



# 6-keto Prostaglandin F<sub>1α</sub> Enzyme Immunoassay Kit

Catalog No. 133-16476

96 Well Kit

## Table of Contents

Description	2
Introduction	2
Precautions	2
Materials Supplied	2
Storage	2
Materials Needed but Not Supplied	2
Sample Handling	3
Reagent Preparation	3
Assay Procedure	4
Data Analysis	5
Assay Performance Characteristics	5
References	7
Limited Warranty	7

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

## Description

Sapphire's 6-keto Prostaglandin F<sub>1α</sub> (6-keto PGF<sub>1α</sub>) kit is a competitive immunoassay for the quantitative determination of 6-keto PGF<sub>1α</sub> in biological samples. This assay is based on the competition between 6-keto PGF<sub>1α</sub> and a 6-keto PGF<sub>1α</sub>-alkaline phosphatase tracer for a limited amount of 6-keto PGF<sub>1α</sub>-specific antiserum. Because of the competition between 6-keto PGF<sub>1α</sub> in the sample and 6-keto PGF<sub>1α</sub> tracer for the 6-keto PGF<sub>1α</sub> antiserum, the signal obtained with the assay will be inversely proportional to the amount of 6-keto PGF<sub>1α</sub> in each sample. This equilibration is performed in the wells of a 96-well plate pre-coated with mouse polyclonal anti-rabbit IgG, which binds all of the 6-keto PGF<sub>1α</sub> antiserum added to the well. After the equilibration step, the plate is washed, and a solution of *para*-nitrophenyl phosphate (*p*NPP), a substrate for alkaline phosphatase, is added. The product of this enzymatic reaction has a distinct yellow color which absorbs at 412 nm, allowing quantification of 6-keto PGF<sub>1α</sub> in each sample.

## Introduction

6-keto PGF<sub>1α</sub> is the stable, non-enzymatic hydration product of prostaglandin I<sub>2</sub> (PGI<sub>2</sub>; prostacyclin), a potent vasodilator and inhibitor of platelet aggregation.<sup>1</sup> PGI<sub>2</sub> does not exist preformed in cells but rather is synthesized in response to immediate need, primarily in the vascular endothelium and renal cortex.<sup>1,2</sup> Following cellular stimulation, arachidonic acid is hydrolyzed from phospholipids stores by one of a family of phospholipase A<sub>2</sub> enzymes and converted to the unstable endoperoxide PGH<sub>2</sub> by either COX-1 or COX-2.<sup>3,4</sup> PGH<sub>2</sub> is rapidly isomerized to PGI<sub>2</sub>, by the enzyme PGI synthase (prostacyclin synthase), and exerts its action within a short distance of its synthesis by binding to the cell surface IP receptor.<sup>5,6</sup> PGI<sub>2</sub> is very unstable, being non-enzymatically hydrated to 6-keto PGF<sub>1α</sub> (t<sub>1/2</sub> = 2-3 minutes), and then quickly converted to the major metabolite, 2,3-dinor-6-keto PGF<sub>1α</sub> (t<sub>1/2</sub> = 30 minutes) prior to excretion in the urine.<sup>7-9</sup> Caution should be exercised when measuring 6-keto PGF<sub>1α</sub> in some biological samples as the majority of urinary 6-keto PGF<sub>1α</sub> is of renal origin and venipuncture may cause release of PGI<sub>2</sub> from the vascular endothelial cells during collection of blood samples (*i.e.* whole blood, plasma and serum).<sup>10</sup> 6-keto PGF<sub>1α</sub> is released into the medium of cultured cells without further metabolism.

## Precautions

1. Please read all instructions carefully before beginning this assay.
2. Some reagents contain azide, which may react with lead or copper plumbing. If disposing of reagents in the sanitary sewer, please flush with a large volume of water to prevent azide build-up.
3. This kit is for research use only. It is not intended for human or diagnostic use.

