF_{2α} ELISA kit is a competitive immunoassay for the quantitative determination of free 8-iso-Prostaglandin F_{2α} in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to 8-iso-PGF_{2α} to bind, in a competitive manner, 8-iso-PGF_{2α} in a sample or an alkaline phosphatase molecule which has 8-iso-PGF_{2α} covalently attached to it. After a simultaneous incubation at room temperature the excess reagents are washed away and substrate is added. After a short incubation time the enzyme reaction is stopped and the yellow color generated is read on a microplate reader at 405 nm. The intensity of the bound yellow color is inversely proportional to the concentration of 8-iso-PGF_{2α} in either standards or samples. The measured optical density is used to calculate the concentration of 8-iso-PGF_{2α}. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard¹ or Tijssen².

INTRODUCTION

The 8-epimer of Prostaglandin F_{2α} (8-iso-PGF_{2α}) is produced in vivo by both non-cyclooxygenase and cyclooxygenase dependant mechanisms from arachidonic acid³⁻⁵. 8-iso-PGF_{2α} has been shown to be a potent vasoconstrictor^{5,6}, a potential mediator of hepatorenal syndrome and atherosclerosis⁵ and a mutagen in 3T3 cells and in vascular smooth muscle cells^{5,6,7}. It has also been postulated to participate as a pathophysiological mediator and is able to modify the fluidity and integrity of membranes⁵. 8-iso-PGF_{2α} has been shown to circulate in plasma and is excreted in urine⁶.

8-iso-Prostaglandin F_{2α}



SAFETY WARNINGS & PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.



Handle
with care

- Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up.
- Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
- The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions. The activity of the conjugate is affected by high concentrations of chelators, such as EDTA and EGTA.
- We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
- The 8-iso-Prostaglandin $F_{2\alpha}$ Standard provided, Catalog No. 80-0110, is supplied in ethanolic buffer at a pH optimized to maintain 8-iso-PGF_{2 α} integrity. Care should be taken handling this material because of the known and unknown effects of prostaglandins.

MATERIALS SUPPLIED

1. **Goat anti-Rabbit IgG Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0060**
A plate using break apart strips coated with goat antibody specific to rabbit IgG.
2. **8-iso-PGF_{2α} ELISA Conjugate, 5ml, Catalog No. 80-0108**
A blue solution of alkaline phosphatase conjugated with 8-iso-PGF_{2α}.
3. **8-iso-PGF_{2α} ELISA Antibody, 5 mL, Catalog No. 80-0109**
A yellow solution of a rabbit polyclonal antibody to 8-iso-PGF_{2α}.
4. **Assay Buffer Concentrate, 27 mL, Catalog No. 80-0011**
Tris buffered saline containing proteins and sodium azide as a preservative.
5. **Wash Buffer Concentrate, 27 mL, Catalog No. 80-1286**
Tris buffered saline containing detergents.
6. **8-iso-Prostaglandin F_{2α} Standard, 0.5ml, Catalog No. 80-0110**
A solution of 1,000,000 pg/mL 8-iso-PGF_{2α}.
7. **pNpp Substrate, 20 mL, Catalog No. 80-0075**
A solution of p-nitrophenyl phosphate in buffer. Ready to use.
8. **Stop Solution, 5 mL, Catalog No. 80-0247.**
A solution of trisodium phosphate in water. Keep tightly capped. **CAUTION: Caustic.**
9. **8-iso-PGF_{2α} Assay Layout Sheet, 1 each, Catalog No. 30-0021**
10. **Plate Sealer, 1 each, Catalog No. 30-0012**



Reagents
require
separate
storage
conditions.

STORAGE

All components of this kit are stable at 4°C until the kit's expiration date.

OTHER MATERIALS NEEDED

1. Deionized or distilled water.
2. Precision pipets for volumes between 5 µL and 1,000 µL.
3. Repeater pipets for dispensing 50 µL and 200 µL.
4. A disposable beaker for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. Microplate reader capable of reading at 405 nm, preferably with correction at between 570 and 590 nm.

SAMPLE HANDLING

The 8-iso-PGF_{2α} ELISA kit is compatible with 8-iso-PGF_{2α} culture supernatants and urine after dilution in Assay Buffer. Tissue may be used after extraction. Serum and plasma are not suitable for use in this assay and should be analyzed in the Direct 8-iso-PGF_{2α} ELISA (catalog number ADI-900-091). Please refer to the Sample Recovery recommendations on page 15 for details of suggested dilutions. However, the end user must verify that the recommended dilutions are appropriate for their samples. Samples containing rabbit IgG may interfere with the assay.

