Human Fibroblast Growth Factor-21 (FGF-21)

96-Well Plate

Cat. # EZHFGF21-19K

HUMAN FIBROBLAST GROWTH FACTOR-21 (FGF-21) ELISA KIT
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HUMAN FGF-21 ELISA KIT
96-Well Plate (Cat. # EZHFGF21-19K)

I. INTENDED USE

This Human FGF-21 ELISA kit is used for the non-radioactive quantification of Human FGF-21 in serum, plasma, and adipocyte extracts or cell culture media samples. This kit specifically measures native Human FGF-21. One kit is sufficient to measure 38 unknown samples in duplicate. ***This kit is for research purpose only.***

II. PRINCIPLES OF PROCEDURE

This assay is a Sandwich ELISA based, sequentially, on: 1) concurrent capture of Human FGF-21 molecules from samples to the wells of a microtiter plate coated with a polyclonal goat anti-human FGF-21 antibody, and binding of a second biotinylated polyclonal goat anti-human antibody to the captured molecules , 2) washing of unbound materials from samples, 3) binding of streptavidin-horseradish peroxidase conjugate to the immobilized biotinylated antibodies, 4) washing of excess of free enzyme conjugates, and 5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbance at 450 nm – 590nm after acidification of formed products. Since the increase in absorbance is directly proportional to the amount of captured Human FGF-21 in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of Human FGF-21.

III. REAGENTS SUPPLIED

Each kit is sufficient to run one 96-well plate and contains the following reagents:

A. Human FGF-21 ELISA Plate

Coated with Goat anti-Human FGF-21 Antibodies
Quantity: 1 strip plate
Preparation: Ready to Use
Note: Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8°C.

B. Adhesive Plate Sealer

Quantity: 2 sheets
Preparation: Ready to Use

C. 10X HRP Wash Buffer Concentrate

10X concentrate of 50 mM Tris Buffered Saline containing Tween-20
Quantity: 2 bottles containing 50 mL each
Preparation: Dilute 1:10 with distilled or deionized water

D. Human FGF-21 Standard

Purified Recombinant Human FGF-21, lyophilized.
Quantity: 0.5 mL upon hydration
Preparation: Reconstitute with 0.5 mL distilled or deionized water. See insert for concentration.

E. Human FGF-21 Quality Controls 1 and 2

One vial each, lyophilized, containing purified recombinant Human FGF-21 at two different levels.
Quantity: 0.5mL/bottle upon hydration
Preparation: Reconstitute each vial with 0.5mL distilled or deionized water.

F. Matrix Solution

Quantity: 0.5mL, 2 bottles
Preparation: Dilute each bottle 1:2 with 0.5mL Assay Buffer and mix well prior to use.

G. Assay Buffer

0.05M PBS, pH 6.8, containing proprietary protease inhibitors, with Tween 20, 0.08% Sodium Azide and 1% BSA.
Quantity: 25 mL
Preparation: Ready to Use

H. Human FGF-21 Detection Antibody

Pre-titered Biotinylated Goat anti-Human FGF-21 Antibody
Quantity: 3 mL
Preparation: Ready to Use

III. REAGENTS SUPPLIED (continued)

I. Enzyme Solution

Pre-titered Streptavidin-Horseradish Peroxidase Conjugate in Buffer
Quantity: 12 mL
Preparation: Ready to Use

J. Substrate (Light sensitive, avoid unnecessary exposure to light)

3, 3', 5, 5'-tetramethylbenzidine in buffer
Quantity: 12 mL
Preparation: Ready to Use.

