

# Human FGF-23 (C-Term) ELISA Kit

Enzyme-Linked ImmunoSorbent Assay (ELISA) for the Determination of Human Fibroblast Growth Factor 23 Levels in Plasma or Cell Culture Media

**Immutopics**

*Immutopics, Inc.*

For RESEARCH Use Only

96 Test Kit  
Cat.# 60-6000

Not for use in diagnostic procedures

Store at 2 - 8°C Upon Receipt

## INTENDED USE

This kit is intended for research use only in the determination of human FGF-23 levels in plasma or cell culture media. Reference ranges and clinical utility have not been established.

## INTRODUCTION

Fibroblast growth factor 23 (FGF-23) is a recently discovered, novel member of a large family of related proteins. Its gene encodes a 251 amino acid protein. The amino-terminal portion of FGF-23 (aa 1-24) is hydrophobic and is likely to serve as a signal peptide allowing its secretion into the blood circulation. Its carboxyl-terminal portion (aa 180-251) shares only limited amino acid homology with other members of the FGF family of proteins. FGF-23 is most closely related to FGF-21 (~24% amino acid sequence homology) and FGF-19 (~22% amino acid sequence homology).

Renal phosphate wasting disorders leading to hypophosphatemia are among the causes of defective mineralization of bone and growth plate development. Patients with autosomal dominant hypophosphatemic rickets (ADHR), a rare genetic disorder, carry one of several different FGF-23 mutations that make the protein resistant to proteolytic cleavage. Furthermore, tumors that cause oncogenic osteomalacia (OOM) have been shown to overexpress FGF-23 mRNA making it likely that elevated concentrations of FGF-23 in the blood are the cause of renal phosphate wasting in this group of patients. Consistent with this conclusion, the application of recombinant FGF-23 to rodents was shown to increase urinary excretion of phosphate thus leading to hypophosphatemia and osteomalacia/rickets. Taken together, all currently available data suggest that FGF-23 is either directly or indirectly involved in the regulation of phosphate homeostasis.

The measurement of human FGF-23 in the blood circulation is likely to provide an important diagnostic tool for the laboratory evaluation of patients with a variety of different hypophosphatemic disorders, including oncogenic osteomalacia, X-linked hypophosphatemic rickets, and autosomal dominant hypophosphatemic rickets. Furthermore, the sensitive measurement of FGF-23 is likely to provide novel insights into the regulation of bone and mineral homeostasis.

## TEST PRINCIPLE

The Human FGF-23 (C-Term) ELISA Kit is a two-site enzyme-linked immunosorbent assay (ELISA) for the measurement of FGF-23 in plasma or cell culture media. Two affinity purified goat polyclonal antibodies have been selected to detect epitopes within the carboxyl-terminal (C-Term) portion of FGF-23. One antibody is immobilized onto the microtiter plate wells for capture. The other antibody is biotinylated for subsequent binding with horseradish peroxidase (HRP) conjugated to avidin for detection.

A sample containing human FGF-23 is incubated simultaneously with the immobilized capture antibody and the biotinylated detection antibody in a microtiter well. FGF-23 contained in the sample is immunologically bound by the capture antibody and the detection antibody to form a "sandwich" complex:

Well/Anti-Human FGF — Human FGF-23 — Biotin/Anti-Human FGF  
(C-terminal) (C-terminal)

At the end of this incubation period, the well is washed to remove any unbound antibody and other components. This immobilized sandwich complex is then incubated with HRP-Avidin in a timed reaction to allow the enzyme to bind to the biotinylated antibody. At the end of this incubation period the well is again washed to remove unbound components. The enzyme bound to the well is incubated with a substrate solution in a timed reaction and then measured in a

spectrophotometric microtiter plate reader. The enzymatic activity of the antibody complex bound to the well is directly proportional to the amount of FGF-23 in the sample. A standard curve is generated by plotting the absorbance versus the respective FGF-23 concentration for each standard on linear or logarithmic scales. The concentration of human FGF-23 in the samples is determined directly from this curve.

## REAGENTS: Preparation and Storage

Store the kit at 2-8°C upon receipt. **Store the standards and controls at -20°C or below after reconstitution.** For the expiration date of the kit refer to the label on the kit box. All components are stable until this expiration date.

**Prior to use allow all reagents to come to room temperature and mix by gentle swirling and inversion.** Reagents from different kit lot numbers should not be combined or interchanged.

