Instructions for Use

Fetuin-A ELISA

Enzyme immunoassay for the *in-vitro diagnostic* quantitative determination of human Fetuin-A in human serum, plasma, cell culture supernatant, tissue extraction and urine.

REF **RE53301**

Σ **12x8**

2-8°C

EU: IVD CE

U.S.: *For research use only.*
Not for use in diagnostic procedures.

I B L I N T E R N A T I O N A L G M B H

Flughafenstrasse 52a
D-22335 Hamburg, Germany

Phone: +49 (0)40-53 28 91-0
Fax: +49 (0)40-53 28 91-11

IBL@IBL-International.com
www.IBL-International.com

1. INTENDED USE

Enzyme immunoassay for the *in-vitro diagnostic* quantitative determination of human Fetuin-A in human serum, plasma, cell culture supernatant, tissue extraction and urine.

2. SUMMARY AND EXPLANATION

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 blood stream, where its concentration in adult mammals ranges from 0.5 – 1.0 g/L. Fetuin-A occurs in high serum concentration during fetal life and involves 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 biologically, studies have demonstrated that Fetuin-A is the major calcification inhibitor found in circulation, where it interferes with calcium salt precipitation. Recent study has indicated that Fetuin-A level drops in uremic patients on hemodialysis in comparison to normal healthy controls. The low Fetuin-A level in patients with chronic kidney failure strongly associates with a higher cardiovascular mortality. On the other hand, it is demonstrated that a higher than normal serum Fetuin-A in older population associates incident diabetes, which is independent from other markers of insulin resistance. Further, a higher Fetuin-A level may be an independent risk marker of patients with for cardiovascular diseases.

3. TEST PRINCIPLE

The assay utilizes the two-site “sandwich” technique with two selected goat antihuman Fetuin-A polyclonal antibodies that bind to different epitopes of human Fetuin-A.
Assay standards, controls and prediluted patient serum samples containing human Fetuin-A is added to microtiter wells of microplate that was coated with a high affinity polyclonal goat anti-human Fetuin-A antibody. After the first incubation period, the antibody on the wall of microtiter well captures human Fetuin-A in the sample and unbound proteins in each microtiter well is 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 tracer antibody” is formed.
The unbound tracer antibody is removed in the subsequent washing step. HRP conjugated tracer antibody bound to the well is then incubated with a substrate solution in a timed reaction and then measured in a spectrophotometric microplate reader.
The enzymatic activity of the tracer antibody bound to the Fetuin-A on the wall of the microtiter well is directly proportional to the amount of Fetuin-A in the sample. A standard curve is generated by plotting the absorbance versus the respective human Fetuin-A concentration for each standard on point-to-point or cubical scales. The concentration of human Fetuin-A in test samples is determined directly from this standard curve.

4. WARNINGS AND PRECAUTIONS

1. For *in-vitro diagnostic* use only. For professional use only.
2. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.
3. In case of severe damage of the kit package please contact IBL or your supplier in written form, latest one week after receiving the kit. Do not use damaged components in test runs, but keep safe for complaint related issues.
4. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
5. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.
6. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details. Material Safety Data Sheets for this product are available on the IBL-Homepage or upon request directly from IBL.
7. Chemicals and prepared or used reagents have to be treated as hazardous waste according to national biohazard and safety guidelines or regulations.
8. Avoid contact with Stop solution. It may cause skin irritations and burns.
9. All reagents of this kit containing human serum or plasma have been tested and were found negative for anti-HIV I/II, HBsAg and anti-HCV. However, a presence of these or other infectious agents cannot be excluded absolutely and therefore reagents should be treated as potential biohazards in use and for disposal.

5. STORAGE AND STABILITY

The kit is shipped at ambient temperature and should be stored at 2-8°C. Keep away from heat or direct sun light. The storage and stability of specimen and prepared reagents is stated in the corresponding chapters.

The microtiter strips are stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2–8°C.

6. SPECIMEN COLLECTION AND STORAGE

Serum and Plasma (EDTA)

Only 10 µL of human serum or plasma is required for human Fetuin-A measurement. No special preparation of 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 (850-1500 xg for 10 minutes). The serum should be separated from the clot within three hours of blood collection and transferred to a clean test tube. Serum samples may be stored at –20°C or below until measurement. Avoid repeated more than three times freezing and thawing of specimen.

