Version 22-05/24

User's Manual

Prostacyclin ELISA Kit

REF **DEIA4105**

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RUO

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

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

Urinary Prostacyclin ELISA Kit is a competitive immunoassay for the quantitative determination of Prostacyclin (PGI2) breakdown products in urine. FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

General Description

Prostacyclin (PGI2) is involved in platelet aggregation, vasoconstriction, and reproductive functions. PGI2 has a half life of 60 minutes in plasma but only 2 to 3 minutes in buffer. The production of PGI2 is typically monitored by measurement of 6k-PGF1α and 2,3d-6k-PGF1α. 6-keto-PGF1α is produced by the non-enzymatic hydration of PGI2, and is further metabolized in urine to the 2,3-dinor derivative. A number of pharmaceuticals alter and/or inhibit the synthesis of PGI2 . Methods to measure PGI2 in urine typically involve HPLC, gas chromatography/mass spectrometry, or radioimmunoassay and enzyme immunoassay for 6k-PGF1α. The conversion of PGI2 into 6k-PGF1α and 2,3d-6k-PGF1α is shown below.

Principles of Testing

The Prostacyclin ELISA kit is a competitive immunoassay for the quantitative determination of Prostacyclin (PGI2) breakdown products in urine. Please read the complete kit insert before performing this assay. The kit measures the two major breakdown products of PGI2 in urine, 6-keto-Prostaglandin F1α (6k-PGF1α) and 2,3-dinor-6-keto-Prostaglandin F1α (2,3d-6k-PGF1α). The kit uses a polyclonal antibody that binds these two molecules with almost equal affinities. This antibody binds in a competitive manner, both the 6k-PGF1α and 2,3d-6k-PGF1α in the sample, or an alkaline phosphatase molecule which has 6k-PGF1α 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 color generated is read on a microplate reader at 405 nm. The intensity of the bound yellow color is inversely proportional to the total concentration of 6k-PGF1 α and 2,3d-6k-PGF1α in either standards or samples. The measured optical density of the 2,3d-6k-PGF1α standards is used to calculate the concentration of both 6k-PGF1α and 2,3d-6k-PGF1α in the sample.

Reagents And Materials Provided

1. One 96 Well Donkey anti-Sheep IgG Plate: A plate using break-apart strips coated with donkey antibody specific to sheep IgG. 96 (8x12) wells
2. Urinary Prostacyclin Conjugate: A blue solution of alkaline phosphatase conjugated with 6-keto-PGF1α. 5 mL
3. Urinary Prostacyclin Antibody: A yellow solution of a sheep polyclonal antibody to 6k-PGF1α and 2,3d-6k-PGF1α. 5 mL
4. Assay Buffer: Tris buffered saline containing proteins and detergents and sodium azide as preservative. 27 mL
5. Wash Buffer Concentrate: Tris buffered saline containing detergents. 27 mL

6. Urinary Prostacyclin Standard: A solution of 100,000 pg/mL 2,3d-6k-PGF1α. 0.5 mL
7. p-Npp Substrate: A solution of p-nitrophenyl phosphate in buffer. Ready to use. 20 mL
8. Stop Solution: A solution of trisodium phosphate in water. Keep tightly capped. Caution: Caustic. 5 mL
9. Plate Sealer: 1 slide

Materials Required But Not Supplied

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. Disposable beaker for diluting Wash Buffer.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.

Storage

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

Specimen Collection And Preparation

The Prostacyclin ELISA Kit is compatible with 6k-PGF1α and 2,3d-6k-PGF1α samples in urine, tissue culture and buffer after dilution in Assay Buffer. Please refer to the Recovery recommendations for details of suggested dilutions. However, the end user must verify that the recommended dilutions are appropriate for their samples. Samples containing sheep 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 tissue culture media instead of Assay Buffer. There will be small change in binding associated with running the standards and samples in media. Users should only use standard curves generated in media or buffer to calculate concentrations of 6k-PGF1α and 2,3d-6k-PGF1α in the appropriate matrix. For urine samples, prostaglandin synthetase inhibitors, such as, indomethacin or meclofenamic acid at concentrations up to 10 µg/mL should be added to the urine samples. Extraction may be necessary for measurement in some situations; a suitable extraction procedure is outlined below:

Materials Needed

Urinary Prostacyclin standard to accurately determine the extraction efficiency.

2 M hydrochloric acid, deionized water, ethanol, hexane and ethyl acetate.

200 mg C18 Reverse Phase Extraction Columns.