### 1. HUMAN FGF-23 ANTIBODY COATED PLATE (40-6015)

One plate with 12 eight well strips (96 wells total) coated with antibody to human FGF-23 plus desiccant. This reagent should be stored at 2 - 8°C and is stable until the expiration date on the kit.

### 2. BIOTINYLATED HUMAN FGF-23 ANTIBODY (40-6020)

One vial containing 5.5 mL biotin labeled anti-human FGF-23 in phosphate buffered saline with protein stabilizers and 0.1% sodium azide. This reagent should be stored at 2 - 8°C and is stable until the expiration date on the kit.

### 3. HRP-AVIDIN (40-6070)

One vial containing 21 mL of horseradish peroxidase (HRP) conjugated to avidin in TRIS buffered saline with protein stabilizers with a non-azide, non-mercury preservative. This reagent should be stored at 2 - 8°C protected from light and is stable until the expiration date on the kit.

### 4. HUMAN FGF-23 STANDARDS (40-6031 to 40-6036)

Six vials, five of which contain human FGF-23 lyophilized in a protein matrix with a non-azide, non-mercury preservative. **Refer to vial label for exact concentration.** Before use reconstitute the vial with the FGF-23 concentration of 0 RU/mL with 2.0 mL of deionized water. Before use reconstitute each of the other five vials of standards with 1.0 mL of deionized water. Allow the vials to sit for approximately 20 minutes with occasional gentle swirling and inversion. Assure complete reconstitution before use.

Use the standards immediately after reconstitution; freeze the unused portion for later use. After reconstitution the standards are stable until the expiration date on the kit when stored at -20°C or below with up to 3 freeze/thaw cycles.

### 5. HUMAN FGF-23 CONTROLS I & II (40-6041 & 40-6042)

Two vials each containing human FGF-23 lyophilized in a protein matrix with a non-azide, non-mercury preservative. **Refer to vial label for control ranges.** Before use reconstitute each control with 1.0 mL of deionized water. Allow the vials to sit for approximately 20 minutes with occasional gentle swirling and inversion. Assure complete reconstitution before use.

Use the controls immediately after reconstitution; freeze the unused portion for later use. After reconstitution the controls are stable until the expiration date on the kit when stored at -20°C or below with up to 3 freeze/thaw cycles.

### 6. ELISA WASH CONCENTRATE (40-0041)

One vial containing 20 mL of a 20 fold concentrate. Before use dilute the contents to 400 mL with deionized water and mix well. Upon dilution this yields a working wash solution containing a surfactant in saline with a non-azide, non-mercury preservative.

The diluted wash solution should be stored at room temperature and is stable until the expiration date on the kit.

#### 7. ELISA HRP SUBSTRATE (40-0022)

One bottle containing 21 mL of tetramethylbenzidine (TMB) with hydrogen peroxide. This reagent should be stored at 2 - 8°C protected from light and is stable until the expiration date on the kit.

#### 8. ELISA STOP SOLUTION (40-0030)

One bottle containing 11 mL of 1 M sulfuric acid. This reagent may be stored at room temperature or at 2 - 8°C and is stable until the expiration date on the kit.

#### 9. PLATE SEALER (20-2000)

Three included in kit.

#### 10. HUMAN FGF-23 SAMPLE DILUENT (Optional reagent, must be ordered separately using catalog # 30-6031)

One bottle containing 50 mL of a protein matrix with 0.1% sodium azide in liquid, ready-to-use form. This reagent should be stored at 2 - 8°C and is stable until the expiration date on the bottle.

### SAFETY PRECAUTIONS

Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid (i.e. ELISA HRP Substrate and ELISA Stop Solution). TMB is dissolved in a solution which contains acetone, an irritant to skin and mucous membranes. In case of contact with any of these reagents, wash thoroughly with water. TMB is a suspected carcinogen. Use Good Laboratory Practices. Wash hands before eating. Do not eat, drink or smoke in the work area.

Some of the reagents in this kit contain sodium azide. Sodium azide may react with lead or copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide buildup (Manual Guide-Safety Management No. CDC-22, Center for Disease Control, Atlanta, Georgia, April 30, 1976).

### MATERIALS REQUIRED BUT NOT PROVIDED

- 1.0 mL volumetric pipette for reconstituting standards and controls.
- Precision pipets capable of delivering 50  $\mu$ L, 150  $\mu$ L and 200  $\mu$ L.
- Aluminum foil.
- Repeating dispenser suitable for delivering 350  $\mu$ L.
- Aspiration device or suitable microtiter plate washer.
- Container for storage of wash solution.
- Spectrophotometric microtiter plate reader capable of reading absorbance at 450 nm and at 595 - 650 nm.
- Deionized water.
- Horizontal rotator capable of maintaining 180 - 220 RPM.
- Timer.