K. Stop Solution (Caution: Corrosive Solution)

0.3 M HCl
Quantity: 12 mL
Preparation: Ready to Use

IV. STORAGE AND STABILITY

Prior to use, all components in the kit can be stored up to 2 weeks at 2-8^{o}C. For longer storage (> 2 weeks), freeze diluted Wash Buffer, Assay Buffer, and reconstituted Standards and Controls at ≤ –20^{o}C. Minimize repeated freeze and thaw of the FGF-21 Standards and Quality Controls. Unused microtiter strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8°C. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

V. REAGENT PRECAUTIONS

A. Sodium Azide

Sodium Azide has been added to certain reagents as a preservative. Although the concentrations are low, Sodium Azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

B. Hydrochloric Acid

Hydrochloric Acid is corrosive and can cause eye and skin burns. It is harmful if swallowed and can cause respiratory and digestive tract burns. Avoid contact with skin and eyes. Do not swallow or ingest.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipettes and Pipette Tips: 10 µL - 20 µL or 20 µL - 100 µL
2. Multi-Channel Pipettes and Pipette Tips: 5 µL ~ 50 µL and 50 µL ~ 300 µL
3. Buffer and Reagent Reservoirs
4. Vortex Mixer
5. Deionized Water
6. Microtiter Plate Reader capable of reading absorbency at 450 nm
7. Orbital Microtiter Plate Shaker
8. Absorbent Paper or Cloth

VII. SAMPLE COLLECTION AND STORAGE

1. To prepare serum samples, whole blood is directly drawn into a centrifuge tube that contains no anti-coagulant. Let blood clot at room temperature for 30 min.

2. Promptly centrifuge the clotted blood at 2,000 to 3,000xg for 15 minutes at 4 ± 2^{o}C.

3. Transfer and store serum samples in separate tubes. Date and identify each sample.

4. Use freshly prepared serum or aliquot and store samples at ≤ –20^{o}C for later use. For long-term storage, keep at -70 ^{o}C. Avoid freeze/thaw cycles.

5. To prepare plasma samples, whole blood should be collected into centrifuge tubes containing enough K_3EDTA to achieve a final concentration of 1.735 mg/mL and centrifuged immediately after collection. Observe the same precautions in the preparation of serum samples.

6. If heparin is to be used as an anticoagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.

7. Avoid using samples with gross hemolysis or lipemia.

VIII. REAGENT PREPARATION

A. Human FGF-21 Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Human FGF-21 Standard with 0.5 mL distilled or deionized water to give a concentration described on the analysis sheet. Invert and mix gently, let sit for 5 minutes then vortex gently.

2. Label six tubes 1, 2, 3, 4, 5, and 6. Add 0.25 mL Assay Buffer (AB-GLPHK) to each of the six tubes. Prepare serial dilutions by adding 0.25 mL of the reconstituted standard to Tube 1, mix well and transfer 0.25 mL of Tube 1 to Tube 2, mix well and transfer 0.25 mL of Tube 2 to Tube 3, mix well and transfer 0.25 mL of Tube 3 to Tube 4, mix well and transfer 0.25 mL of Tube 4 to Tube 5, mix well and transfer 0.25 mL of Tube 5 to Tube 6 and mix well.

   Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with Standard before dispensing. Unused portions of standard should be stored at ≤ -20°C. Avoid multiple freeze/thaw cycles.

Volume of Deionized Water to Add	Volume of Standard to Add	Standard Concentration (pg/mL)
0.5 mL	0	X (refer to analysis sheet for exact concentration)

Tube #	Volume of Assay Buffer (AB-GLPHK) to Add	Volume of Standard to Add	Standard Concentration (pg/mL)
Tube 1	0.25 mL	0.25 mL of reconstituted standard	X/2
Tube 2	0.25 mL	0.25 mL of Tube 1	X/4
Tube 3	0.25 mL	0.25 mL of Tube 2	X/8
Tube 4	0.25 mL	0.25 mL of Tube 3	X/16
Tube 5	0.25 mL	0.25 mL of Tube 4	X/32
Tube 6	0.25 mL	0.25 mL of Tube 5	X/64

B. Human FGF-21 Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Using a pipette, reconstitute each of the Human FGF-21 Quality Control 1 and Quality Control 2 with 0.5 mL distilled or deionized water into the glass vials. Invert and mix gently, let sit for 5 minutes then mix well.

