Quantikine® ELISA

Human Fetuin A Immunoassay

Catalog Number DFTA00

For the quantitative determination of human Fetuin A concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Human Fetuin A, also known as α2-Heremans-Schmid glycoprotein, is encoded by the AHSG gene on chromosome 3. Based on the amino acid sequence similarity and conserved structure, it is classified as a member of the cystatin superfamily of cysteine protease inhibitors. Fetuin A is mainly produced by hepatocytes and monocytes/macrophages. It is most abundant in the plasma and in the bone. In the plasma, Fetuin A is a 59 kDa, disulfide bond-linked two chain polypeptide consisting of two N-terminal cystatin domains and a smaller C-terminal domain (1-3). In the bone, Fetuin A is the most abundant non-collagenous protein. It accumulates in the bone through strong binding to apatite (4, 5).

Fetuin A is a well-known negative acute-phase reactant, whose amounts fall in response to endogenous and exogenous stimulation (6, 7). The major physiologic functions of Fetuin A are not fully understood. Several lines of evidence have suggested that it may play an important role in blood vessel calcification. Purified bovine Fetuin A, when added into a solution supersaturated with calcium phosphate, is able to inhibit the precipitation of calcium phosphate mineral (8). In mice with high serum concentrations of calcium and phosphate, Fetuin A is an important systemic inhibitor of apatite formation in serum. Gene knockout mice bearing no Fetuin A protein have increased risk for etopic calcification in blood vessels (9). Furthermore, serum levels of Fetuin A-mineral complex correlate with artery calcification in rats (10, 11). In humans, a number of investigations have revealed similar findings as those found in the animal studies. It has been shown that serum levels of Fetuin A are significantly lower in end stage renal failure patients on long-term hemodialysis than in healthy controls (12-16). Sera from these patients show an impaired capacity to inhibit calcium phosphate precipitation. This impairment is corrected when purified Fetuin A is added. Therefore, decreased serum Fetuin A levels may contribute to accelerated vascular calcification in uremic patients (12). Fetuin A serum levels may have potential value as a biomarker to predict artery calcification and mortalities in renal failure patients (12-16). Fetuin A is also involved in inflammation. It has been observed that Fetuin A can suppress tumor necrosis factor (TNF) release from lipopolysaccharide (LPS)-stimulated macrophages. In agreement with this observation, administration of Fetuin A to pregnant rats significantly prevents the onset of abortion induced by LPS (17-19). These studies indicate that Fetuin A may act as a counter-regulator of LPS-induced inflammatory response. In the AHSG gene promoter region, there are several potential interleukin 6-response elements, and the synthesis of Fetuin A is down-regulated during injury and inflammation (20).

The Quantikine Human Fetuin A Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Fetuin A in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant human Fetuin A and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Fetuin A showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring human Fetuin A.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Fetuin A has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Fetuin A present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Fetuin A is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Fetuin A bound. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Fetuin A Microplate	893822	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Fetuin A.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human Fetuin A Standard	893824	Recombinant human Fetuin A in a buffered protein base with preservative; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and store at ≤ -20 °C for up to 1 month. Avoid repeated freeze-thaw cycles.
Human Fetuin A Conjugate	893823	21 mL of a monoclonal antibody specific for human Fetuin A conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1S	895137	11 mL of a buffered protein base with preservative. *For cell culture supernate samples.*	
Assay Diluent RD1X	895121	11 mL of a buffered protein base with preservative. *For serum/plasma samples. May contain crystals. Warm to room temperature to dissolve.*	
Calibrator Diluent RD5-26 Concentrate	895525	21 mL of a concentrated buffered protein base with preservatives. *Used diluted 1:10 in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 50 mL and 500 mL graduated cylinders.
- Test tubes for dilution of standards and samples.
- Human Fetuin A Controls (optional; available from R&D Systems).

PRECAUTIONS

Fetuin A is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

SAMPLE PREPARATION

Serum and plasma samples require a 4000-fold dilution. A suggested 4000-fold dilution can be achieved by adding 10 µL of sample to 990 µL of Calibrator Diluent RD5-26 (diluted 1:10).* Complete the 4000-fold dilution by adding 25 µL of the diluted sample to 975 µL Calibrator Diluent RD5-26 (diluted 1:10).