### SPECIMEN COLLECTION

The FGF-23 molecule appears to be unstable resulting in decreased immunoreactivity over time. Sample collection and storage procedures should be carried out in an expeditious manner. **Due to the variable lability of the molecule measurement of the FGF-23 concentration should be made using EDTA plasma or cell culture media. (Serum is no longer recommended as an appropriate sample.)** Three hundred microliters of plasma or culture media are required to assay the sample in duplicate. A morning, 12 hour fasting sample is recommended. Centrifuge the sample and separate the plasma or media from the cells. Samples should be assayed immediately or stored frozen at -20°C or below. Avoid repeated freezing and thawing of specimens.

### ASSAY PROCEDURE

- Place a sufficient number of Antibody Coated Strips in a holder to run FGF-23 standards, controls and unknown samples.
- Pipet 150  $\mu$ L of standard, control, or sample into the designated or mapped well. Freeze the remaining standards and controls as soon as possible after use.
- Pipet 50  $\mu$ L of Biotinylated Antibody into each well, and cover the plate with one plate sealer.
- Incubate plate at room temperature for 10 minutes on a horizontal rotator set at 180 - 220 RPM; then continue

to incubate stationary for 18 - 24 hrs.

- Remove the plate sealer. Aspirate the contents of each well. Wash each well five times by dispensing 350  $\mu$ L of working wash solution into each well then completely aspirating the contents. Preferably, an automated microtiter plate washer should be used.
- Pipet 200  $\mu$ L of HRP-Avidin reagent into each of the wells.
- Re-cover the plate with a plate sealer and aluminum foil. Incubate at room temperature for 60 minutes on a horizontal rotator set at 180 - 220 RPM.
- Remove the aluminum foil and plate sealer. Aspirate the contents of each well. Wash each well five times by dispensing 350  $\mu$ L of working wash solution into each well and then completely aspirating the contents. Preferably, an automated microtiter plate washer should be used.
- Pipet 200  $\mu$ L of ELISA HRP Substrate into each of the wells.
- Re-cover the plate with a plate sealer and aluminum foil. Incubate at room temperature for 30 minutes on a horizontal rotator set at 180 - 220 RPM.
- Remove the aluminum foil and plate sealer. Read the absorbance at 595 nm (see Note) within 5 minutes in a microtiter plate reader against the 0 RU/mL Standard wells as a blank.
- Immediately pipet 50  $\mu$ L of ELISA Stop Solution into each of the wells. Mix on horizontal rotator for 1 minute.
- Read the absorbance at 450 nm within 10 minutes in a microtiter plate reader against a reagent blank of 200  $\mu$ L of Substrate and 50  $\mu$ L of Stop Solution.  
*If wavelength correction is available, set the instrument to dual wavelength measurement at 450 nm with background wavelength correction set to absorbance used in step #11.*

**NOTE: Absorbance may be read at wavelengths from 595 nm to 650 nm depending upon available filters.**

### PROCEDURAL NOTES

- It is recommended that all standards, controls and samples be assayed in duplicate. The average absorbance reading of each duplicate should then be used for data reduction and the calculation of results.
- Keep light sensitive reagents (i.e. HRP-Avidin and ELISA HRP Substrate) in the original amber bottles or other suitable container which is well protected from light.
- Store any unused Antibody Coated Strips in the resealable aluminum pouch with desiccant to protect from moisture.
- The sample and all reagents should be pipetted carefully to minimize air bubbles in the wells.
- The sequence and timing of each reagent addition is important as both the immunological and enzymatic reactions are in kinetic modes. The washing step is also an important part of the total assay procedure. The use of an automated microtiter plate washer is strongly recommended. All pipeting and washing steps should be performed such that the timing is as consistent as possible.
- Samples with values greater than the highest standard should be diluted 1:10 or greater with the 0 RU/mL Standard or optional Sample Diluent reagent and reassayed. Multiply the result by the dilution factor. (See Limitations, # 2)
- Plasma or cell culture media samples may contain fibrin clots or cellular debris. Freeze/thaw of plasma samples may accelerate clot formation. These samples must be centrifuged and decanted prior to assay to remove all particulate material which can cause random high non-specific binding on well surface.

