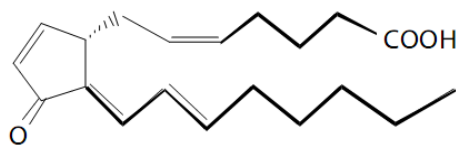


Introduction and Background

A. Overview

15-deoxy- $\Delta^{12,14}$ -Prostaglandin J₂ is one of the ultimate dehydration products of PGD₂. PGD₂ is formed from PGH₂ which itself is synthesized from arachidonic acid by the enzyme prostaglandin synthase¹⁻⁴. In the aqueous solution PGD₂ forms PGJ₂₅. Albumin or other serum proteins present in the sample will convert PGD₂ to the isomeric compound, Δ^{12} -PGJ₂⁶⁻⁸. In males about 152 ng of Δ^{12} -PGJ₂ is excreted in urine per day, and in women about half this amount is excreted⁹. Excellent recent reviews^{10,11} of the biological actions of these compounds have been published. 15-d-PGJ₂ has been shown as an inducer of adipogenesis and an activator of the γ isoform of the peroxisome proliferation activated receptor (PPAR γ)¹².

15-deoxy- $\Delta^{12,14}$ -Prostaglandin J₂



B. Test Principle

The 15-deoxy- $\Delta^{12,14}$ -PGJ₂ ELISA Kit is a competitive immunoassay for the quantitative determination of 15-deoxy- $\Delta^{12,14}$ -Prostaglandin J₂ (15-d-PGJ₂) in biological fluids. The kit uses a polyclonal antibody to 15-d-PGJ₂ to bind, in a competitive manner, the 15-d-PGJ₂ in the sample or an alkaline phosphatase molecule which has 15-d-PGJ₂ covalently attached to it. After a simultaneous incubation 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 read on a microplate reader at 405nm. The intensity of the bound yellow color is inversely proportional to the concentration of 15-d-PGJ₂ in either standards or samples. The measured optical density is used to calculate the concentration of 15-d-PGJ₂. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard¹³ or Tijssen¹⁴.

C. Additional Required Materials

1. Deionized or distilled water.
2. Precision pipets for volumes between 5 μ L and 1,000 μ L.
3. Repeater pipets for dispensing 50 μ L and 200 μ L.
4. Disposable beaker for diluting buffer concentrates.
5. Graduated cylinders.
6. A Microplate shaker.
7. Adsorbent paper for blotting.
8. A 37°C Incubator.
9. Microplate reader capable of reading at 405 nm, preferably with correction at between 570 and 590 nm.

D. Procedural Guidelines

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

1. 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.
2. Stop solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
3. The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg²⁺ and Zn²⁺ ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA.
4. 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.
5. The 15-deoxy- Δ 12,14-PGJ2 standard provided, is supplied in ethanolic buffer at a pH optimized to maintain 15-d-PGJ2 integrity. Care should be taken handling this material because of the known and unknown effects of prostaglandins.

Material and Method

A. List of component

Component	Description	Amount
Goat anti-Rabbit IgG Microtiter Plate	A plate using break-apart strips coated with goat antibody specific to rabbit IgG.	One Plate 96 Wells
15-d-PGJ2 EIA Conjugate	A blue solution of alkaline phosphatase conjugated with 15-d-PGJ2	5 mL
15-d-PGJ2 EIA Antibody	A yellow solution of a polyclonal antibody to 15-d-PGJ2	5 mL
Assay Buffer	Tris buffered saline, containing proteins and sodium azide as preservative.	27 mL
Wash Buffer Concentrate	Tris buffered saline containing detergents.	27 mL
15-deoxy- Δ 12,14-Prostaglandin J2 Standard	A solution of 1,000,000 pg/mL 15-d-PGJ2	0.5 mL
p-Npp Substrate	A solution of p-nitrophenyl phosphate in buffer. Ready to use.	20 mL
Stop Solution	A solution of trisodium phosphate in water. Keep tightly capped. Caution: Caustic.	5 mL
Plate Sealer	-	2 each

B. Preparation of reagent

Sample preparation

The 15-d-PGJ2 EIA is compatible with 15-d-PGJ2 samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer can be read directly from the standard curve. Please refer to the Sample Recovery recommendations 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. Some samples normally have very low levels of 15-d-PGJ2 present and extraction may be necessary for accurate measurement. A suitable extraction procedure is outlined below:

Materials Needed

1. 15-d-PGJ2 Standard to allow extraction efficiency to be accurately determined.
2. 2M hydrochloric acid, deionized water, ethanol, hexane and ethyl acetate.
3. 200 mg C₁₈ Reverse Phase Extraction Columns.

Procedure

1. Acidify the plasma, urine or tissue homogenate by addition of 2M HCl to pH of 3.5. Approximately 50 μ L of HCl will be needed per mL of plasma. 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 10 mL of deionized water.
3. Apply the sample under a slight positive pressure to obtain a flow rate of about 0.5 mL/minute. Wash the column with 10 mL of water, followed by 10 mL of 15% ethanol, and finally 10 mL hexane. Elute the sample from the column by addition of 10 mL ethyl acetate.
4. If analysis is to be carried out immediately, evaporate samples under a stream of nitrogen. Add at least 250 μ L of Assay Buffer to the dried samples. Vortex well then allow to sit 5 minutes at room temperature. 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 15-18 for details of extraction protocols.

