Human Intact FGF-23 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.

96 Test Kit

For RESEARCH Use Only

Cat.# 60-6500

Not for use in diagnostic procedures

Store at 2 - 8ºC Upon Receipt

INTENDED USE

This kit is intended for research use only in the determination of human intact 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 Intact 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 Intact FGF-23 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 isolated and selected to detect epitopes within the amino-terminal and the carboxyl-terminal portions of FGF-23. One antibody is immobilized onto the microtiter plate wells for capture. The other antibody is conjugated with horseradish peroxidase (HRP) for detection.

A sample containing FGF-23 is incubated simultaneously with the immobilized capture antibody and the HRP conjugated detection antibody in a microtiter well. Intact 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 Intact FGF-23 — HRP/Anti-Human FGF
(C-terminal) (NH_2-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 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 intact FGF-23 in the sample. A standard curve is generated by plotting the absorbance versus the respective intact FGF-23 concentration for each standard on linear or logarithmic scales. The concentration of human intact FGF-23 in the samples is determined directly from this curve.

REAGENTS: Preparation and Storage

Store the kit at 2-8ºC upon receipt. **Store the standards and controls at -20ºC 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 INTACT FGF-23 ANTIBODY COATED PLATE (40-6515)**
   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. **HRP ANTIBODY DILUTING BUFFER (40-6560)**
   One vial containing 4 mL of TRIS buffered saline with protein stabilizers and a non-azide, non-mercury preservative. This reagent should be stored at 2 - 8°C and is stable until the expiration date on the kit.
3. **HRP CONJUGATED HUMAN INTACT FGF-23 ANTIBODY (40-6520)**
   One vial containing 2 mL of horseradish peroxidase conjugated anti-human FGF-23 in a stabilized protein solution 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.

   NOTE: Make a Working HRP Antibody Solution by pipetting 1 part HRP Conjugated Human Intact FGF-23 Antibody and 2 parts HRP Antibody Diluting Buffer prior to use. Mix only the volume required for immediate use. Mix well to ensure homogeneity.
4. **HUMAN INTACT FGF-23 STANDARDS (40-6531 to 40-6536)**
   Six vials, five of which contain Human Intact 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 intact FGF-23 concentration of 0 pg/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 -20ºC or below with up to 2 freeze/thaw cycles.
5. **HUMAN INTACT FGF-23 CONTROLS I & II (40-6541 & 40-6542)**
   Two vials each containing Human Intact 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 of the controls 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 -20ºC or below with up to 2 freeze/thaw cycles.

6. **ELISA WASH CONCENTRATE (40-0041)**
   One bottle 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 (10-2016)**
   Two included in kit.

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.

MATERIALS REQUIRED BUT NOT PROVIDED

1. 1.0 mL volumetric pipette for reconstituting standards and controls.
2. Precision pipets capable of delivering 50 µL ,150 µL and 200 µL.
3. Aluminum foil.
4. Automated microtiter plate washer OR
5. Repeating dispenser for delivering 350 µL and suitable aspiration device.
6. Container for storage of wash solution.
7. Spectrophotometric microtiter plate reader capable of reading absorbance at 450 nm and at 595 - 650 nm.
8. Deionized water.
9. Horizontal rotator capable of maintaining 180 - 220 RPM.
10. Timer.

SPECIMEN COLLECTION

The intact FGF-23 molecule appears to be highly unstable resulting in decreased immunoreactivity over time. Specimen collection and assay or storage procedures should be carried out in an expeditious manner. Measurement of the intact FGF-23 concentration should be made using EDTA plasma or cell culture media. 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 -20ºC or below. Avoid repeated freezing and thawing of specimens.

ASSAY PROCEDURE

1. Place a sufficient number of Antibody Coated Strips in a holder to run FGF-23 standards, controls and unknown samples.
2. Pipet 150 µL of standard, control, or sample into the designated or mapped well. Freeze the remaining standards and controls as soon as possible after use.
3. Pipet 50 µL of the Working HRP Antibody Solution consisting of 1 part HRP Conjugated Intact FGF-23 Antibody and 2 parts HRP Antibody Diluting Buffer into each well.
4. Cover the plate with one plate sealer, then cover with aluminum foil to avoid exposure to light.
5. Incubate plate at room temperature for three (3) hours on a horizontal rotator set at 180 - 220 RPM.
6. Remove the aluminum foil and plate sealer. Using an automated microtiter plate washer aspirate the contents of each well. Wash each well five times by dispensing 350 µL of working wash solution into each well and then completely aspirate the contents. A suitable aspiration device may also be used.
7. Pipet 200 µL of ELISA HRP Substrate into each of the wells.
8. Re-cover the plate with the plate sealer and aluminum foil. Incubate at room temperature for 30 minutes on a horizontal rotator set at 180 - 220 RPM.
9. Remove the aluminum foil and plate sealer. Read the absorbance at 620 nm (see Note) within 5 minutes in a microtiter plate reader against the 0 pg/mL Standard wells as a blank.
10. Immediately pipet 50 µL of ELISA Stop Solution into each of the wells. Mix on horizontal rotator for 1 minute.
11. Read the absorbance at 450 nm within 10 minutes in a microtiter plate reader against a reagent blank of 200 µL of Substrate and 50 µL 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 #9.

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

PROCEDURAL NOTES

1. 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.

2. Keep light sensitive reagents (i.e. HRP Conjugated Antibody, the Working HRP Antibody Solution consisting of combined HRP Conjugated Antibody and HRP Antibody Diluting Buffer, and ELISA HRP Substrate) in the original amber bottles or other suitable container which is well protected from light.

