



Correlate-CLIA™

High Sensitivity 6-keto-Prostaglandin F_{1α} Chemiluminescence Enzyme Immunoassay Kit

Catalog No. 910-004

96 Well Kit

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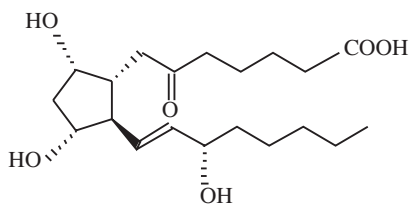
Description

The Assay Designs Correlate-CLIA™ 6-keto-Prostaglandin $F_{1\alpha}$ (6-keto-PGF $_{1\alpha}$) kit is a competitive immunoassay for the quantitative determination of 6-keto-PGF $_{1\alpha}$ in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to 6-keto-PGF $_{1\alpha}$ to bind, in a competitive manner, the 6-keto-PGF $_{1\alpha}$ in the sample or an alkaline phosphatase molecule which has 6-keto-PGF $_{1\alpha}$ covalently attached to it. After a simultaneous incubation at room temperature the excess reagents are washed away and chemiluminescent substrate is added. The substrate reacts with the bound alkaline phosphatase conjugate to produce light emission at approximately 530nm. The intensity of the emitted light is inversely proportional to the concentration of 6-keto-PGF $_{1\alpha}$ in either standards or samples. The measured chemiluminescence is used to calculate the concentration of 6-keto-PGF $_{1\alpha}$ in the samples. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard¹ or Tijssen².

Introduction

Prostacyclin (PGI $_2$) is involved in platelet aggregation, vasoconstriction, and reproductive functions²⁻⁵. However, PGI $_2$ has a half life of 60 minutes in plasma but only 2 to 3 minutes in buffer⁵. The production of PGI $_2$ is typically monitored by measurement of 6-keto-PGF $_{1\alpha}$. 6-keto-PGF $_{1\alpha}$ is produced by the non-enzymatic hydration of PGI $_2$, and has been shown to be stable⁵. A number of pharmaceuticals alter and/or inhibit the synthesis of PGI $_2$ ⁶ and methods to measure PGI $_2$ in blood and urine typically involve HPLC⁷, gas chromatography/mass spectrometry⁸, radioimmunoassay^{9,10}, or enzyme immunoassay¹¹.

6-keto-Prostaglandin $F_{1\alpha}$



Precautions

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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. 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.
3. 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.
4. The 6-keto-prostaglandin $F_{1\alpha}$ Standard provided, Catalog No. 80-0044, is supplied in ethanolic buffer at a pH optimized to maintain 6-keto-PGF $_{1\alpha}$ integrity. Care should be taken handling this material because of the known and unknown effects of prostaglandins.

Materials Supplied

- 1. Donkey anti-Sheep IgG White Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0048**
A white plate using break-apart strips coated with donkey antibody specific for sheep IgG.
- 2. 6-keto-PGF_{1α} CLIA Conjugate, 5 mL, Catalog No. 80-0099**
A blue solution of alkaline phosphatase conjugated with 6-keto-PGF_{1α}.
- 3. 6-keto-PGF_{1α} CLIA Antibody, 5 mL, Catalog No. 80-0100**
A yellow solution of a polyclonal antibody to 6-keto-PGF_{1α}.
- 4. Assay Buffer, 30 mL, Catalog No. 80-0010**
Tris buffered saline, containing proteins, with sodium azide as preservative.
- 5. Wash Buffer Concentrate, 30 mL, Catalog No. 80-1286**
Tris buffered saline containing detergents.
- 6. 6-keto-Prostaglandin F_{1α} Standard, 0.5mL, Catalog No. 80-0044**
A solution of 500,000 pg/mL 6-keto-PGF_{1α}.
- 7. Lumiphos 530™ CLIA Substrate*, 21 mL, Catalog No. 80-0134**
Alkaline Phosphatase substrate in diethanolamine buffer at pH 9.5, containing fluorescent enhancers, with sodium azide as preservative.
- 8. 6-keto-PGF_{1α} Plate CLIA Assay Layout Sheet, 1 each, Catalog No. 30-0018**
- 9. Plate Sealer, 1 each, Catalog No. 30-0012**

Storage

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

Materials Needed but Not Supplied

1. Deionized water. No difference in assay results is seen with 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 beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Absorbant paper for blotting.
8. Plate luminometer capable of reading glow chemiluminescence. Some radiation counters may be suitable. Please refer to the counter instruction manual for recommendations on suitability for chemiluminescence measurements.