C. Matrix Solution Preparation

Dilute each bottle of Matrix Solution with 0.5ml of Assay Buffer. Combine contents of both bottles together and invert several times to mix prior to use.

IX. ASSAY PROCEDURE

Pre-warm all reagents to room temperature prior to setting up the assay.

1. Dilute the 10X Wash Buffer concentrate 10 fold by mixing the entire content of each bottle of Wash Buffer with 450 mL deionized water (dilute both bottles with 900 mL deionized water).

2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8°C. Assemble the strips in an empty plate holder and wash each well 3 times with 300 µl of diluted Wash Buffer per wash. Decant wash buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. **Do not let wells dry before proceeding to the next step. If an automated machine is used for the assay, follow the manufacturer's instructions for all washing steps described in this protocol.**

3. Add in duplicate 50 µL of 1:2 diluted Matrix Solution to blank wells, Standard wells, and Quality Control wells.

4. Add in duplicate 50 µL of Assay Buffer to blank wells and sample wells.

5. Add in duplicate 50 µL Human FGF-21 Standards in the order of ascending concentration to the appropriate wells. Add in duplicate 50 µL QC1 and 50 µL QC2 to the appropriate wells. Add 50 µL of the unknown samples in duplicate to the remaining wells.

6. Add 25 µL Detection Antibody to all wells. **For best result all additions should be completed within 30 minutes.** Cover the plate with plate sealer and incubate at room temperature for 2 hours on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 400 to 500 rpm.

7. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the wells.

8. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap firmly after each wash to remove residual buffer.

9. Add 100 µL Enzyme Solution to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker.

10. Remove sealer, decant solutions from the plate, and tap plate to remove the residual fluid.

11. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap firmly after each wash to remove residual buffer.

IX. ASSAY PROCEDURE (continued)

12. Add 100 µL of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker for **approximately** 5 to 20 minutes. Blue color should be formed in wells of the FGF-21 standards with intensity proportional to increasing concentrations of FGF-21.

Note: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.

13. Remove sealer and add 100 µL Stop Solution **[CAUTION: CORROSIVE SOLUTION]** and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification. Read absorbance at 450 nm and 590nm in a plate reader within 5 minutes and ensure that there are no air bubbles in any well. Record the difference of absorbance units. The absorbance of the highest FGF-21 standard should be approximately 2.0 - 3.0, or not to exceed the capability of the plate reader used.

Assay Procedure for Human FGF-21 ELISA kit (Cat. # EZHFGF21-19K)

	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 6-8	Step 9	Step 10-11	Step 12	Step 12	Step 13	Step 13
Well #	**Dilute each bottle of 10X Wash Buffer with 450mL Deionized Water.**	**Wash plate 3X with 300 µl Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels**	Matrix Solution	Assay Buffer	Standards/ Controls/ Samples	Detection Ab	**Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µl Wash Buffer**	Enzyme Solution	**Seal, Agitate, Incubate 30 minutes at Room Temperature. Wash 3X with 300 µl Wash Buffer**	Substrate	**Seal, Agitate, Incubate 5 - 20 minutes at Room Temperature.**	Stop Solution	**Read Absorbance at 450 nm and 590 nm.**
A1, B1			50 µl	50 µl	0 µl	25 µl		100 µl		100 µl		100 µl	
C1, D1			50 µl	0 µl	50 µl of Tube 6								
E1, F1			50 µl	0 µl	50 µl of Tube 5								
G1, H1			50 µl	0 µl	50 µl of Tube 4								
A2, B2			50 µl	0 µl	50 µl of Tube 3								
C2, D2			50 µl	0 µl	50 µl of Tube 2								
E2, F2			50 µl	0 µl	50 µl of Tube 1								
G2, H2			50 µl	0 µl	50 µl of Reconstituted Standard								
A3, B3			50 µl	0 µl	50 µl of QC 1								
C3, D3			50 µl	0 µl	50 µl of QC 2								
E3, F3			0 µl	50 µl	50 µl of Sample								
G3, H3			0 µl	50 µl	50 µl of Sample								
A4, B4 ↓			0 µl	50 µl	50 µl of Sample	▼		▼		▼		▼	