## Materials Supplied

1. 6-keto Prostaglandin F <sub>1α</sub> Antiserum	1 vial	133-64763
2. 6-keto Prostaglandin F <sub>1α</sub> AP Tracer	1 vial	133-64762
3. 6-keto Prostaglandin F <sub>1α</sub> Standard	1 vial	133-64761
4. Mouse Anti-rabbit IgG Coated Plate	1 plate	133-00006
5. Tris Buffer Concentrate	2 vials	133-00003
6. AP Wash Buffer Concentrate	1 vial	133-00004
7. DEA Buffer Concentrate	1 vial	133-00001
8. <i>p</i> NPP Tablets	5 tablets	133-00002
9. Plate Cover	1 cover	133-00005

## Storage

This kit should be stored at -20°C and used before the expiration date printed on the box.

## Materials Needed but Not Supplied

1. A source of Millipore water
2. Adjustable pipettors
3. Materials used for sample preparation
4. An orbital shaker
5. A plate reader capable of measuring absorbance between 405-420 nm

## Sample Handling

[*Note: Samples should be stored at -80°C if unable to be assayed immediately following collection.*]

### Materials needed for sample purification

1. 6-keto Prostaglandin  $F_{1\alpha}$  standard to use in determination of recovery
2. 1.0 M HCl, methanol, Millipore water, hexane and ethyl acetate
3. 6 ml SPE C-18 cartridges

### Purification Procedure

While the following purification protocol works well for many samples, it may not be sufficient for the purification of 6-keto  $PGF_{1\alpha}$  from all samples.

1. Split samples into two equal parts and place each sample into clean test tubes.
2. Spike one set of samples with 6-keto  $PGF_{1\alpha}$ .
3. Perform the following steps on all samples:
  - a. Acidify the sample to pH < 4.0 by the addition of 1.0 M HCl.
  - b. Prepare a C-18 SPE cartridge by conditioning it first with 5 ml of methanol followed by 5 ml Millipore water.
  - c. Apply the sample and allow it to flow through the column.
  - d. Rinse the column with 5 ml Millipore water, followed by 5 ml hexane. Allow the column to become dry following the hexane wash.
  - e. Elute the 6-keto  $PGF_{1\alpha}$  with 5 ml ethyl acetate containing 1% methanol. If unable to run the assay at this time, store the samples in the ethyl acetate/methanol at -80°C.
  - f. Dry the sample under a stream of nitrogen. Reconstitute the sample in Tris Buffer and assay both spiked and unspiked samples in the assay.

## Reagent Preparation

All diluted buffers should be stored at 4°C. When stored in this manner, they will be stable for approximately two months.

### 1. Tris Buffer

Dilute the contents of one vial of Tris Buffer Concentrate with 90 ml of Millipore water. It is common for the concentrated buffer to contain crystalline salts after thawing. It is important to rinse the vial to obtain any salts that may have precipitated.

### 2. Wash Buffer

Dilute the 5 ml vial of Wash Buffer to a final volume of 750 ml with Millipore water.

### 3. DEA Buffer

Dilute the 2.5 ml vial of DEA Buffer Concentrate to a final volume of 25 ml with Millipore water.

### 4. 6-keto Prostaglandin $F_{1\alpha}$ Standard

[*Note: If assaying culture medium samples that have not been diluted with Tris Buffer, the culture medium rather than Tris Buffer should be used for dilution of the standard curve.*]

Equilibrate a pipet tip in ethanol, by repeatedly filling and expelling. Use the equilibrated pipet tip to transfer 100  $\mu$ l of the 6-keto Prostaglandin  $F_{1\alpha}$  standard into a clean test tube. Dilute with 900  $\mu$ l Millipore water. The concentration of this standard is 50 ng/ml. Label eight glass test tubes #1 - #8. Pipet 900  $\mu$ l of Tris Buffer into tube #1, and 500  $\mu$ l into tubes #2 - #8. Transfer 100  $\mu$ l of the 50 ng/ml standard into tube #1 and vortex to mix. Transfer 250  $\mu$ l from tube #1 to tube #2. Vortex to mix. Transfer 250  $\mu$ l from tube #2 to tube #3. Vortex to mix. Continue this process for standard tubes #4 - #8. These diluted standards should be used within twenty-four hours.