*Lumiphos 530 is the trademark of Lumigen Inc., Southfield, MI, USA and supplied under US patents 4,857,652; 4,983,779; 4,959,182; 5,004,565; 4,962,192, & 5,386,017; European patents 254051B1 & 352713B1; Japanese patent 5-45590; Australian patent 603,736; Korean patent 69,259 and Taiwanese patent 46,563.

Sample Handling

The Assay Designs' Correlate-CLIA™ 6-keto-PGF_{1α} enzyme immunoassay is compatible with 6-keto-PGF_{1α} samples in a wide range of matrixes after dilution in Assay Buffer. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions. **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 a 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 6-keto-PGF_{1α} in the appropriate matrix. For tissue, urine and serum samples, prostaglandin synthetase inhibitors, such as, indomethacin or meclofenamic acid at concentrations up to 10 µg/mL should be added to either the tissue homogenate or urine and serum samples. Samples may be used in the assay directly by dilution in the range of 1:10 to 1:1000 in Assay Buffer. Some samples normally have very low levels of 6-keto-PGF_{1α} present and extraction may be necessary for accurate measurement. A suitable extraction procedure is outlined below:

Materials Needed

1. High Specific Activity Tritiated 6-keto-PGF_{1α}. Activity should be >3.5 TBq/mmol. Sufficient labeled 6-keto-PGF_{1α} should be added 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 µL 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 10mL 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 0.5mL/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 50 µL ethanol to the dried samples and 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. Evaporate the organic solvent under a stream of nitrogen prior to running assay and reconstitute as above.

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

Procedural Notes

1. Do not mix reagents from different lot numbers or use reagents beyond the 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 the 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 addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**
10. **The chemiluminescent signal generated is read after 60 minutes. The signal is still being generated and the wells must be read in the order in which the substrate was added. If you do not have a luminometer that will precisely time the substrate incubation, the following protocol must be followed. We suggest adding the substrate at 10 second intervals between wells and reading the generated chemiluminescence for 1 seconds at 10 second intervals for consistency. If luminometer injection is not used, we suggest using a repeater type syringe, such as an Eppendorf™ Repeater™ Pipette, Catalog Number 2226000-6 and a 5 mL repeater Combitip™ set for delivery of 200 µL. Use the repeater to add substrate to the wells in the order in which they will be read. Please be aware that some plate luminometers read up and down columns in sequence.**

Reagent Preparation

1. 6-keto-PGF_{1α} Standard

Allow the 500,000 pg/mL 6-keto-PGF_{1α} standard solution to warm to room temperature. Label eight 12 x 75 mm glass tubes #1 through #8. Pipet 0.98 mL of Assay Buffer into tube #1. Pipet 0.75 mL of Assay Buffer into tubes #2-8. Add 20 µL of the 500,000 pg/mL standard to tube #1. Vortex thoroughly. Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes # 3-8.

The concentration of 6-keto-PGF_{1α} in tubes #1 through #8 will be 10,000, 2,500, 625, 156, 39, 9.8, 2.4 and 0.6 pg/mL respectively. See 6-keto-PGF_{1α} Plate CLIA Assay Layout Sheet for dilution details.

Diluted standards should be used within 60 minutes of preparation.

2. 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 Assay Buffer 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 Blank and TA 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. Add 5 µL of the blue Conjugate to the TA wells.
12. Add 200 µL of the substrate solution to each well. Incubate at room temperature for 60 minutes with shaking. Read each well in the same order that substrate was added, for 2 seconds on a suitable luminometer.

Calculation of Results

Several options are available for the calculation of the concentration of 6-keto-PGF_{1α} in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program such as "AssayZap", sold by Biosoft [Tel: (314) 524-8029] for Macintosh and IBM-compatible computers. If this sort of data reduction software is not readily available, the concentration of 6-keto-PGF_{1α} can be calculated as follows:

1. Calculate the average net Relative Light Units (RLU) bound for each standard and sample by subtracting the average NSB RLU from the average RLU bound:

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

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

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

3. Using Logit-Log paper plot Percent Bound versus Concentration of 6-keto-PGF_{1α} for the standards. Approximate a straight line through the points. The concentration of 6-keto-PGF_{1α} in the unknowns can be determined by interpolation.