Note:

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 the assay results.

Preparation of 15-d-PGJ2

Allow the 1,000,000 pg/mL 15-d-PGJ2 standard solution to warm to room temperature. Label six 12 x 75 mm glass tubes #1 through #6. Pipet 800 µL of standard diluent (Assay Buffer or Tissue Culture Media) into tube #1. Pipet 750 µL of standard diluent into tubes #2 through #6. Add 200 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. Continue this for tubes #2 through #6.

The concentration of 15-d-PGJ2 in tubes #1 through #6 will be 200,000, 50,000, 12,500, 3,125, 781 and 195 pg/mL respectively. See 15-d-PGJ2 Assay Layout Sheet for dilution details.

Diluted Standards should be used within 60 minutes of preparation.

15-d-PGJ2 Conjugate

Allow the conjugate to warm to room temperature. Any unused conjugate should be aliquoted and re-frozen at or below -20 °C. Avoid repeated freeze/thaws of the aliquots.

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 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.

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.

Protocol

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 #6 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 remaining wash buffer.
11. Add 5 µL of the light blue Conjugate 1:10 Dilution (see Step 3, Reagent Preparation) to the TA wells.
12. Add 200 µL of the pNpp Substrate solution to every well. Cover plate with the plate sealer provided and incubate at 37°C for 3 hours.
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.

Calculations

Several options are available for the calculation of the concentration of 15-d-PGJ₂ in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If this sort of data reduction software is not readily available, the concentration of 15-d-PGJ₂ 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:
Average Net OD = Average Bound OD - 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:
Percent Bound = Net OD/ Net Bo OD x 100
3. Using Logit-Log paper plot Percent Bound versus Concentration of 15-d-PGJ₂ for the standards. Approximate a straight line through the points. The concentration of 15-d-PGJ₂ in the unknowns can be determined by interpolation.