3. Store any unused Antibody Coated Strips in the resealable aluminum pouch with desiccant to protect from moisture.

4. The sample and all reagents should be pipetted carefully to minimize air bubbles in the wells.

5. 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.

6. Samples with values greater than the highest standard should be diluted 1:10 with saline and reassayed. Multiply the result by 10. (See Limitations, # 2)

7. 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 intact 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 pg/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 intact 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 pg/mL Standard and the next highest standard should be calculated by the formula:

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

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 intact FGF-23 concentration of samples reading greater than the fifth standard are read directly from the standard curve.

EXAMPLE DATA AND STANDARD CURVE

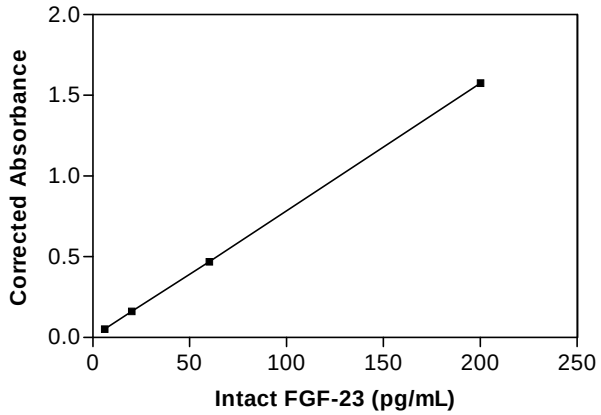
The following are representative examples of data and the resulting standard curves from the primary and secondary procedures. **These curves should not be used in lieu of a standard curve run with each assay.**

PRIMARY ASSAY - 450 nm

WELL I.D.	ABS	AVERAGE ABS	CORRECTED ABS	RESULTS pg/mL
Reagent Blank	0.000			
	0.000	0.000		
0 pg/mL	0.030			
	0.029	0.030	0.000	
6 pg/mL	0.085			
	0.081	0.083	0.053	
20 pg/mL	0.194			
	0.189	0.192	0.162	
60 pg/mL	0.496			
	0.490	0.493	0.463	
200 pg/mL	1.586			
	1.626	1.606	1.576	
Control I	0.130			
	0.133	0.132	0.102	12
Control II	1.186			
	1.210	1.198	1.168	149
Sample 1	0.257			
	0.253	0.255	0.225	28
Sample 2	1.967			
	1.995	1.981	1.951	*

* > 200 pg/mL. Calculate using secondary assay.

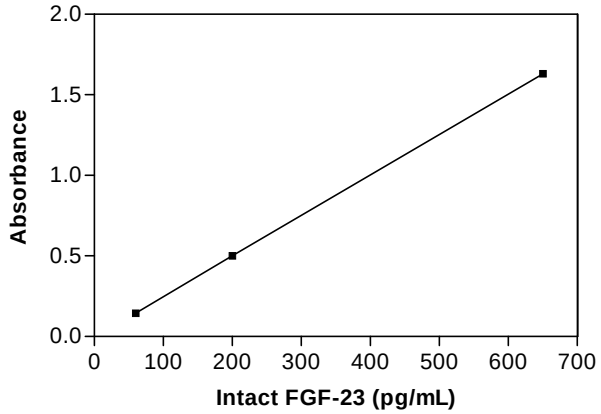
Human Intact FGF-23 ELISA
Primary Assay



SECONDARY ASSAY - 620 nm

WELL I.D.	ABS	AVERAGE ABS	RESULTS pg/mL
0 pg/mL	0.000		
	0.000	0.000	
60 pg/mL	0.163		
	0.155	0.159	
200 pg/mL	0.522		
	0.510	0.516	
650 pg/mL	1.674		
	1.618	1.646	
Sample 2	0.846		
	0.851	0.849	332

Human Intact FGF-23 ELISA
Secondary Assay



LIMITATIONS OF THE PROCEDURE

1. The lowest concentration of human intact FGF-23 measurable is 1.0 pg/mL (assay sensitivity) and the highest concentration of human intact FGF-23 measurable without dilution is the value of the highest standard.
2. The reagents in this Human Intact FGF-23 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 100,000 pg/mL will read greater than the highest standard and should be diluted 1:10 or greater with saline and reassayed for correct values.
3. Grossly lipemic plasma samples may affect the immunological response and it is recommended that results obtained with such samples be scrutinized accordingly.

4. 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.

QUALITY CONTROL

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

PERFORMANCE CHARACTERISTICS:

SENSITIVITY

The sensitivity of the Human Intact FGF-23 ELISA as determined by the 95% confidence limit on 20 duplicate determinations of the 0 pg/mL Standard is 1.0 pg/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 (pg/mL)	Coefficient of Variation
14.6	4.4 %
148	2.6 %

To assess inter-assay precision the mean and coefficient of variation were calculated from duplicate determinations of two samples performed in 20 assays.

Mean Value (pg/mL)	Coefficient of Variation
15.6	6.1 %
166	6.5 %

PARALLELISM

The multiple molecular forms and fragments of FGF-23 circulating in both normal patients and those with various phosphate wasting disorders are as yet undefined therefore making inconsistencies in serial dilution studies difficult to interpret.

Some patients show excellent parallelism while others yield a significant under-recovery upon serial dilution.

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

RECOVERY

Variable instability of the intact molecule in different matrices apparent in both samples and calibrators combined with the fragment issues described under Parallelism render typical recovery studies also very difficult to interpret.

In general when matrices are identical and protected from proteases the observed recovery of intact peptide measured with this ELISA is 90 - 110%.

WARRANTY

This product is warranted to perform as described in its labeling and literature when used in accordance with all instructions. Immutopics, Inc. DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, and in no event shall Immutopics, 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.

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CLIENT SERVICES

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

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