X. MICROTITER PLATE ARRANGEMENT

Human FGF-21 ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Tube 3	QC 1	Etc.								
B	Blank	Tube 3	QC 1	Etc.								
C	Tube 6	Tube 2	QC2									
D	Tube 6	Tube 2	QC2									
E	Tube 5	Tube 1	Sample 1									
F	Tube 5	Tube 1	Sample 1									
G	Tube 4	Reconstituted Standard	Sample 2									
H	Tube4	Reconstituted Standard	Sample 2									

XI. CALCULATIONS

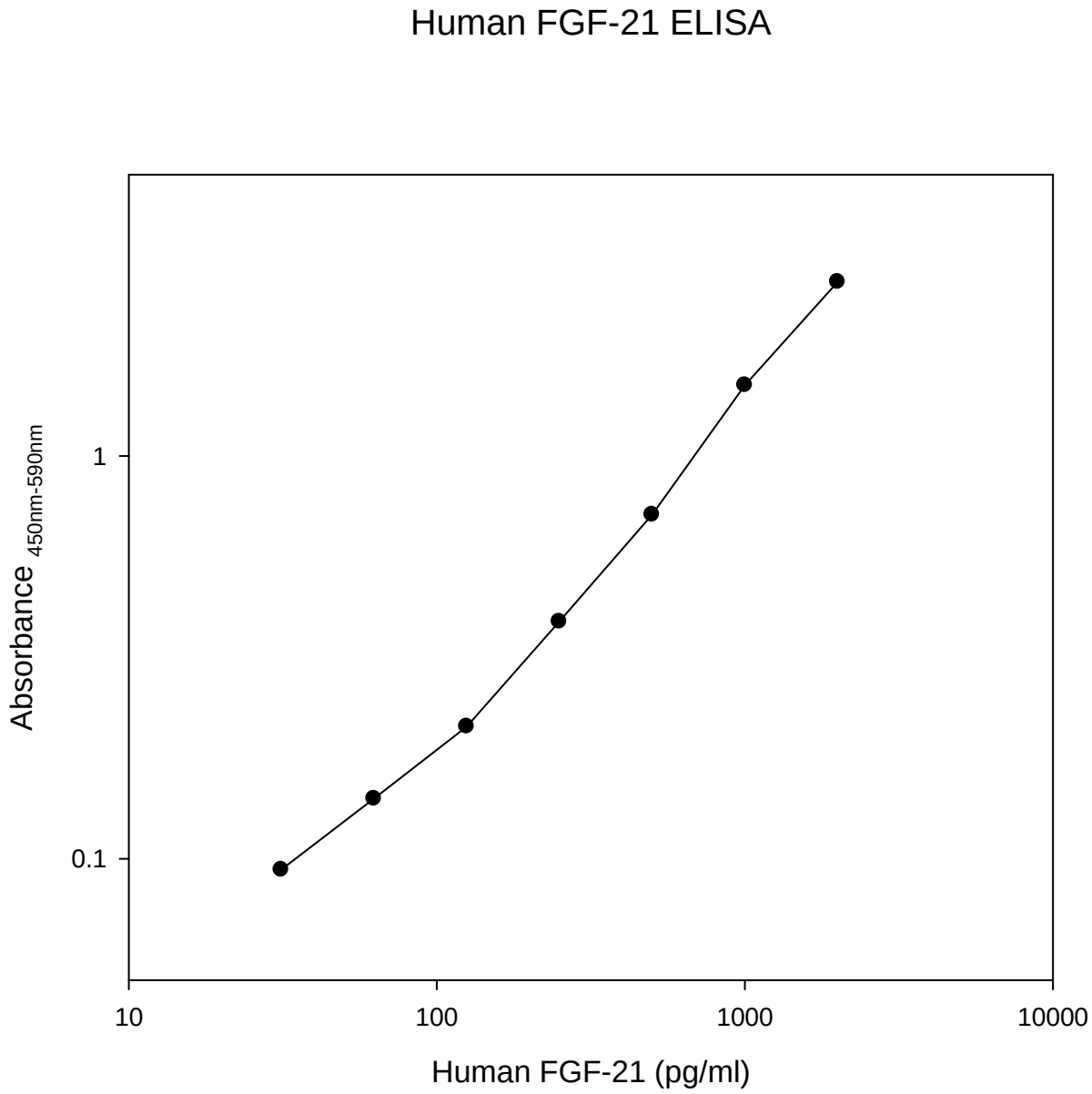
The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function