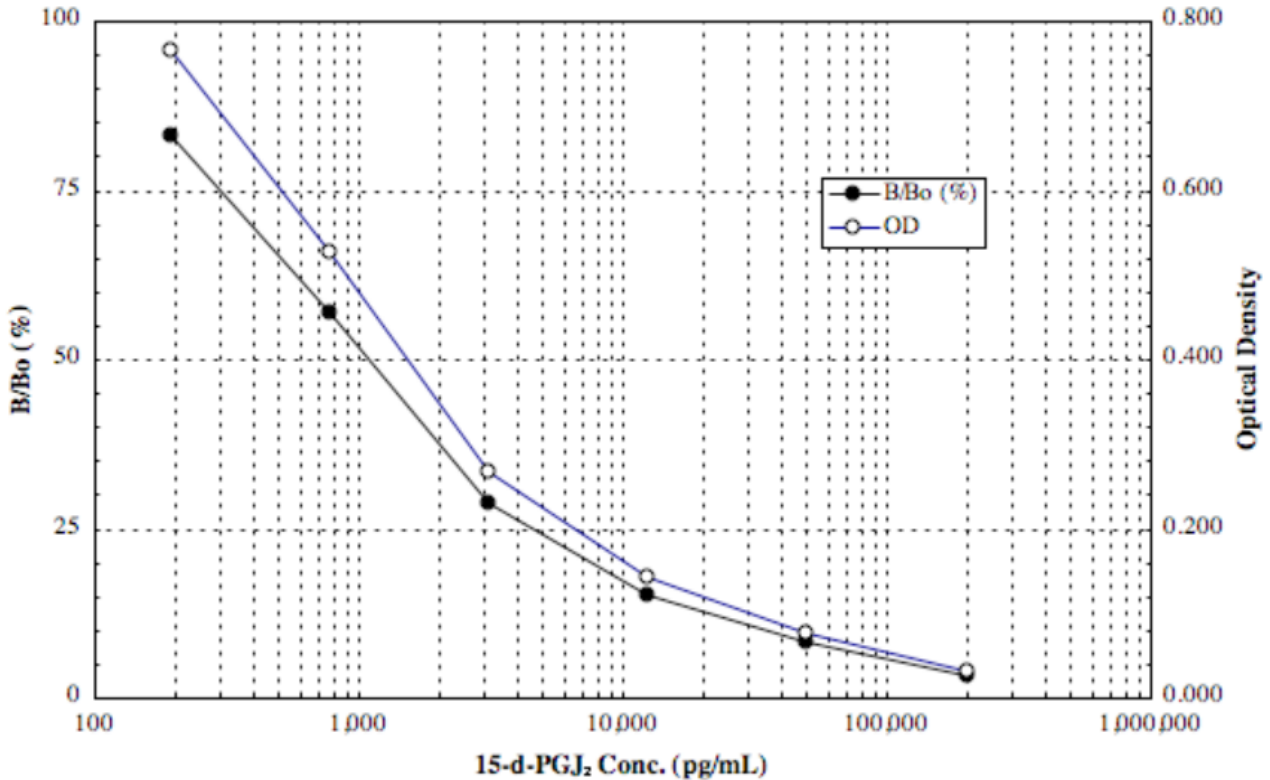
Typical Results

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

	Mean	Average	Percent	15-d-PGJ ₂
Sample	OD (-Blank)	Net OD	Bound	(pg/mL)
Blank OD	(0.134)			
TA	2.696			
NSB	0.009	0.000	0.00%	
Bo	0.932	0.923	100%	0
S1	0.034	0.031	3.4%	200,000
S2	0.086	0.077	8.3%	50,000
S3	0.151	0.142	15.4%	12,500
S4	0.276	0.267	28.9%	3,125
S5	0.535	0.526	57.0%	781
S6	0.775	0.766	83.0%	195
Unknown 1	0.624	0.615	66.6%	486
Unknown 2	0.102	0.093	10.1%	23,638

Typical Standard Curve

A typical standard curve is shown below. This curve must not be used to calculate 15-d-PGJ₂ concentrations; each user must run a standard curve for each assay.



Typical Quality Control Parameters

Total Activity Added	=	2.696 x 10 x 10 = 269.6
%NSB	=	0.0%
%Bo/TA	=	0.3%
Quality of Fit	=	0.9997 (Calculated from 4 parameter logistic curve fit)
20% Intercept	=	7,636 pg/mL
50% Intercept	=	1,104 pg/mL
80% Intercept	=	247 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 Protocols¹⁹.

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 #6. The detection limit was determined as the concentration of 15-d-PGJ2 measured at two (2) standard deviations from the zero along the standard curve.

Average Optical Density for the Bo	=	1.300 ± 0.030 (2.3%)	
Average Optical Density for Standard #6	=	0.983 ± 0.050 (5.1%)	
Delta Optical Density (0-195 pg/mL)	=	0.318	
2 SD's of the Zero Standard	=	2 x 0.030	= 0.060
Sensitivity	=	0.060/0.318 x 195 pg/mL	= 36.8 pg/mL

Linearity

A sample containing 13,011 pg/mL 15-d-PGJ2 was diluted 4 times 1:2 in the kit Assay Buffer and measured in the assay. The data was plotted graphically as actual 15-d-PGJ2 concentration versus measured 15-d-PGJ2 concentration.

The line obtained had a slope of 0.974 and a correlation coefficient of 0.998.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of 15-d-PGJ2 and running these samples multiple times (n=16) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of 15-d-PGJ2 in multiple assays (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of 15-d-PGJ2 determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	15-d-PGJ2 (pg/mL)	Intra-assay %CV	Inter-assay %CV
Low	1,136	5.6	
Medium	3,062	5.7	
High	4,394	7.4	
Low	1,195		15.7
Medium	4,406		13.0
High	7,219		14.5

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 500,000 to 5 pg/mL. These samples were then measured in the 15-d-PGJ2 assay, and the measured 15-d-PGJ2 concentration at 50% B/Bo 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
15-deoxy - Δ 12,14-PGJ2	100%
PGJ2	49.2%
Δ 12-PGJ2	5.99%
PGD2	4.92%
Arachidonic Acid	0.03%
PGE2	<0.01%
PGF2 α	<0.01%
9 α ,11 β -PGF2 α	<0.01%
Thromboxane B2	<0.01%
2-Arachidonoylglycerol	<0.01%
Anandamide	<0.01%

Sample Recoveries

Please refer to 4 and 5 for Sample Handling recommendations and Standard preparation. 15-d-PGJ2 concentrations were measured in a variety of different samples including tissue culture media, human saliva, human urine and porcine plasma. 15-d-PGJ2 was spiked into the samples which were diluted with the appropriate diluent and assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Tissue Culture Media	97.4	1:2
Human Saliva	94.4	None
Human Urine	113.9	1:16
Porcine Plasma	105.0	\geq 1:4

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15-d-PGJ2 Plate Layout:

A1 Blank	A2 Std1	A3 Std5	A4	A5	A6	A7	A8	A9	A10	A11	A12
B1 Blank	B2 Std1	B3 Std5	B4	B5	B6	B7	B8	B9	B10	B11	B12
C1 TA	C2 Std2	C3 Std6	C4	C5	C6	C7	C8	C9	C10	C11	C12
D1 TA	D2 Std2	D3 Std6	D4	D5	D6	D7	D8	D9	D10	D11	D12
E1 NSB	E2 Std3	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F1 NSB	F2 Std3	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G1 Bo	G2 Std4	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H1 Bo	H2 Std4	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

Kit Lot No. _____ Exp. Date _____ Date _____ Tech. _____

1st Incub.: Start Time _____ Temp. _____

Notes: _____

End Time _____ Temp. _____

2nd Incub.: Start Time _____ Temp. _____

End Time _____ Temp. _____