### 5. 6-keto Prostaglandin $F_{1\alpha}$ Alkaline Phosphatase Tracer

Reconstitute the 6-keto Prostaglandin  $F_{1\alpha}$  Alkaline Phosphatase Tracer with 6 ml of Tris buffer. Vortex to mix.

Store this reconstituted tracer at 4°C and use within four weeks.

### 6. 6-keto Prostaglandin $F_{1\alpha}$ Antiserum

Reconstitute the 6-keto Prostaglandin  $F_{1\alpha}$  Antiserum with 6 ml of Tris Buffer. Vortex to mix.

Store this reconstituted antibody at 4°C and use within four weeks.

## Assay Procedure

*[Note: All reagents should be allowed to warm to room temperature before use.]*

**1. Tris Buffer**

Pipet 150 µl Tris Buffer into non-specific binding (NSB) wells, and 100 µl Tris Buffer into zero standard (B<sub>0</sub>) wells. If tissue culture medium was used to dilute the standard curve, substitute 100 µl of this same medium for the Tris Buffer in the NSB and B<sub>0</sub> wells.

**2. 6-keto Prostaglandin F<sub>1α</sub> Standard**

Pipet 100 µl of standards into the appropriate wells.

**3. Samples**

Pipet 100 µl of samples into the appropriate wells. Each sample should be assayed in duplicate or triplicate.

**4. 6-keto Prostaglandin F<sub>1α</sub> Alkaline Phosphatase Tracer**

Pipet 50 µl of tracer into each well except the blank wells and total activity (TA) wells.

**5. 6-keto Prostaglandin F<sub>1α</sub> Antiserum**

Pipet 50 µl of antiserum into each well except the blank wells, TA wells and NSB wells.

Well	Tris Buffer	Std/Sample	Tracer	Antiserum
Blank				
TA			5 µl (at development)	
NSB	150 µl		50 µl	
B <sub>0</sub>	100 µl		50 µl	50 µl
Std/Sample		100 µl	50 µl	50 µl

*Pipetting Summary*

**6. Incubate the plate**

Cover each plate with a plate cover and incubate for two hours at room temperature on an orbital shaker.

**7. Prepare the pNPP Solution**

Dissolve 5 pNPP tablets in 25 ml DEA Buffer (25 ml is sufficient to develop 100 wells). *[Note: Reconstituted pNPP is not stable, so we recommend that you make only the amount that you need at any one time.]*

**8. Wash the plate**

Empty the wells and rinse five times with Wash Buffer. After the final wash, firmly tap the inverted plate on a paper towel to remove any recalcitrant drops of buffer.

**9. Develop the plate**

Add 200 µl pNPP solution to each well including blank and TA wells. Add 5 µl of tracer to the TA wells. Cover the plate and allow to develop in the dark on an orbital shaker. This assay typically develops in approximately 60-90 minutes.

**10. Read the plate**

Wipe the bottom of the plate with a paper towel to remove any finger prints, smudges or dirt which may interfere with obtaining an accurate reading of absorbance. Remove the plate cover, and read the plate at a wavelength between 405 and 420 nm.

## Data Analysis

Most plate readers come with data reduction software that plots data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as %B/B<sub>0</sub> versus log concentration of standard using either a 4-parameter logistic or a log-logit curve fit.

### 1. Prepare the Data

The following procedure is recommended to prepare the data prior to graphing (subtract the absorbance of the blank wells from all wells on the plate, if not already done).

- a. Average the absorbance readings from the NSB wells.
- b. Average the absorbance readings from the B<sub>0</sub> wells.
- c. Subtract the average NSB from the average B<sub>0</sub>. This is the corrected B<sub>0</sub>.

$$\text{Corrected } B_0 = \text{Average } B_0 - \text{Average NSB}$$

- d. Calculate the %B/B<sub>0</sub> for each standard and sample. To do this, subtract the average NSB absorbance from the absorbance and divide by the corrected B<sub>0</sub> (from step c). Multiply by 100 to obtain %B/B<sub>0</sub>. Repeat for all wells.

$$\%B/B_0 = \left[ \frac{\text{Absorbance} - \text{Average NSB}}{\text{Corrected } B_0} \right] \times 100$$

