Mouse/Rat FGF-23 (C-Term) ELISA Kit

Immutopics

Immutopics, Inc.

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

96 Test Kit
Cat. #60-6300

For RESEARCH Use Only

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 quantitative determination of Mouse FGF-23 levels in plasma or cell culture media. **This assay is also useful in the determination of Rat FGF-23 levels.**

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.

Renal phosphate wasting disorders leading to hypophosphatemia are among the causes of defective mineralization of bone and growth plate development. Autosomal dominant hypophosphatemic rickets (ADHR), a rare genetic disorder, results from 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. Consistent with this conclusion, the administration of recombinant FGF-23 to rodents was shown to increase urinary excretion of phosphate thus leading to hypophosphatemia and osteomalacia/rickets. Recent studies with chronic kidney disease (CKD) patients have shown FGF-23 to be an early predictor of abnormal renal tubular function, bone mineralization, disease severity and over-all mortality risk.

Taken together, all currently available data suggest that the measurement of FGF-23 levels may provide an important diagnostic tool for the evaluation of hypophosphatemic and hyperphosphatemic disorders.

TEST PRINCIPLE

This Mouse FGF-23 (C-Term) ELISA Kit is a homologous, 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) region of mouse FGF-23. One antibody is biotinylated for capture and the other antibody is conjugated with the enzyme horseradish peroxidase (HRP) for detection.

A sample containing mouse FGF-23 is incubated simultaneously with the biotinylated capture antibody and the HRP conjugated detection antibody in a streptavidin coated 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/Avidin— Biotin Anti-mFGF23 (C-terminal) — Mouse FGF23 — HRP Anti-mFGF23 (C-terminal)

At the end of this incubation period, the well is washed to remove any unbound antibody and other 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 mouse 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. **STREPTAVIDIN COATED MICROTITER PLATE (40-0010)**
   One plate with 12 eight well strips (96 wells total). This reagent should be stored in the foil pouch with desiccant at 2 - 8°C and is stable until the expiration date on the kit.
2. **BIOTINYLATED MOUSE FGF-23 ANTIBODY (40-6310)**
   One vial containing 2.7 mL of biotin labeled anti-mouse FGF-23 in 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 MOUSE FGF-23 ANTIBODY (40-6320)**
   One vial containing 2.7 mL of horseradish peroxidase (HRP) conjugated to anti-mouse 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 Antibody Solution by pipetting equal volumes of Biotinylated Mouse FGF-23 Antibody and HRP Conjugated Mouse FGF-23 Antibody prior to use. Mix only the volume required for immediate use. Mix well to ensure homogeneity.
4. **MOUSE FGF-23 STANDARDS (40-6331 to 40-6336)**
   Six vials each containing recombinant mouse 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 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 3 freeze/thaw cycles.
5. **MOUSE FGF-23 CONTROLS I & II (40-6341 & 40-6342)**
   Two vials each containing recombinant mouse 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 -20ºC 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 phosphate buffered 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 (40-0026)**
   One bottle containing 11 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.
10. **SAMPLE DILUENT (Optional reagent, must be ordered separately using catalog #30-6631)**
    One bottle containing 10 mL of a lyophilized protein matrix with a non-azide, non-mercury preservative. This reagent should be stored at 2 - 8ºC and is stable until the expiration date on the bottle. Before use reconstitute with 10 mL of deionized water. Allow the bottle to sit for approximately 20 minutes with occasional gentle swirling and inversion. Assure complete reconstitution before use. Aliquot and freeze (-20°C or below) any unused portion for later use.

    NOTE: Human FGF-23 Sample Diluent, Cat: #30-6631 is suitable for use as a diluent in this mouse/rat assay.

SAFETY PRECAUTIONS

Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid (i.e. ELISA HRP Substrate and ELISA Stop Solution). 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 and 2.0 mL volumetric pipettes for reconstituting standards and controls.
2. Precision pipets capable of delivering 25 µL, 50µL and 100 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 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.** Fifty microliters of plasma or culture media are required to assay the sample in duplicate. 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 Streptavidin Coated Strips in a holder to run FGF-23 standards, controls and samples.
2. Pipet 25 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 Antibody Solution consisting of 1 part Biotinylated Antibody and 1 part HRP Antibody 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 aspirating the contents.** A suitable aspiration device may also be used.
7. Pipet 100 µL of ELISA HRP Substrate into each of the wells.
8. 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.
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 100 µL of Substrate and 50 µL of Stop Solution.