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High concentrations of Fetuin A are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

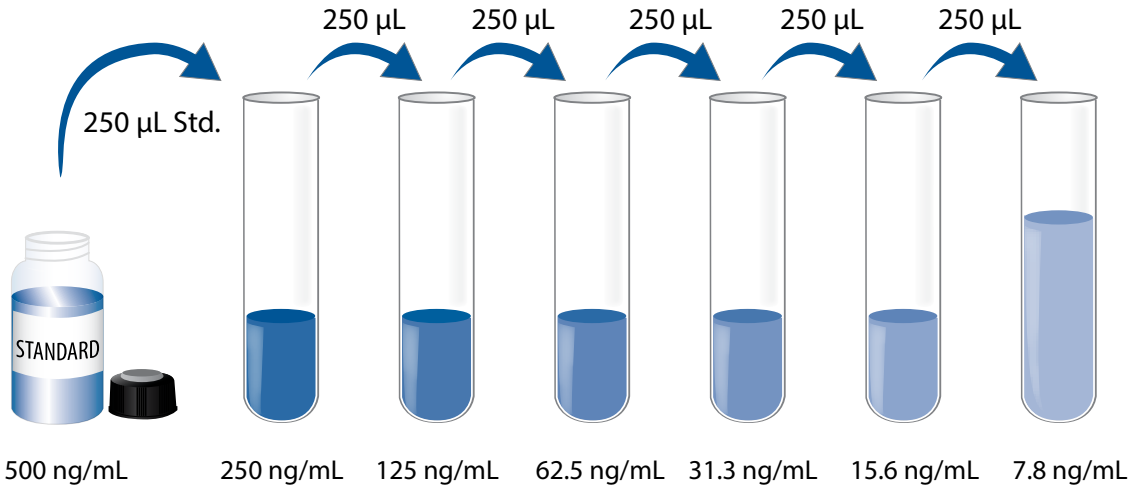
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-26 (diluted 1:10) - Add 5 mL of Calibrator Diluent RD5-26 Concentrate into 45 mL of deionized or distilled water to prepare 50 mL of Calibrator Diluent RD5-26 (diluted 1:10).

Human Fetuin A Standard - **Refer to the vial label for recontitution volume.** Reconstitute the Human Fetuin A Standard with Calibrator Diluent RD5-26 (diluted 1:10). This reconstitution produces a stock solution of 500 ng/mL. Allow the standard to sit for a minimum of 5 minutes with gentle agitation prior to making dilutions.

Pipette 250 µL of Calibrator Diluent RD5-26 (diluted 1:10) into six tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Human Fetuin A Standard (500 ng/mL) serves as the high standard. Calibrator Diluent RD5-26 (diluted 1:10) serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

Note: *High concentrations of Fetuin A are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. **For Culture Supernate Samples:** Add 100 µL of Assay Diluent RD1S to each well.
   For Serum/Plasma Samples: Add 100 µL of Assay Diluent RD1X to each well. *May contain crystals. Warm to room temperature to dissolve.*
4. Add 50 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human Fetuin A Conjugate to each well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

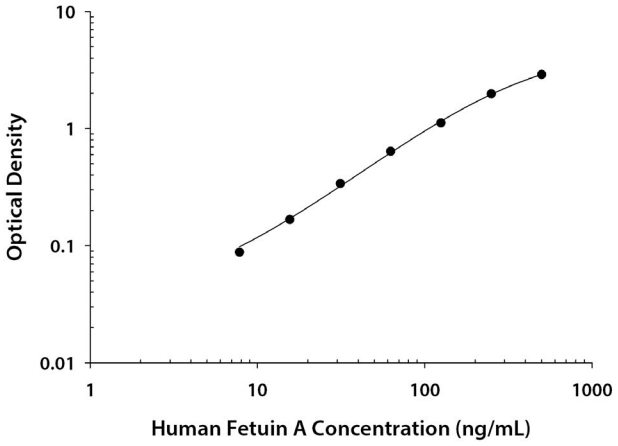
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human Fetuin A concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