### 2. Plot the Standard Curve

Plot %B/B<sub>0</sub> for all standards *versus* 6-keto PGF<sub>1α</sub> concentration using log (x) and linear (y) axes, and fit the data to a four parameter logistic equation. Alternatively, the data can be linearized using a logit transformation. [**Note:** Do not use %B/B<sub>0</sub> in this calculation.]

$$\text{Logit } (B/B_0) = \ln [B/B_0 / (1 - B/B_0)]$$

Plot the data as logit (B/B<sub>0</sub>) *versus* log concentration of standard and perform a linear regression fit.

### 3. Determine the Concentration of your Samples

Calculate the %B/B<sub>0</sub> for each sample. Determine the concentration of each sample using the equations obtained from the analysis of the standard curve. Remember to account for any dilutions made to the sample prior to addition to the well. %B/B<sub>0</sub> values of greater than 80% or less than 20% should be re-assayed as they generally fall outside of the linear range of the standard curve.

### 4. Correct for Recovery (if purification was performed)

Divide the concentration determined in step #3 by the recovery factor. Correct for any volume changes of the sample which may have occurred during purification.

Recovery Factor =

$$\frac{\text{EIA value of spiked sample (pg/ml)} - \text{EIA value of unspiked sample (pg/ml)}}{\text{Concentration of spike (pg/ml)}}$$

## Assay Performance Characteristics

### 1. Precision

Intra-assay precision was determined by measuring samples containing low, medium, and high concentrations of PGF<sub>1α</sub> multiple times in the same assay (eight samples per plate on a total of five plates). Inter-assay precision was determined by measuring low, medium, and high concentrations of the samples eight times using different reagents.

	PGF <sub>1α</sub> (pg/ml)	Intra-assay %CV	Inter-assay %CV
Low	61.7	9.3	21.3
Medium	185.2	8.9	21.0
High	555.6	6.1	16.6

## 2. Specificity

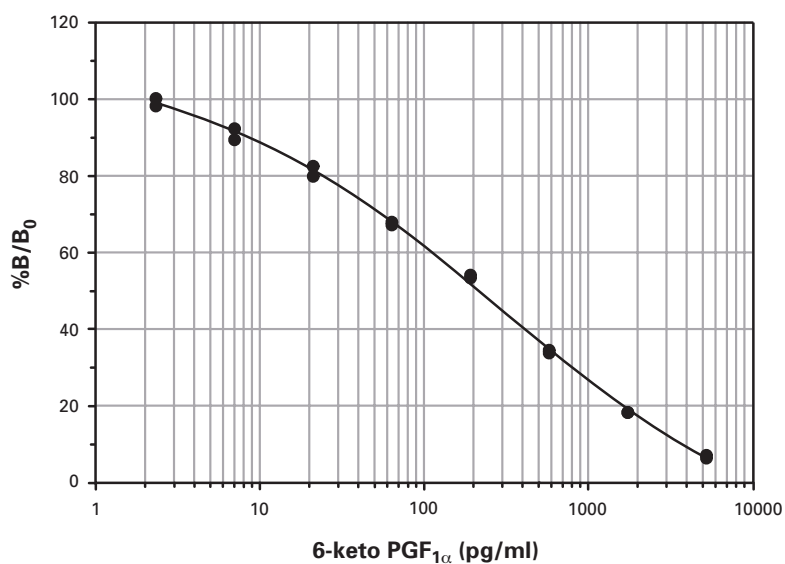
Analyte	Cross-Reactivity	Analyte	Cross-Reactivity
6-keto Prostaglandin F <sub>1α</sub>	100%	6,15-diketo-13,14-dihydro Prostaglandin F <sub>1α</sub>	2%
6-keto Prostaglandin E <sub>1</sub>	151%	Prostaglandin F <sub>2α</sub>	0.4%
Prostaglandin F <sub>1α</sub>	11%	Thromboxane B <sub>2</sub>	0.05%
2,3-dinor-6-keto Prostaglandin F <sub>1α</sub>	5%	Prostaglandin D <sub>2</sub>	<0.01%
13,14-dihydro-15-keto Prostaglandin F <sub>1α</sub>	3%	Tetranor PGEM	<0.01%
Prostaglandin E <sub>2</sub>	2%	Tetranor PGFM	<0.01%

## 3. Typical Results

The standard curve shown here is an example of data typically produced by this kit. Your results will vary from these, and it is therefore important that you run a standard curve each time you use the kit.