Samples in the majority of tissue culture media, including those containing fetal bovine serum, can also be read in the assay, provided the standards have been diluted into the diluted non-conditioned tissue culture media instead of Assay Buffer. There will be a small change in binding associated with running the standards and samples in media. Tissue culture media must be diluted at least 1:10 in the assay buffer. Users should only use standard curves generated in media or buffer to calculate concentrations of 8-iso-PGF_{2α} in the appropriate matrix. For measurement of free isoprostane in tissue homogenates or urine, prostaglandin synthetase inhibitors, such as indomethacin or meclofenamic acid at concentrations up to 10 µg/mL should be added to the samples. Urine samples may be used in the assay directly after diluting in Assay Buffer. If low levels of urinary 8-iso-PGF_{2α} are expected, adjust the pH to 3.0. Add 3 parts ice-cold methanol to 1 part urine. Vortex the samples and incubate at -80°C for 30 minutes, then centrifuge samples in a microcentrifuge for 10 min. The supernatant may then be diluted 1:10-1:50 in Assay Buffer. Samples that have very low levels of 8-iso-PGF_{2α} must be extracted for accurate measurement. A suitable extraction procedure is outlined below:

Materials Needed

1. 8-iso-PGF_{2α} Standard to allow extraction efficiency to be accurately determined.
2. 2 M hydrochloric acid, deionized water, ethanol, hexane and ethyl acetate.
3. 200 mg C18 Reverse Phase Extraction Columns.

Procedure

1. Acidify the sample by addition of 2M HCl to pH of 3.5. Allow to sit at 4°C for 15 minutes. Centrifuge samples in a microcentrifuge for 2 minutes to remove any precipitate.
2. Prepare the C18 reverse phase column by washing with 10 mL of ethanol followed by 10ml of deionized water.
3. Apply the sample under a slight positive pressure to obtain a flow rate of about 0.5ml/ minute. Wash the column with 10 mL of water, followed by 10ml of 15% ethanol, and finally 10 mL hexane. Elute the sample from the column by addition of 10ml ethyl acetate.
4. If analysis is to be carried out immediately, evaporate samples under a stream of nitrogen. Add at least 250 µL of Assay Buffer to the dried samples. Vortex will then allow to sit at room temperature for five minutes. Repeat twice more. If analysis is to be delayed, store samples as the eluted ethyl acetate solutions at -80°C until the immunoassay is to be run. Evaporate the organic solvent under a stream of nitrogen prior to running assay and reconstitute as above.

Please refer to references 8-11 for details of extraction protocols.

PROCEDURAL NOTES

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided.
8. Care must be taken to minimize contamination by endogenous alkaline phosphatase. Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.
9. Prior to the addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.

REAGENT PREPARATION

1. Assay Buffer

Just before use, prepare the assay buffer by diluting 10 mL of the supplied Assay Buffer Concentrate with 90 mL of deionized water. Discard unused buffer or add up to 0.09% sodium azide (w/v) for storage.

2. 8-iso-PGF_{2α} Standard

Allow the 1,000,000 pg/mL 8-iso-PGF_{2α} standard solution to warm to room temperature. Label eight 12 x 75 mm glass tubes #1 through #8. Pipet 900 µL of standard diluent (Assay Buffer or Tissue Culture Media) into tube #1. Pipet 750 µL of standard diluent into tubes #2 through #8. Add 100 µL of the 1,000,000 pg/mL standard to tube #1. Vortex thoroughly. Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Add 250 µL of tube #2 to tube #3 and vortex. Continue this for tubes #4 through #8. The concentration of 8-iso-PGF_{2α} in tubes #1 through #8 will be 100,000, 25,000, 6,250, 1,562.5, 390.6, 97.7, 24.4 and 6.1 pg/mL respectively. See the 8-iso-PGF_{2α} Assay Layout Sheet for dilution details. Diluted Standard should be used within 60 minutes of preparation.

3. Conjugate 1:10 Dilution for Total Activity Measurement

Prepare the conjugate 1:10 Dilution by diluting 50 µL of the supplied conjugate with 450 µL of the Assay Buffer. The dilution should be used within 3 hours of preparation. This 1:10 dilution is intended for use in the Total Activity Wells ONLY.

4. Wash Buffer

Prepare the Wash Buffer by diluting 5 mL of the supplied concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

ASSAY PROCEDURE

Bring all reagents to room temperature for at least 30 minutes prior to opening. All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4°C.
2. Pipet 100 µL of standard diluent (Assay Buffer or Tissue Culture Media) into the NSB and the Bo (0 pg/mL Standard) wells.
3. Pipet 100 µL of Standards #1 through #8 into the appropriate wells.
4. Pipet 100 µL of the Samples into the appropriate wells.
5. Pipet 50 µL of Assay Buffer into the NSB wells.
6. Pipet 50 µL of blue Conjugate into each well, except the Total Activity (TA) and Blank wells.
7. Pipet 50 µL of yellow Antibody into each well, except the Blank, TA and NSB wells.