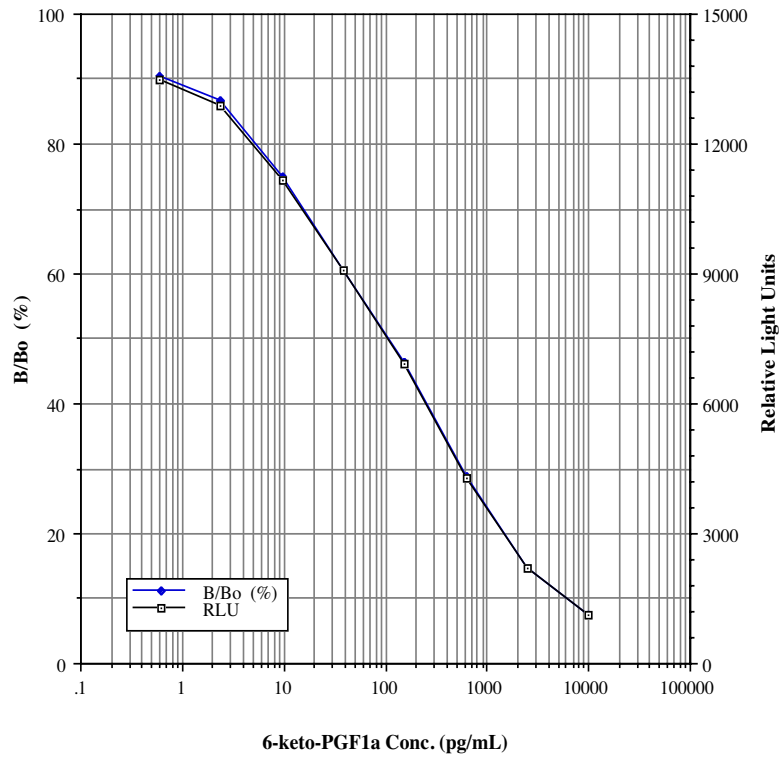
Typical Results

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

<u>Sample</u>	<u>Average RLU</u>	<u>Net RLU</u>	<u>Percent Bound</u>	<u>6-k-PGF_{1α} (pg/mL)</u>
Blank	(204.0)			
TA	51,750.0	51,546.0		
NSB	419.0	215.0	0.00%	
S1	1,329.0	1,125.5	7.56%	10,000
S2	2,393.5	2,189.5	14.72%	2,500
S3	4,470.0	4,266.0	28.67%	625
S4	7,127.5	6,923.5	46.53%	156.25
S5	9,212.5	9,008.5	60.55%	39.06
S6	11,348.5	11,144.5	74.90%	9.77
S7	13,085.0	12,881.0	86.57%	2.44
S8	13,671.0	13,467.0	90.51%	0.61
Bo	15,083.0	14,879.0	100%	0
Unknown 1	120,578	117,268	65.36%	59.4
Unknown 2	40,337	37,027	20.64%	1675.5

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate 6-keto-PGF_{1α} concentrations; each user must run a standard curve for each assay.



Typical Quality Control Parameters

Total Activity Added = 51,546.0 x 10 = 515,460.0

%NSB = 0.04 %

%Bo/TA = 2.89 %

Quality of Fit = 0.9998917 (Calculated from 4 parameter logistic curve fit)

20% Intercept = 1762.4 pg/mL

50% Intercept = 105.5 pg/mL

80% Intercept = 5.7 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 Protocols¹⁶.

Sensitivity

Sensitivity was calculated by determining the average RLU signal bound for sixteen (16) wells run as Bo, and comparing to the average RLU signal for sixteen (16) wells run with Standard #8. The detection limit was determined as the concentration of 6-keto-PGF_{1α} measured at two (2) standard deviations from the zero along the standard curve.

$$\begin{aligned} \text{Average RLU for the Bo} &= 12,024 \pm 1,502 \text{ (12.5\%)} \\ \text{Average RLU for Standard \#8} &= 11,096 \pm 922 \text{ (8.3\%)} \\ \text{Delta RLU's (0-0.6 pg/mL)} &= 12,024 - 11,096 = 928 \\ \text{2 SD's of the Zero Standard} &= 2 \times 1,502 = 3,003 \\ \text{Sensitivity} &= \frac{3,003}{928} \times 0.6 \text{ pg/mL} = \mathbf{1.94 \text{ pg/mL}} \end{aligned}$$

Linearity

A sample containing 50,000 pg/mL 6-keto-PGF_{1α} was diluted serially 5 times 1:5 in the kit Assay Buffer and measured in the Correlate-CLIA™ assay. The data was plotted graphically as actual 6-keto-PGF_{1α} concentration versus measured 6-keto-PGF_{1α} concentration.