Urine

Twenty four hour urine sample is recommended to be used for the determination of urine Fetuin-A concentration. Spot urine from the second morning urination may be used if strenuous physical activity shortly before sample collection has been ruled out and polyuric renal dysfunction is not present. Intra-individual day-to-day fluctuations in the concentration of urine proteins caused by diurisis may be reduced by relating to the urinary creatinine concentration. For cell culture supernatant, tissue extracts, one should serial dilute test sample and measure multiple diluted samples for a more accurate Fetuin-A test result.

7. MATERIALS SUPPLIED

Quantity	Symbol	Component
1 x 12 x 8	**MTP**	**Microtiter Plate** Ready to use. Break apart strips. Coated with antibodies against human Fetuin-A.
1 x 5 x 0.5 mL	**CAL A-E**	**Standard A-E** Contains: human Fetuin-A in a liquid bovine serum based matrix with a non-azide preservative. Exact concentrations see vial labels or QC certficate.
1 x 2 x 0.5 mL	**CONTROL 1** **CONTROL 2**	**Control 1+2** Contains: human Fetuin-A in a liquid bovine serum based matrix with a non-azide preservative. Exact concentrations see vial labels or QC certficate.
1 x 0.6 mL	**ENCONJ**	**Enzyme Conjugate** Ready to use. Contains: anti-human Fetuin-A tracer antibody in a stabilized protein matrix conjugated to concentrated HRP.
1 x12 mL	**ENZBUF**	**Enzyme Buffer** Ready to use. Contains: Trizma Hydrochloride based buffer.
1 x 11 mL	**ASSAYBUF** **CONC**	**Assay Buffer** Concentrate (10x) Ready to use. Contains: phosphate buffer, BSA.
1 x 20 mL	**WASHBUF** **CONC**	**Wash Buffer** Concentrate (30x) Contains: surfactant in phosphate buffered saline with a nonazide preservative.
1 x 12 mL	**TMB SUBS**	**TMB Substrate Solution** Ready to use. Contains: TMB (tetramethylbenzidine).
1 x 12 mL	**TMB STOP**	**TMB Stop Solution** Ready to use. Contains: 0.5 M H_2SO_4.

8. MATERIALS REQUIRED BUT NOT SUPPLIED

1. Micropipettes (Multipette Eppendorf or similar devices, < 3% CV). Volume: 10 µL; 25 µL; 100µL, 1000 µL
2. Repeating dispenser suitable for delivering 100 µL.
3. Disposable pipette tips suitable for above volume dispensing.
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. Vortex mixer
8. Plastic microtiter well cover or polyethylene film.
9. 8-Channel Micropipettor with reagent reservoirs
10. Wash bottle, automated or semi-automated microtiter plate washing system
11. Microtiter plate reader capable of reading absorbance at 450 nm (reference wavelength 600-650 nm)
12. Bidistilled or deionised water
13. Paper towels, pipette tips and timer

9. PROCEDURE NOTES

1. Any improper handling of samples or modification of the test procedure may influence the results. The indicated pipetting volumes, incubation times, temperatures and pretreatment steps have to be performed strictly according to the instructions. Use calibrated pipettes and devices only.
2. Once the test has been started, all steps should be completed without interruption. Make sure that required reagents, materials and devices are prepared ready at the appropriate time. Allow all reagents and specimens to reach room temperature (18-25°C) and gently swirl each vial of liquid reagent and sample before use. Mix reagents without foaming.
3. Avoid contamination of reagents, pipettes and wells/tubes. Use new disposable plastic pipette tips for each component and specimen. Do not interchange caps. Always cap not used vials. Do not reuse wells/tubes or reagents.
4. Some components contain ≤ 250 µL solution. Take care that the solution is completely on the bottom of the vial before opening.
5. It is advised to determine samples in duplicate to be able to identify potential pipetting errors.
6. Use a pipetting scheme to verify an appropriate plate layout.
7. Incubation time affects results. All wells should be handled in the same order and time sequences. It is recommended to use an 8-channel Micropipettor for pipetting of solutions in all wells.
8. Microplate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or an automatic microplate washing system. Do not allow the wells to dry between incubations. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled precisely with Wash Buffer, and that there are no residues in the wells.
9. Humidity affects the coated wells/tubes. Do not open the pouch until it reaches room temperature. Unused wells/tubes should be returned immediately to the resealed pouch including the desiccant.