NOTE: Every well used should be Green in color except the NSB wells which should be Blue. The Blank and TA wells are empty at this point and have no color.

8. Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired.
9. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 2 more times for a total of 3 Washes.
10. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
11. 11. Add 5 µL of the light blue Conjugate 1:10 Dilution (see Step 3, Reagent Preparation, on Page 9) to the TA wells.
12. Add 200 µL of the pNpp Substrate solution to every well. Incubate at room temperature for 45 minutes without shaking.
13. Add 50 µL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.
14. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

CALCULATION OF RESULTS

Several options are available for the calculation of the concentration of 8-iso-PGF_{2α} in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of 8-iso-PGF_{2α} can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

$$\text{Average Net OD} = \text{Average Bound OD} - \text{Average NSB OD}$$

2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

$$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net Bo OD}} \times 100$$

3. Plot Percent Bound versus Concentration of 8-iso-PGF_{2α} for the standards. Approximate a straight line through the points. The concentration of 8-iso-PGF_{2α} in the unknowns can be determined by interpolation.

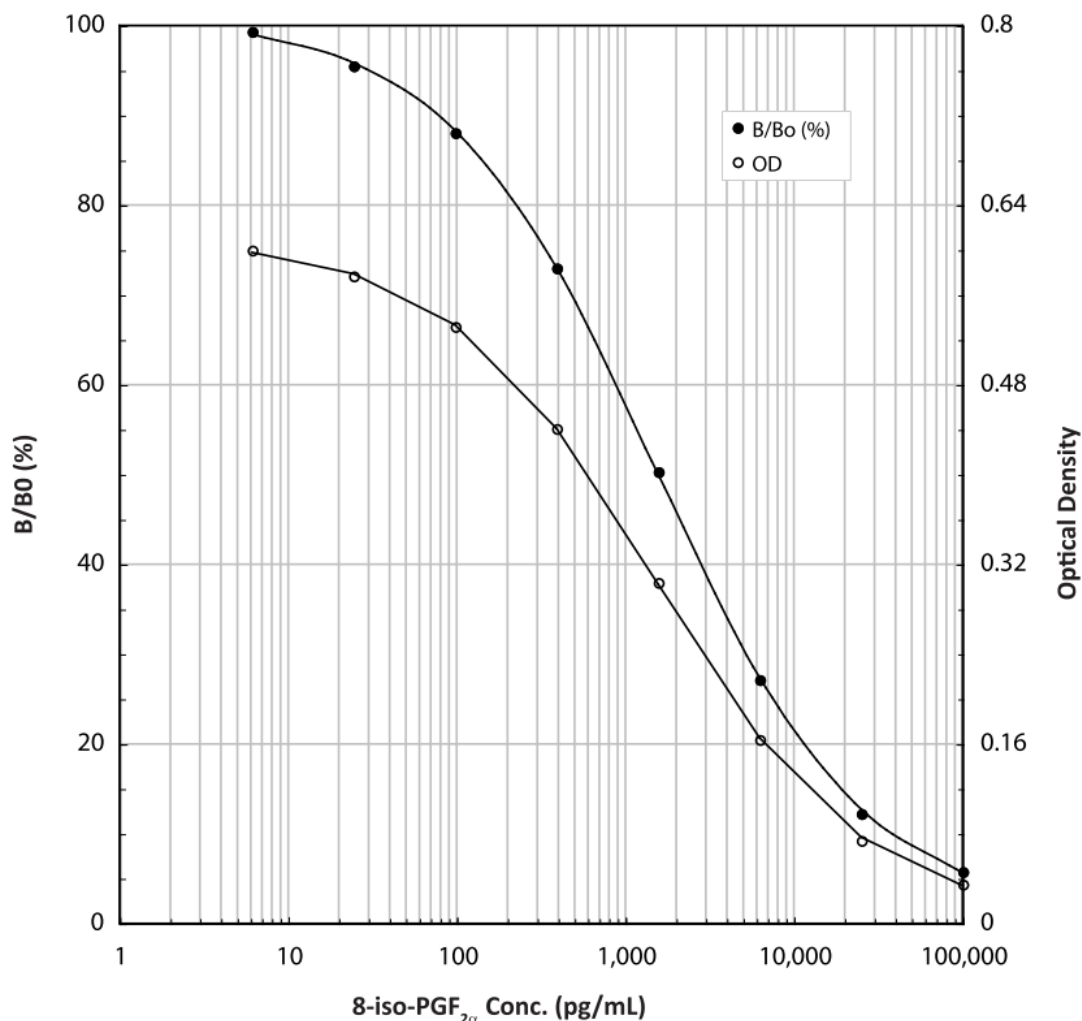
TYPICAL RESULTS

The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Mean OD (-Blank)	Average Net OD	Percent Bound	8-iso-PGF _{2α} (pg/mL)
Blank OD	(0.089)			
TA	0.401	0.401		
NSB	0.001	0.001	0.00%	
Bo	0.605	0.604	100%	0
S1	0.037	0.036	6.0%	100,000
S2	0.076	0.075	12.4%	25,000
S3	0.166	0.165	27.3%	6,250
S4	0.305	0.304	50.3%	1,562.5
S5	0.442	0.441	73.0%	390.6
S6	0.534	0.533	88.2%	97.7
S7	0.579	0.578	95.7%	24.4
S8	0.602	0.601	99.5%	6.1
Unknown 1	0.171	0.170	28.2%	5,944
Unknown 2	0.355	0.354	58.6%	965

TYPICAL STANDARD CURVES

A typical standard curve is shown below. This curve must not be used to calculate 8-iso-PGF_{2α} concentrations; each user must run a standard curve for each assay.



Typical Quality Control Parameters

Total Activity Added =	0.401 x 10 x10 =40.1
%NSB =	0.001%
%Bo/TA =	15.06
Quality of Fit =	1.000 (Calculated from 4 parameter logistic curve fit)
20% Intercept =	11,286 pg/mL
50% Intercept =	1,563 pg/mL
80% Intercept =	229 pg/mL

PERFORMANCE CHARACTERISTICS

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols¹².

Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run as Bo, and comparing to the average optical density for sixteen (16) wells run with Standard #8. The detection limit was determined as the concentration of 8-iso-PGF_{2α} measured at two (2) standard deviations from the zero along the standard curve.