The line obtained had a slope of 1.441 with a correlation coefficient of 1.000.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of 6-keto-PGF_{1α} and running these samples multiple times (n=8) in the same assay.

The precision numbers listed below represent the percent coefficient of variation for the concentrations of 6-keto-PGF_{1α} determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	6-keto-PGF _{1α} Concentration (pg/mL)	Intra Assay <u>%CV</u>
Low	39.81	5.7
Medium	749.5	9.1
High	18,440.0	3.5

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 39 pg/mL. These samples were then measured in the 6-keto-PGF_{1α} Correlate-EIA assay, and the measured 6-keto-PGF_{1α} 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.

<u>Compound</u>	<u>Cross Reactivity</u>	<u>Compound</u>	<u>Cross Reactivity</u>
6-keto-PGF _{1α}	100%	PGD ₂	0.60%
PGF _{2α}	1.67%	PGE ₁	0.20%
PGF _{1α}	0.60%	13,14-dihydro-15-keto-PGF _{1α}	<0.01%
6,15-diketo-13,14-dihydro-PGF _{1α}	0.12%	PGA ₂	<0.01%
15-keto-PGF _{2α}	<0.01%	PGE ₂	<0.01%
PGB ₁	<0.01%	15-HETE*	<0.01%
Thromboxane B ₂	<0.01%	2-Arachidonoylglycerol	<0.01%
2,3-dinor-6-keto-PGF _{1α}	3.17%	Anandamide	<0.01%

*Data from S. Rawlinson, Royal Veterinary College, London,U.K.

Sample Recoveries

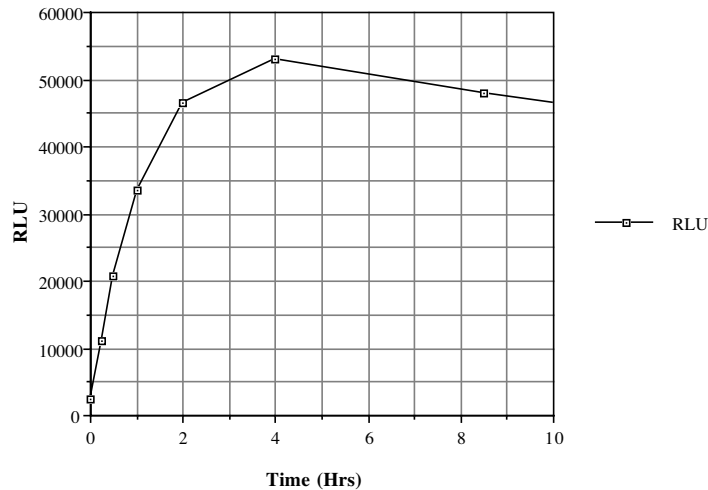
6-keto-PGF_{1α} concentrations were measured in a variety of different samples including tissue culture media, human saliva, serum, and urine. For samples in tissue culture media, ensure that the standards have been diluted into the same media (refer to page 4). 6-keto-PGF_{1α} was spiked into the undiluted samples of these media, which were diluted with the kit Assay Buffer and then assayed in the Correlate-EIA™ kit. The following results were obtained:

<u>Sample</u>	<u>% Recovery</u>	<u>Recommended Dilution*</u>
Tissue Culture Media	98.8	None
Human Saliva	110.7	1:10-1:100
Human Urine	108.7	1:100-1:1000
Human Serum	108.4	1:100-1:1000

* See Sample Handling instructions on page 4 for details.

Time Course of Chemiluminescent Emission

The chemiluminescent signal generated from the reaction of the alkaline phosphatase conjugate and the CLIA substrate is a kinetic reaction that reaches a maximum light output after approximately 4 hours. The chemiluminescent emission will last for several hours. The data is presented below.



References

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LIMITED WARRANTY

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Material Safety Data Sheet (MSDS) available on our website or by fax.

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