## CALCULATION OF RESULTS

The two absorbance readings taken before and after the addition of the ELISA Stop Solution allow for the construction of two standard curves using the human FGF-23 standards contained in the kit. **Refer to the individual vial label for exact concentration.** The primary curve used for calculation of results is the second reading taken after the addition of the ELISA Stop Solution and read at 450 nm. This data utilizes the absorbance values obtained with the first five standards. The first reading taken before the addition of the ELISA Stop Solution and read at 595 nm - 650 nm is intended to extend the analytical range to the value of the sixth (highest) standard provided in the kit. It should be utilized only if sample results extend beyond the value of the fifth standard. Results obtained with the first reading should not replace the on-scale reading at 450 nm. Each curve should be generated as follows:

### Primary Procedure — Read at 450 nm

1. Calculate the average absorbance for each pair of duplicate assay wells.
2. Subtract the average absorbance of the 0 RU/mL Standard from the average absorbance of all other readings to obtain corrected absorbance.
3. The standard curve is generated by plotting the corrected absorbance of the first five standard levels on the ordinate against the standard concentration on the abscissa using linear-linear or log-log paper. Appropriate computer assisted data reduction programs may also be used for the calculation of results.

The FGF-23 concentration of the controls and samples are read directly from the standard curve using their respective corrected absorbance. If log-log graph paper or computer assisted data reduction programs utilizing logarithmic transformation are used, samples having corrected absorbance between the 0 RU/mL Standard and the next highest standard should be calculated by the formula:

$$\text{Value of unknown} = \frac{\text{Corrected Absorbance (unknown)}}{\text{Corrected Absorbance (2}^{\text{nd}} \text{ Std.)}} \times \text{Value of the 2}^{\text{nd}} \text{ Std.}$$

### Secondary Procedure — Read at 595 nm - 650 nm

1. Calculate the average absorbance for each pair of duplicate assay wells.
2. The standard curve is generated by plotting the absorbance of the three highest standards on the ordinate against the standard concentration on the abscissa using linear-linear or log-log graph paper.
3. The FGF-23 concentration of samples reading greater than the fifth standard are read directly from the standard curve.

## EXAMPLE DATA AND STANDARD CURVE

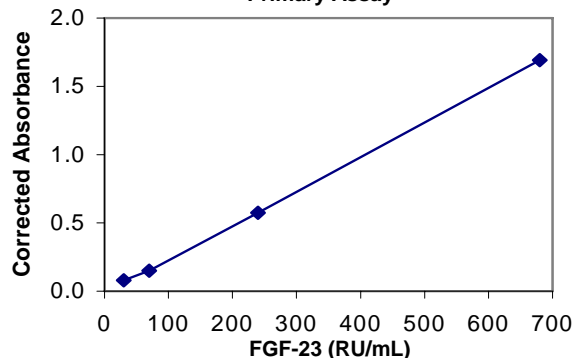
The following are representative examples of data and the resulting standard curves from the primary and secondary procedures. **These curves should not be used in lieu of a standard curve run with each assay.**

WELL I.D.	PRIMARY ASSAY - 450 nm			RESULTS RU/mL
	ABS	AVERAGE ABS	CORRECTED ABS	
Reagent Blank	0.000 0.000	0.000		
0 RU/mL	0.109 0.111	0.110	0.000	
30 RU/mL	0.183 0.194	0.191	0.081	
70 RU/mL	0.256 0.266	0.261	0.151	
240 RU/mL	0.684 0.683	0.684	0.574	
680 RU/mL	1.853 1.748	1.801	1.691	

Control I	0.234 0.235	0.235	0.125	55.3
Control II	0.434 0.447	0.441	0.331	157
Sample 1	0.577 0.589	0.583	0.473	219
Sample 2	2.028 2.101	2.065	1.955	*

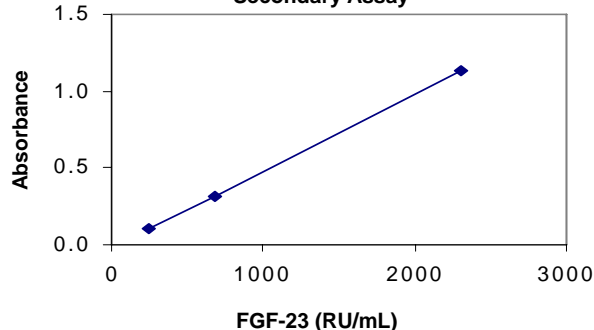
\* > 680 RU/mL. Calculate using secondary assay

