Hu Fetuin-A ELISA

KAPEPKT800

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Human Fetuin-A ELISA Kit

Enzyme Linked ImmunoSorbent Assay (ELISA) for the measurement of Human Fetuin-A Levels in Serum

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IN VITRO DIAGNOSTIC

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INTENDED USE

This ELISA (enzyme-linked immunosorbent assay) kit is intended for the quantitative determination of human Fetuin-A, also known as alpha-2-HS glycoprotein (AHSG), in serum. The measurement of Fetuin-A can help in the diagnosis of some cancers and genetically inherited deficiencies of this serum protein. This Fetuin-A ELISA kit is for laboratory professional use.

SUMMARY OF PHYSIOLOGY

Fetuin-A, also known as alpha-2-HS glycoprotein, is a 59 kDa glycoprotein that consists of two amino-terminal cystatin domains and a smaller carboxyl-terminal domain. Fetuin-A is synthesized by the liver and secreted into the blood stream. The Fetuin-A concentration in adult men ranges from 0.5 – 1.5 g/L. During fetal life, high serum concentrations are found. Fetuin-A is involved in protease inhibitory activities and development-associated regulation of calcium metabolism and osteogenesis. It accumulates in bones and teeth as a major fraction of noncollagenous bone proteins. Studies have demonstrated that Fetuin-A is the major calcification inhibitor found in serum, where it interferes with calcium salt precipitation. Recent studies have shown that Fetuin-A levels drop in uremic patients on hemodialysis in comparison to normal healthy controls. The low Fetuin-A level may be associated with a higher cardiovascular mortality in patients on dialysis.

ASSAY PRINCIPLE

This ELISA is designed, developed and produced for the quantitative measurement of human Fetuin-A in serum samples. The assay utilizes the two-site "sandwich" technique with two selected goat anti-human Fetuin-A polyclonal antibodies that bind to different epitopes of human Fetuin-A.

Assay calibrators, controls and prediluted patient serum samples containing human Fetuin-A are added to the microtiter wells of a microplate that was coated with a high affinity polyclonal goat anti-human Fetuin-A antibody. After the first incubation period, the antibody fixed on the wall of the microtiter well captures human Fetuin-A in the sample. Remaining unbound proteins in the microtiter wells are washed away. Then a horseradish peroxidase (HRP) conjugated polyclonal anti-human Fetuin-A antibody is added to each microtiter well and a "sandwich" of "capture antibody - human Fetuin-A - HRP conjugated detecting antibody" is formed. The unbound detecting antibodies are removed in the subsequent washing step. The wells are incubated with a substrate solution in a timed interval, the reaction is stopped and the developed colour is then measured in a spectrophotometric microplate reader. The enzymatic activity of the detecting antibodies bound to the Fetuin-A on the wall of the microtiter wells is directly proportional to the amount of Fetuin-A in the sample. A calibration curve is generated by plotting the absorbance versus the respective human Fetuin-A concentration for each calibrator (point-to-point or cubical scales). The concentration of human Fetuin-A in test samples is determined directly from this calibration curve.

REAGENTS: Preparation and Storage

This test kit must be stored at 2 – 8°C upon receipt. For the expiration date of the kit refer to the label on the kit box. All components are stable at 2 – 8°C until this expiration date.

Prior to use, allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.

1. **Fetuin-A Antibody Coated Microplate**

One microplate with 12 x eight strips (96 wells total) coated with antibody to human Fetuin-A. The plate is framed and sealed in a foil Ziploc bag with a desiccant. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

2. Ab | HRP | CONC — **Detecting Antibody**

One vial containing 0.6 mL concentrated horseradish peroxidase (HRP) conjugated anti-human Fetuin-A detecting antibody in a stabilized protein matrix. This reagent must be diluted 1: 21 with dilution buffer before use. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

3. DIL | BUF — **Dilution buffer**

One vial containing 12 mL ready to use Trizma Hydrochloride based buffer. It should be only used for the dilution of the detecting antibody according to the assay procedures. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

4. INC | BUF | CONC — **Incubation buffer concentrate**

One vial containing 11 mL of concentrated incubation buffer based on phosphate buffered saline with bovine serum albumin. This concentrated incubation buffer must be diluted 1:10 with distilled or deionized water (11 mL concentrate plus 99 mL distilled water) before use. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

5. WASH | SOLN | CONC — **Washing buffer**

One bottle containing 30 mL of a 30-fold concentrate. Before use the contents must be diluted with 870 mL of distilled water and mixed well. Upon dilution this yields a working wash solution containing a surfactant in phosphate buffered saline with a non-azide preservative. The diluted solution should be stored at room temperature and is stable until the expiration date on the kit box.