Note: When sample volumes assayed differ from 50 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 25 µL of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 50 µL, compensate the volume deficit with assay buffer.

XII. INTERPRETATION

1. The assay will be considered accepted when all Quality Control values fall within the calculated Quality Control Range. If any QC's fall outside the control range, review results with a supervisor.
2. If the difference between duplicate results of a sample is >15% CV, repeat the sample.
3. The limit of sensitivity of this assay is 15.6 pg/mL Human FGF-21 (50 µL sample size).
4. The appropriate range of this assay is 31.25 pg/mL to 2000 pg/mL Human FGF-21 (50 µL sample size). Any result greater than 2000 pg/mL in a 50 µL sample should be diluted using assay buffer, and the assay repeated until the results fall within range.

XIII. STANDARD CURVE



Typical Standard Curve, not to be used to calculate data.

XIV. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of FGF-21 that can be detected by this assay is 15.6 pg/mL when using a 50 µL sample size.

B. Specificity

The antibody pair used in this assay is specific to Human FGF-21 and does not significantly cross-react to the following molecules/hormones tested:

EGF, Eotaxin, FGF-2, Flt-3 Ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFNa2, IFNg, IL-1ra, IL-1a, IL-1b, IL-2, sIL-2Ra, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MCP-3, MDC, MIP-1a, MIP-1b, PDGF-AA, PDGF-AB/BB, RANTES, sCD40L, TGFa, TNFa, TNFb, VEGF, Leptin, GLP-1 (Active), C-Peptide, Insulin, Glucagon, Amylin (Active), and Amylin (Total).

The antibody pair used in this assay cross-react 0.05% to Human FGF-23 and 0.02% to FGF-19.

C. Precision

Intra-Assay Variation

Sample No.	Mean FGF-21 Levels (pg/mL)	Intra-Assay % CV
1	163	5.8
2	210	1.9
3	328	3.0

The assay variations of Millipore Human FGF-21 ELISA Kits were studied on three human serum samples with varying concentrations of endogenous FGF-21. The mean intra-assay variation was calculated from the results of eight replicate determinations in each assay for the indicated samples.

XIV. ASSAY CHARACTERISTICS (continued)

Inter-Assay Variation

Sample No.	Mean FGF-21 Levels (pg/mL)	Inter-Assay % CV
1	80	9.0
2	133	1.2
3	151	8.4
4	191	3.5
5	242	4.8
6	306	1.2

The assay variations of Millipore Human FGF-21 ELISA Kits were studied on six human serum samples with varying concentrations of endogenous FGF-21.The mean inter-assay variations of each sample were calculated from the results of three separate assays with duplicate samples in each assay.

D. Recovery

Spike & Recovery of Human FGF-21 in Serum

Sample No.	FGF-21 Added pg/mL	Expected pg/mL	Observed pg/mL	% of Recovery
1	0	26.0	26.0	
	62.5	88.5	81.0	92
	250	276.0	247.0	89
	500	526.0	436.0	83
2	0	88.0	88.0	
	62.5	150.5	145.0	96
	250	338.0	319.0	94
	500	588.0	551.0	94
3	0	144.0	144.0	
	62.5	206.5	200.0	97
	250	394.0	371.0	94
	500	644.0	615.0	96
4	0	302.0	302.0	
	62.5	364.5	358.0	90
	250	552.0	523.0	88
	500	802.0	765.0	93
5	0	519.0	519.0	
	62.5	581.5	573.0	99
	250	769.0	715.0	93
	500	1019.0	885.0	87

Varying amounts of Human FGF-21 were added to five human serum samples and the FGF-21 content was determined in two separate assays. The % of recovery = observed FGF-21 concentrations/expected FGF-21 concentrations x 100%.