10. PRE-TEST SETUP INSTRUCTIONS

10.1. Preparation of concentrated components

Dilute / dissolve	Component		Diluent	Relation	Remarks	Storage	Stability
20 mL	WASHBUF CONC	ad 600 mL	bidist. water	1:30	Warm up at 37°C to dissolve crystals, if necessary.	18-25°C	until Exp. date
11 mL	ASSAYBUF CONC	ad 110 mL	bidist. water	1:10		2-8°C	until Exp. date

	The contents of the kit for 96 determinations can be divided into 3 separate runs. The volumes stated below are for one run with 4 strips (32 determinations).				
Dilute / dissolve	**Component**		**Diluent**	**Relation**	**Remarks**
200 µL	ENZCONJ	with 4 mL	ENZBUF	1:21	Prepare freshly and use only once.

10.2. Dilution of Samples

Serum/Plasma

Patient serum or plasma sample need to be diluted 1:10.000 with assay buffer before being measured.

(1) Label 2 test tubes (12x75 mm) with 1A and 1B.
(2) Add 1 mL of assay buffer to each tube (both 1A and 1B).
(3) Pipet 10 µL of patient serum or plasma sample to tube 1A and mix well (1:100 dilution).
(4) Pipet 10 µL of diluted patient sample from 1A to tube 1B mix well (1:10.000 dilution).

Note: It is recommended to use a precision/calibrated pipette and careful technique to perform the dilution in order to get precise results! We recommend using Eppendorf Repeat Pipette with 12.5 mL combitip for adding 1 mL assay buffer and don't use 50 mL combitip.

Urine

Patient urine sample need to be diluted 1:100 with assay buffer before being measured.

(1) Label 1 test tubes (12x75 mm) with 1.
(2) Add 1 mL of assay buffer to each tube.
(3) Pipet 10 µL of patient urine sample to tube 1 and mix well (1:100 dilution).

Note: If a higher than standard level 5 of Fetuin-A test result is obtained, a further dilution of urine sample (e.g. 1:500) should be measured for reporting a more accurate test result.

11. TEST PROCEDURE

1.	Pipette **25 µL** of each **Standard, Control and diluted patient sample** into the respective wells of the Microtiter Plate.
2.	Pipette **100 µL** of **Assay Buffer** into each well.
3.	Mix gently. Cover plate with one plate sealer and also with aluminum foil to avoid exposure to light. **Incubate 120 min** at **RT (18-25°C).**
4.	Remove the aluminum foil and the plate sealer. Discard incubation solution. Wash plate **5 x** with **350 µL** of **diluted Wash Buffer.** Remove excess solution by tapping the inverted plate on a paper towel.
5.	Pipette **100 µL** of freshly prepared **Enzyme Conjugate** into each well.
6.	Mix gently. Cover plate with one plate sealer and also with aluminum foil to avoid exposure to light. **Incubate 30 min** at **RT (18-25°C).**
7.	Remove the aluminum foil and the plate sealer. Discard incubation solution. Wash plate **5 x** with **350 µL of diluted Wash Buffer.** Remove excess solution by tapping the inverted plate on a paper towel.
8.	For adding of Substrate and Stop Solution use, if available, an 8-channel Micropipettor. Pipetting should be carried out in the same time intervals for Substrate and Stop Solution.
9.	Pipette **100 µL** of **TMB Substrate Solution** into each well.
10.	Mix gently. **Incubate 20 min** at **RT (18-25°C).**
11.	Remove the aluminum foil. Stop the substrate reaction by adding **100 µL** of **TMB Stop Solution** into each well. Briefly mix contents by gently shaking the plate. Color change from blue to yellow.
12.	**Measure** optical density with a photometer at **450 nm** (Reference-wavelength: 600-650 nm) within **10 min** after pipetting of the Stop Solution.