Average Optical Density for the Bo = 0.364 ± 0.012 (3.298%)

Average Optical Density for Standard #8 = 0.355 ± 0.009 (2.535%)

Delta Optical Density (0-6.1 pg/mL) = $0.364 - 0.355 = 0.009$

2 SD's of the Zero Standard = $2 \times 0.012 = 0.024$

Sensitivity = $\frac{0.024}{0.009} \times 6.1 \text{ pg/mL} = 16.3 \text{ pg/mL}$

Linearity

A sample containing 10,000 pg/mL 8-iso-PGF_{2α} was diluted 8 times 1:2 in the kit Assay Buffer and measured in the assay. The data was plotted graphically as actual 8-iso-PGF_{2α} concentration versus measured 8-iso-PGF_{2α} concentration.

The line obtained had a slope of 1.01 and a correlation coefficient of 0.991.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of 8-iso-PGF_{2α} and running these samples multiple times (n=12) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of 8-iso-PGF_{2α} in multiple assays (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of 8-iso-PGF_{2α} determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	8-iso-PGF2 α (pg/mL)	Intra-assay %CV	Inter-assay %CV
Low	232	11.0	
Medium	1,128	4.4	
High	8,263	5.8	

Low	183		11.0
Medium	855		8.8
High	6,239		5.0

Cross Reactivities

The cross reactivities for a number of related eicosanoid compounds was determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer at concentrations from 100,000 to 6 pg/ml. These samples were then measured in the 8-iso-PGF_{2 α} assay and the measured 8-iso-PGF_{2 α} concentration at 50% B/Bo was calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Compound	Cross Reactivity
8-iso-PGF2 α	100%
PGF1 α	4.6%
PGF2 α	1.85%
PGE1	0.19%
TXB2	0.023%
PGB1	0.02%
PGE3	0.012%
6-keto-PGF1 α	0.008%
13,14-dihydro-15-keto-PGF2 α	0.008%
6,15-keto-13,14-dihydro-PGF1 α	0.005%
8-iso-PGE1	<0.001%
PGA2	<0.001%
PGJ2	<0.001%
2-Arachidonoylglycerol	<0.001%
Anandamide	<0.001%

SAMPLE RECOVERIES

Please refer to pages 6-9 for Sample Handling recommendations and Standard preparation. 8-iso-PGF_{2α} concentrations were measured in a variety of different samples including tissue culture media and urine. 8-iso-PGF_{2α} was spiked into the undiluted samples of these media, which were diluted into the assay buffer and then assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Tissue Culture Media	129.9	1:10
Human Urine	101.58	Neat - 1:60

* See Sample Handling on page 6 for details.

Serum and plasma are not suitable for analysis in this kit and should be measured in Direct 8-isoPGF_{2α} kit ELISA (catalog number ADI-900-091).

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NOTES



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