Procedure

1. Add sufficient Urinary Prostacyclin Standard to a typical sample for the determination of extraction efficiency.

2. Acidify the sample by addition of 2 M 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.
3. Prepare the C18 reverse phase column by washing with 10 mL of ethanol followed by 10 mL of deionized water.
4. 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.
5. If analysis is to be carried out immediately, evaporate samples under a stream of nitrogen. Reconstitute sample with at least 200 µL of Assay Buffer. If analysis is to be delayed, store samples as the eluted ethyl acetate solutions at -80°C until the immunoassay is to be run. Prior to analysis, evaporate the organic solvent under a stream of nitrogen and reconstitute as above.

*Please refer to references 12-15 for details of extraction protocols.

Plate Preparation

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Std 1	Std 5	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
B	Blank	Std 1	Std 5	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
C	TA	Std 2	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
D	TA	Std 2	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
E	NSB	Std 3	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
F	NSB	Std 3	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
G	Bo	Std 4	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
H	Bo	Std 4	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample

Reagent Preparation

1. Urinary Prostacyclin Standard: Allow the 100,000 pg/mL 2,3d-6k-PGF1α standard solution to warm to room temperature. Label five 12 × 75 mm glass tubes #1 through #5. Pipet 1 mL of standard diluent (Assay Buffer or Tissue Culture Media) into tube #1. Pipet 750 µL of standard diluent into tubes #2 through #5. Remove 20 µL of diluent from tube #1. Add 20 µL of the 100,000 pg/mL stock 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 #5. The concentration of 2,3d-6k-PGF1α in tubes #1 through #5 will be 2,000, 500, 125, 31.25 and 7.81 pg/mL, respectively. Diluted standards should be used within 60 minutes of preparation.

Std.	Diluent Vol. (µL)	Vol. Added (µL)	Urinary Prostacyclin Conc. (pg/mL)
1	980	20, Stock	2,000
2	750	250, Std. 1	500
3	750	250, Std. 2	125
4	750	250, Std. 3	31.25
5	750	250, Std. 4	7.81

2. 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.
3. 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 for 3 months.

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 Plate Layout 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 #5 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 the 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 the wells and tap the plate dry on a lint free paper towel.
11. Add 5 µL of the blue Conjugate 1:10 Dilution (see step Conjugate 1:10 Dilution for Total Activity Measurement, Reagent Preparation) to the TA wells.
12. Add 200 µL of the p-Npp 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.

Short protocol

Well I.D.	Blank	TA	NSB	B0	Stds.	Samples
	A1, B1	C1, D1	E1, F1	G1, H1	A2-F3	C3-H12
Standard Diluent	---	---	100 µL	100 µL	---	---
Assay Buffer	---	---	50 µL	---	---	---
Std. and/or Sample	---	---	---	---	100 µL	100 µL
Conjugate	---	---	50 µL	50 µL	50 µL	50 µL
Antibody	---	---	---	50 µL	50 µL	50 µL
Incub. 2 hours at RT, shaking	→	→	→	→	→	→
Asp. & Wash 3 x 200 µL	→	→	→	→	→	→
Conjugate	---	5 µL*	---	---	---	---
Substrate	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL
Incub. 45 min at RT	→	→	→	→	→	→
Stop Solution	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL

Note: Conjugate for TA must be diluted 1:10 in Assay Buffer. Add 500 µL of Assay Buffer to a clean test tube. From this volume, remove 50 µL of Assay Buffer and, using the same measuring device, add 50 µL of Urinary Prostacyclin Conjugate.

Quality Control

Typical Quality Control Parameters

Total Activity Added = 0.443 × 100 = 44.3

%NSB = 0.0%

%Bo/TA = 2.5%

Quality of Fit = 1.0000 (Calculated from 4 parameter logistic curve fit)

20% Intercept = 270.0 pg/mL

50% Intercept = 62.9 pg/mL

80% Intercept = 14.6 pg/mL

Calculation

Several options are available for the calculation of the Urinary Prostacyclin (UP) concentration 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 UP 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 OD — 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) × 100

3. Using Logit-Log paper plot Percent Bound versus Concentration of UP for the standards. Approximate a straight line through the points. The concentration of UP in the unknowns can be determined by

interpolation.