6. CHROM | TMB — **TMB-Substrate solution**

One bottle containing 12 mL of tetramethylbenzidine (TMB) with hydrogen peroxide. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

7. STOP | SOLN — **Stop Solution**

One bottle containing 12 mL of 0.5 M sulfuric acid. This reagent should be stored at 2 – 8°C or room temperature and is stable until the expiration date on the kit box.

8. CAL | N — **Calibrators 0 - 4**

Five vials each containing human Fetuin-A in a liquid bovine serum based matrix with a non-azide preservative. **Refer to the vials for the exact concentration of each calibrator.** All the calibrators should be stored at -20°C or below after the first use with up to 3 freeze cycles.

9. CONTROL | N — **Controls 1 - 2**

Two vials each contains human Fetuin-A in a liquid bovine serum based matrix with a non azide preservative. **Refer to the vials for the exact concentration range of each control.** Both controls should be store at -20°C or below after the first use with up to 3 freeze cycles.

SAFETY PRECAUTIONS

For in vitro diagnostic use.

Source material from which reagents of bovine serum was derived in the contiguous 48 United States. It was obtained only from donor health animals maintained under veterinary supervision and found free of contagious diseases. Wear gloves while performing this assay and handle these reagents as if they are potential infectious. Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid. TMB may cause irritation to skin and mucous membranes and cause an allergic skin reaction. TMB is a suspected carcinogen. Sulfuric acid may cause severe irritation on contact with skin. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at lease 15 minutes. Use Good Laboratory Practices.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Precision single channel pipettes capable of delivering 10 µL, 25 µL, 100 µL, and 1000 µL.
2. Repeating dispenser suitable for delivering 100 µL.
3. Disposable pipette tips suitable for dispensing of the volumes indicated above.
4. Disposable 12 x 75 mm or 13 x 100 glass or plastic tubes.
5. Disposable plastic 100 mL and 1000 mL bottle with caps.
6. Aluminum foil.
7. Deionized or distilled water.
8. Plastic microtiter well cover or polyethylene film.
9. ELISA multichannel wash bottle or automatic (semi-automatic) washing system.
10. Spectrophotometric microplate reader capable of reading absorbance at 450 nm.

SPECIMEN COLLECTION

Only 10 µL of human serum is required for human Fetuin-A measurement. No special preparation of the individual is necessary prior to specimen collection. Whole blood should be collected and must be allowed to clot for minimum 30 minutes at room temperature before the serum is separated by centrifugation (850 – 1500xg for 10 minutes). The serum should be separated from the clot within three hours after blood collection and transferred to a clean test tube. Serum samples may be stored at –20ºC or below until measurement. Avoid repeated (more than three times) freezing and thawing of specimens.

ASSAY PROCEDURE

1. Patient Sample Preparation

Patient sample need to be diluted 1:10000 with incubation buffer before being measured.

(1) Label 2 test tubes (12x75 mm) with 1A and 1B
(2) Add 1 mL of incubation buffer to each tube (both 1A and 1B)
(3) Pipet 10 µL of patient sample to tube 1A and mix well (1:100 dilution)
(4) Transfer 10 µL of the diluted patient sample from tube 1A to tube 1B , mix well (1:10,000 dilution)

Note: It is recommended to use calibrated precision pipettes and to perform the dilution carefully in order to get precise results! We recommend to use an Eppendorf Repeat Pipette with a 12.5 mL combitip for adding 1 ml incubation buffer in stead of a 50 mL combitip.

2. Reagent Preparation

(1) Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.
(2) Incubation buffer Concentrate and Washing buffer must be diluted to working solutions prior to use. Please see REAGENTS section for details.