    If dual wavelength measurement is available set Measurement wavelength to 450 nm and Reference wavelength 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 Antibody Solution consisting of combined Biotinylated Antibody and HRP Conjugated Antibody, and ELISA HRP Substrate) in the original amber bottles or other suitable container which is well protected from light.
3. Store any unused Streptavidin 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 or greater with the 0 pg/mL Standard or optional Sample Diluent reagent and reassayed. Multiply the result by the dilution factor. (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.
8. Rarely, upon opening the streptavidin plate, small white crystals may be observed in some of the wells. This is entirely cosmetic and will not affect the assay. This condition is reported by other kit manufacturers and results from the final stabilizing buffer used in the coating process.

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 mouse 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 used only for sample results that fall between the value of the fifth and sixth standard.** Results obtained with this 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 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:

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

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 only between the fifth and sixth standard are read directly from this standard curve.

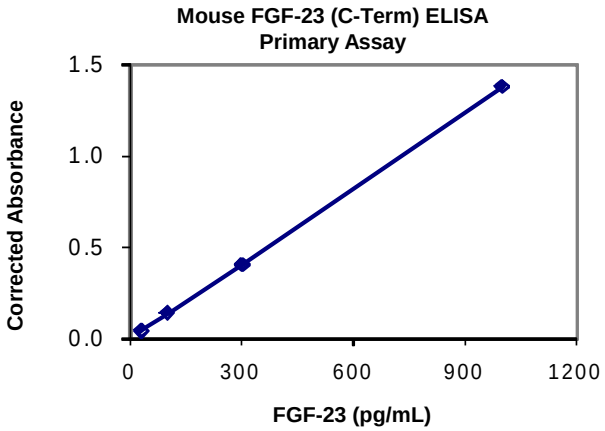
EXAMPLE DATA AND STANDARD CURVE

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

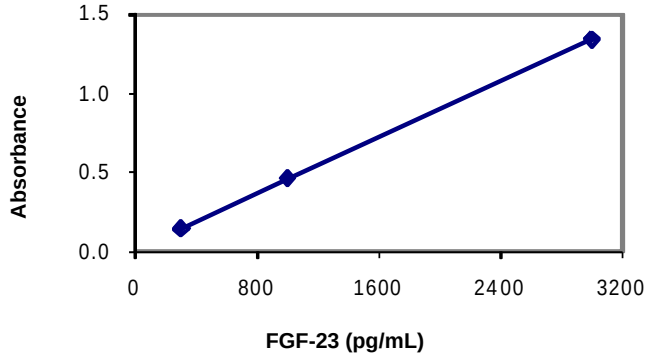
WELL I.D.	ABS	AVERAGE ABS	CORRECTED ABS	RESULTS pg/mL
Reagent Blank	0.000			
	0.000	0.000		
0 pg/mL	0.043			
	0.043	0.043	0.000	
30 pg/mL	0.087			
	0.091	0.089	0.046	
100 pg/mL	0.191			
	0.185	0.188	0.145	
300 pg/mL	0.453			
	0.449	0.451	0.408	
1000 pg/mL	1.446			
	1.407	1.427	1.384	
Control I	0.146			
	0.142	0.144	0.101	69.3
Control II	0.338			
	0.320	0.329	0.286	207
Sample 1	0.636			
	0.624	0.630	0.587	429
Sample 2	3.346			
	3.236	3.291	3.248	*

* > 1000 pg/mL. Calculate using secondary assay



SECONDARY ASSAY - 620 nm

WELL I.D.	ABS	AVERAGE ABS	RESULTS pg/mL
0 pg/mL	0.000		
	0.000	0.000	
300 pg/mL	0.146		
	0.147	0.147	
1000 pg/mL	0.471		
	0.459	0.465	
3000 pg/mL	1.376		
	1.310	1.343	
Sample 2	1.172		
	1.216	1.194	2606



LIMITATIONS OF THE PROCEDURE

1. The lowest concentration of mouse FGF-23 measurable is 4 pg/mL (assay sensitivity) and the highest concentration of mouse FGF-23 measurable without dilution is the value of the highest standard.
2. The reagents in this Mouse 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 500,000 pg/mL will read greater than the highest standard and should be diluted 1:10 or greater with the 0 pg/mL Standard or optional Sample Diluent reagent and reassayed for correct values.