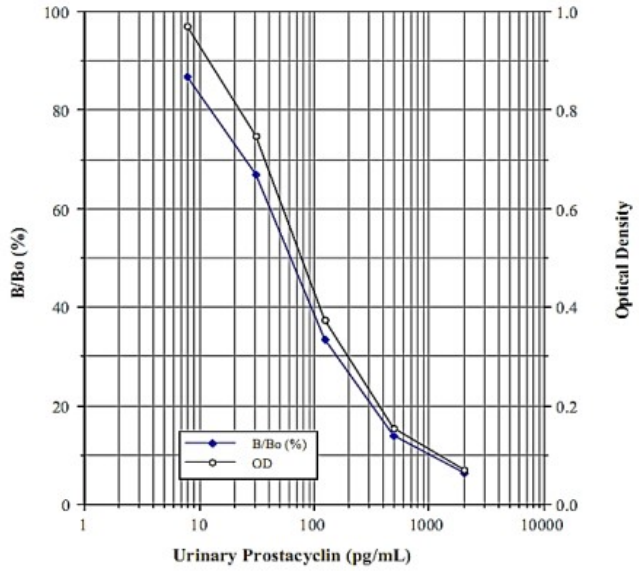
Typical Standard Curve

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

	Mean	Average	Percent	Urinary Prostacyclin
Sample	OD (-Blank)	Net OD	Bound	(pg/mL)
Blank OD	(0.137)			
TA	0.443	0.444		
NSB	-0.001	0.000	0.00%	
Bo	1.117	1.118	100%	0
S1	0.069	0.070	6.3%	2,000
S2	0.154	0.155	13.9%	500
S3	0.372	0.373	33.4%	125
S4	0.746	0.747	66.8%	31.25
S5	0.969	0.970	86.8%	7.81
Unknown 1	0.241	0.240	22.2%	255
Unknown 2	0.665	0.666	61.5%	36.9

The Prostacyclin ELISA Kit measures both 6k-PGF1α and 2,3d-6k-PGF1α in most samples. If the levels of either 6k-PGF1α or 2,3d-6k-PGF1α are to be determined independently, then the samples should be measured in other ELISA Kit. Please contact the CD technician for inquiry.

A typical standard curve is shown below. This curve must not be used to calculate Urinary Prostacyclin concentrations; each user must run a standard curve for each assay.



Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of 2,3d-6k-PGF1α and running these samples multiple times (n=24) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of 2,3d-6k-PGF1α in multiple assays (n=8). The precision numbers listed on the next page represent the percent coefficient of variation for the concentrations of 2,3d-6k-PGF1α determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	2,3d-6k-$PGF_{1\alpha}$ (pg/mL)	Intra-assay %CV	Inter-assay %CV
Low	23	9.1	
Medium	66	3.4	
High	200	2.9	
Low	23		9.6
Medium	72		5.4
High	223		3.4

Sensitivity

Sensitivity was calculated in Assay Buffer 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 #5. The detection limit was determined as the concentration of 2,3d-6k-PGF1α measured at two (2) standard deviations from the zero along the standard curve.

Average Optical Density for the Bo = 0.731 ± 0.016 (2.18%)

Average Optical Density for Standard #5 = 0.693 ± 0.020 (2.94%)

Delta Optical Density (0-7.81 pg/mL) = 0.038

2 SD's of the Zero Standard = 2 × 0.016 = 0.032

Sensitivity = (0.032/0.038) × 7.81 pg/mL = 6.58 pg/mL

Specificity

The cross reactivities for a number of related eicosanoid compounds were 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 39 pg/mL. These samples were then measured in the UP assay, and the measured 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
2,3-dinor-6-keto-$PGF_{1\alpha}$	100%
6-keto-$PGF_{1\alpha}$	89.14%
$PGF_{1\alpha}$	13.21%
$PGF_{2\alpha}$	4.99%
13,14 dihydro-$PGF_{1\alpha}$	2.55%
15-keto-$PGF_{2\alpha}$	0.32%
PGE_1	0.08%
PGD_2	0.03%
6,15-diketo-13,14 dihydro-$PGF_{1\alpha}$	0.03%
PGB_1	0.01%
PGE_2	0.01%
PGA_2	0.01%
TXB_2	0.01%

Linearity

A sample containing 400 pg/mL 2,3d-6k-PGF1α was diluted 6 times 1:2 in the kit Assay Buffer and measured in the assay. The data was plotted graphically as actual 2,3d-6k-PGF1α concentration versus measured 2,3d-6k-PGF1α concentration. The line obtained had a slope of 1.069 and a correlation coefficient of 0.995.

Recovery

Please refer to Sample Handling recommendations and Standard preparation. UP concentrations were measured in tissue culture media (TCM) and urine. PGI2 was spiked into the undiluted samples of these media which were then diluted with the appropriate diluent and assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Tissue Culture Media	109.2	1:2
Human Urine	101.9	1:10

Precautions

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 Mg_{2+} and Zn_{2+} ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA.
4. The Urinary Prostacyclin standard provided is supplied in ethanolic buffer at a pH optimized to maintain integrity. Care should be taken handling this material because of the known and unknown effects of prostaglandins.
5. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
6. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
7. Standards can be made up in either glass or plastic tubes.
8. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
9. Pipet standards and samples to the bottom of the wells.
10. Add the reagents to the side of the well to avoid contamination.
11. 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.
12. 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.
13. Prior to addition of substrate, ensure that there is no residual wash buffer in wells. Any remaining wash buffer may cause variation in assay results.