3. Assay Procedure

(1) Place a sufficient number of antibody coated microwell strips in a holder to run human Fetuin-A calibrators, controls and unknown samples in duplicate.
(2) Test Configuration

ROW	STRIP 1	STRIP 2	STRIP 3
A	CAL 0	CAL 4	SAMPLE 2
B	CAL 0	CAL 4	SAMPLE 2
C	CAL 1	C 1	SAMPLE 3
D	CAL 1	C 1	SAMPLE 3
E	CAL 2	C 2	
F	CAL 2	C 2	
G	CAL 3	SAMPLE 1	
H	CAL 3	SAMPLE 1	

(3) Add 25 µL of the calibrators, controls and 1:10,000 diluted patient samples into the designated microwells.
(4) Add 100 µL of incubation buffer to each well
(5) Mix gently and cover the plate with a plate sealer and with an aluminum foil to avoid exposure to light.
(6) Incubate the plate at room temperature for 2 hours.
(7) Prepare Detecting Antibody Working Solution by diluting the detecting antibody 1:21 with the Dilution buffer. For each strip, 1 mL of Dilution buffer with 50 µL of Detecting Antibody is required, in a clean test tube.
The table below shows the amount of antibody working solution that is required for a number of strips.

Strip no.	Dilution buffer	Detecting Antibody
1	1 mL	50 µL
2	2 mL	100 µL
3	3 mL	150 µL
4	4 mL	200 µL
5	5 mL	250 µL
6	6 mL	300 µL
7	7 mL	350 µL
8	8 mL	400 µL
9	9 mL	450 µL
10	10 mL	500 µL
11	11 mL	550 µL
12	12 mL	600 µL

Note: this detecting antibody working solution should be freshly prepared right before running the assay.

(8) Remove the aluminum foil and the plate sealer. Aspirate the contents of each well. Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirate the contents. Alternatively, an automated microplate washer can be used.
(9) Add 100 µL of the diluted detecting antibody working solution to each well.
(10) Cover the plate with a plate sealer and with an aluminum foil to avoid exposure to light.
(11) Incubate the plate at room temperature for 30 minutes.
(12) Remove the aluminum foil and the plate sealer. Aspirate the contents of each well. Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirate the contents. Alternatively, an automated microplate washer can be used.
(13) Add 100 µL of TMB-Substrate solution into each well.
(14) Cover the plate with aluminum foil to avoid exposure to light.
(15) Incubate the plate at room temperature for 20 minutes
(16) Remove the aluminum foil. Add 100 µL of Stop Solution into each well. Mix gently.
(17) Read the absorbance at 450 nm within 10 minutes in a microplate reader

NOTE: to reduce the background, one can set the instrument to dual wavelength measurement at 450 nm with background wavelength correction set at 595 nm or 620 nm or 630 nm.

PROCEDURAL NOTES

1. It is recommended that all calibrators, controls and unknown samples are assayed in duplicate. The average absorbance reading of each duplicate should be used for data reduction and the calculation of the results.
2. Keep light sensitive reagents in the original amber bottles.
3. Store any unused antibody coated strips in the foil Ziploc bag with desiccant to protect from moisture.
4. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
5. Incubation times or temperatures other than those stated in this insert may affect the results.
6. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate reading
7. All reagents should be mixed gently and thoroughly prior to use. Avoid foaming.

INTERPRETATION OF RESULTS

1. Calculate the average absorbance for each pair of duplicate test results.
2. Subtract the average absorbance of the CAL 0 (0 ng/mL) from the average absorbance of all other readings to obtain corrected absorbance.
3. The calibration curve is generated by the corrected absorbance of all calibrator levels on the ordinate against the calibrator concentration on the abscissa using point-to-point or log-log paper. Appropriate computer assisted data reduction programs may also be used for the calculation of results.

The human Fetuin-A concentrations for the controls and 1:10000 diluted samples are read directly from the calibration curve using their respective corrected absorbance.

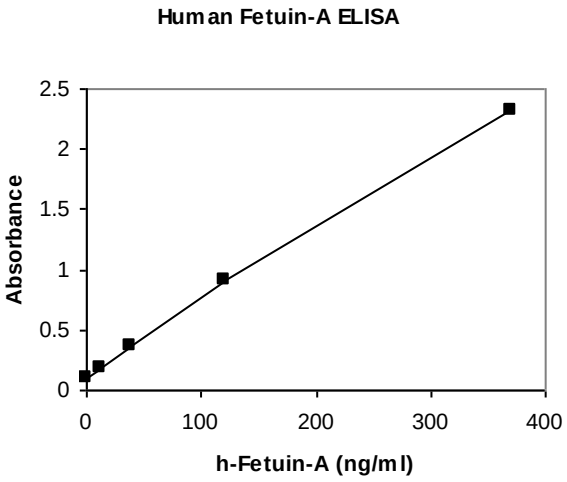
Each sample result must be multiplied with the dilution factor (10000) to obtain the Fetuin-A concentration in the undiluted sample.