Human FGF-23 (C-Term) ELISA  
Primary Assay



WELL I.D.	SECONDARY ASSAY - 595 nm		RESULTS RU/mL
	ABS	AVERAGE ABS	
0 RU/mL	0.000 0.000	0.000	
240 RU/mL	0.103 0.097	0.100	
680 RU/mL	0.314 0.318	0.316	
2300 RU/mL	1.148 1.119	1.134	
Sample 2	0.619 0.649	0.633	1,307

Human FGF-23 (C-Term) ELISA  
Secondary Assay



## LIMITATIONS OF THE PROCEDURE

1. The lowest concentration of human FGF-23 measurable is 3.0 RU/mL (assay sensitivity) and the highest concentration of human FGF-23 measurable without dilution is the value of the highest standard.

- The reagents in this Human FGF-23 (C-Term) ELISA kit have been optimized so that the high dose "hook effect" is not a problem for samples with elevated FGF-23 values. Samples with levels between the highest standard and 600,000 RU/mL will read greater than the highest standard and should be diluted 1:10 or greater with the 0 RU/mL Standard or optional Sample Diluent reagent and reassayed for correct values.
- Grossly lipemic serum or plasma samples may affect the immunological response and it is recommended that results obtained with such samples be scrutinized accordingly.
- Differences in protein concentration and protein type between samples and standards in an immunoassay contribute to "protein effects" and dose biases. When measuring low protein concentration culture media samples against high protein concentration standards, it is recommended that like samples be assayed together in the same assay to minimize this bias.

SAMPLE	ORIG. VALUE	AMOUNT ADDED	OBSERVED VALUE	EXPECTED VALUE	% O/E
1	43.9	240	281	280	100
		480	539	515	105
		720	802	751	107
2	45.1	240	306	281	109
		480	543	516	105
		720	864	752	115
3	36.7	240	311	273	114
		480	508	509	100
		720	666	746	89

## QUALITY CONTROL

To assure the validity of the results each assay should include adequate controls with known levels of human FGF-23. Immotopics recommends that all assays include the laboratory's own human FGF-23 controls in addition to those provided with this kit.

## PERFORMANCE CHARACTERISTICS:

### Accuracy

At this time there is no synthetic or purified natural full length FGF-23 available for use as a standard. This kit utilizes dilutions of a cell culture supernatant into which FGF-23 has been secreted as the antigen source for its calibrators. This secreted FGF-23 is known to be present in multiple molecular forms; the identity, quantity and ratio of each is unknown.

The concentration of the standards and controls are therefore expressed in Reference Units/mL (RU/mL) relative to this specific lot of cell culture supernatant.

### SENSITIVITY

The sensitivity of the Human FGF-23 (C-Term) ELISA as determined by the 95% confidence limit on 20 duplicate determinations of the 0 RU/mL Standard is 3.0 RU/mL.

### PRECISION

To assess intra-assay precision the mean and coefficient of variation were calculated from 20 duplicate determinations of two samples each performed in a single assay.

Mean Value (RU/mL)	Coefficient of Variation
52.7	5.0 %
140	5.0 %

To assess inter-assay precision the mean and coefficient of variation were calculated from duplicate determinations of two samples performed in 18 assays.

Mean Value (RU/mL)	Coefficient of Variation
50.9	5.0 %
153	7.3 %

### PARALLELISM

The multiple molecular forms and fragments of FGF-23 circulating in patients with various phosphate wasting disorders are as yet undefined and therefore makes serial dilution studies difficult to interpret because of the nature of the present kit calibrators. (See Accuracy section).

Some patients show excellent parallelism while others with excessive fragments yield an under-recovery upon serial dilution.

Therefore at this time results are best interpreted as relative relationships among samples.

### RECOVERY

Various amounts of FGF-23 were added to three different human serum or plasma samples and assayed. Results in RU/mL are as follows:

## WARRANTY

This product is warranted to perform as described in its labeling and literature when used in accordance with all instructions. Immotopics, Inc. DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, and in no event shall Immotopics, Inc. be liable for consequential damages. Replacement of the product or refund of the purchase price is the exclusive remedy for the purchaser. This warranty gives you specific legal rights and you may have other rights which vary from state to state.

## REFERENCES

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## CLIENT SERVICES

To place an order or for technical assistance, contact Immotopics International at (800) 681-6665 or (949) 369-9207 or FAX to (949) 369-9405 or e-mail: info@immutopicsintl.com.

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