3. Grossly lipemic 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 mouse FGF-23. Immutopics recommends that all assays include the laboratory's own mouse FGF-23 controls in addition to those provided with this kit.

PERFORMANCE CHARACTERISTICS:

SENSITIVITY

The sensitivity of the Mouse FGF-23 (C-Term) ELISA as determined by the 95% confidence limit on 20 duplicate determinations of the 0 pg/mL Standard is 4 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
62	6.2 %
206	4.5 %

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
66.6	5.9 %
201	4.4 %

PARALLELISM

Mouse serum samples were diluted with 0 pg/mL Standard and assayed. Results in pg/mL are as follows:

SAMPLE	DILUTION	OBSERVED VALUE	EXPECTED VALUE	% O/E
1	undiluted	85.7		
	1:2	40.0	42.9	93
	1:4	20.3	21.4	95
	1:8	12.8	10.7	119
2	undiluted	102		
	1:2	43.4	51	85
	1:4	20.8	25	83
	1:8	10.3	12.5	82

RECOVERY

Various amounts of FGF-23 were added to two different mouse serum samples and assayed. Results in pg/mL are as follows:

SAMPLE	ORIG. VALUE	AMOUNT ADDED	OBSERVED VALUE	EXPECTED VALUE	% O/E
1	95	529	417	600	70
		1059	881	1106	80
		1588	1187	1612	74
2	19	529	517	543	95
		1059	1074	1068	100
		1588	1533	1593	96

CROSSREACTIVITY

Mouse and rat FGF-23 have an overall amino acid sequence homology of 94%. Within the binding region of the capture antibody used in this assay the homology with rat is 95% and for the detection antibody it is 90%.

When assayed in this kit, samples from rats with induced, escalating renal failure exhibited serum concentrations of approximately 100 pg/mL (control) through 50,000 pg/mL.

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.

REFERENCES

1. Farrow E, YuX, Summers L, et.al. "Iron deficiency drives an autosomal dominant hypophosphatemic rickets (ADHR) phenotype in fibroblast growth factor-23 (Fgf23) knock-in mice", *PNAS,* 2011; 108: E1146-E1155.
2. Kawata T, Imanishi Y, Kobayashi K, Miki T, Arnold A, Inaba M, Nishizawa Y. "Parathyroid Hormone Regulates Fibroblast Growth Factor-23 in a Mouse Model of Primary Hyperparathyroidism." *J Am Soc Nephrol,* 2007; 18: 2683-2688.
3. Perwad F, Azam N, Zhang M, Yamashita T, Tenenhouse H, Portale A. "Dietary and Serum Phosphorus Regulate Fibroblast Growth Factor 23 Expression and 1, 25-Dihydroxyvitamin D Metabolism in Mice." *Endocrinology* 2005; 146(12): 5358-5364.
4. Shimada T, Kakitani M, Yamazaki Y, Hasegawa H, Takeuchi Y, Fujita T, Fukumoto S, Tomizuka K, Yamashita T. "Targeted ablation of *Fgf23* demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism". *JClin Invest,* 2004; 113 (4): 561-568.
5. Jonsson KB, Zahradnik R, Larsson T, White K, Sugimoto T, Imanishi Y, Yamamoto T, Hampson G, Koshiyama H, Ljunggren Ö, Oba K, Yang IM, Miyauchi A, Econs MJ, Lavigne J, Jüppner H. "Fibroblast Growth Factor 23 in Oncogenic Osteomalacia and X-Linked Hypophosphatemia." *N Engl J Med,* 2003; 348:1656-63.
6. Shimada T, Mizutani S, Muto T, Yoneya T, Hino R, Takeda S, Takeuchi Y, Fujita T, Fukumoto S, Yamashita T. "Cloning and Characterization of FGF23 As a Causative Factor of Tumor-induced Osteomalacia". *Proc. Natl. Acad. Sci. USA*, 2001; 98:6500-05.

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.

Developed and Manufactured by: ***Immutopics, Inc.*** *San Clemente, CA 92673*

Distributed by: ***Immutopics International*** *San Clemente, CA 92673*

www.**immutopicsintl**.com

Catalog # 60-6300
90-6300
Effective: 09/14