12. QUALITY CONTROL

The test results are only valid if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable standards/laws. All kit controls must be found within the acceptable ranges as stated on the vial labels. If the criteria are not met, the run is not valid and should be repeated. Each laboratory should use known samples as further controls.

In case of any deviation the following technical issues should be proven: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.

13. CALCULATION OF RESULTS

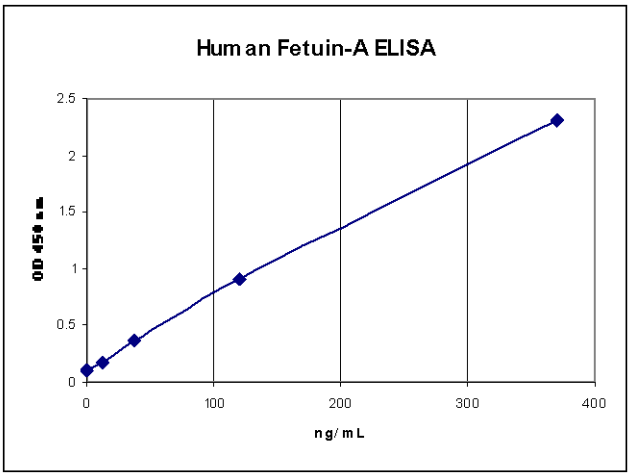
The obtained OD of the standards (y-axis, linear) are plotted against their concentration (x-axis, logarithmic) either on semi-logarithmic graph paper or using an automated method. A good fit is provided with cubic spline, 4 Parameter Logisitcs or Logit-Log.

For the calculation of the standard curve, apply each signal of the standards (one obvious outlier of duplicates might be omitted and the more plausible single value might be used).

Typical Calibration Curve

(Example. Do not use for calculation!)

Standard	ng/mL	OD_{Mean}
A	0.0	0.098
B	12.5	0.179
C	38	0.371
D	120	0.913
E	370	2.312



The human serum or plasma Fetuin-A concentrations for the controls and 1:10.000 diluted samples are read directly from the standard curve using their respective corrected absorbance. If log-log graphic paper or computer assisted data reduction program utilizing logarithmic transformation are used, samples having corrected absorbance between the 1 ng/mL standard and the next highest standard should be calculated by the formula:

$$\text{Value of unknown} = \frac{\text{Corrected absorbance (unknown)}}{\text{Corrected absorbance (2}^{nd}\text{ STD)}} \times \text{Value of the 2}^{nd}\text{ STD}$$

14. INTERPRETATION OF RESULTS

The results themselves should not be the only reason for any therapeutical consequences. They have to be correlated to other clinical observations and diagnostic tests.

15. EXPECTED VALUES

Seventy normal adult sera 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.
The 10.00-fold dilution factor must be added to each sample for the original sample Fetuin-A concentration. For example, a 1/10.000 fold diluted sample value is 24.3 ng/mL directly from the standard curve, the original sample Fetuin-A concentration should be 24.3 ng/mL x 10.000 = 243000 ng/mL = 0.243 g/L

16. LIMITATIONS OF THE PROCEDURE

a. The lowest concentration of human Fetuin-A directly measurable is 5.0 ng/mL (assay analytical sensitivity). After back calculation for the 1/10.000 fold dilution of patient serum sample, the assay measures the lowest serum Fetuin-A concentration at 50 µg/mL of original serum sample.

b. Since there is no Gold Standard concentration available for human Fetuin-A measurement, the values of assay standards were established by diluting a highly purified recombinant human Fetuin-A in a protein matrix.

c. For unknown sample value read directly from the assay is greater than 350 ng/mL, it is recommend to measure a further diluted sample for more accurate measurement.

d. Bacterial or fungal contamination of serum specimens or reagents, or cross contamination between reagents may cause erroneous results.

e. Water deionized with polyester resins may inactive the horseradish peroxidase enzyme.