For example, the concentration of a 1/10000 diluted sample read from the calibration curve is 24.3 ng/mL. The Fetuin A concentration of the undiluted sample is: 24.3 ng/mL x 10000 = 243000 ng/mL = 0.243 g/L

EXAMPLE DATA AND CALIBRATION CURVE

Typical absorbance data and the resulting calibration curve from human Fetuin-A ELISA are rshown below. **This curve should never be used instead of the real time calibration curve.**

Well I.D.	OD 450 nm Absorbance			Results ng/mL
	Readings	Average	Corrected	
0 ng/mL	0.097 0.099	0.098	0.000	
12.5 ng/mL	0.173 0.185	0.179	0.081	
38 ng/mL	0.364 0.379	0.371	0.273	
120 ng/mL	0.876 0.949	0.913	0.815	
370 ng/mL	2.298 2.325	2.312	2.214	
Control 1	0.471 0.498	0.484	0.386	55.1 ng/mL
Control 2	1.697 1.700	1.690	1.592	258.9 ng/mL



EXPECTED VALUES

Seventy serum samples from normal adult men were measured with this human Fetuin-A ELISA. The ninety-five percentile normal range was found to be 0.35 to 0.95 g/L with a mean value of 0.57 g/L and a standard deviation of 0.13 g/L.

LIMITATION OF THE PROCEDURE

1. The detection limit of the assay is 5.0 ng/mL (assay analytical sensitivity). This corresponds to a Fetuin A concentration of 50 µg/ml in the original sample (5.0 ng/ml x 10000).
2. Since there is no Calibrator available for human Fetuin-A measurement, the values of the calibrators were established by diluting a highly purified recombinant human Fetuin-A in a protein matrix.
3. If the assay result is higher than 350 ng/ml, it is recommended to measure a further diluted sample.
4. If your microplate reader is unable to read beyond 2.0 at OD 450 nm, one can run the assay without the calibrator level 4 from the calibrator set.
5. Bacterial or fungal contamination of serum specimens or reagents, or cross contamination between reagents may cause erroneous results.
6. Water deionized with polyester resins may inactivate the horseradish peroxidase enzyme.

QUALITY CONTROL

To assure the validity of the results each assay should include adequate controls with known Fetuin-A levels. We recommend that all assays include laboratory's own Fetuin-A controls in addition to those provided with this kit.

PERFORMANCE CHARACTERISTICS

Sensitivity

The analytical sensitivity of the human Fetuin-A ELISA, as determined by the 95% confidence limit on 20 duplicate determinations of the zero calibrator, is 5.0 ng/mL.

Precision

The intra-assay precision is validated by measuring two samples in a single assay with 20-replicate determinations.

Mean Fetuin-A Value (ng/mL)	CV (%)
33.6	5.5
121.1	4.8

The inter-assay precision is validated by measuring two samples in duplicate in 12 individual assays.

Mean Fetuin-A Value (ng/mL)	CV (%)
32.4	6.8
123.7	5.7

Linearity

Two human serum samples were diluted with incubation buffer and assayed. The results expressed in ng/mL are as follows:

#	DILUTION	OBSERVED VALUE	EXPECTED VALUE	RECOVERY %
1	1:10,000	21.9		
	1:20,000	11.9	11.0	108
	1:40,000	5.3	5.5	96
	1:80,000	2.9	2.7	107
	1:160,000	1.6	1.4	114
2	1:10,000	192		
	1:20,000	99.9	96	104
	1:40,000	45.2	48	94
	1:80,000	22.2	24	93
	1:160,000	13.4	12	112

Recovery

Two patient samples were spiked with various amounts of human Fetuin-A and assayed. The results expressed in ng/mL are as follows:

#	Orig. Value	Amount Spiked	Observed Value	Expected Value	Recovery %
1	33.6	21	25.1	27.3	92
		63	44.4	48.3	92
		200	120.1	116.8	103
2	121.1	21	68.9	71.1	97
		63	88.6	92.1	96
		200	157.1	160.6	98

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