XIV. ASSAY CHARACTERISTICS (continued)

E. Linearity and Dilution

Sample No.	Volume Sampled	Expected pg/mL	Observed pg/mL	% Of Expected
1	50µl	322.0	322.0	
	25µl	161.0	146.0	91
	12.5µl	80.5	89.0	111
	6.25µl	40.3	45.0	112
2	50µl	377.0	377.0	81
	25µl	188.5	152.0	100
	12.5µl	94.3	94.0	83
	6.25µl	47.1	39.0	
3	50µl	472.0	472.0	
	25µl	236.0	209.0	89
	12.5µl	118.0	99.0	84
	6.25µl	59.0	56.0	95
4	50µl	765.0	765.0	
	25µl	382.5	347.0	91
	12.5µl	191.3	166.0	87
	6.25µl	95.6	87.0	91

Four human serum samples with the indicated sample volumes were assayed in two separate experiments. Required amounts of matrix solution were added to compensate for lost volumes below 50 µL. The resulting dilution factors of 1.0, 2.0, 4.0, and 8.0 representing 50 µL, 25 µL, 12.5 µL, and 6.25 µL sample volumes assayed, respectively, were applied in the calculation of observed FGF-21 concentrations. % expected = observed/expected x 100%.

XV. QUALITY CONTROLS

The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Millipore website www.millipore.com/bmia.

XVI. TROUBLESHOOTING GUIDE

1. To obtain reliable and reproducible results the operator should carefully read this manual and fully understand all aspects of each assay step before attempting to run the assay.
2. Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
3. Have all necessary reagents and equipment ready on hand before starting. Once the assay has been started all steps should be completed with precise timing and without interruption.
4. Avoid cross contamination of any reagents or samples to be used in the assay.
5. Make sure all reagents and samples are added to the bottom of each well.
6. Careful and complete mixing of solutions in the well is critical. Poor assay precision will result from incomplete mixing or cross well contamination due to inappropriate mixing.
7. Remove any air bubble formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
8. Do not let the absorbency reading of the highest standard reach 3.0 units or higher after acidification.
9. High absorbance in background or blank wells could be due to 1) cross well contamination by standard solution or sample or 2) inadequate washing of wells with Wash Buffer or 3) overexposure to light after substrate has been added.

XVII. REPLACEMENT REAGENTS

Reagents	**Cat. #**
Human FGF-21 ELISA Plate	EP19
10X HRP Wash Buffer Concentrate (50 mL)	EWB-HRP
Human FGF-21 Standards	E8019-K
Human FGF-21 Quality Controls 1 and 2	E6019-K
Matrix Solution	EMTX-PS5
Assay Buffer	AB-GLPHK
Human FGF-21 Detection Antibody	E1019
Enzyme Solution	EHRP-4
Substrate	ESS-TMB2
Stop Solution	ET-TMB

XVIII. ORDERING INFORMATION

A. To place an order:

For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size

TELEPHONE ORDERS:
Toll Free US (800) MILLIPORE
FAX ORDERS: (636) 441-8050
MAIL ORDERS: Millipore
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:
To best serve our international customers, it is Millipore's policy to sell our products through a network of distributors. To place an order or to obtain additional information about Millipore products, please contact your local distributor.

B. Conditions of Sale

All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to human or animals. All products are intended for *in vitro* use only.

C. Material Safety Data Sheets (MSDS)

Material safety data sheets for Millipore products may be ordered by fax or phone. See Section A above for details on ordering.