	Raw Data		Average	Corrected
Total Activity	1.534	1.480	1.507	
NSB	0	0	0	
B <sub>0</sub>	0.410	0.422	0.421	0.421
	0.421	0.429		

Concentration Std (pg/ml)	Raw Data		Corrected		%B/B <sub>0</sub>	
5,000	0.032	0.029	0.032	0.029	7.6	6.9
1,667	0.079	0.079	0.079	0.079	18.8	18.8
555.6	0.144	0.147	0.144	0.147	34.2	35.0
185.1	0.229	0.226	0.229	0.226	54.4	53.8
61.7	0.284	0.287	0.284	0.287	67.5	68.3
20.6	0.348	0.337	0.348	0.337	82.8	80.1
6.9	0.389	0.377	0.389	0.377	92.5	90
2.3	0.414	0.422	0.414	0.422	98.5	100.4



## **References**

1. Whorton, A.R., Smigel, M., Oates, J.A., *et al.* Regional differences in prostacyclin formation by the kidney. Prostacyclin is a major prostaglandin of renal cortex. *Biochim. Biophys. Acta* **529**, 176-180 (1978).
2. Moncada, S., Gryglewski, R., Bunting, S., *et al.* An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature* **263**, 663-665 (1976).
3. Smith, W.L., DeWitt, D.L., and Garavito, R.M. Cyclooxygenases: structural, cellular, and molecular biology. *Annu. Rev. Biochem.* **69**, 145-182 (2000).
4. Funk, C.D. Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science* **294**, 1871-1875 (2001).
5. Nakagawa, O., Tanaka, I., Usui, T., *et al.* Molecular cloning of human prostacyclin receptor cDNA and its gene expression in the cardiovascular system. *Circulation* **90**, 1643-1647 (1994).
6. Ogawa, Y., Tanaka, I., Inoue, M., *et al.* Structural organization and chromosomal assignment of the human prostacyclin receptor gene. *Genomics* **27**, 142-148 (1995).
7. Samuelsson, B., Goldyne, M., Granström, E., *et al.* Prostaglandins and thromboxanes. *Annu. Rev. Biochem.* **47**, 997-1029 (1978).
8. Rosenkranz, B., Fischer, C., Reimann, I., *et al.* Identification of the major metabolite of prostacyclin and 6-keto prostaglandin F<sub>1α</sub> in man. *Biochim. Biophys. Acta* **619**, 207-213 (1980).
9. Dusting, G.J., Moncada, S., and Vane, J.R. Prostacyclin (PGX) is the endogenous metabolite responsible for relaxation of coronary arteries induced by arachidonic acid. *Prostaglandins* **13**, 3-15 (1977).
10. Murphy, R.C. and Fitzgerald, G.A. Current approaches to estimation of eicosanoid formation *in vivo*. *Adv. Prostaglandin Thromboxane Leukot. Res.* **22**, 341-348 (1994).

## **Warranty and Limitation of Remedy**

Sapphire Bioscience Pty Ltd ("Sapphire") makes no representation or warranty of any kind, express or implied, which extends beyond the description of the product contained herein, except that the material will meet Sapphire's specifications at the time of delivery. Sapphire's sole liability hereunder shall be limited to refund of the purchase price of, or at Sapphire's option the replacement of, all material that does not meet Sapphire's specifications. Sapphire shall not be liable otherwise or for incidental or consequential damages, including but not limited to, the costs of handling. The said refund or replacement is conditional on the Buyer giving written notice to Sapphire within thirty (30) days after arrival of the material at its destination. Failure of the buyer to give the said notice within the said thirty (30) days shall constitute a waiver by the Buyer of all claims hereunder with respect to the said material.

13/12/07