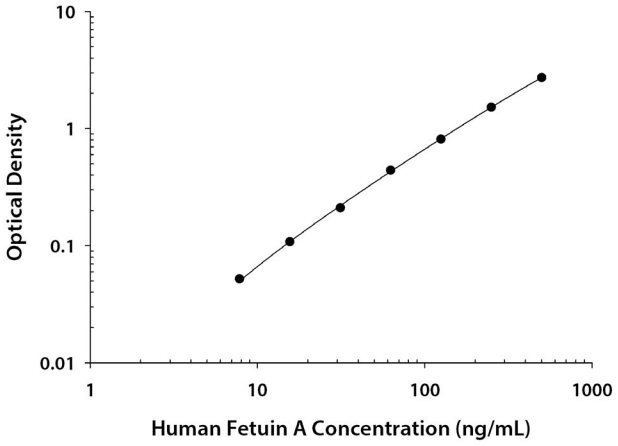
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.026 0.026	0.026	—
7.8	0.110 0.118	0.114	0.088
15.6	0.192 0.194	0.193	0.167
31.3	0.358 0.372	0.365	0.339
62.5	0.656 0.674	0.665	0.639
125	1.120 1.168	1.144	1.118
250	1.982 2.033	2.008	1.982
500	2.853 2.979	2.916	2.890

SERUM/PLASMA ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.033 0.045	0.039	—
7.8	0.089 0.092	0.091	0.052
15.6	0.145 0.148	0.147	0.108
31.3	0.241 0.258	0.250	0.211
62.5	0.460 0.500	0.480	0.441
125	0.830 0.873	0.852	0.813
250	1.538 1.586	1.562	1.523
500	2.741 2.786	2.764	2.725

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	45.0	147	344	47.1	149	334
Standard deviation	3.32	10.6	19.6	3.00	10.7	28.2
CV (%)	7.4	7.2	5.7	6.4	7.2	8.4

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	43.3	124	264	44.9	127	269
Standard deviation	1.69	6.08	10.6	3.67	9.34	22.6
CV (%)	3.9	4.9	4.0	8.2	7.4	8.4

RECOVERY

The recovery of human Fetuin A spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	103	93-109%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Fetuin A were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	98	99	103	100
	Range (%)	91-107	95-106	92-113	96-106
1:4	Average % of Expected	101	98	99	103
	Range (%)	91-109	94-100	94-102	98-107
1:8	Average % of Expected	96	94	96	98
	Range (%)	85-102	91-99	88-104	89-109
1:16	Average % of Expected	———	90	96	103
	Range (%)	———	86-93	85-106	90-111

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

Eighty assays were evaluated and the minimum detectable dose (MDD) of human Fetuin A ranged from 0.16-1.74 ng/mL. The mean MDD was 0.62 ng/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human Fetuin A produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human Fetuin A in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (µg/mL)	Range (µg/mL)	Standard Deviation (µg/mL)
Serum (n=35)	473	303-671	95
EDTA plasma (n=35)	450	274-631	83
Heparin plasma (n=35)	454	285-617	72

Cell Culture Supernates - HepG2 human hepatocellular carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate until confluent. An aliquot of the cell culture supernate was removed, assayed for natural human Fetuin A, and measured 30.4 µg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human Fetuin A.

The factors listed below were prepared at 5 mg/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 5 mg/mL in a mid-range recombinant human Fetuin A control were also assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Cathepsin A
Cathepsin B
Cathepsin C
Cathepsin F
Cathepsin L
Cathepsin O
Cathepsin S
C-Reactive Protein
Cystatin A
Cystatin B
Cystatin C
Cystatin D
Cystatin E/M
Cystatin F
Cystatin S
Cystatin SA
Cystatin SN
Fetuin B
Serpin A1

Recombinant mouse:

Fetuin A

Natural proteins:

human α1-Acid Glycoprotein
human Fibronectin

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