17. PERFORMANCE

Precision	Mean (ng/mL)		CV (%)		
	Sample 1	Sample 2	Sample 1	Sample 2	
Intra-Assay	33.6	121.1	5.5	4.8	
Inter-Assay	32.4	123.7	6.8	5.7	
Analytical Sensitivity (Limit of Detection)	Mean signal (Zero-Standard) + 2SD (as read from the standard curve)				5 ng/mL
Linearity	Range (ng/mL)		Serial dilution up to		Range (%)
	1.6 – 192		1/ 160.000		93 - 112
Recovery	Mean recovery after spiking: 96 %				92 – 103

18. PRODUCT LITERATURE REFERENCES

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Symbols / Symbole / Symbôles / Símbolos / Símbolos / Σύμβολα

REF	Cat.-No.: / Kat.-Nr.: / No.- Cat.: / Cat.-No.: / N.º Cat.: / N.–Cat.: / Αριθμός-Κατ.:
LOT	Lot-No.: / Chargen-Bez.: / No. Lot: / Lot-No.: / Lote N.º: / Lotto n.: / Αριθμός -Παραγωγή:
	Use by: / Verwendbar bis: / Utiliser à: / Usado por: / Usar até: / Da utilizzare entro: / Χρησιμοποιείται από:
	No. of Tests: / Kitgröße: / Nb. de Tests: / No. de Determ.: / N.º de Testes: / Quantità dei tests: / Αριθμός εξετάσεων:
CONC	Concentrate / Konzentrat / Concentré / Concentrar / Concentrado / Concentrato / Συμπύκνωμα
LYO	Lyophilized / Lyophilisat / Lyophilisé / Liofilizado / Liofilizado / Liofilizzato / Λυοφιλιασμένο
IVD	In Vitro Diagnostic Medical Device. / In-vitro-Diagnostikum. / Appareil Médical pour Diagnostics In Vitro. / Dispositivo Médico para Diagnóstico In Vitro. / Equipamento Médico de Diagnóstico In Vitro. / Dispositivo Medico Diagnostico In vitro. / Ιατρική συσκευή για In-Vitro Διάγνωση.
	Evaluation kit. / Nur für Leistungsbewertungszwecke. / Kit pour évaluation. / Juego de Reactivos para Evaluació. / Kit de avaliação. / Kit di evaluazione. / Κιτ Αξιολόγησης.
	Read instructions before use. / Arbeitsanleitung lesen. / Lire la fiche technique avant emploi. / Lea las instrucciones antes de usar. / Ler as instruções antes de usar. / Leggere le istruzioni prima dell'uso. / Διαβάστε τις οδηγίες πριν την χρήση.
	Keep away from heat or direct sun light. / Vor Hitze und direkter Sonneneinstrahlung schützen. / Garder à l'abri de la chaleur et de toute exposition lumineuse. / Manténgase alejado del calor o la luz solar directa. / Manter longe do calor ou luz solar directa. / Non esporre ai raggi solari. / Να φυλάσσεται μακριά από θερμότητα και άμεση επαφή με το φως του ηλίου.
	Store at: / Lagern bei: / Stocker à: / Almacene a: / Armazenar a: / Conservare a: / Αποθήκευση στους:
	Manufacturer: / Hersteller: / Fabricant: / Productor: / Fabricante: / Fabbricante: / Παραγωγός:
	Caution! / Vorsicht! / Attention! / ¡Precaución! / Cuidado! / Attenzione! / Προσοχή!

Symbols of the kit components see MATERIALS SUPPLIED.
Die Symbole der Komponenten sind im Kapitel KOMPONENTEN DES KITS beschrieben.
Voir MATERIEL FOURNI pour les symbôles des composants du kit.
Símbolos de los componentes del juego de reactivos, vea MATERIALES SUMINISTRADOS.
Para símbolos dos componentes do kit ver MATERIAIS FORNECIDOS.
Per i simboli dei componenti del kit si veda COMPONENTI DEL KIT.
Για τα σύμβολα των συστατικών του κιτ συμβουλευτείτε το ΠΑΡΕΧΟΜΕΝΑ ΥΛΙΚΑ.

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IBL International GmbH Flughafenstr. 52A, 22335 Hamburg, Germany	Tel.: E-MAIL: WEB:	+ 49 (0) 40 532891 -0 Fax: -11 IBL@IBL-International.com http://www.IBL-International.com
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